Lymphocytic Choriomeningitis Virus and Other Arenaviruses

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Foreword

Four decades ago, lymphocytic choriomeningitis virus was discovered, at about the same time and independently, in 3 different localities in the U.S.A. Armstrong and Lillie encountered the agent in a monkey when they passaged a recent isolate of the St. Louis encephalitis virus; Rivers and Scott isolated 5 strains from patients with meningitis; and Traub revealed the virus in a colony of albino mice. Already in these first isolations mice were incriminated, and later observations proved beyond doubt that M. musculus is the principal reservoir of the virus in nature.

For some time LCM virus was regarded as the sole etiologic agent of Wallgren's "méningite aseptique aiguë". Soon, however, it became clear that Wallgren's syndrome had a multitude of causes, among which the LCM virus was of little relevance, and in subsequent years it disappeared from the sight of most virologists. Indeed, it might have fallen into oblivion had it not been for Erich Traub who, practically all by himself, continued to investigate the intricate relationship between this virus and its natural host, the common house mouse.

Interest in LCM virus was revived by Burnet and Fenner who based their immunological concept of self-recognition on Owen's observations on erythrocyte chimerism in cattle twins and on the phenomenon of persistent infection of the mouse with LCM virus. It was also Burnet who pointed out that the coexistence of host and virus depended on the property of the latter to spare the cell in which it multiplies, and John Hotchin must be given credit for pioneering the idea that the experimental LCM disease in adult mice represents a virus-induced immune disease. Today, the interaction between LCM virus and mouse is considered an excellent model for the study of a variety of phenomena of biological and medical relevance, such as immunological tolerance, viral immunopathology, slow virus diseases, and latent virus infections, and interest in LCM virus is rapidly growing again.

Other developments of the more recent past have further stimulated research on this virus. In 1969, Wallace P. Rowe called upon interested investigators to select a name for a group of viruses with certain properties in common with LCM virus. Arenovirus (later changed to Arenavirus and considered a genus) was the chosen name; the type species is LCM virus, and other approved members are Amapari virus, Junin virus, Lassa virus, Latino virus, Machupo virus, Parana virus, Pichinde virus, Pistillo virus, Tamiami virus, and Tacaribe virus. The biological as well as structural similarities of these viruses are close, and the establishment of the group has proved to be a most effective impetus for

the study of the properties of many arenaviruses including those of LCM virus. The initiative in 1969 not only created a new group of viruses. It also led to the foundation of a "club" of investigators with common interests who for more than 2 years had the peculiar attribute to have never actually met. When my colleagues and I at the Heinrich-Pette-Institut decided to act as hosts for the first meeting, we were, fortunately, unaware of the organizational difficulties which lay ahead. If those who have contributed towards overcoming these difficulties were all to be named, the list would be prohibitively long. However, even with the help of so many we would have failed without our secretary, Miss Evelyne Danckers. She was the heart and soul of our efforts before, during and after the meeting, and she deserves the sincere gratitude of all of us.

Together with my colleagues of this institute I wish to thank all participants – speakers and discussants – who gave the symposium the scientific quality it had. We hope that the anticipated increase of the size of our "club" will not prevent us from meeting anew, and we trust that this will happen once again in an atmosphere based as much on personal friendship as on scientific achievements.

Fritz Lehmann-Grube

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General Introduction

LCM Virus Research, Retrospect and Prospects

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To an old friend of LCM virus it is gratifying to see that a large number of scientists are now actively engaged in LCM research and that great progress has been made in recent years. In the 1930's, there was just a handful of LCM investigators scattered over a few countries. Now there are numerous groups of research workers on different continents interested in this subject. The disease, especially that in mice, appears to have obtained the reputation of being a good research model for several basic problems.

INTRODUCTION

For those in the audience who are not very familiar with LCM, I would like to make a few introductory remarks. LCM virus was isolated in 1933 by Armstrong and Lillie in Bethesda, Maryland, from a monkey used for passage of the St. Louis encephalitis virus. It was not certain whether the virus originated in man or monkey. Human cases of LCM were encountered soon thereafter by Armstrong and co-workers as well as by Rivers and Scott in New York. In 1934, I found the virus in the mouse colony of the Princeton Section of the Rockefeller Institute. A causal relationship was established between the infection in mice and one of the human cases of Rivers and Scott. More human infections were reported by several investigators in the following years but, on the whole, the incidence of the disease in man has remained low. A number of human laboratory infections with LCM virus, few of them fatal, have been reported.

A search for the virus in trapped wild mice did not give conclusive results at Princeton, but Armstrong and Sweet were able in 1939 to detect LCM virus in gray house mice and to correlate the murine infection with human cases of LCM occurring on the same premises. This geographic relationship between murine and human infections was later confirmed by numerous investigators, notably by Scheid and his colleagues at the Universitäts-Nervenklinik, Cologne, and wild house mice are now generally regarded as the main reservoir in nature of LCM virus. For this, mice are admirably equipped, as I will point out later.

It is not yet clear how the virus is transmitted from mice to man. Some investigators consider it as an arthropod-borne agent, since experimental transmission from animal to animal was successful with a variety of arthropods, such as mosquitoes, bed bugs, and ticks, and since besides certain arboviruses only LCM virus could be shown by Rehácek in 1965 to grow in tick cells in vitro. Positive results were also obtained by others with larvae of Trichinella spiralis. However,

none of these experimental vectors have thus far been shown to play a role in nature.

Besides man and mice the natural host spectrum of LCM virus at present includes hamsters and possibly monkeys. Experimental infections were successful with several other animal species, including guinea-pigs, rats, cotton rats, rabbits, dogs, and chick embryos.

LCM VIRUS

Compared with the large amount of work done on pathogenetic, immunological and epidemiological aspects of LCM, the study of the virus itself was somewhat neglected in the past. According to present knowledge, the LCM agent is a medium-sized RNA virus. Electron micrographs show virus particles budding from the cell membrane. Virions contain several electron-dense, sand grain-like particles, presumably consisting of RNA. For this and other reasons, LCM virus, Tacaribe, Machupo, and Junin viruses from South America as well as Lassa virus from Africa, which show similar ultramicroscopic structures, were included in a new taxonomic group, the arenaviruses, in which the LCM virus represents the prototype. LCM virus is ether-sensitive and generally very labile. Specific antigen(s) demonstrable by complement fixation and by fluorescent staining occur in infected cells and tissues. An interesting feature of infected cells are clusters of ribosomes which were shown by Abelson and co-workers to contain specific antigen.

LCM virus appears to be quite stable serologically but variable with regard to its pathogenic properties. Consequently, it is often not possible to repeat experimental results exactly with other virus strains. Since different strains of laboratory mice also vary in their behavior towards this virus, the duplication of experimental work becomes even more difficult.

In cultures, LCM virus was found to grow in a large variety of mammalian cells. As a rule, little or no CPE is produced, and this is a handicap for titrating the virus. No cell type fully suitable for plaque assay is thus far known. This difficulty has been circumvented by Lehmann-Grube and his colleagues and by Oldstone and Dixon who based infectivity assays on demonstrating the appearance of CF antigen or immunofluorescing antigen, respectively, in infected cell cultures.

MURINE LCM

I shall now turn to the disease in mice which has been one of my main fields of interest for many years. Unfortunately, the work was repeatedly interrupted for long periods of time by circumstances beyond my control.

Studies conducted from 1935 to 1939 showed that there are 2 main types of virus-host relationship in LCM virus-infected mice, depending on the age at the time of infection: 1) Lifelong persistent infection present in mice infected congenitally. Such animals show no signs of disease for many months. They have a lifelong solid resistance against intracerebral infection. In spite of this, no neutralizing antibody was demonstrable in their blood, although CF antibody at very low levels was occasionally found. 2) Acute adult infection causing disease, followed by relatively rapid elimination of infectious virus in survivors. CF antibody was readily demonstrable in the sera of such

animals, but the presence of neutralizing antibody was questionable for a long time. The cerebral immunity was of relatively short duration in such cases.

These results, which showed the LCM virus to be capable of persistently infecting mice following vertical transmission, were later confirmed and extended by Haas. They formed, together with Owen's work on erythrocyte chimerism in bovine twins published in 1945, the basis for Burnet and Fenner's thoughts on self-recognition which were to develop later into the concept of immunological tolerance. Since then, much more basic work on the pathogenesis and the immunology of murine LCM has been reported by a number of investigators so that today a reasonably clear picture exists of the events taking place after chronic congenital and acute adult infection in mice.

I will now try to give a brief description of the two conditions.

Mice Infected Congenitally

Mice infected congenitally, that is, in the mother's body, become permanent virus carriers. They show what Hotchin and colleagues have called a persistent tolerant infection. Their immunity was designated as "tolerant immunity" in contrast to the "active immunity" present in adult mice after acute infection.

In the colony with infected mice at Princeton, where about 50% of the breeding mice were infected or immune when the disease was recognized in 1934, congenital infection became the only mode of virus transmission in the course of 2 years, and this no doubt is important in wild mice as well. It contributes effectively to the maintenance of the virus in nature.

Congenitally infected mice look like normal individuals for many months, most of them for their entire life, in spite of the fact that they carry large amounts of virus in their organs and blood and discharge virus continuously in their nasal secretions, urine, feces, milk, and sperm. They have no effective mechanism for virus clearance. Leukocyte count is essentially normal. In contrast to mice with acute adult infection, they can readily pass the virus to normal mice by contact (nose to nose), with the milk or by sexual intercourse. In tolerant females every successive litter becomes infected congenitally, no matter whether the animal was mated with a normal male or a tolerant one. All embryos in each successive litter are infected. There is evidence for virus transmission via the ovum, which comes from a heavily infected milieu. Normal females mated with tolerant males do not always produce infected litters, and in infected litters viral antigen is often not demonstrable in all embryos. We have had cases in which only 1 out of 10 or 12 embryos was infected. When such females are again bred to a tolerant male, the progeny of the following litter is never infected because the female has in the meantime become actively immune. Much later, however, when its active immunity has subsided, mating with a tolerant male may again produce infected litters.

Lymph node cells taken from tolerant mice will grow normally in vitro. The growth curve of the virus in them resembles a horizontal straight line in contrast to the curve obtained from normal cells infected in vitro which shows repeated peaks and remissions for several weeks but later tends to become a straight line also. Lehmann-Grube produced persistent infection in L cell cultures in vitro and observed a simi-

lar pattern with alternating phases of high production and low production of infectious virus.

Immunity in congenitally infected mice is characterized by a lifelong absolute resistance to intracerebral infection. Inoculated virus becomes undemonstrable within a short time. Newborn mice are already fully resistant to intracerebral infection with a "neurotropic" virus strain which is virulent for normal baby mice. Since embryos and newborn mice are not known to be capable of a humoral or cellular immune response, the only plausible explanation is that inoculated virus is prevented from infecting cells by some sort of interference mechanism in the absence of demonstrable interferon. This is what I meant when I spoke of "cellular immunity" in my earlier papers on LCM (1938) and of "interference immunity" in later publications (1960-1963). The inactivation of inoculated virus may be effected by the body temperature. This is not unlikely in view of Lehmann-Grube's studies in which a half-life at 37°C of only 16 to 20 min for LCM virus was found as compared with 28 h for poliovirus type 1. The hypothetical interference or blocking mechanism or whatever you want to call it is only weakly active against other viruses in congenitally infected mice.

Neutralizing antibody has not been demonstrated in such mice, but very low levels of CF antibody were occasionally detected. Other investigators found low levels of antibody demonstrable by the fluorescence technique. Pollard and co-workers reported increased levels of γ-globulin in congenitally infected gnotobiotic mice.

The immunological tolerance present in such animals is of long duration. It is virus-specific since tolerant mice are fully capable of making antibody against other antigens. They are also capable of mounting a normal homograft response. The tolerance concept has recently been challenged by Oldstone and Dixon, but other eminent experts in the field are still in favor of it.

Results similar to the ones with congenitally infected mice have been obtained with mice infected neonatally. These were widely used in pathogenetic and immunological studies in recent years. Own work still in progress has revealed a difference between mice infected neonatally and mice infected congenitally. We found that the LCM virus strain WCC, which Dr. Hotchin would call "aggressive", is highly virulent for newborn mice when inoculated intracerebrally, whereas congenitally infected baby mice born of persistently infected females show no signs of disease in spite of the fact that they carry large amounts of WCC virus in their viscera at birth. We also noticed that very low levels of CF antibody are more frequent in neonatally infected mice than in congenitally infected individuals at a time when maternal antibody has disappeared from their blood. An interesting discussion of the "neonatal versus congenital" problem can be found in Lehmann-Grube's recent monograph.

Several investigators reported fruitless attempts to break the tolerance of persistently infected mice using different procedures. Remarkably successful were the experiments of Volkert and colleagues (1962-1965) with adoptive immunization achieved by transplanting isogeneic lymphoid cells from actively immunized donors. The transplants effected a 10,000-fold reduction of the virus titer within 5 weeks. Spleen and lymph nodes, which usually have high infectivity titers, could be completely cleared, but virus remained in the kidneys. Mice receiving the transplants developed 100-fold higher antibody titers than the cell donors. There was no parallelism between antibody formation and virus suppression. Neutralizing antibody, CF antibody, and

infectious virus were present in the serum concurrently. Cellular immunity was obviously more important for virus clearance than humoral antibody.

An interesting phenomenon of great importance in neonatally infected mice is the "late onset disease" described by Hotchin in 1962 and studied by him and Collins in 1963 and 1964. Such animals appeared healthy for about 10 months and then began to show signs of disease reminiscent of the runting syndrome in newborn mice. All of the mice finally succumbed to this condition. Chronic glomerulonephritis was found in 34% of the Albany and 16% of the Swiss mice used. This condition was believed to be due to an autoimmune process caused by a gradual waning of virus tolerance. In the affected glomeruli deposits of antibody-containing material were found which stained with fluorescent rabbit anti-mouse γ-globulin antiserum. Since the lesions are similar to those occurring in Aleutian mink disease, it is possible that virus-induced antigen-antibody complexes are responsible.

These findings qualify "late disease" as an example of a slow virus infection or, better expressed, slow virus disease, since infection with the LCM virus does not at all appear to be slow. The observations of Hotchin and Collins were confirmed by several other investigators. It seems that the lesions described develop more readily in neonatally infected mice than in those infected congenitally. In own experiments, gradual lymphoid hypertrophy with increasing age was seen in congenital carrier mice.

Acute Infection of Adult Mice

As compared with the congenital infection, the study of the acute infection in adult mice was somewhat neglected in the early years of LCM research because not much of a difference seemed to exist between the events following infection with LCM virus and those following infections with other viruses. An unusual feature was the difficulty to detect neutralizing antibody in recovered mice. It was not yet known then that such mice are fully capable of making neutralizing antibody as reported by Hotchin and co-workers and by Lehmann-Grube. Moreover, the foot pad test and modern tissue culture methods were not yet available.

The acute adult disease attracted much more interest after Rowe had shown in 1952 that pre-irradiation with x-rays could prevent symptoms and lesions in experimentally infected adult mice without depressing multiplication of the virus. This observation indicated that the virus itself is harmless for mature mice as it is for mouse embryos. Since x-irradiation with suitable doses is known to reduce the number of blood leukocytes drastically, the result suggested that these cells, especially the lymphocytes which are found in great numbers in pathological infiltrates of infected mice, might be responsible for the disease syndrome. Confirmation and extension of the findings came from several investigators. Similar results were later obtained by using chemical immunosuppressants, anti-mouse lymphocyte serum or anti-mouse thymocyte serum. Neonatal thymectomy would also prevent the acute disease, as shown by Rowe and colleagues and by Sikora in 1963.

The essence of the experiments with physical, chemical and biological immunosuppressive measures appears to be that the cellular rather than the humoral immune response causes virus elimination as well as acute disease.

In 1958, Hotchin proposed the hypothesis, widely accepted today, that the disease of the adult mouse is due to an immunological conflict resembling the homograft response. As in the latter phenomenon, migrating cells from the lymphoreticular system appear to play the dominant role. There is increasing evidence that their activity is directed against a new antigen being formed at the surface of infected cells.

Basic Mechanism of Immunity in LCM Virus-Infected Mice

Whereas sensitized lymphoid cells no doubt play an important part in the disease process and in virus clearance, they do not seem to be of primary importance for protective immunity in murine LCM for the following reasons: 1) Infected embryos in which a specific cellular immune response appears to be either missing or minimal are, with few exceptions, solidly immune at birth against intracerebral inoculation with a "neurotropic" virus strain, for instance WCC, which will produce severe disease in all newborn normal controls, killing approximately 80% of them. 2) Subcutaneously infected adult mice show cerebral immunity for a few months but, in the stage of waning immunity, many of them respond to an intracerebral virus inoculation with an "accelerated reaction". In the light of newer knowledge, sensitized lymphoid cells seem to be responsible for this reaction which I had interpreted as being an allergic phenomenon. The interesting feature is that it is usually no longer possible to demonstrate infectious virus in mice which have reached the "accelerated stage". Evidently, the cellular immune response had been suppressed in such animals by persisting virus before its concentration had fallen below a critical level. 3) "High dose immune paralysis", resembling the classical phenomenon caused in mice by large doses of pneumococcal polysaccharide, was first mentioned in 1936 in intracerebrally infected adult mice by Bengtson and Wooley and later studied more extensively by Hotchin and Benson and by Hannover Larsen. In this condition, high titers of CF antibody and antibody demonstrated by immunofluorescence but not protective antibody coexisted with virus in the blood. There were significant levels of anti-complementary activity pointing to the presence of antigen-antibody complexes in the circulation. Twenty months later, such mice had suppressed the virus and developed high titers of neutralizing antibody.

It seems likely that in mice with high dose immune paralysis more cells are infected initially than in animals receiving smaller virus doses and that the immune paralysis caused by the virus is therefore strong enough to inhibit a leukocytic immune response immediately. It is noteworthy that the phenomenon seems to be observed only with "viscerotropic" virus strains which have a greater affinity for the lymphoreticular system than "neurotropic" strains.

Thus, the basic disease-preventing mechanism appears to be the same in congenitally infected mice and in those infected as adults, namely, the inhibition of the leukocytic immune response by persisting virus. As numerous titrations have shown, small amounts of active virus persisting in the lymphoreticular system can produce the effect. This may also explain why it is so difficult or nearly impossible to immunize mice against disease with inactivated LCM virus.

The "sterile immunity" of long duration which can be produced in adult mice by repeated intracerebral inoculations of virus following, for instance, a primary subcutaneous injection is more difficult to analyze. One possible explanation is that repeated virus inoculations

may desensitize lymphoid cells so that they do no longer react with the hypothetical new antigen and thereby cause disease. Increased formation of neutralizing antibody may also play a role in such animals.

Persistent LCM Virus Infection and Leukemia

The interaction between persistent LCM virus infection and leukemia in mice and, to a lesser extent, in guinea-pigs has been the subject of numerous publications. We reported in 1941 that lymphatic leukemia was more frequent and appeared at a younger age in persistently infected mice from the Princeton colony than in LCM virus-free controls derived from the same stock. Later, leukemia was also seen in 1 of 2 wild mice persistently infected with LCM virus which Dr. Haas had sent me from the U.S.A.

Contamination of several strains of leukemia virus with LCM virus was described by other investigators. Sometimes the severity of the leukemia was markedly reduced by the contaminating LCM virus. In contrast, Hotchin reported that infection of L cells with LCM virus increased their oncogenicity in mice. In own experiments published in 1962, persistent LCM virus infection appeared both to increase the incidence of leukemia and to moderate its severity to such a degree that one was tempted to conclude that LCM virus might have caused the rather benign tumors. (I was not fully convinced of this, however, and therefore put a question mark on the title of the paper.)

More light was recently shed on the interaction of LCM and leukemia viruses by Oldstone, Aoki, and Dixon who reported stimulation of the production of Gross leukemia antigen by LCM virus. This effect was seen in inbred mice with high or low leukemia incidence as well as in cultures of embryonic cells obtained from the different mouse strains. The results indicate that the effect is independent of genetic host factors. They can explain the higher incidence of leukemia in mice with persistent LCM virus infection but not the reduction of the severity of the leukemia. The authors drew attention to the possibility that enhancement of leukemia antigen production by LCM virus may influence the incidence of autoimmune disease in mice.

CONCLUSION

In concluding, I would like to say that in spite of the vast amount of research work carried out on LCM in recent years enough stimulating problems remain for further work. For instance, the question should be settled once and for all whether the concept of self-recognition is applicable to the persistent infection of Mus musculus with LCM virus, as proposed by Burnet and Fenner, or whether Oldstone and Dixon's view is correct. Possibly both parties are right, the first as far as the symptom-free phase in persistently infected mice is concerned, the second with respect to the late phase sometimes culminating in pathologic alterations resembling autoimmune disease. The basic question of whether or not a self-replicating agent is at all capable of inducing true immunological tolerance will have to be answered. Such studies will throw more light on the mechanism by which persisting virus inhibits the cellular immune response.

Another problem of great interest concerns the antigens involved in autoimmune disease and the role which antigen-antibody complexes may play. Needless to say, more information is desirable on the virus

itself and on the epidemiology of LCM in the field. The study of the LCM-leukemia interaction has just passed into a more productive phase, and further results of scientific interest and perhaps practical applicability may be anticipated.

These are just a few of the problems which will keep the LCM wagon rolling.

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Lymphocytic Choriomeningitis Virus

The Virus: Purification, Chemical and Physical Properties, Complement-Fixing Antigen (s)

Chairman: I. R. Pedersen

LCM Virus: Its Purification and its Chemical and Physical Properties

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SUMMARY

Procedures for optimal infection of BHK21 cells by LCM virus are described. LCM virus could be purified by ammonium sulfate precipitation of the virus-containing medium followed by centrifugation in sucrose gradients, whereby the buoyant density of the virus was estimated to be 1.18 g/ml. The nucleic acids of the LCM virus were studied by centrifugation in sucrose gradients and by acrylamide gel electrophoresis. These analyses have led to the detection of 7 different RNA molecules in LCM virus with sedimentation values of 31S, 28S, 23S, 18S, 5.5S, 5S and 4S. Only the 31S and the 23S RNA's appear to be virus-specific; the 28S and the 18S RNA's originate from ribosomes located inside the virion and the small 5.5S, 5S and 4S RNA's are probably host RNA's associated with these ribosomes. Preliminary protein analysis revealed 1 major and 5 smaller components. Thus, both the nucleic acid and the protein composition of the LCM virus, which is the prototype of the arenavirus group, seem to be different from other enveloped RNA viruses.

INTRODUCTION

LCM virus has been known for almost 4 decades but only during the last few years have sufficient chemical, physical and serological data been obtained to place LCM virus and related viruses in a distinct group of enveloped RNA viruses (19,20,32,33). This group was given the name "arenaviruses" due to the sand grain-like structures observed electron microscopically in the interior of the virus particles (9,19,20,32), and LCM virus has been chosen as the prototype (32). This report on LCM virus, its purification and its chemical and physical properties, is based mainly on some of the author's more recent experiments. For a general description, reference is made to 3 new reviews on arenaviruses (12,16,28). It is a prerequisite for many experiments with LCM virus in vitro that all cells are infected. Since viral growth often is influenced by homologous interference, means to counteract the interference are dealt with. The purification of the LCM virus by ammonium sulfate precipitation and centrifugation in sucrose gradients is described in detail, although other methods could be used. The biochemical properties of LCM virus agree well with those of Pichinde virus, which is a member of the same virus group (7,18,30), but very few data are available for comparison with other arenaviruses. Indeed, some of these are so dangerous to handle that their chemical properties probably never will be examined.

RESULTS AND DISCUSSION

Growth conditions of the LCM virus. There are many different strains of LCM virus (e.g. WE, WCP, E-350, CA 1371, Traub). Differences exist between them with regard to growth characteristics, pathogenicity and other properties (12,16), and it remains to be determined whether these differences will also hold at the biochemical level. One explanation for the differences could be the presence, within these strains, of varying numbers of interfering particles which gradually develop during and interfere with viral multiplication (13,17,35,39,40). This interference is restricted to influencing the growth of arenaviruses and does not influence the growth of a number of other viruses (35,39,40). It has been proposed that these particles are defective viruses which contain only part of the viral genome as has been demonstrated for other viruses (14). In this study, only the Traub strain was employed.

Very often, infection of cells in culture by LCM virus was improved if virus was diluted, due to dilution of interfering material. Since virus titers per se do not tell much about the degree of interference in a virus pool, we suggest that virus should be titrated, in addition, by the fluorescent antibody method. Thus, as illustrated in Figure 1,

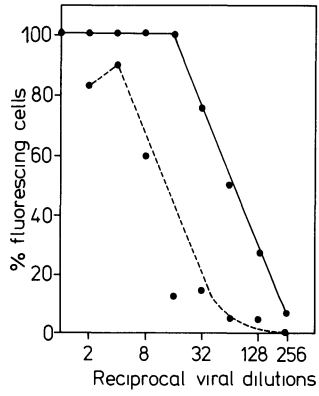

Fig. 1. Titration of 2 different LCM virus preparations by immunofluorescence. When tested intracerebrally in mice, both preparations had titers of 10^7 LD50/0.03 ml. For the immunofluorescence assay 6 x 10^5 BHK21 cells in 2 ml of Stoker's medium containing 1% fetal calf serum were seeded on coverslips in Leighton tubes. The cells were infected with 0.1 ml of 2-fold dilutions of LCM virus, and the cultures were incubated at 37°C for 20 h. The cells were then stained by the fluorescent antibody method (26).

when 2 different LCM virus preparations were titrated in mice, they both had LD50 titers of 10^7/0.03 ml. However, they differed markedly when quantitation was done by the fluorescent antibody method. One preparation could not infect 100% of the cells, and upon dilution the infectivity was increased. Although defective interfering LCM virus particles also contribute to viral antigen as detectable by the fluorescent antibody method, they probably infect cells only in association with intact LCM virions (14). Thus, these titrations are easy to perform and useful in determining the optimal concentration of LCM virus for infection of the cells.

In the present work, LCM virus was grown in BHK21 cells, but other cell lines may be used since LCM virus has a broad host range (16). How-

ever, one must be cautious. We have had great difficulties during purification of LCM virus, grown in L cells, in distinguishing between incorporation of 3H-uridine in material isolated from LCM virus-infected and non-infected cell cultures. These difficulties can be explained easily by the reported presence of a latent RNA tumor virus in L cells (10,15). Consequently, results from LCM virus-infected L cells can be difficult to interpret. We have had the same difficulties with KB cells, but no attempts have been made to determine whether this was also due to a latent virus.

Suspension cultures of BHK21 cells containing 1% fetal calf serum were infected with input multiplicities of about 5 intracerebral mouse LD50/cell. After incubation for 20 to 24 h at 37°C, 100% of the cells were found to be infected when examined by the immunofluorescence method (23,26). Thereafter, the virus-containing medium was isolated by low speed centrifugation in the cold.

Purification of LCM virus. A combination of conventional techniques can be used to purify the LCM virus, and satisfactory recovery of the virus can be obtained if the proper precautions are taken, mainly with respect to pH, temperature, and the presence of protective proteins in the virus solutions.

If large quantities of virus were to be prepared, purification was started by precipitation of virus from the cell-free medium either by the addition of cold methanol to a final concentration of 25 to 30% (22), or by the addition of an equal volume of saturated ammonium sulfate (23), although precipitation with polyethylene glycol may also be used (7). Precipitation at 0 to 5°C with ammonium sulfate was preferred, since for methanol precipitation maintenance of the temperature below 5°C was critical. Furthermore, smaller methanol precipitates were formed if fetal calf serum was used instead of ordinary calf serum. It was important that the ammonium sulfate solution was neutralized first by NaOH since acid solutions decreased infectivity. It was also advisable to limit the serum concentration to about 1% in the growth medium, if virus was precipitated with ammonium sulfate, in order to avoid too large precipitates. Precipitation was allowed to proceed for 1 h at 0°C and, after centrifugation at 0°C for 10 min at about 7,000 x G, the precipitate was collected. The sediment was washed with ammonium sulfate, 50% saturated in 0.01 M tris, 0.1 M NaCl, 0.001 M EDTA pH 7.4 (TES pH 7.4 buffer) and again centrifuged. The resulting precipitate was redissolved in a small volume of cold TES pH 7.4 buffer, and bovine serum albumin was added to a final concentration of 0.1% for stabilization. The infectivity recovered at this step varied from 20 to 50%.

To obtain pure preparations of LCM virus, further centrifugation is necessary. Differential centrifugation has been recommended (27), but this procedure may lead to loss of infectivity; also centrifugation in different salt gradients has been used, but recovery is variable and in some cases several bands are formed (6). Therefore, virus material centrifuged in discontinuous sucrose gradients was preferred.

Initially, virus which had been redissolved after ammonium sulfate precipitation was clarified by centrifugation at 10,000 rev/min for 10 min at 0°C. The supernatant was centrifuged in a discontinuous gradient consisting of 5 ml 65% sucrose and 5 ml 15% sucrose in TES pH 7.4 buffer at 20,000 to 25,000 rev/min at 4°C in Spinco SW 25.1 or SW 27 rotors for 1 to 2 h. After centrifugation, the virus was located in a band which formed in the interface between the 2 layers of sucrose.

This material was collected in a volume of about 1 ml, and bovine serum albumin was then added to a final concentration of 0.1%.

Before the last purification step, the virus solution must be dialyzed against cold TES buffer, pH 7.4 or 8.4, or diluted 4-fold in TES buffer to lower the sucrose concentration. The last step consisted of centrifugation to equilibrium in a 15 to 65% sucrose gradient in TES pH 7.4 buffer at 20,000 to 26,000 rev/min for about 16 h at 4°C in a Spinco SW 25.1 or SW 27 rotor. The virus appeared in 1 sharp band which had a buoyant density of 1.18 g/ml, as illustrated in Figure 4.

Attempts were made to purify the virus by rate zonal centrifugation in 5 to 20% sucrose gradients in TES pH 7.4 buffer by centrifugation in the Spinco 25.1 rotor at 20,000 rev/min for 75 min (23). However, because of its heterogeneity in size (9), the virus tends to band more broadly than by equilibrium centrifugation. The sedimentation value of LCM virus was estimated to be 470 to 500 S (23).

After the last purification step, the virus band was collected and the material was stored at -20°C after the addition of bovine serum albumin to a final concentration of 0.1%. For chemical analysis I recommend to avoid too long storage since breakdown of viral RNA occurs after 4 months at -20°C.

Viral RNA. Various inhibitors of nucleic acid synthesis have been used to show indirectly that the LCM virus is an RNA-containing virus (2,4, 5,29). In these studies it was demonstrated that compounds which preferentially affect DNA synthesis, such as BUdR, FUdR or arabinosyl cytosine, have no influence on the multiplication of LCM virus. Other RNA viruses, namely the RNA tumor viruses, are affected by DNA inhibitors. This is explained by assuming that DNA synthesis is a necessary step in viral replication which is mediated by reverse transcriptase contained in the virion (37). No reverse transcriptase activity has been detected in LCM virus preparations (34). Contrary to expectation, actinomycin D reduces the multiplication of LCM virus. However, this effect is only seen when high concentrations are employed. Lower concentrations of the drug may even enhance yield of virus (4,16,24,36), and it may safely be concluded that DNA synthesis is not involved in the replication of LCM virus.

Further evidence in favor of LCM virus containing RNA comes from electron microscopic studies (9), but most conclusive is the direct analysis of the viral RNA isolated from highly purified LCM virus. These analyses were performed by zonal centrifugation in 5 to 20% sucrose gradients (23) and by electrophoresis in acrylamide gels (24).

The results from acrylamide gel separation of viral RNA are illustrated in Figure 2. Four RNA components were found; 2 of these migrate together with the cellular 18S and 28S ribosomal RNA and 2 other components localize on either side of the 28S component. The 18S and the 28S RNA isolated from virus probably originate from ribosomes, as will be discussed later. The other 2 components are virus-specific; their sedimentation values were estimated as 23S and 31S by rate zonal centrifugation, which corresponds to molecular weights of 1.1×10^6 and 2.1×10^6, respectively (24). All 4 RNA components are single-stranded, since they are sensitive to pancreatic RNase and sediment more slowly in low salt gradients (23).

Three small RNA components with sedimentation constants of 4S, 5S and 5.5S can be detected in 3H-uridine-labeled LCM virus preparations if electrophoresis is performed in 10% gels, which gives optimal separa-

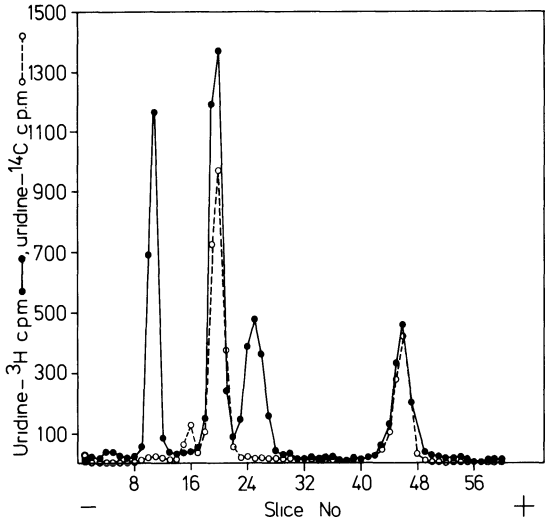

Fig. 2. Acrylamide gel electrophoresis of LCM virus RNA. 3H-uridine-labeled RNA was liberated from purified LCM virus by treatment with 1% SDS for 10 min at room temperature. 14C-uridine-labeled BHK21 cell RNA was added as a marker, and the sample was applied to a 6 cm 2.7% acrylamide gel column and electrophoresed for 4.5 h at 6 m Amp/gel and 50 V, after which the gel was frozen and sliced into 1 mm fractions. Further technical details have been described elsewhere (24).
●——● 3H-uridine-labeled viral RNA;
○- - -○ 14C-uridine-labeled BHK21 cell RNA.

tion of small RNA's. The 5.5S component cannot be detected unless the RNA preparation has been heated to 70°C. Figure 3 shows the electrophoretic separation of these small RNA's on a tandem gel column. The larger RNA's are separated simultaneously except for the 31S RNA, which barely enters the gel. Figure 3A illustrates the separation of the 4S and the 5S component, and Figure 3B shows the appearance of the 5.5S component after the RNA sample was heated to 70°C, which causes the liberation of the 5.5S RNA (28S-associated RNA) from the 28S ribosomal RNA (38). Like the 18S and the 28S RNA's isolated from LCM virus, the 4S, 5S and 5.5S RNA's all originate from the host cell and they are associated with the cellular ribosomes contained in the virion (25). Together they constitute about 7 to 10% of the ribosomal RNA from virus (25).

The 23S RNA and the 31S RNA can also be extracted with phenol from LCM virus-infected BHK21 cells in which ribosomal RNA synthesis has been suppressed by 0.05 µg/ml of actinomycin D. Thereafter they can be separated by electrophoresis on 2.5% acrylamide gel columns (24). In this case, the amount of labeled 23S and 31S corresponds to the 3H-uridine-labeled mitochondrial RNA's, which means that virus-specific RNA in the cell amounts to about 1% of the total cellular RNA after a labeling period of 24 h.

Ribosomes in the LCM virion. As mentioned above, considerable amounts of 18S and 28S RNA's could be detected in purified LCM virus preparations. These RNA species are not distinguishable from host ribosomal RNA by sedimentation in sucrose gradients (23,24) and acrylamide gel electrophoresis (24,25). Their synthesis is completely blocked by low

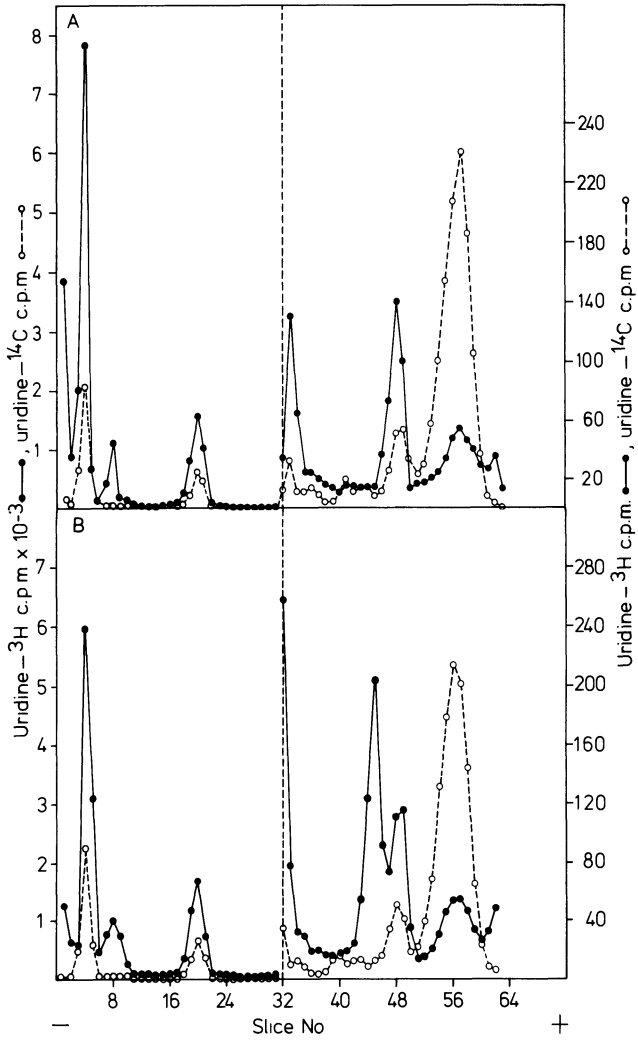

Fig. 3. Acrylamide gel electrophoresis of LCM RNA unheated (A) and heated to 70°C for 3 min (B). 3H-uridine-labeled viral RNA was prepared for electrophoresis, as outlined in the legend to Figure 2. It was then mixed with 14C-uridine-labeled BHK21 cell RNA and electrophoresed in a tandem gel consisting of 5 cm 3% and 5 cm 10% acrylamide gel column. Electrophoresis was performed at 6 m Amp/gel and 78 V for 4 h. The gels were then frozen and sliced into 1.6 mm portions. For other conditions, see legend to Figure 2.
●——● 3H-uridine-labeled viral RNA;
○– – –○ 14C-uridine-labeled BHK21 cell RNA.

concentrations of actinomycin D, which is characteristic for ribosomal RNA, whereas the synthesis of the viral 23S RNA and 31S RNA is unaffected by this drug when employed in low concentrations (24). Thus, the ribosomal origin of the 18S and the 28S RNA's is demonstrated. The next obvious question is whether the viral ribosomal RNA which constitutes about 25 to 50% of the labeled RNA in the virus is due to con-

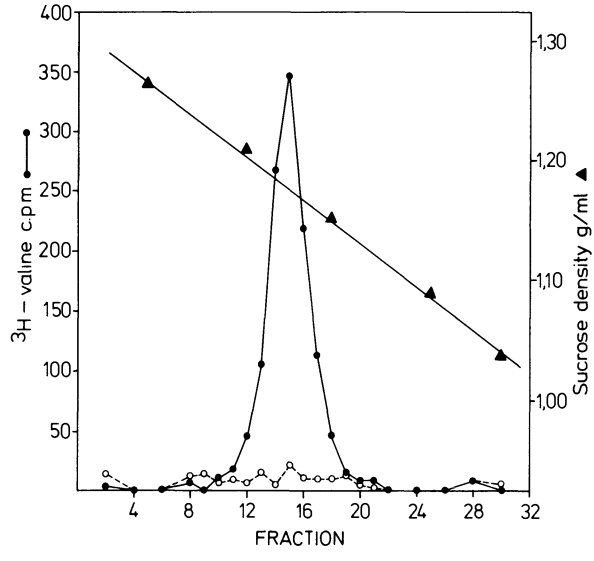

Fig. 4. Equilibrium density gradient centrifugation of 3H-valine-labeled LCM virus and control 3H-valine-labeled material from a similar mock-infected cell culture centrifuged simultaneously in a separate tube. Virus was centrifuged in a 33 ml preformed 15 to 65% sucrose gradient in TES buffer in a Spinco SW 27 rotor at 26,000 rev/min for 17 h at 5°C. Fractions were collected from the bottom of the tube, precipitated with TCA and counted as described before (24).
●——● 3H-valine-labeled LCM virus;
○- - -○ 3H-valine-labeled mock-infected material;
▲——▲ sucrose density.

tamination of the virus preparation with free host RNA or with RNA in intact ribosomes. RNA of the intact LCM virus was found to be insensitive to pancreatic RNase in EDTA-containing solutions (23), and reconstruction experiments show that purified virus preparations are contaminated with less than 3% cellular RNA. Furthermore, the 18S and 28S RNA's from LCM virus are labeled in the right molar ratio, and the most likely explanation is that they originate from ribosomes inside the virion. This hypothesis is supported by the presence of ribosome-like or sand grain-like particles in electron micrographs of arenaviruses (9,19,20). Also, other enveloped RNA viruses, such as influenza (8) and RNA tumor viruses (3,21,31), are associated with ribosomal RNA, although less is present relative to the virus-specific RNA than we observe in LCM virus. Finally, it is noteworthy that ribosome-like particles can also be seen in electron micrographs of mouse mammary tumor virus (11).

Specificity of viral ribosomes. At the present time very few data are available regarding the specificity of the ribosomes which must be contained in the LCM virus particle. It has been demonstrated that the ribosomal RNA which enters the virus can be synthesized both before and after virus infection (24,25). This observation does not support specificity of the viral ribosomes, unless one assumes that cellular ribosomes are altered by the addition or depletion of certain proteins which may transform them into a specific class of ribosomes which then enter the virus. One observation pointing in this direction is the formation of aggregates of ribosome-like particles in cells at sites of virus formation (1). Furthermore, the ribosomes or monosomes iso-

lated from avian myeloblastosis virus were found to sediment differently as compared with host cell ribosomes in sucrose gradients (31), but further analysis of the ribosomal proteins would be required before differences between ribosomes can be well documented. Whether viral ribosomes are necessary for the replication of LCM virus or other RNA viruses is not known.

23S viral RNA and interference. Since homologous interference develops during infection of cells with arenaviruses (17,39,40) and since it has been demonstrated that interfering virus particles can be incomplete or defective (13), one could imagine that the smaller 23S viral RNA component of LCM virus originates from defective interfering particles. Sedimentation of LCM virus by rate zonal centrifugation resulted in only 1 virus band, and no subviral particles which could be defective were detected (23). Furthermore, virus grown under conditions which should increase or decrease the formation of interfering LCM virus particles followed by analysis of the viral RNA's by acrylamide gel electrophoresis contained similar relative amounts of 23S and 31S RNA's (25).

Thus, there is no indication that the 23S viral RNA originates from defective interfering LCM virus particles.

The 23S RNA could be a breakdown product of the larger 31S viral RNA, but heating to 70°C, as illustrated in Figure 2, did not convert the 31S RNA into 23S RNA (25), although such heating liberated the 5.5S RNA from the 28S RNA (25) and converted tumor virus RNA into smaller pieces (37).

Viral proteins. The viral proteins were examined by labeling of LCM virus-infected BHK21 suspension cultures with 3H-valine for 24 h. Pu-

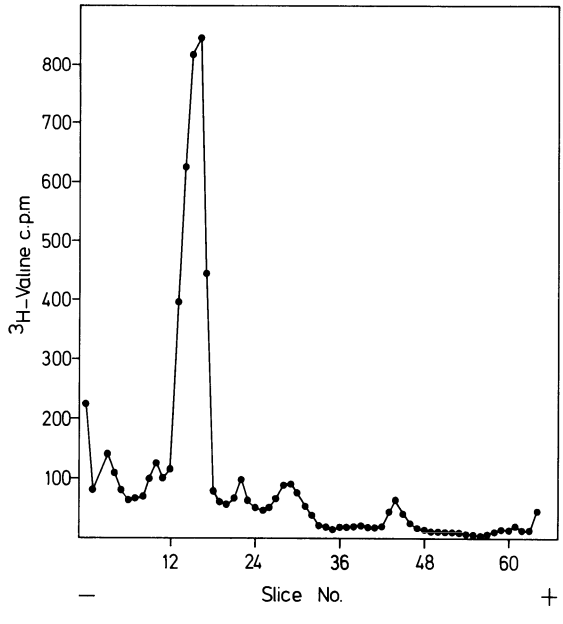

Fig. 5. Acrylamide gel electrophoresis of 3H-valine-labeled LCM virus proteins. Electrophoresis was performed in a 10 x 0.6 cm gel for 18 h at 2 m Amp/gel. The gels were frozen, sliced into 1.6 mm portions and counted as described before (24).

rification of the virus was performed as outlined above. By equilibrium centrifugation in 15 to 65% sucrose, the virus could be located in 1 distinct band having a buoyant density of 1.18 g/ml (Fig. 4), corresponding to the density of 3H-uridine-labeled LCM virus (23). Analysis of the viral proteins by electrophoresis in 7% and 10% acrylamide gel columns resulted in 1 large protein component and 5 smaller components, as illustrated in Figure 5. These results are preliminary and will need confirmation using virus labeled with other amino acids or glucosamine.

CONCLUDING REMARKS

Some of the biochemical analyses of LCM virus as reported here, in essence, are confirmed by the recent biochemical analysis of another arenavirus, Pichinde virus (7,18,30). Thus, arenaviruses appear to be enveloped RNA viruses containing ribosomes, 2 virus-specific single-stranded RNA's with molecular weights of 2.1×10^6 and 1.1×10^6, respectively, and 3 small host RNA's which are associated with the ribosomes in the virion. It is of interest that some other enveloped RNA viruses, such as the RNA tumor viruses and influenza virus, also contain ribosomes or ribosomal RNA and several viral genes (8,11,37). As in the case of LCM virus, however, the function of these structures remains to be determined.

ACKNOWLEDGMENT

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Antigenic Properties of the LCM Virus: Virion and Complement-Fixing Antigen

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SUMMARY

Two procedures for the concentration of LCM virus without loss of infectivity are described: precipitation by zinc acetate and concentration by polyethylene glycol. Further purification is achieved by steric chromatography on controlled pore glass. At a pore size of 43.7 nm all the infectivity appears in the exclusion volume. This material is free of complement-fixing activity.

Non-infectious complement-fixing antigen is extracted with butanol from infected guinea-pig organs or L cells and concentrated by evaporation. Preparations with titers of 40,000 or more can thus be obtained from either source. The extracted material (ECFA) by itself does not induce antibodies or specific protection in animals. When emulsified with Freund's complete adjuvant, complement-fixing antibody is induced in guinea-pigs or rabbits. This antibody can be boosted to high titers with soluble ECFA. Not a trace of neutralizing activity is demonstrable in the same sera. Guinea-pigs repeatedly inoculated with large quantities of high-titered ECFA with or without adjuvant are not protected against subsequent challenge with infectious virus. Sera specifically reacting with ECFA do not react with purified virus. The neutralizing activity of anti-LCM virus hyperimmune serum is not blocked by ECFA. Upon disintegration of the purified virus with urea and sodium dodecyl sulfate, complement-fixing activity is released, provided the starting material contained $10^{9.5}$ ID50 (L)/ml or more.

It is concluded that complement-fixing antigen as extracted with butanol from infected tissues or cells is not represented on the surface of the virus. Whether it is a structural component of the virion remains to be determined.

INTRODUCTION

Lymphocytic choriomeningitis of the mouse may serve as a model for investigating pathologic immune phenomena in virus diseases (4,5,7). Although the immunologic nature of LCM is not disputed, the antigen (allergen) which is responsible for disease and death in virus-infected adult mice is not identified. There are numerous possibilities (7), and an answer depends on a detailed knowledge of the antigens which appear in infected cells and tissues.

Previous attempts to study the LCM virus were rendered difficult by the extraordinary lability of viral infectivity as well as the lack of an assay procedure. In the past we have established conditions for stabilizing the infectivity (6) and developed a simplified assay procedure for the LCM virus (9). We have now begun to study viral antigens in detail. First results are reported in this communication.

MATERIALS AND METHODS

Cell cultures. L cells are grown at 37°C in large cylindrical bottles which have a surface available for cell growth of approximately 2,000 cm^2; they are rolled with 0.5 rev/min. Growth medium (GM) consists of minimum essential medium (1) plus nonessential amino acids (10) supplemented with 0.5% lactalbumin hydrolysate and 3% heated calf serum. Medium for maintenance of cells after infection (MM) has the same basic composition, but calf serum is reduced to 1.5% and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer at pH 7.2 is added to a final concentration of 0.01 M. Conditions for the cultivation of Vero cells are essentially identical, except that GM and MM contain 10% and 3% calf serum, respectively. RK-13 (rabbit) cells (obtained from Flow Laboratories) are grown and maintained with rabbit serum instead of calf serum.

Virus. LCM virus, strain WE (14), is used throughout. Titrations are done by means of a micro-assay which is based on the detection of CF antigen released from L cells grown in the wells of plastic culture trays; this is a modification of a method previously described (9), the details of which will be published elsewhere. Though L cell cultures are less sensitive than mice - 1 ID50 (L) corresponds to 5 ID50 (mouse) - the new method is more accurate and much easier to perform, thus allowing the titration of great numbers of virus samples without difficulty.

Propagation of virus. Only virus released into the medium is utilized. After monolayers have formed, GM is replaced by 250 ml of MM containing approximately 10^5 ID50 (L) of LCM virus. Two days later, 5.0 ml of 0.5 M EDTA solution made pH 7.8 by the addition of 1.0 M tris are added. Within 2 to 5 h most cells come off the glass. Those still adhering are loosened by gentle shaking. Cells are separated from the medium by centrifugation for 20 min at 2,000 x G.

Preparation of virus concentrates

a) Precipitation by zinc acetate (19): Culture fluid is cooled below 4°C in an ice bath and to each liter 20 ml of 1.0 M zinc acetate are admixed under vigorous stirring. Taking into account the EDTA which had been added to the culture, this makes the mixture 0.01 M or more in free zinc ions. Upon stirring for 20 min at 4°C, a heavy precipitate is formed which is collected by low speed centrifugation. It is dissolved in 1/100 to 1/80 the original volume with 0.5 M EDTA in 1.0 M tris. Because of the voluminous precipitate, the solution comprises 1/50 to 1/40 of the original volume of culture fluid.

b) Concentration by polyethylene glycol (PEG) (13). To each liter of infectious culture fluid 60.0 g of NaCl and 40.0 g of PEG 40,000 are added. At room temperature and with stirring the compounds dissolve within 10 min. The solution, now faintly turbid, is filled into centrifuge bottles, cooled in an ice bath and stored overnight at 4°C. After centrifugation at 2,000 x G for 1 h, the clear supernatant is discarded and the bottle walls are carefully dried. The scarcely vis-

ible sediment is resuspended in the smallest possible volume of 0.1 M NaCl, 0.1 M tris, and 0.05 M EDTA in water at pH 7.8. Starting with liter quantities of culture fluid, concentration factors of up to 500-fold can be obtained.

Steric chromatography. Columns filled with controlled pore glass (CPG) are prepared and used as already described (2,3). In a Stabilock-Column type 11 (H. Hölzel, 825 Dorfen, Bundesrepublik Deutschland) filled with 39 g of 43.7 nm CPG (Corning CPG, 10-370) the exclusion volume is 44.2 ml and the total free solute volume is 109.5 ml.

Preparation of concentrated CF antigen. Infected L cells are collected by centrifugation and resuspended at 1/100 the original volume in a solution containing in water 0.01 M tris and 0.005 M EDTA. They are disrupted by repeated freezing and thawing. Cellular debris is diluted with water 2-fold and then homogenized with an equal volume of n-butanol. The milky homogenate is stirred overnight at 4°C and then centrifuged for 30 min at 2,000 x G, after which a clear butanol phase, a viscous layer of cell material, and a turbid water phase are well separated. The water phase is isolated and the cell debris is extracted once again with fresh water. Butanol is evaporated under vacuum from the combined water phases, and 1 M citric acid is added under vigorous stirring to a pH of ca 4.8, which results in the formation of a heavy precipitate. This is spun down by low speed centrifugation and the supernatant fluid is concentrated 40-fold in a rotary evaporator at 35° to 40°C; alternatively it is dried and redissolved. The concentrate is centrifuged at 120,000 x G for 1 h and the supernatant is exhaustively dialyzed against 0.1 M NaCl, 0.01 M tris, 0.001 M EDTA (NTE-buffer) adjusted to pH 7.4 by HCl, followed by further concentration in the evaporator. The last steps may be repeated until volumes become too small to be handled.

Analytical procedures. Protein concentrations are determined according to Lowry et al. (11) using bovine serum albumin as a standard.

Complement fixation tests are done with the "Microtiter System". Two units of complement are employed. The unit of complement is that amount of guinea-pig serum which causes partial lysis of 0.05 ml of sensitized sheep red cells, the concentration of sheep red cells being such that when measured as cyano-hemoglobin at 546 nm an optical density of 0.400 is read.

The neutralizing activities of sera are assayed either by the "constant virus, variable serum" procedure or by its opposite, the "constant serum, variable virus" procedure. The latter is performed essentially as described previously (8). For the former, sera are diluted 2-fold and heated for 20 min at 56°C. Serial dilutions are mixed with virus diluted such as to give 100 ID50/inoculum. After incubation for 2 h at 37°C the reaction mixtures are tested for infectivity in L cell micro-cultures.

Immunoprecipitation tests in agar are performed as standardized by Ouchterlony (12).

RESULTS

In order to facilitate separate studies of virion and CF antigen(s), LCM virus had to be prepared free of CF activity. Therefore, only tissue culture medium was used as the source for virus because no CF

antigen is demonstrable in the medium during the first 2 days after infection (7).

LCM virus is grown in L cell monolayers. As described in Materials and Methods, EDTA is added to the culture 2 to 5 h before harvesting, which results in a 10-fold increase of the infectious titer as compared with cultures not treated with EDTA. Concentrations of $10^{8.5}$ ID50 (L)/ml of medium are thus obtained. The enhancing effect of EDTA is unexplained. Since the cell-associated infectivity is also significantly higher in treated cultures, EDTA cannot act solely by hastening the release of cell-bound virus. In contrast to infectivity, CF activity is not significantly increased either in the culture medium or in the final virus concentrate.

Both the zinc acetate precipitation and the phase exclusion by PEG 40,000 yield approximately 100% of the initial infectivity. Either concentrate contains about 3 times more protein than MM. However, due to the higher concentration factor obtainable, the specific infectivity is much higher in the PEG concentrate.

Further purification is achieved by steric chromatography on CPG. Figure 1 shows the elution profile of 9.5 ml zinc acetate-precipitated LCM virus containing $10^{9.9}$ ID50 (L)/ml. NTE-buffer was pumped at a rate of 5.6 ml/min. The solid line, which is a continuously recorded transmission profile at 278 nm, demonstrates that the bulk of small contaminants is separated from the infectivity (broken line). With a

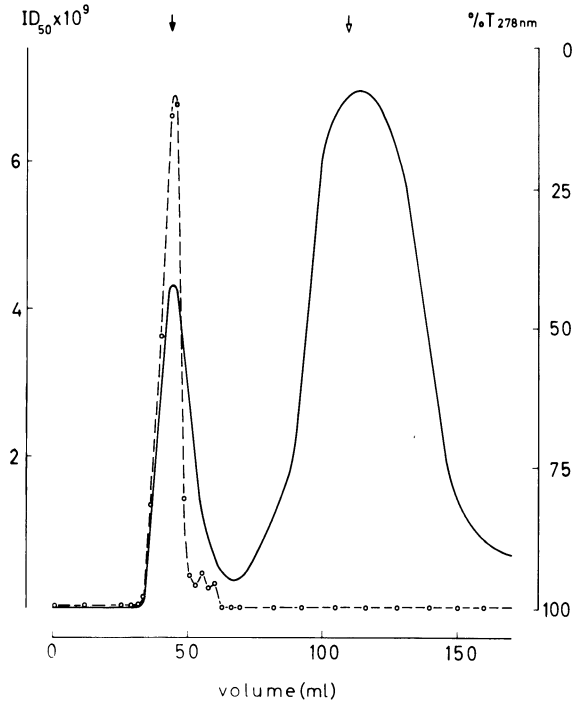

Fig. 1. Zinc acetate-concentrated LCM virus chromatographed on a column of controlled pore glass with a pore size of 43.7 nm. Infectivity: o--o--o ; % transmission at 278 nm: ———— ; exclusion volume: closed arrow; total buffer volume: open arrow.

dilution factor of 2, over 90% of infectivity is recovered in the exclusion volume. Figure 2 shows the elution profile of 14 ml of a PEG-concentrated LCM virus suspension chromatographed on the same column. With $10^{9.9}$ ID50 (L)/ml in the starting concentrate, over 90% of slightly diluted infectivity was recovered in the exclusion volume. PEG concentrates contain less low molecular weight proteins than do zinc acetate precipitates, which is obvious from a comparison of the transmission lines in Figures 1 and 2. It should be emphasized that residual PEG is separated from the virus fraction, which is not apparent from the graph because this compound does not absorb at 278 nm. Actually, the virus is solubilized from the PEG sediment during chromatography.

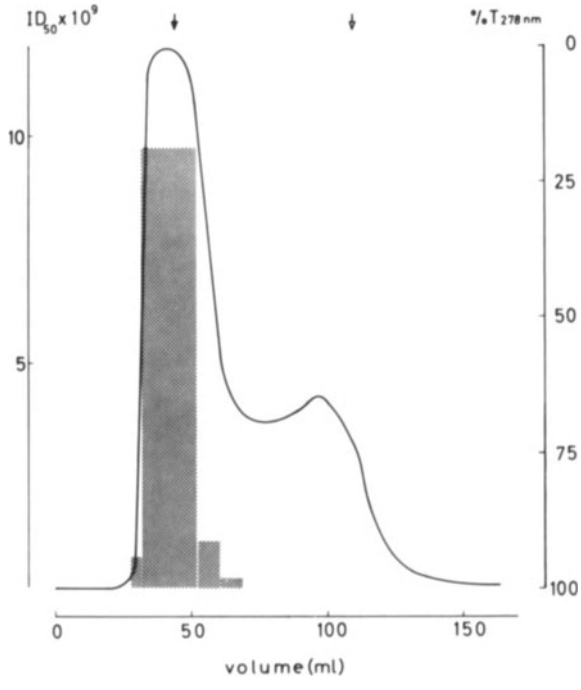

Fig. 2. Polyethylene glycol-concentrated LCM virus chromatographed with controlled pore glass. Further details see Figure 1.

When the results obtained with the 2 methods of concentration are compared, it is obvious that high concentration factors of up to 500-fold are only achieved by combining PEG treatment with steric chromatography. This is a striking example of the value of CPG as compared with organic gels such as Sephadex, which would fail to work under these extreme conditions. Furthermore, because of the high flow rates achieved with CPG chromatography cooling is not necessary. CPG-purified PEG concentrates contain at the most 10% of the protein present in maintenance medium. In contrast, the CPG-purified zinc acetate-precipitated virus contains at least 50% of the MM proteins. Taking into account infectivity and protein, purification factors are 1,000 or more and 100 or less for PEG CPG-processed and zinc acetate CPG-processed fractions, respectively.

The procedure of solubilizing and concentrating CF antigen from L cells has been described in the section on Materials and Methods.

The same procedure is also used for antigen from other cells grown in vitro as well as from homogenates of infected guinea-pig organs. Approximately half the activity present in the crude cell lysate is solubilized and extracted with butanol. No doubt CF activity is always discarded with the solid phase, and we do not know whether our preparation contains part of the total activity or just one antigen species out of several. In order to stress this ignorance, we call the material derived by treatment with butanol "extracted complement-fixing activity" (ECFA).

Following the described procedure, antigen can be concentrated to titers of 40,000 or more as measured in a micro-assay. ECFA from cell cultures contains less than 1.0 mg protein/ml, which contrasts with 50 mg and more protein/ml, in preparations from guinea-pig organs with comparable titers. Therefore, cell cultures are preferred as the source of antigen despite the greater difficulties in procuring sufficient starting material.

It is one of the biological characteristics of the WE strain of LCM virus to be absolutely lethal for guinea-pigs. In other words, 1 infectious unit kills the animal (7). Despite several injections of undiluted high titer ECFA in ml quantities no guinea-pig died. Nor was it possible to detect complement-fixing or neutralizing activities in the sera of these animals even after weeks. Furthermore, all guinea-pigs succumbed with a typical LCM disease within 7 to 10 days when challenged with 50 ID_{50} (mouse) LCM virus, strain WE. Thus, we conclude that high titer ECFA is free of infectious virus and not immunogenic on intravenous, intraperitoneal, or subcutaneous application.

In contrast, if ECFA was emulsified with Freund's complete adjuvant before inoculation, CF antibody was induced in mice, guinea-pigs, and rabbits, the concentrations of which were boosted to titers of 512 or more by subsequent intravenous or intraperitoneal injections of ECFA. However, not a trace of protection against challenge inoculation with virulent virus became demonstrable, and neutralizing antibody in the sera of these animals was entirely absent although a battery of neutralization tests performed both in cell cultures and in mice was employed. Thus, these sera are specific for ECFA and do not contain

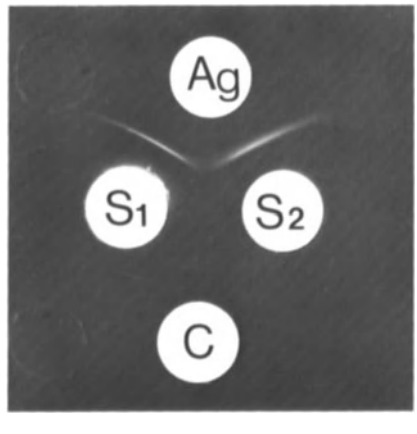

Fig. 3. Immunodiffusion test. S1: anti-ECFA rabbit antiserum; S2: anti-LCM virus hyperimmune rabbit antiserum; Ag: ECFA from L cells; C: control "antigen" from uninfected L cells.

antibodies directed against the intact virus. Evidently, ECFA does not contain components of the virion surface in an immunogenic form.

Studies with Ouchterlony double diffusion tests revealed the following. The anti-ECFA activity in anti-ECFA antiserum formed a line of identity with the anti-ECFA activity in anti-LCM virus hyperimmune serum (Fig.3). This precipitation was completely abolished by absorption of sera with ECFA but not with material made under identical conditions from non-infected normal cells (Fig. 4). No difference between ECFA prepared from L cells, Vero cells, or RK-13 cells could be revealed by immuno-diffusion tests (Fig. 5). Usually we immunize rabbits with ECFA as

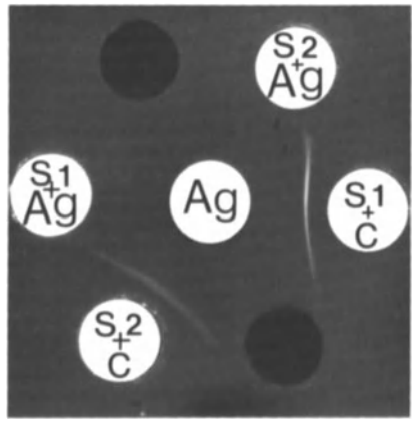

Fig. 4. Immunodiffusion test. S1: anti-ECFA rabbit antiserum; S2: anti-LCM virus hyperimmune rabbit antiserum; Ag: ECFA from L cells; C: control "antigen" from uninfected L cells.

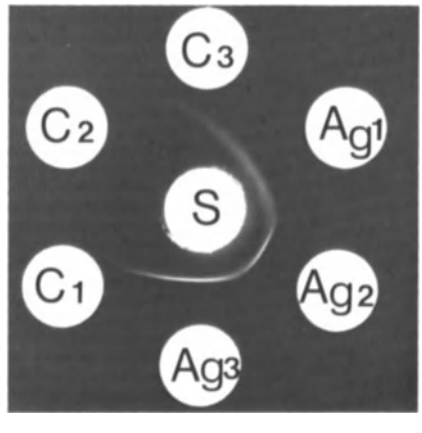

Fig. 5. Immunodiffusion test. S: anti-ECFA rabbit antiserum; Ag1: ECFA from Vero cells; Ag2: ECFA from L cells; Ag3: ECFA from RK-13 cells; C1, C2, and C3: control "antigens" from uninfected Vero, L, and RK-13 cells.

well as virus from primary and permanent rabbit cells grown and maintained in rabbit serum, and no reaction against normal cell constituents was demonstrable in immune sera raised by ECFA from homologous cell sources.

Attempts to absorb anti-ECFA activity from both antiserum specific for ECFA and hyperimmune serum directed against all viral components by highly concentrated and CPG-purified virion preparations failed, which indicates that ECFA is not represented on the surface of the infectious virus. This conclusion is strengthened by the inability of high titer ECFA to block the neutralization of viral infectivity by anti-LCM virus rabbit antiserum (Table 1). It will be shown during this symposium by Rutter (15) that LCM virus-infected L cells contain at least 2 types

Table 1. Effect of virus-specific complement-fixing antigen from L cells and guinea-pig organs on neutralization of LCM virus by rabbit antiserum

	Mouse titer (ID50/ml)				
	Diluent	L cell		Guinea-pig	
Serum (1:10)		LCM Ag	Co Ag	LCM Ag	Co Ag
Immune	5.02	5.65	5.66	6.19	6.19
Control	8.15	8.65	8.52	8.23	8.19
Index	3.13	3.00	2.86	2.04	2.00

of antigen which have different specificities and are also distinguishable by their pattern of distribution inside and on the surface of the cell. One of these reacts with antiserum specific for ECFA; the other one may be a structure of the virus surface, although this latter assumption is at present not much more than a working hypothesis.

Complement fixation, though of low degree, always occurs when CPG-purified virion preparations containing $10^{9.5}$ ID50 (L)/ml or more are reacted with antiserum prepared against non-purified virus. However, we never observed binding of complement when the same virus preparation was tested against ECFA-specific antiserum.

Upon disruption of the virions by dialysis against a solution containing 9 M urea, 2% SDS, 1% 2-mercaptoethanol, 0.05 M EDTA, and 0.1 M tris at pH 7.8, the turbid material turned clear within 2 h. Upon redialysis against a solution of 4 M urea, 0.1% mercaptoethanol, 0.001 M EDTA, and 0.002 M tris at pH 7.4, slight turbidity returned. This material, which had been negative before treatment, was tested against anti-ECFA antiserum and was found to bind complement at titers as high as 128. Rise of specific activity was also sometimes found when purified and disrupted virions were tested against hyperimmune anti-LCM virus antiserum.

DISCUSSION

More than 30 years ago, a thorough account of the properties of CF antigen from LCM virus-infected guinea-pig organs was given by Smadel et al. (16,17,18). In respect of its immunologic properties, it was noted that concentrated and partially purified antigen was precipitated by hyperimmune serum. Conversely, complement-fixing and precipitating antibodies could be absorbed beyond detectability from such sera by the antigen. Significantly, neutralizing titers were not affected. When non-infectious CF antigen was repeatedly inoculated into guinea-pigs, neither complement-fixing nor neutralizing activities could later be demonstrated. Nor were these animals immune when challenged with virulent virus. However, pre-existing complement-fixing antibody was boosted to high titers with solubilized CF antigen.

Our results confirm and extend the observations of Smadel and his coworkers. During infection of a cell with the LCM virus, CF antigen is produced in addition to infectious progeny. Part of the material with CF activity can be extracted from the cell in a soluble form by butanol and other solvents. ECFA thus obtained is not represented on the surface of the virus. This conclusion is based on the inability of butanol-extracted complement-fixing material with high specific activity to induce protection of guinea-pigs or the formation of neutralizing antibody in guinea-pigs and rabbits, although it stimulates the formation of high concentrations of antibody which specifically combines with ECFA. Also, this antigen does not at all block neutralization of infectivity by hyperimmune antiserum. Rutter and Gschwender (15) have shown by immunofluorescence and immuno-electron microscopy studies that cell membranes of infected cells contain antigen specific for the virus surface but not for ECFA. While it can safely be concluded that ECFA is not part of the virus surface, we do not yet know whether it is a structural component situated inside the whole particle. Our experiments clearly show that CF activity is released from the virion by its disruption. However, antigen thus obtained is not necessarily identical with ECFA. An answer to this question seems of major relevance, and attempts are being made at present to compare antigen(s) released from purified virions with material obtained by extracting infected cells with solvents.

It is also obvious from our results that antigens of the virus surface which induce neutralizing antibody and/or immunity lose their immunogenicity when subjected to the extraction procedure. Although the starting material used for butanol extraction contains high concentrations of infectious virus, the final product is free of infectivity and does not induce protective immunity or neutralizing antibody in recipient animals. Apparently, not only the virions are disrupted by the treatment but also their surface antigens, at least to the extent that antigenic sites lose their structural characteristics.

A peculiar property of ECFA is its lack of immunogenicity in the absence of Freund's adjuvant, which is unexpected because this material is a potent antigen when tested by CF or immunodiffusion tests. No explanation can be offered at present, and this paradox needs further exploration.

ACKNOWLEDGMENTS

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Electron Microscopy of LCM Virus-Infected L Cells

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SUMMARY

L cells, infected with strain WE LCM virus, were examined by electron microscopy. Whereas the ultrastructure of nuclei was essentially normal, both cytoplasm and plasma membrane were markedly altered, which was demonstrable as early as 16 h after infection. Changes consisted of increasing density of mitochondria, the formation of membranous structures appearing as multilayered concentrically arrayed lamellae or as irregular convolutions, and the appearance of granules either singly or accumulated in masses which were sometimes as large and delineated as cytoplasmic inclusions. These granules resembled closely those which characteristically are found in the interior of arenavirus particles. Higher magnification revealed that they were composed of granular and/or rod-like subunits.

The most striking feature of the surface of infected cells was the presence of budding particles which could be separated on the basis of size and density into 2 different types. By use of rabbit anti-LCM virus antiserum in combination with peroxidase-conjugated pig anti-rabbit γ-globulin antibody, these particles were identified as LCM virus. Viral antigen was also located on the surface of infected cells at sites where morphologic alterations were not apparent.

INTRODUCTION

LCM virus is reputed to leave the host cell in which it multiplies morphologically and functionally intact (7). While this statement may be accepted as a general rule, there are exceptions. Thus, certain cultured cells lose their ability to take up vital dyes when infected with the virus. In a few instances disturbances are so severe that morphological alterations or even frank cytolysis may be observed by light microscopy.

Infections of our line of L cells follow the general rule; light microscopic morphology remains normal, although virus replicates readily in almost all cells as demonstrated by the appearance of both viral antigen and infectious progeny (6,9). This investigation was undertaken to answer the question of whether ultrastructural changes might occur in these cells without progression to a character or magnitude which would be visible by light microscopy. The results show that the WE strain of LCM virus does alter the fine structure of both cytoplasmic organelles and plasma membranes of our L cells.

MATERIALS AND METHODS

L cells were grown with Eagle's minimum essential medium supplemented with non-essential amino acids (10) and 5% calf serum.

LCM virus, strain WE, was passaged twice and propagated in L cells. Titration of viral infectivity was done in L cell tube cultures as previously described (8). For electron microscopy, 1.25 x 10^6 cells in 5 ml of medium were seeded into 5 cm plastic Petri plates (Greiner, Nürtingen, Germany) and incubated for 24 h at 37°C under a humidified atmosphere containing 5% CO_2. The cells were washed, 0.1 ID50/cell of virus contained in 0.5 ml Eagle's medium (no serum) was added, and adsorption was allowed to proceed for 1 h at 22°C. The cells were then washed once again, medium with 10% calf serum, prewarmed at 37°C, was added (time 0), and incubation was continued at 37°C.

At intervals of 16, 24, 36 and 48 h cells were prepared for electron microscopy. Monolayers were washed with phosphate-buffered saline, stabilized with 1.7% glutaraldehyde in Na-cacodylate buffer, removed from the dish, fixed in Dalton's 2% chrome osmium solution and embedded in Epon or ERL (20). Sections were stained with alcoholic uranyl acetate and lead citrate solutions. Non-infected cultures treated in parallel served as controls.

Identification of virus-specific antigen was achieved by means of a modification (17) of the peroxidase labeling procedure originally applied to LCM virus-infected cells by Abelson et al. (1). In brief, cells were stained with rabbit anti-LCM virus antiserum with high neutralizing and CF activities, followed by peroxidase-conjugated pig anti-rabbit immunoglobulin antibody without prior treatment with alcohol. By this regimen only antigen on the cell surface is stained. Its advantage lies in the fact that structural details of the interior of the cell remain largely intact. In contrast to Abelson and his colleagues, we found treatment of cells with alcohol not to be necessary for obtaining optimal staining of antigen on virus and cell surface.

RESULTS

Ideally, the infectious process should have been synchronized, and in our first experiments we employed multiplicities of 5 L cell ID50/cell (corresponding to ca 50 mouse ID50/cell). However, under these conditions interference phenomena intervened and in order to achieve maximal virus multiplication as well as enhancement of ultrastructural alterations, the virus dose had to be lowered to 0.1 L cell ID50/cell (corresponding to ca 1 mouse ID50/cell).

In Figure 1, a typical non-infected L cell is illustrated. Mitochondria, endoplasmic reticulum and Golgi membranes, and a characteristic pattern of mono- and polyribosomes are evident. First virus-induced alterations in a few cells were seen 16 h after infection. The nuclear membrane was more extensively folded which, occasionally, led to lobulation. Nuclear pores were enlarged. The most prominent changes occurred in the cytoplasm of some infected cells. New structures appeared which consisted either of membranes arranged concentrically around fine granular material (Fig. 2) and, sometimes, mitochondria or of membranous masses which were directly contiguous with the external lamina of the nuclear envelope (Fig. 3). This latter type of network appeared to enlarge with time and eventually it was found in a less compact form surrounding the nucleus. Alternatively, in a few cells it occupied the entire cytoplasmic space. The majority of mitochon-

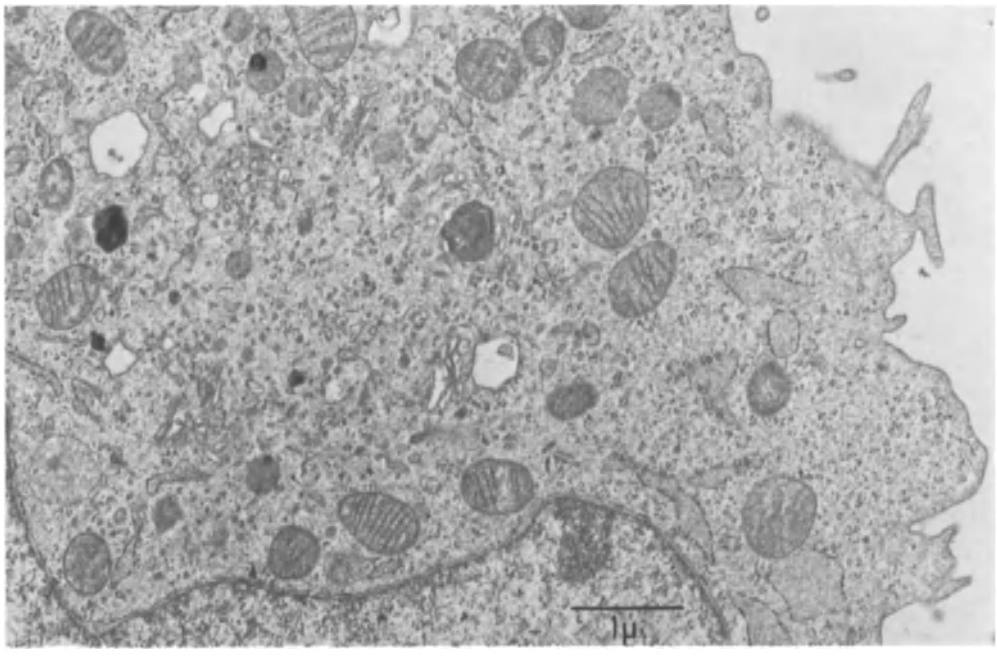

Fig. 1. Uninfected L cell prepared in parallel with a 16 h post-infection harvest.

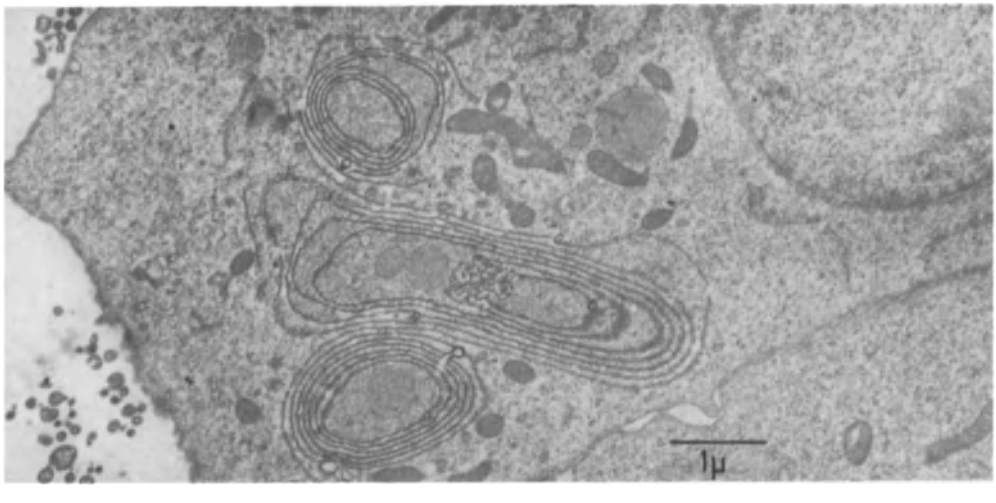

Fig. 2. Multilayered membranous structure in an L cell 48 h after infection of the culture.

dria from seemingly infected cells were more densely structured than normal (Fig. 2 and 3). The abnormally monodispersed ribosomes were increased in number (Fig. 2, 3, 4 and 6). They were interspersed with

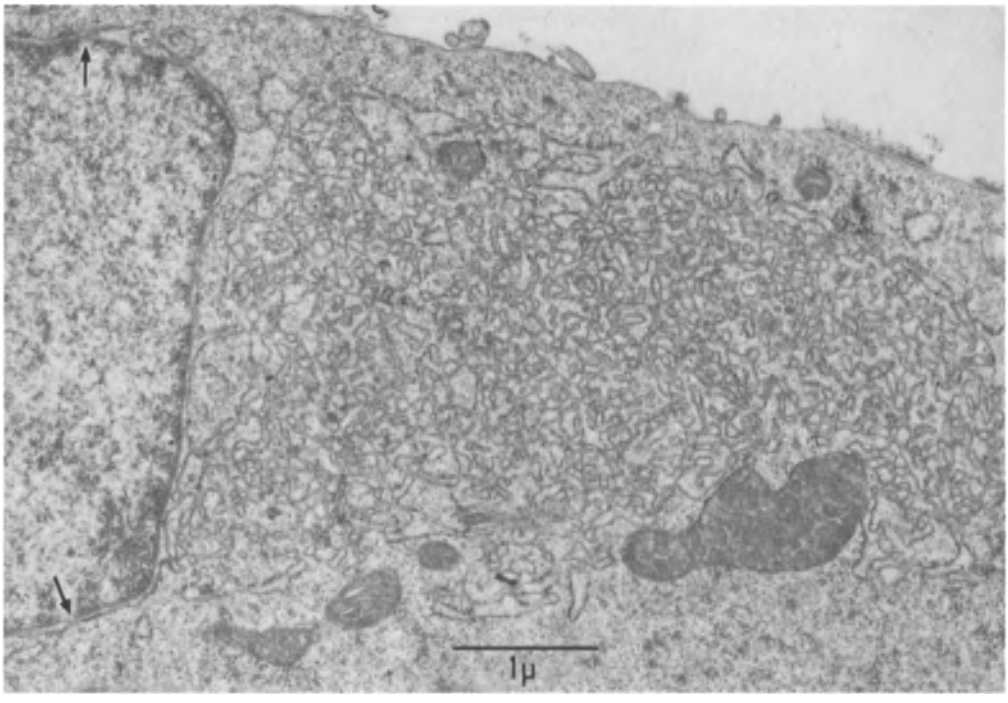

Fig. 3. Dense mitochondria, massive convolutions of membranes, and enlarged nuclear pores (arrows) in a cell 48 h after infection.

denser and somewhat larger granules (compare Fig. 4b with 4c), resembling the ones which characteristically are found in the interior of arenavirus particles. At higher magnification they were resolved into subunits which themselves appeared to be granular or rod-like (Fig. 4b). These granules occurred either singly or in aggregates of varying size; these were randomly distributed throughout the cytoplasm (Fig. 3, 4a and 6). Occasionally they were condensed to form large aggregates with the appearance of delineated cytoplasmic inclusions. Elsewhere the same material was observed in areas beneath the plasma membrane (Fig. 4a and 4d) in association with modification of normal bilaminar plasma membrane structure.

The number, size, and shape of viral particles which were released from the plasma membrane of infected cells varied considerably. At some sites they were rather uniform, round to oval in shape, measuring 90 to 120 nm in diameter (Fig. 5). High resolution revealed a surface layer of delicate processes resembling spikes (Fig. 5). Virus particles were released by a well-defined budding process, and where they still adhered to the cell, continuity of the respective membranes was readily observed. In many areas the plasma membrane lost its bilaminar structure and became fuzzy in appearance (Fig. 5); these alterations were considered due to nascent budding. The rather unstructured interior of viral particles contained varying numbers of dense granules. These resembled the granules which are found either free in the cytoplasm or as small to large aggregates, as described above. Again, they were not homogeneously compact. When resolution was increased, they were seen to be composed of small subunits which were granular to

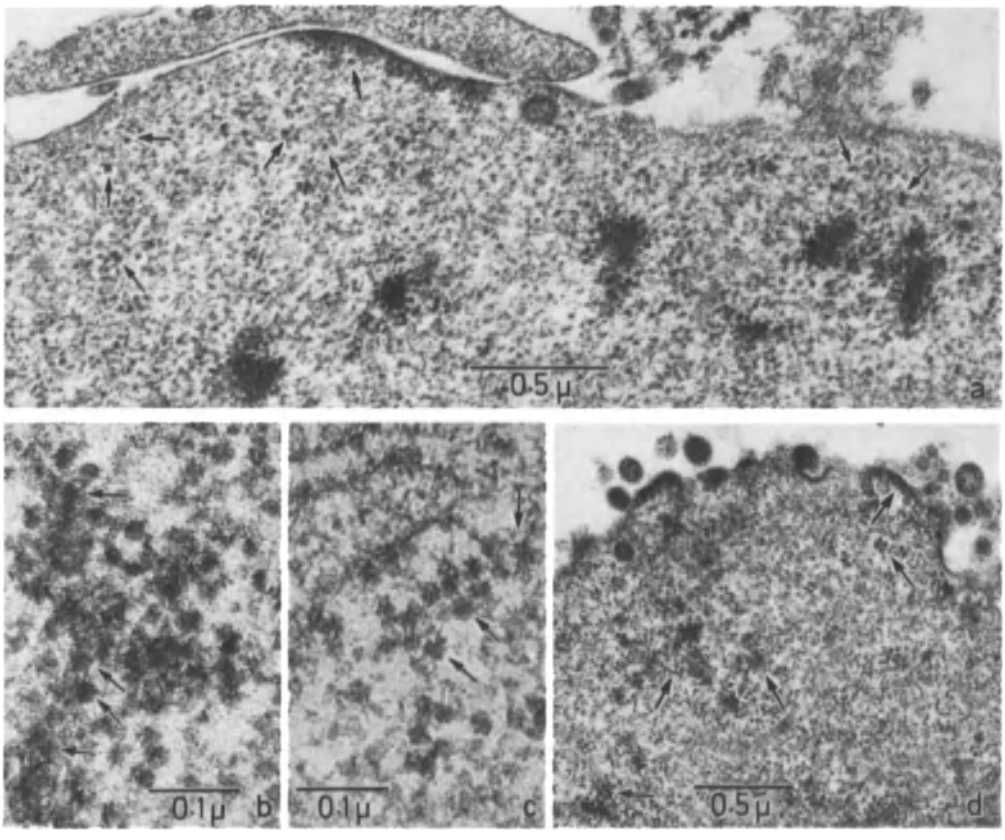

Fig. 4. (a) Dense granules (arrows) in a cell 36 h after infection of the culture. Morphologically identical forms are seen as aggregates lying either free in the cytoplasm or in close proximity to the plasma membrane. (b) Higher magnification of a small inclusion as seen in Figure 4a. Arrows point to rod-like subunits. For comparison, normal L cell ribosomes are depicted at the same magnification (Fig. 4c). (d) Small dark spots in the cytoplasm, dense viral particles on the cell surface, and loss of bilaminar structure of the plasma membrane of an L cell from a culture infected 36 h previously.

rod-shaped (Fig. 5). Virus particles were released from the plasma membrane individually or in small groups. At some sites, however, they were set free in such large numbers that the entire cell surface seemed to dissolve (Fig. 6 and 8). Similar events took place in association with the membranes of large vacuoles.

Free virus particles were not always as uniform in size and shape as the ones just described, nor was their structure always as well preserved. In some groups they were smaller and denser (Fig. 4d). In others, considerable polymorphism was found (Fig. 6 and 8). Sometimes both particles and plasma membrane seemed to be in the process of disintegrating (Fig. 7), and in such zones the latter lost its typical bilamellar structure. At these sites, virus particles were embedded in a cloud of finely dispersed granules and filaments.

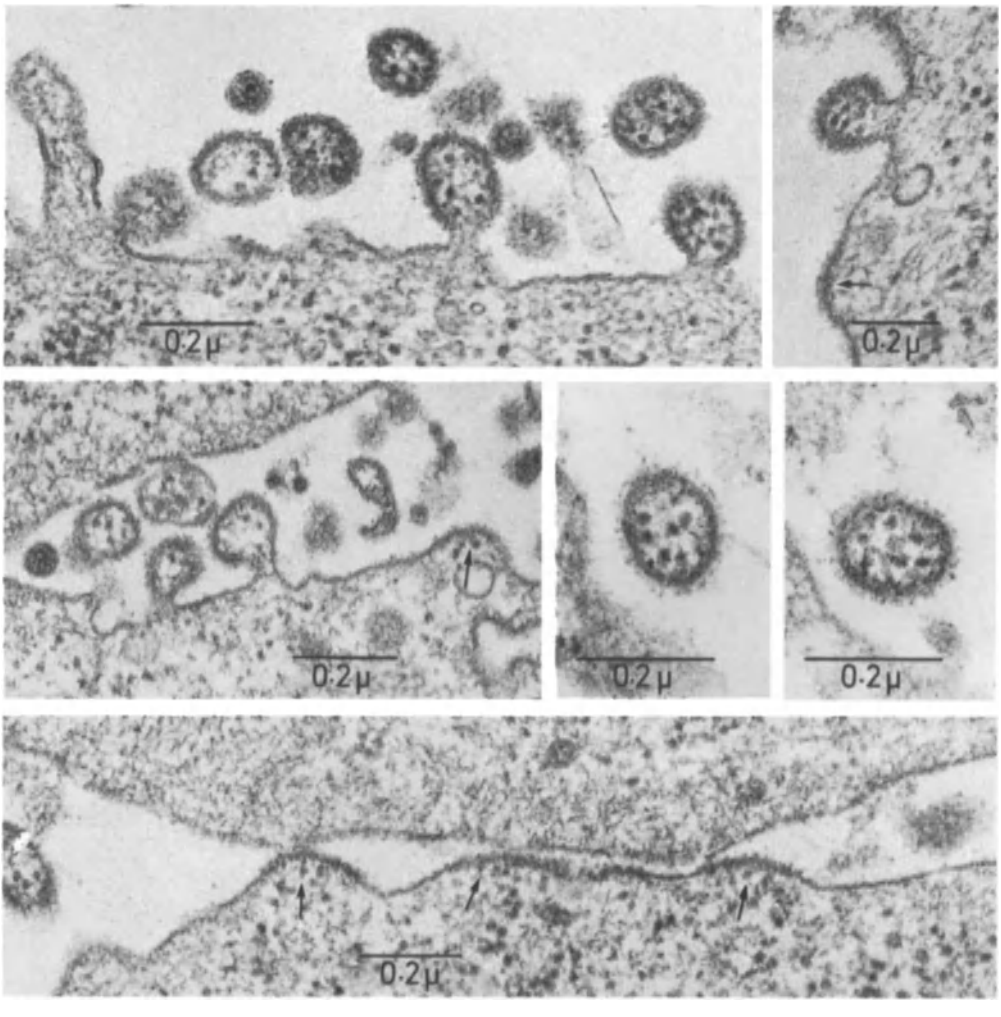

Fig. 5. Budding and release of typical arenavirus particles. Internal granules and fuzzy surfaces due to spike-like projections are recognized. Arrows point to loss of bilaminar structure of the membrane caused by early stages of budding.

In addition to these relatively loosely structured viral particles, another type of extracellular particle was found; it was only 50 to 65 nm in diameter and was more densely structured. In the center of such particles 1 or 2 granules were occasionally detected (Fig. 8). This type occurred singly in some instances; however, in others it comprised up to 10% of all particles.

In addition to these structures, which were only found in LCM virus-infected cells, there were others resembling type A particles (Fig. 10); these were observed in infected as well as in uninfected cells although in the former their number seemed to be higher. L cells are known to be persistently infected with a murine leukovirus.

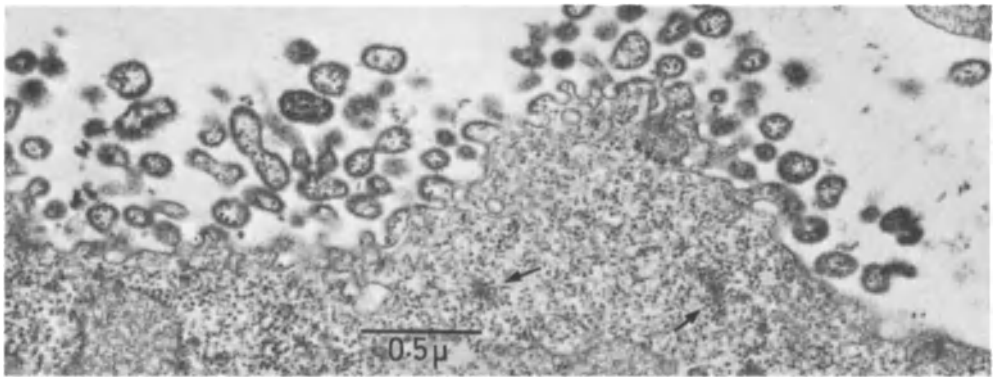

Fig. 6. Numerous LCM virus particles in association with the plasma membrane of an L cell 48 h after infection of the culture. Cytoplasmic condensations of granules are indicated by arrows.

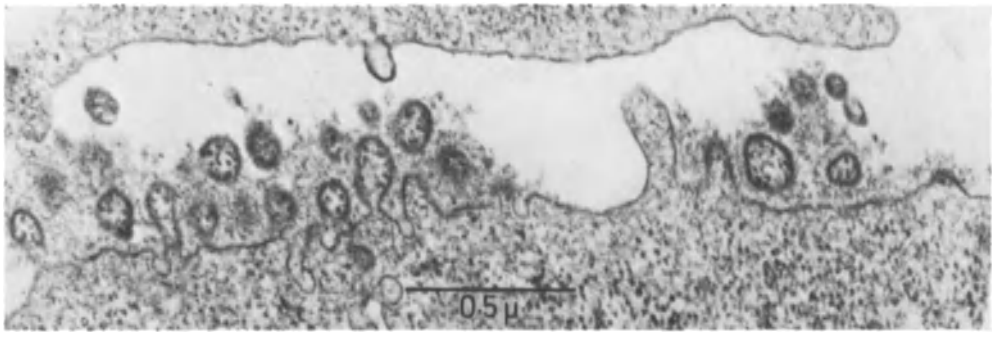

Fig. 7. Disintegration of both plasma membrane and LCM virus particles 48 h after infection of the culture.

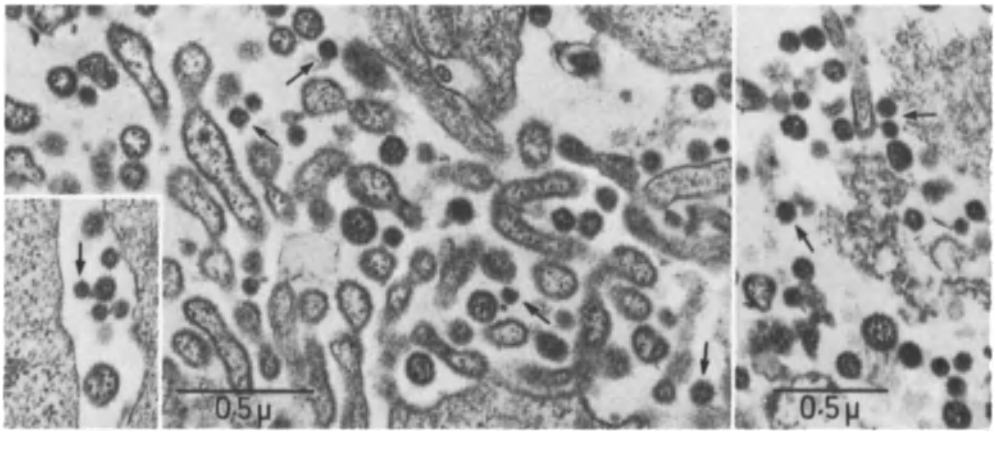

Fig. 8. Typical LCM virus particles interspersed with smaller more densely structured forms (arrows) not previously recognized in electron microscopic pictures of arenaviruses.

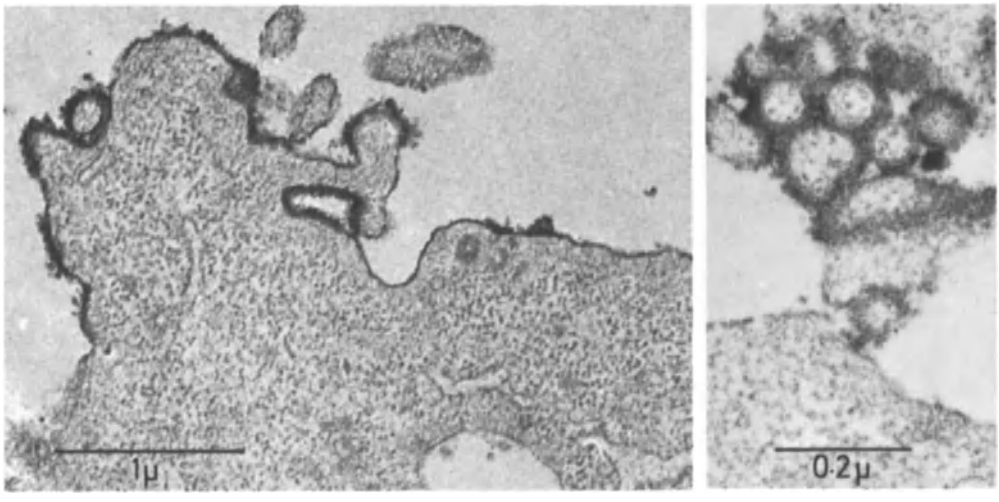

Fig. 9. Immuno-peroxidase staining of LCM virus-specific antigen on the surface of an L cell 48 h after infection of the culture; staining of free virus particles is also evident.

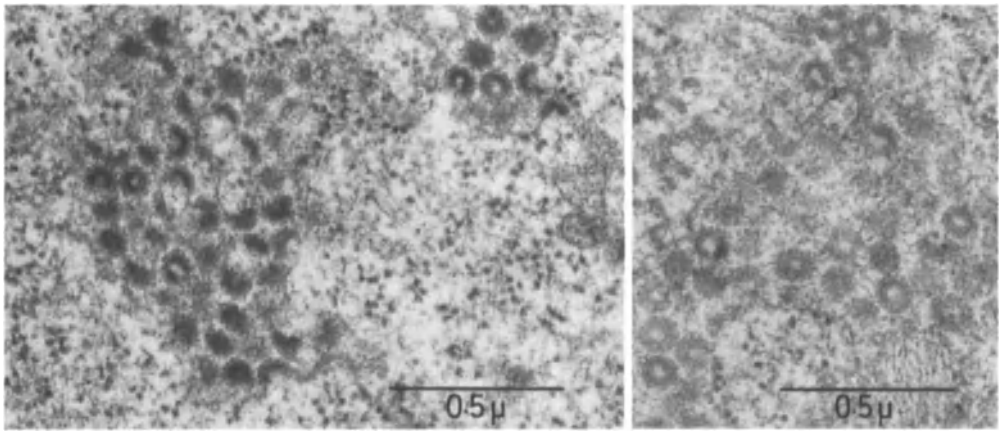

Fig. 10. L cell 36 h after infection. Particles of this kind were occasionally observed in both infected and uninfected cells.

When the immuno-peroxidase method was used to localize antigen on the surface of LCM virus-infected cells, extracellular as well as budding virus particles were labeled (Fig. 9). In addition, antigen was present in distinct small to large areas of the membrane which were not otherwise altered. Such labeling was never observed on uninfected cells. (It must be stressed that the peroxidase method as used here only localizes viral antigen on surface structures; the interior of virus particles and cells remains unstained; hence our results do not permit conclusions as to the relation of structural alterations to viral synthetic processes inside infected cells.)

DISCUSSION

Pathology in adult mice infected with the LCM virus is thought to be an allergic phenomenon (4) and it is generally assumed that direct damage of the cell caused by the replicating agent is absent. Previous studies on strain L mouse fibroblasts seemed to bear out this assumption; neither cytopathology nor decrease of cellular multiplication was observed after primary infection with WE and E-350 strains of LCM virus (6,9).

The present study shows that in spite of the apparent integrity of the infected L cell ultrastructural alterations are present and demonstrable as early as 16 h after infection. Of course, not all of these are pathologic in the sense that they represent true damage. Thus, increased lobulation of the nucleus, enlargement of nuclear pores and increased numbers of ribosomes are indicative of enhanced metabolic activity. Less easy to interpret are the cytoplasmic structures consisting of either regularly arranged multilayered lamellae surrounding fine granular material (Fig. 2) or irregular convolutions of membranes (Fig. 3). It is not clear whether these membranes participate in the synthesis of viral material or whether they are signs of degenerative processes. The function of the densely structured granules occurring either free in the cytoplasm or in aggregates, sometimes reaching the dimensions of large cytoplasmic inclusions (Fig. 4a and 6), is likewise uncertain. Previous authors have described similar structures in arenavirus-infected cells (1,12,13), and Abelson et al. (1), using immunofluorescence and immuno-peroxidase methods, concluded that these patches represent accumulations of viral antigen. We, too, believe that some sort of a relationship exists. However, since particles resembling virus or viral subunits have not been identified in the interior of the cell, the spatial arrangement remains uncertain.

Virus particles are only seen in association with the cell surface. Two general types can be distinguished. One has been described previously and is assumed to represent the arenavirus virion (1,3,5,11, 12,13,19). Granules consisting of smaller subunits lie in an unstructured matrix which is enclosed by a unit membrane studded with spike-like projections; release from the cell is by budding, whereby host components are incorporated (5). Usually these granules, which account for the name arenavirus (15), are said to be ribosome-like. In our study, they could be differentiated from normal L cell ribosomes which are less dense and somewhat smaller. However, morphologically related forms occur singly or as aggregates in the cytoplasm of infected cells. Dalton and his colleagues noted that the granules contained in the virus particles are considerably more resistant to the action of RNase than true cytoplasmic ribosomes (3). Virus particles as released from the surface of infected cells vary considerably in size. We did not, however, encounter the large forms exceeding 300 nm which have been documented by others (3,11); we are reluctant to consider the few elongated particles (Fig. 8) in these measurements. Abelson and his colleagues (1) too noted a relatively narrow range of particle size. They, as well as we, used cultures not later than 48 h after infection, whereas Dalton and co-workers (3) and Murphy and his colleagues (11) prepared cells for electron microscopy at 3 and 7 days. In a subsequent study, Murphy et al. (12) mentioned that in later stages of infection the number of large particles was increased and it appears that a wide size distribution of viral elements is characteristic of harvests from older cultures.

Although the particles just mentioned are variable in both size and shape, they can be clearly distinguished from another particle type

with which they are interspersed. This latter type is smaller, more densely structured and contains at most 2 arenavirus granules.

Labeling procedures prove that all these particles contain LCM virus antigen. However, are all these different structures - small or large, dense or light - true virions? Before electron microscopy was available for visualization, the size of the infectious unit had been determined by filtration and centrifugation experiments. Rivers and Scott (14) arrived at diameters not exceeding 150 nm, and later figures ranged between 33 and 60 nm (2,18). These were minimal estimates and they are in good agreement with the size of the smaller forms as seen on electron micrographs. The question of whether the larger particles are also infectious cannot be answered from morphological studies alone.

Inasmuch as the cell membrane is assumed to play a central role in the pathogenesis of the murine LCM disease, knowledge of alterations of the surface of LCM virus-infected cells may be the key to a better understanding of the processes which turn an infection into a disease. Needless to say, a single budding virus alters the plasma membrane, if only temporarily. Our micrographs show that budding of new virus can be so extensive as to cause complete dissolution of the cell surface. Other morphologic changes of plasma membranes occur independent of virus budding. Inclusion-like condensations are occasionally seen directly beneath the surface of the cell (Fig. 4a). To us, an exciting finding is the demonstration, by an indirect peroxidase labeling procedure, of virus-specific antigen in distinct areas of the plasma membrane where morphologic alterations are not apparent (Fig. 9). They seem to correlate in size and number with LCM virus-specific patches of antigen on infected cells as demonstrated with the help of the indirect immunofluorescence procedure by Rutter and Gschwender (16). It seems pertinent to point out that the demonstration of viral antigen on both plasma membrane and virus particles was accomplished by a peroxidase labeling procedure which avoided treatment of the cells with alcohol; we may conclude that drastic alterations of surfaces are not a prerequisite for revealing the existence of viral antigen as claimed by others (1).

We cannot yet exclude the possibility that in these areas of antigenic alteration budding of virus is imminent. We believe, however, that at these sites new virus-induced antigen is accumulated which is not represented on the surface of viral particles. Admittedly, this statement reflects at present not much more than a working hypothesis based on its own attractiveness rather than on solid evidence. We are, however, hopeful that these antigenic areas represent the pathogenic allergens which many of us are anxiously looking for.

ACKNOWLEDGMENTS

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Interaction of LCM Virus with Cells, Including New Virus-Induced Cellular Antigens

Chairman: F. Lehmann-Grube

Antigenic Alteration of Cells in Vitro Infected with LCM Virus

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SUMMARY

New antigens in LCM virus-infected L cells were studied by means of immunofluorescence and cytotoxicity procedures. With hyperimmune antisera directed against all viral components and containing both neutralizing and complement-fixing antibodies, indirect immunofluorescent staining of fixed cells reveals cytoplasmic antigen which is distributed in a finely diffuse as well as a coarsely granular pattern. Employing both immunofluorescent staining and cytotoxicity assays, these sera also detect viral antigen on the surface of LCM virus-infected cells.

In contrast, only granular fluorescence and no surface antigen can be demonstrated when antisera specific for a complement-fixing antigen extracted from infected cultures are employed. Persistently infected L cells, whose cytoplasm contains masses of brightly fluorescing material, fail to display detectable surface antigen using either type of antisera.

It is concluded that at least 2 new antigens are produced in the interior of an LCM virus-infected L cell, one of which may also appear on its surface. The other antigen, which is produced in large quantities in infected cells and which is characterized by its complement-fixing potential, is not represented on the cell surface.

INTRODUCTION

Acute lymphocytic choriomeningitis of the mouse has become a valuable model for obtaining knowledge of virus-induced pathologic immune phenomena and the role they may play in converting a virus infection into a virus disease (5,7). Indeed, it is becoming increasingly obvious that immunopathology may significantly contribute to the pathogenesis of a number of virus diseases, and great interest is currently directed towards studying virus-specific allergic mechanisms (6). However, relatively little is known as to the viral antigens produced during infection which may be responsible in eliciting immune-mediated disease.

As an extension of our recent progress in purifying and concentrating LCM virus-induced antigens (4), the present report deals with a study of the distribution of viral antigens in and on LCM virus-infected L

cells using antisera with restricted specificities. Both immunofluorescence and cytotoxicity procedures were employed.

MATERIALS AND METHODS

Virus. The WE strain of LCM virus, which was used throughout, was passaged and propagated in L cells. The method for titrating the virus in L cell tube cultures has been described (9). It is based on the detection of CF antigen which is released from infected cells.

Cell cultures. L cells were cultivated in Eagle's minimum essential medium plus non-essential amino acids (11) and 5% calf serum. Origin and properties of L(Arm) cells (L cells persistently infected with Armstrong's strain E-350 of LCM virus) have been described (10).

Antisera. LCM virus-specific immune sera against all virus constituents (anti-LCM virus antisera) or against "ECFA", a complement-fixing material extracted with butanol from infected cells (anti-ECFA antisera), were prepared in rabbits and guinea-pigs as described in the companion paper by Gschwender and Lehmann-Grube (4). In this context, it is pertinent to stress that guinea-pigs were immunized with antigens prepared from infected guinea-pig organs and rabbits with antigens prepared from infected rabbit cell cultures grown and maintained with rabbit serum. Anti-LCM virus antiserum contains both complement-fixing and neutralizing activities, while anti-ECFA antiserum lacks the latter quality though it fixes complement in the presence of either ECFA or unpurified virus preparations at high dilutions. Fluorescein isothiocyanate-conjugated pig anti-guinea-pig and anti-rabbit γ-globulin antisera were obtained from Sevac, Prague.

Immunofluorescence method. Culture tubes containing coverslips were seeded with 4 to 6 x 10^5 L cells in 2 ml of medium. After incubation for 24 h at 37°C, the medium was decanted and the cells were overlaid with 0.5 ml medium containing virus corresponding to input MOI 0.1 to 0.01 ID50. After 15 min at room temperature the inoculum was removed, the cells were rinsed with balanced salt solution, new medium was added, and incubation at 37°C was continued. At various intervals the cells were washed 4 times with warm balanced salt solution, dried at room temperature, fixed with acetone for 10 min at -20°C, and dried again. They were stained either immediately or after storage at -70°C; the indirect method (16) was employed.

For demonstrating virus-induced membrane antigens, cells were rinsed and incubated with diluted serum for 5 min at room temperature. They were washed twice again and then fixed by immersion for 15 min at 4°C in 4% formaldehyde buffered with phosphate at pH 7.2 (3). The cells were incubated for 30 min at 37°C with fluorescein-labeled anti-γ-globulin antiserum, washed, and mounted on slides in buffered glycerol for examination under a fluorescent microscope type Zetopan (Reichert, Vienna, Austria). The instrument was equipped with an HBO 200 mercury vapor lamp; the excitation filters were either BG12/1,5 or BG12/2 and the barrier filter consisted of a combination of OG1/1,5 plus GG9/1.

Cytotoxicity assay. LCM virus-infected L cell coverglass cultures were incubated for 30 min with heat-inactivated 20-fold diluted antiserum from the rabbit together with fresh guinea-pig serum, diluted 10-fold, as source of complement. The cells were examined with the help of a phase contrast microscope.

For control purposes, non-infected cultures were treated with antise-

rum and complement and infected cultures were treated with normal rabbit serum and complement.

RESULTS

By means of the indirect immunofluorescence method, rabbit anti-LCM virus antiserum reveals 2 clearly distinguishable types of antigen in fixed LCM virus-infected cells. Of these, one is characterized by diffusely distributed fine dust-like fluorescence, while the other consists of coarse brightly fluorescing granules. Most cells contain both types of antigen, although visualization of the larger granules may be partially obscured by the abundance of fine material. Early in the infectious process, a few cells contain only the coarse granules (Fig. 1).

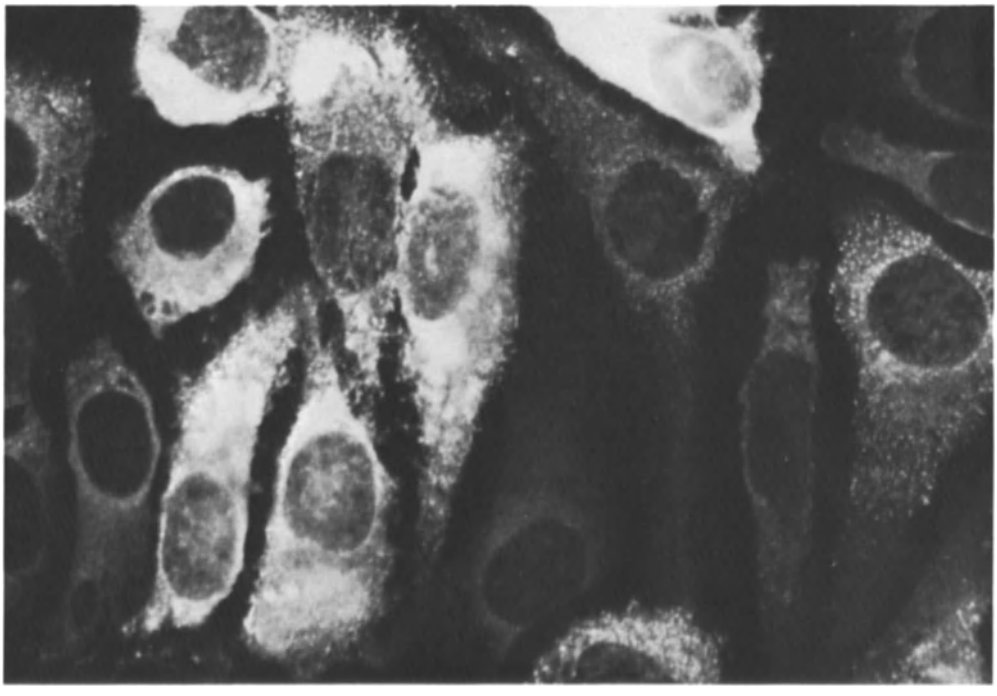

Fig. 1. Demonstration of virus-induced antigen inside L cells, 48 h after infection with LCM virus. Cells were fixed with acetone and stained with rabbit anti-LCM virus antiserum in combination with fluorescing anti-rabbit γ-globulin antiserum. Darkfield condensor. Objective x40; ocular x6.3.

Using antisera raised either in rabbits or in guinea-pigs and directed against the complement-fixing material extracted from infected cells or tissues (ECFA), only 1 type of antigen is demonstrable, which morphologically is indistinguishable from the larger type of granular structures just described. Numerous granules of different sizes and densities are observed which are distributed in an irregular pattern

throughout the cytoplasm; they differ in number from cell to cell (Fig. 2).

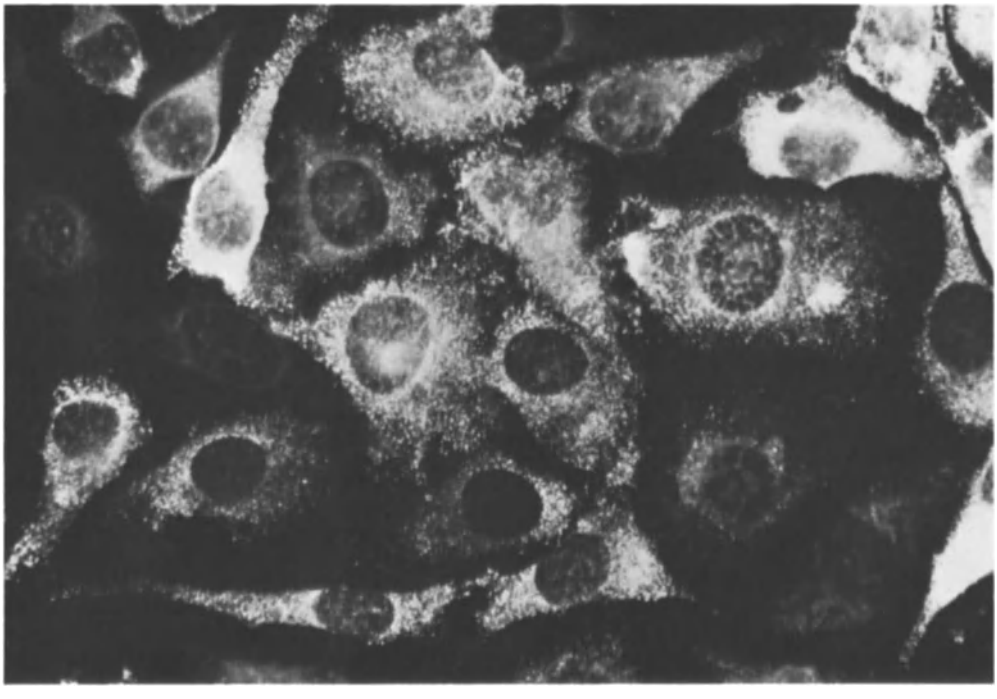

Fig. 2. Same as Figure 1 except that anti-ECFA antiserum was used.

When fixed infected cells are treated with guinea-pig anti-ECFA antiserum prior to staining with rabbit anti-LCM virus antiserum (in combination with fluorescein-labeled pig anti-rabbit γ-globulin antiserum) only the fine dust-like fluorescence remains which differs in intensity from cell to cell.

Absorption of both types of sera with concentrated and highly potent ECFA leads to different results. Whereas the ability of an anti-ECFA antiserum to mark infected cells is completely abolished, the staining potential of anti-LCM virus antiserum is reduced; it retains its property of staining the fine dust-like material, but its ability to stain the coarse structures disappears (Fig. 3).

Application of the immunofluorescence technique to unfixed viable cells permitted the visualization of antigen associated with the cell membrane. After infection, the number of cells carrying new surface antigen gradually increases and reaches a maximum of approximately 90% at 48 h. On the margin of these cells, predominantly along cellular processes, antigen may be so dense as to completely obliterate structural details. On the remainder of the cell surfaces, especially above the nuclei, the distribution of antigen is less compact and fine irregularly distributed granules can be seen (Fig. 4). Demonstration of surface antigen is only possible with anti-LCM virus antiserum. No specific staining occurs with anti-ECFA antiserum, which is also incapable of blocking antiserum directed against all viral constituents.

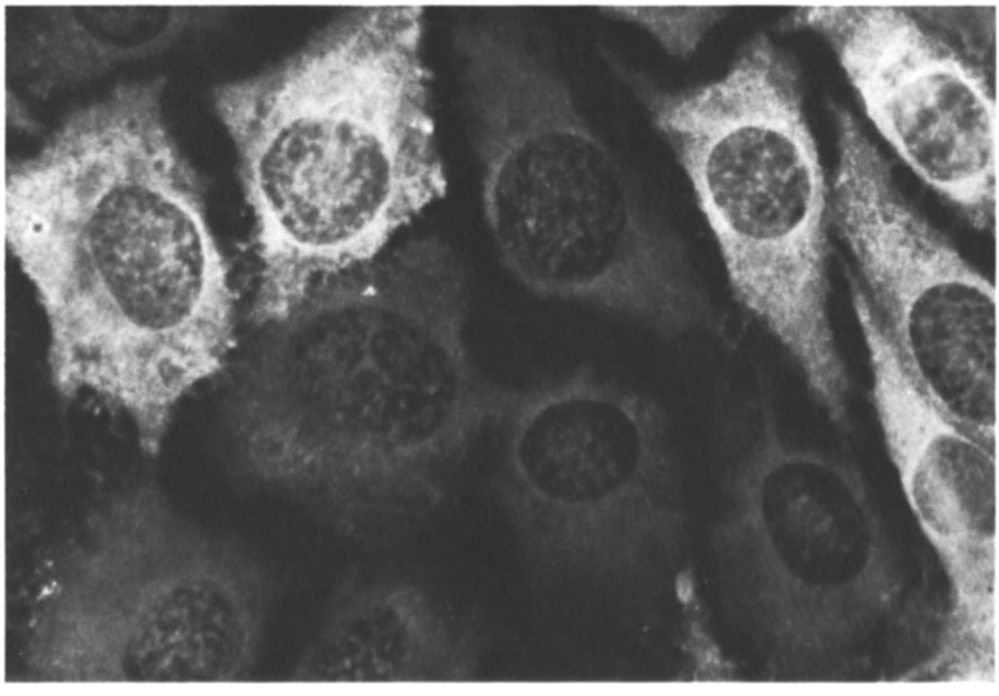

Fig. 3. Same as Figure 1 except that the anti-LCM virus antiserum had been absorbed with ECFA.

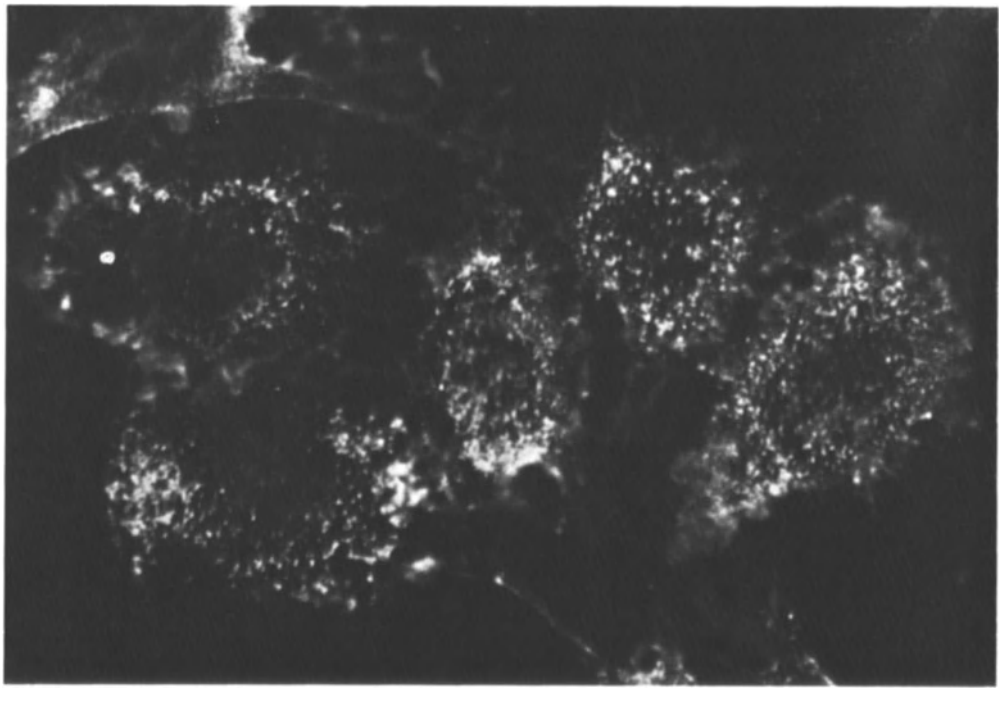

Fig. 4. Demonstration of LCM virus-induced surface antigen on L cells 48 h after infection. Viable (unfixed) cells were stained and visualized as described for Figure 1.

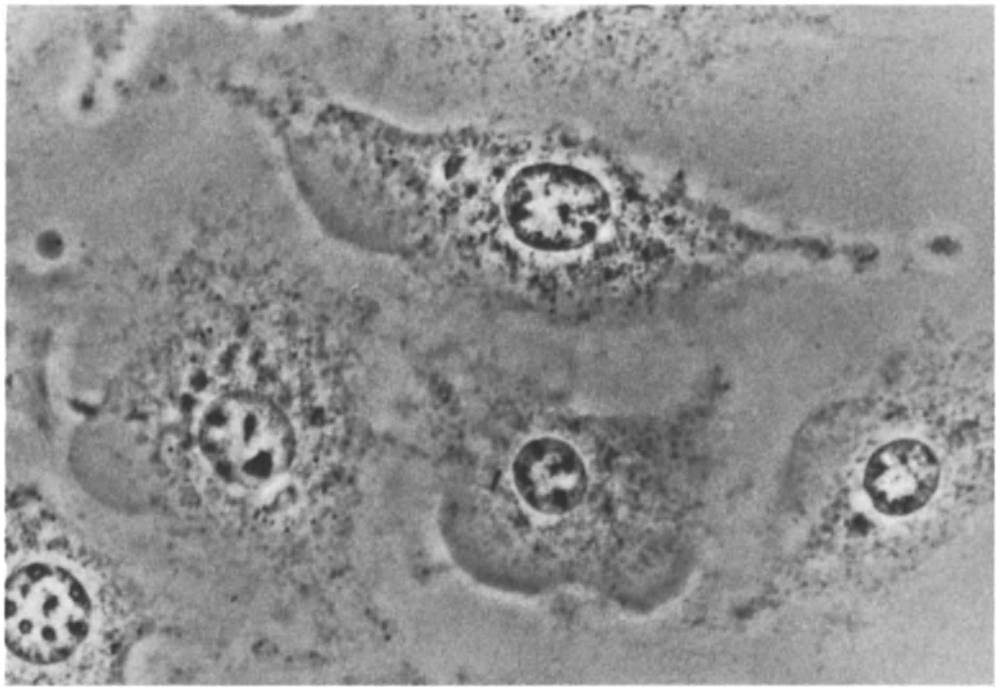

Fig. 5. Cytolysis of LCM virus-infected L cells caused by antiserum and complement. Cells were incubated 48 h after infection for 30 min at 37°C with anti-LCM virus antiserum and guinea-pig serum as a source of complement. Phase contrast. Objective x40; ocular x6.3.

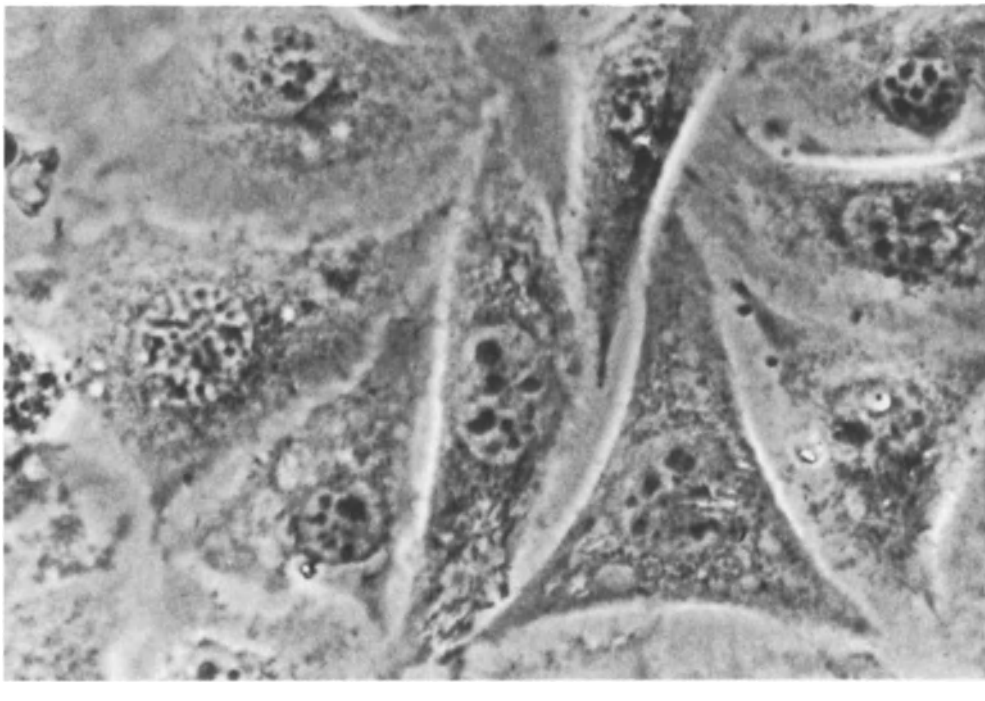

Fig. 6. Same as Figure 5 except that cells were incubated with anti-LCM virus antiserum only (no complement).

Pre-incubation with ECFA does not reduce the ability of anti-LCM virus antiserum to stain the plasma membranes of infected cells.

In contrast to L cells infected rather recently with LCM virus, immunofluorescent staining does not reveal binding of antibody from either anti-ECFA or anti-LCM virus antisera onto the surfaces of persistently infected cells from L(Arm) carrier cultures.

The results obtained with the indirect immunofluorescence method were corroborated by cytotoxicity experiments. L cells recently infected with LCM virus are lysed by anti-LCM virus antiserum plus complement (Fig. 5), the number of lysed cells being proportional to the number of cells containing surface antigen as demonstrated by the immunofluorescence method. Lysis is not caused by anti-ECFA antiserum. Furthermore, absorption of anti-LCM virus antiserum by an equal amount of ECFA having a CF titer of 20,000 as determined in a micro-assay (4) does not cause a decrease of its lytic activity.

DISCUSSION

In studies reported previously from other laboratories, immunofluorescence methods for the detection of virus-induced antigens in LCM virus-infected cells, both in vivo and in vitro, have been successfully employed (7,17). With respect to cultivated cells there is agreement that immunofluorescent antigen becomes demonstrable about the time infectious progeny virus begins to appear. Depending on the MOI, the number of fluorescing cells then increases, but most authors seem to agree that not all cells eventually become positive. Usually fluorescence does not involve the nucleus although exceptions have been noted (10,14).

Although the development, location, and distribution of immunofluorescent LCM virus-induced antigen has been described previously, little was known of its chemical nature or even its antigenic specificities. Our findings show quite clearly that antigens with at least 2 different specificities are induced in L cells infected with the LCM virus. One component, which is distributed in a coarsely granular pattern, is detected by antisera directed against both a mixture of all viral components and ECFA, a complement-fixing material extracted from LCM virus-infected cells or tissues (4). Since this coarse fluorescence disappears upon absorption of antiserum with ECFA and is blocked by prior application of anti-ECFA antiserum to the cell, it is concluded that these granules represent accumulations of ECFA. Though we cannot yet exclude the possibility that ECFA is a structural part of the virion, it is immunologically not expressed on the virus surface (4,8). Furthermore, ECFA does not appear to be represented on the membranes of infected cells and, therefore, is probably not an antigen involved in eliciting acute lymphocytic choriomeningitis of the mouse. By immunofluorescence, ECFA in LCM virus-infected cells is clearly distinguishable, both in morphology and specificity, from material which is diffusely distributed throughout the cytoplasm of the infected cell and which can only be visualized by antiserum directed against all viral components.

The appearance of virus-induced cell surface antigens is not unique for L cells infected with LCM virus and many examples are known (13, 15). It is probable that such virus-induced membrane alterations play an important role in both the pathogenesis and the termination of many viral diseases, including LCM of the mouse. In the past, new antigens on LCM virus-infected mouse cells were demonstrated by functional tests

only and in our opinion not enough attention was paid to quantitative aspects. In addition, the commonplace that any virus infection in any host is accompanied by induction of numerous different antigens, which may be localized in different compartments of the infected cell and which may or may not be incorporated into the virion, was all but ignored. Although our experiments deal with LCM virus infection in vitro, they are thought to open the way to a more critical approach toward understanding the immunology of the LCM virus infection of the mouse.

At the present time, we can say little about the nature of the new antigen(s) on the surface of LCM virus-infected cells. These could represent complete virions which are known to be released by a budding process (1,2,12). The fact that surface antigen cannot be detected on persistently infected L(Arm) cells, which also do not produce or release infectious virus in significant quantities, points in this direction. Alternatively, it could be a non-virion cell surface antigen. It is hoped that experiments now under way in this laboratory, which employ specifically labeled antisera and both light and electron microscopy, will soon provide us with more definitive information concerning the nature of LCM virus-induced cell surface antigen(s).

ACKNOWLEDGMENT

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In Vitro Correlates of LCM Virus-Induced Immune Response

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SUMMARY

LCM virus-specific immune responses of virus-immune and virus carrier BALB/c mice were studied by an in vitro assay involving the lysis of ^{51}Cr-labeled virus-infected "target" L cells by either splenic lymphocytes or antibody in the presence of complement. Lysis by immune lymphocytes was inhibited by anti-θ serum but could not be blocked by anti-viral complement-fixing antibody. The susceptibility of target cells to lymphocyte-mediated lysis was closely related to the density of immunofluorescent staining virus-specific antigen at their surfaces. The number of cells with demonstrable surface antigen decreased with continued passage in culture without a concomitant decrease in intracytoplasmic viral antigen.

Lysis by antibody and complement occurred relatively independent of the density of target cell surface antigen. This observation, together with the demonstration that antibody could not interfere with cell-mediated lysis, suggested that the specificity of anti-viral complement-fixing antibody is different from that of immune lymphocytes. Virus carrier mice spleens contained little or no cytolytic lymphocytes, but serum from these mice contained measurable amounts of cytolytic antibody.

These data fail to implicate a role for blocking antibody in the maintenance of the virus carrier state and support the notion that viral persistence is related to a deficit in virus-specific effector T-lymphocytes.

INTRODUCTION

The development of disease following chronic or acute infection of the mouse with LCM virus has been shown to be the result of the infected host's anti-viral immune response. During neonatally or congenitally established chronic carrier infections, the antibody component of this response together with circulating virus and complement form immune complexes which are deposited in the renal glomeruli and eventually lead to progressive glomerulonephritis (15).

In contrast, the more classical central nervous system (CNS) disease, acute lethal choriomeningitis, is mainly, if not entirely, cell mediated (2,4).

Recent in vivo studies have shown that, in the mouse, thymus-derived (T) lymphocytes are required for the elicitation of CNS immunopathology. When spleen cells obtained from LCM virus-immune mice were specifically depleted of T-lymphocytes by treatment with anti-θ serum, the treated cells lost their ability to produce either clinical or histological choriomeningitis when adoptively transferred to syngeneic adult mice with carrier infections established by cyclophosphamide-mediated immunosuppression (2). However, these same cells retained their capacity to engage in the production of circulating antibody to LCM virus. Immunofluorescent staining of brains from carrier mice given T-lymphocyte-depleted immune spleen cells revealed the presence of both viral antigen and bound IgG at identical sites in the meninges and choroid plexus suggesting that immune complex formation within the CNS is not a primary factor in the production of choriomeningitis.

This report describes a technique for demonstrating and measuring the cytolytic activity of LCM virus-specific T-lymphocytes in vitro and emphasizes some of the experimental conditions under which this activity is maximally expressed. Evidence is presented for possible differences in the antigenic specificity between the humoral and cellular components of the LCM virus-induced immune response, a difference which may have some bearing on the analysis of mechanisms for maintaining the virus carrier state or producing cell-mediated immunopathology.

MATERIALS AND METHODS

Donor mice. BALB/c mice of either sex (Flow Laboratories, Virginia) were used throughout these studies. To prepare virus-immune mice, 8 to 10-week-old animals were given 3 to 4 weekly intraperitoneal injections of a clarified brain suspension containing approximately 10^4 adult mouse intracerebral LD50 of the Armstrong (E-350) strain of LCM virus (3,4). Virus carrier mice were prepared by the intracerebral inoculation of newborn mice with approximately 10^3 LD50 of virus.

Lymphoid cells. Lymphoid cell suspensions in Eagle's medium (MEM, Grand Island Biological, New York) were prepared from the spleens of immune, carrier, and normal mice following previously published procedures (3,4). The number of splenic lymphocytes present in the suspensions was determined by hemocytometer-counting with Türk's solution. Viable lymphocytes were calculated by trypan blue exclusion.

Antisera. The sources of antibody to LCM virus were either the same groups of immune or carrier mice used as spleen cell donors or a single pool of "hyperimmune" serum from adoptively immunized carrier mice (4). Serum pools from virus-immune donor mice usually had CF antibody levels ranging from 25 to 100; the pool from adoptively immunized carriers titered 500 but had no significant virus-neutralizing activity. Carrier mice sera contained no demonstrable CF antibody as measured in a standardized microtiter test (4). Prior to use, sera were heated at 56°C for 30 min. The anti-θ serum was the same as that used in previously published reports (2,7).

Virus-infected target cells. Stationary cultures of normal L cells (clone 929) and L cells acutely or chronically infected with LCM virus were used as a source of targets. Disposable 250 ml tissue culture flasks (Falcon Plastics, California) containing nearly confluent monolayers were inoculated with 3.0 ml of MEM fortified with 10% fetal calf serum and containing virus at a multiplicity ratio of 0.1 mouse LD50/cell. Following a 3 h adsorption period at 37°C in air containing 5% CO_2, the flasks were washed twice with normal saline and 15.0

ml of growth medium was added. After 2 days, the cells in each flask were removed with 0.25% trypsin (Grand Island Biological, New York) and redistributed to 3 new flasks. These singly passaged, acutely infected cells were used in the cytotoxicity test upon reaching confluency. Chronically infected L cell cultures were prepared in an identical fashion but were maintained for periods of 3 to 4 months (16 to 20 passages) before use as targets. Following trypsinization of cell monolayers and the addition of ovomucoid trypsin inhibitor (Sigma Chemical, Missouri) suspensions of normal and infected L cells were washed and suspended in tris-saline buffer pH 7.4 and labeled with sodium 51chromate (specific activity 100 to 200 μCi/μg Cr, "Rachromate", Abbot Laboratories, Illinois) as reported previously (6).

Cytolytic assay. The details of the cytolytic assay have been published by Henney (6). Tubes containing known numbers of lymphoid cells and 51Cr-labeled targets were incubated for varying periods at 37°C in air containing 5% CO_2. Routinely, 10^7 spleen cells and 10^5 L cells, each contained in a 0.1 ml volume, were added to 0.8 ml of MEM containing 10% fetal calf serum and antibiotics. After incubation, the reaction mixture was centrifuged in the cold and the cell-free supernate assayed for 51Cr content. For demonstrating lysis by antiserum and guinea-pig complement, 0.1 ml of each was added, undiluted, to 10^5 target cells, keeping the total volume of the reaction mixture at 1.0 ml. Control tubes consisted of target cells in medium to which were added either complement in the absence of antibody or heat-inactivated complement and antibody. In experiments to determine blocking of lymphocyte-mediated cytolysis by anti-viral antibody, target cells were preincubated for 30 min with antiserum (without complement) before the addition of lymphocytes. When anti-θ serum was used to inhibit T-lymphocyte activity, 0.1 ml, undiluted, was added directly to the reaction mixture without complement.

The percentage of specific cytolysis of target cells was calculated by subtracting from the percentage 51Cr released in the presence of either "sensitized" lymphocytes or antibody and complement, that percentage of label released in the presence of an equal number of normal lymphocytes or an equal amount of homologous normal serum. (Percentage cytolysis = counts in cell-free supernate ÷ total counts x 100.)

Immunofluorescent staining. The indirect method was used, employing antiserum to LCM virus obtained by adoptive immunization of carrier mice (as above) and a fluoresceinated goat anti-mouse IgG (Meloy, Virginia). Companion monolayer cultures, grown in tissue culture chamber slides (Lab-Tek, Illinois), were prepared for each lot of normal or infected L cells used for targets and either stained alive or after methanol fixation to determine the approximate numbers of cells with, respectively, surface and intracytoplasmic viral antigen (details to be published, Cole et al.).

RESULTS

Cell-mediated lysis. In preliminary experiments, a considerable variability was noted between the susceptibility of different target cell preparations to lymphocyte-mediated lysis. It was found that this variability was related to the interval between infection of cells and their use as targets. Cells from cultures recently infected were lysed much more readily than those from cultures maintained for longer periods of time by repeated passage. This phenomenon was illustrated by experiments in which both acutely and chronically infected cells were tested for their relative susceptibility to lysis by immune spleen cell

Table 1. Lysis of LCM virus-infected cells by immune lymphocytes[a]

Lymphocyte donor[b]	Cytotoxicity of spleen cells							
	Expt. 1		Expt. 2		Expt. 3		Expt. 4	
	A[c]	C[c]	A	C	A	C	A	C
Normal	69.0	-	64.1	64.3	23.5	24.9	51.6	59.3
Immune	81.3	-	72.9	68.5	35.0	25.0	71.4	62.5
% specific lysis[d]	12.3	-	8.8	4.2	11.5	0.1	19.8	3.2

[a] Percent lysis after 18 to 20 h incubation of 10^7 spleen cells with 10^5 targets (Expts. 1 to 3) and 5 x 10^5 targets (Expt. 4).

[b] Pooled spleen cell suspensions from 3 to 5 normal or immune mice.

[c] Acutely (A) or chronically (C) infected L cells.

[d] Difference between percentage of target cells lysed by normal and immune lymphocytes.

preparations (Table 1). Although specific lysis could be measured after 2 h, incubation periods of 18 to 20 h were required to demonstrate a maximal lytic effect. Longer incubation periods tended to diminish the differences in ^{51}Cr release between normal and immune lymphocytes because of spontaneous leak of label from target cells dying from ad-

verse culture conditions. The commonly employed effector lymphocyte/target cell ratio of 100:1 resulted in a situation of lymphocyte excess since increasing the proportion of target cells usually resulted in a higher percentage of specific lysis (Expt. 4, Table 1). Reducing the number of effector lymphocytes resulted in a proportional decrease in lysis. Results of additional experiments, to be published elsewhere, indicated that effector lymphocyte activity could be demonstrated in the spleens of mice 6 days after a single intraperitoneal dose of virus increasing to a maximum by about the 11th day and falling to barely detectable levels at 20 days. Spleens from mice on prolonged immunization schedules (6 to 7 weekly doses) contained relatively low lytic activity, although these animals had higher levels of CF antibody in their serum. Traces of cytolytic activity were infrequently demonstrable in spleens from virus carrier mice and only when acutely infected L cells were used as targets. The amounts of specific lysis recorded in 5 different assays of spleens from 12-week-old carrier mice (10^7 lymphocytes versus 10^5 targets) ranged from <1% to 6%.

Immunofluorescent viral antigen. Because of their differing susceptibility to cell-mediated lysis, acutely and chronically infected viable L cells were compared by immunofluorescent staining of their surfaces for viral antigen. Acutely infected populations were shown to contain numerous cells (30 to 60%) exhibiting brightly fluorescent, punctate granules located at the plasma membrane (Fig. 1a). Similar, although fainter, granular fluorescence could also be seen on chronically infected cell surfaces, but the number of such fluorescing cells rarely exceeded 5%. In contrast, immunofluorescent staining of both acutely and chronically infected L cells post-fixation revealed similar percentages of cells (80 to 95%) containing a relatively uniform distribution of intracytoplasmic antigen (Fig. 1b).

Lysis by antibody. Antibody cytotoxicity tests were usually performed with pooled serum obtained from the same groups of 3 to 5 normal or immune mice used as spleen donors. As shown in Table 2 (Expt. 1), acutely and chronically infected L cells were both lysed to about the same extent by antibody in the presence of complement. In general, the amount of lysis by an immune serum pool could be correlated with its CF titer (Table 2). Serum pools obtained from animals shortly after being given a single intraperitoneal immunizing dose of virus had low CF titers (5 to 10) and lysed a small percentage of infected targets (3 to 5%). The "hyperimmune" serum pool prepared from adoptively immunized virus carrier mice had the greatest amount of cytolytic activity which corresponded with its high level of CF antibody. Specific antibody-mediated lysis only occurred in the presence of complement and was maximally expressed by about 9 to 12 h after the addition of serum to target cells.

Sera from virus carrier mice, although containing no detectable CF antibody, invariably lysed a small but significant percentage of infected target cells (Table 2).

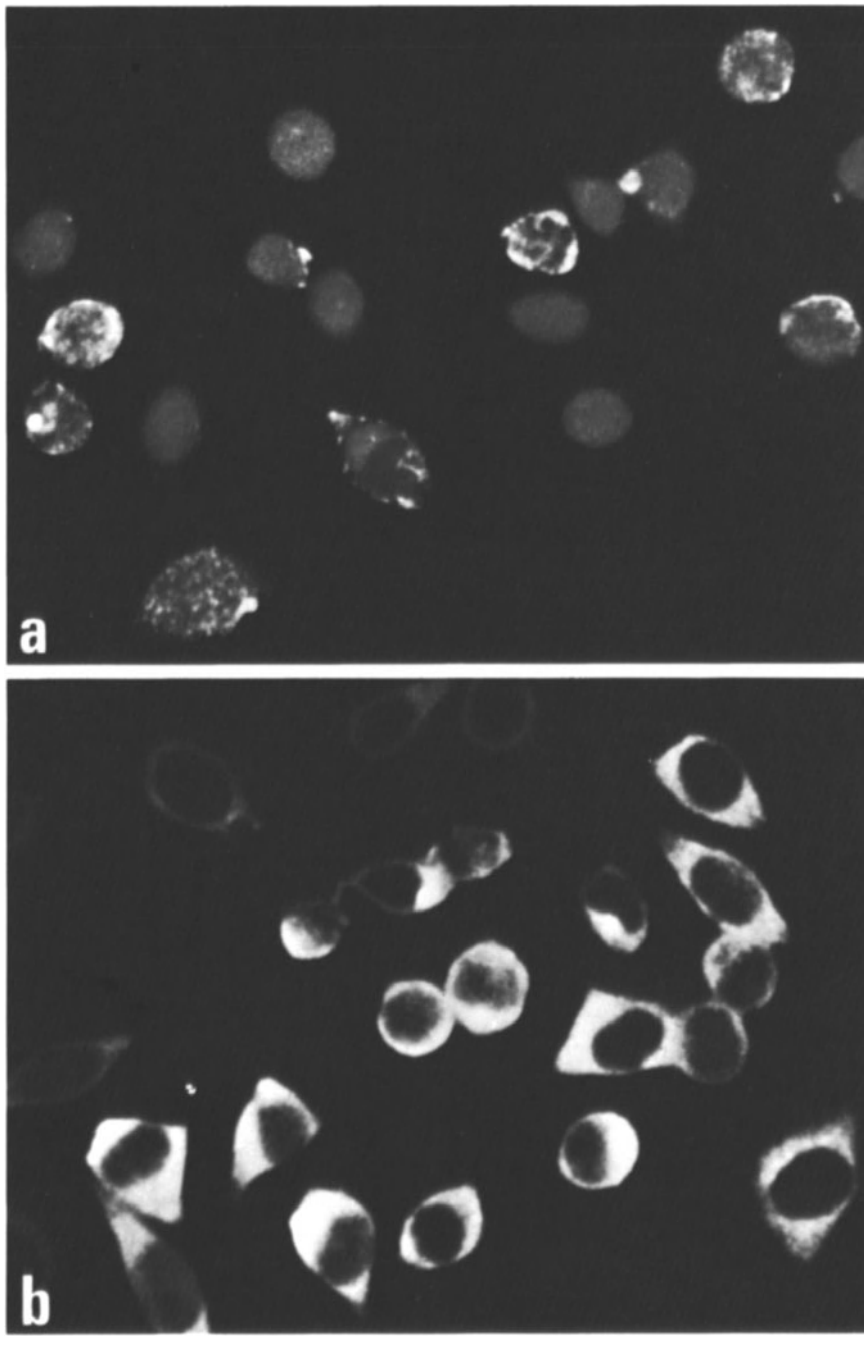

Fig. 1. Indirect immunofluorescent staining of L cells acutely infected with LCM virus. (a) Viral antigen at the surface of cells stained while viable. (b) Antigen in the cytoplasm of cells stained after methanol fixation.

Table 2. Antibody-mediated lysis of LCM virus-infected L cells[a]

Serum donor	Target cells[b]	Percent specific lysis					
		Expt. 1		Expt. 2		Expt. 3	
Immune	A	5.3	(5)[c]	-	(50)	9.2	(100)
	C	5.1		8.7		-	
Hyperimmune	A	23.6	(500)	-		-	
	C	21.6		-		-	
Carrier	A	1.8	(<5)	3.5	(<5)	-	(<5)
	C	1.2		-		1.6	

[a] After 9 to 12 h incubation with serum and complement.
[b] 10^5 acutely (A) or chronically (C) infected targets.
[c] Numbers in parenthesis are CF titers.

Effect of anti-θ serum and antibody on cell-mediated lysis. The ability of immune lymphocytes to lyse infected target cells was virtually eliminated by the addition of anti-θ serum to the reaction mixture (Fig. 2), an effect which was independent of the presence of complement. Lysis of target cells by antibody was unaffected by the addition of anti-θ serum.

The susceptibility of target cells to cell-mediated lysis did not change either by preincubating them in antibody (either immune or hyperimmune serum) prior to the addition of immune lymphocytes or by incorporating antibody into the reaction mixture.

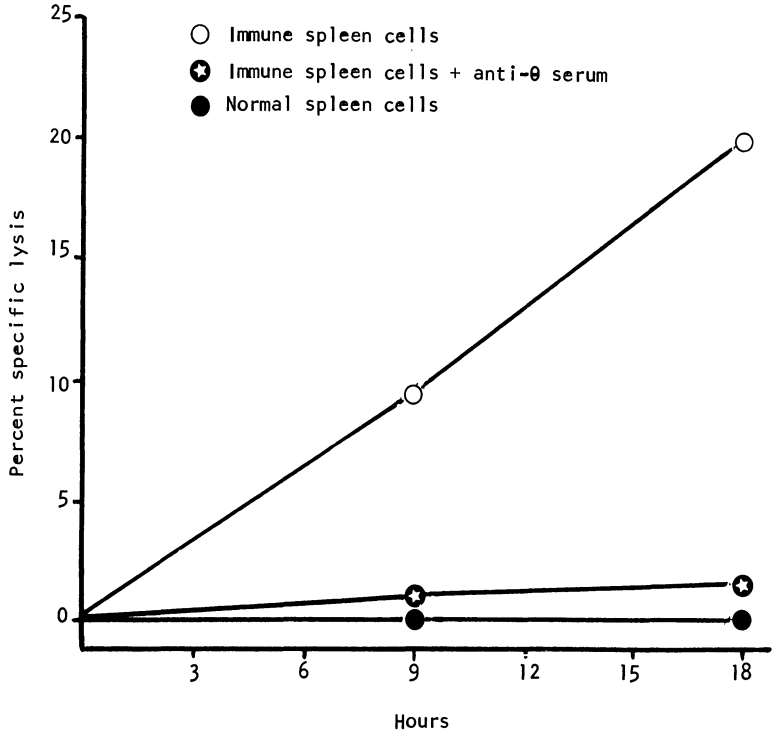

Fig. 2. Inhibition by anti-θ serum of cell-mediated lysis of L cells acutely infected with LCM virus (10^7 lymphocytes and 5×10^5 target cells).

DISCUSSION

The present study demonstrated that L cells acutely infected with LCM virus were readily lysed by either lymphocytes or antibody (in the presence of complement) from virus-immune mice. In contrast, chronically infected cells were relatively resistant to lysis by lymphocytes but about as susceptible to antibody-mediated lysis as acutely infected cells. The marked difference in the density of immunofluorescent surface viral antigen between the 2 types of infected targets provided an acceptable explanation for this phenomenon. A more significant interpretation of this difference in surface staining is that antibody possesses a specificity for a viral antigen which is not recognized by the immune lymphocyte. A seeming contradiction to this interpretation is the fact that immunofluorescent staining of surface viral antigen(s) is accomplished by employing an antibody which in the presence of complement does not discriminate between acutely or chronically infected target cells. However, it is reasonable to assume that acutely infected L cells express greater amounts of several antigens at their surfaces; some being the structural proteins of infectious virions and others which are non-structural or "soluble" (10,18). This assumption is supported by the observation that continued passage of L cells infected with the Armstrong strain of LCM virus leads to a sharp decrease in their production of infectious virus without a corresponding decrease in intra-cytoplasmic antigen (11).

The immune sera used in this study, although containing CF activity, usually failed to neutralize significant amounts of infectious virus. Since high-titered CF antibody did not block cell-mediated lysis of acutely infected L cells despite its ability to lyse these cells in the presence of added complement, we postulate that acutely infected cells must display at least 2 antigenically distinct surface antigens; one recognized by CF antibody ("soluble" antigen?) and the other, perhaps associated with the viral envelope, which is recognized by the immune lymphocyte. If this postulate is correct, it would predict that virus neutralizing antibody might block lysis by immune lymphocytes either by competition for the same antigenic determinants or by steric hindrance. Preliminary experiments indicate that serum from mice, multiply immunized after having suckled their neonatally infected virus carrier offspring, contains antibody which neutralizes virus (1.5 $\log_{10}$ protection) and also blocks lymphocyte-mediated lysis.

The experiments with anti-Θ serum clearly demonstrated that cell-mediated lysis is a T-lymphocyte-dependent phenomenon. The fact that spleen cells from virus carrier mice only occasionally had significant effector cell activity suggests that a relative deficit of virus-specific T-lymphocytes is a factor contributing to the maintenance of the virus carrier state.

The finding of trace amount of cytolytic antibody in serum from carrier mice is consistent with the reports of others (1,14). However, since this antibody appears to be of the type which only fixes complement (15), the possibility that it plays a role during carrier infections by blocking T-lymphocyte function (5,13) seems unlikely.

With a few exceptions, our findings essentially agree with those from similar studies previously published (8,9,11,12,16,17) dealing with either cell- or antibody-mediated lysis of LCM virus-infected murine cells. Holtermann and Majde (8) failed to report a difference in susceptibility to cell-mediated lysis between acutely or chronically infected cells. This discrepancy may relate to the different strain (CA1371) of virus employed and also to the fact that cytotoxicity was calculated after co-culturing lymphocytes and target cells for 48 to 72 h, a method which is generally less quantitative and which may measure, in addition to the specific activity of terminally differentiated effector T-lymphocytes, that associated with toxic lymphocyte by-products (16). Lehmann-Grube and co-workers (11) were unable to detect antibody-mediated lysis of L cells chronically infected with LCM virus. However, the cells had been passaged approximately 200 times and, therefore, were not directly comparable to the chronically infected cells used in the present study. Oldstone and Dixon (17), using ^{51}Cr-labeled acutely infected (CA1371) target cells, found that susceptibility to antibody-mediated lysis was a function of the percentage of cells with immunofluorescent staining surface antigen, a correlation which we observed only with respect to cell-mediated lysis. Since cytolysis of infected cells, whether by antibody or lymphocytes, must obviously depend on the presence of surface antigen(s), the variance in results may relate to differences in the specificities of the anti-viral antisera used in the immunofluorescent staining procedure.

The work presented here has additional bearing on the requirements for eliciting cell-mediated immunopathology in vivo. Besides the participation of T-lymphocytes, it would seem that a prime requisite is the presence of infected cells at a tissue site, such as the central nervous system, with the appropriate viral antigen(s) at their surfaces.

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Histocompatibility and Lymphocytic Choriomeningitis Virus Infection

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SUMMARY

Transfer of skin grafts from mice chronically infected with LCM virus to uninfected syngeneic recipients resulted consistently in graft rejection. Second-set skin grafts from infected donors were rejected in an accelerated fashion. Also rejected were cells of a transplantable adenocarcinoma when transferred from infected donors to uninfected recipients. Lymph node cells of mice immunized with LCM virus were cytotoxic in vitro towards mouse L cells infected with the virus.

It is suggested that cells infected with LCM virus exhibit viral or virus-directed antigens in or on the cytoplasmic membrane. An immune response directed towards such antigens is considered to be responsible for the observed transplant rejections and the in vitro cytotoxicity of lymph node cells of immunized mice. It is probable that the observed immune phenomena play a role in the pathogenesis of LCM disease.

INTRODUCTION

A contribution by immunity to disease pathogenesis has been recognized in a number of microbial infections. In viral infections this contribution may have two major facets, one related to extracellular viral antigenic material, and another related to virus-derived antigenic determinants associated with infected cells.

Immune complexes consisting of extracellular viral antigen and specific antibody have been incriminated in the pathogenesis of glomerulonephritis in chronic infections with lymphocytic choriomeningitis virus (10,11). Since virus-infected cells may exhibit viral or virus-directed antigens in or on the cytoplasmic membrane, it is conceivable that an immune response directed against such antigens may also be a factor in disease pathogenesis.

The possibility that host immunity may have detrimental effects on tissues or cells infected with LCM virus was investigated in vivo by the use of skin transplantation and tumor cell transplantation as well as in vitro by the interaction of infected cells with lymphocytes of donors immunized with LCM virus. The results of these investigations are presented below.

MATERIALS AND METHODS

The materials and methods used have been described in details elsewhere (5,6).

Virus. LCM virus, strain CA 1371, was obtained from Dr. C.J. Pfau, University of Massachusetts. Stock virus was prepared in mouse L cell tissue culture. Virus titrations were carried out by intracerebral inoculation of weanling Swiss-Webster mice.

Animals. Inbred mice of the strains SWR ($H\text{-}2^b$) and C57BL/6J ($H\text{-}2^b$) were obtained from the Jackson Laboratory, Bar Harbor, Maine. C3H/He ($H\text{-}2^k$) and CFW mice were of Lobund stock. LCM virus carrier animals were established by injection of less than 24 h old mice with the virus. Congenitally infected mice were the offspring of neonatally infected parents.

Skin grafting and tumor transplantation. Skin grafting was carried out essentially as previously described (6). Generally each recipient mouse received an autograft on one side of the back and an experimental graft on the other.

An adenocarcinoma which arose in a normal SWR female breeder was used for the tumor transplantation studies. Two lines of the tumor were established, one by passage in normal uninfected mice, and a second by passage in mice congenitally infected with LCM virus. Serial 10-fold dilutions of single cell suspensions were injected via the subcutaneous route and the animals observed for at least 90 days for the appearance of tumors.

In vitro interaction of lymph node cells and infected target cells. Lymph nodes were harvested from axillae, mesenterium, and the retroperitoneal space. Cells, predominantly lymphocytes, were collected after teasing the nodes against a metal grid. Mouse L cells were used after exposure to LCM virus for 48 h or after they had carried the virus for more than 4 months. Lymph node cells and L cells were cocultivated in a water-saturated atmosphere containing 5% CO_2 for 48 or 72 h. The remaining L cells were counted in a hemocytometer.

RESULTS

Skin grafting. As a first approach to the question of a possible effect of an immune response on tissues or cells infected with LCM virus, skin grafts were transferred from virus carrier SWR donors to uninfected syngeneic recipients. In addition to an experimental graft, each recipient also received an autograft as a technical control. Other uninfected mice received skin grafts from uninfected syngeneic donors. The results of these experiments are presented in Table 1. It will be seen that isografts from infected donors were consistently rejected by uninfected recipients. This rejection was evident on the 10th day after grafting and by day 14 the infected grafts were generally necrotic and sloughing off. Isografts from non-infected donors were generally accepted. In a number of recipients of infected isografts the autografts were also rejected. The rejection of these autografts was thought to be due to cross infection from the infected isografts. This possibility was tested by injecting LCM virus in close proximity of autografts of uninfected animals. Of a total of 8 such autografts 4 were rejected.

Table 1. Survival of skin grafts from LCM virus carrier donors or uninfected donors to uninfected syngeneic recipients[a]

Status of isograft donors	No. of isografts accepted/No. grafted	No. of autografts accepted/No. grafted
Uninfected	18/18	18/18
Infected	0/20	7/20

[a] Each mouse received 2 grafts: an autograft and an isograft.

An immune reaction was considered likely to be the basis for the observed graft rejections. Further support for this view was obtained by regrafting animals which previously had rejected an infected isograft with isografts from infected donors. These grafts were observed for evidence of accelerated graft rejection. Criteria for accelerated graft rejection were lack of vascularization and necrotic edges on the 6th day after grafting. The results of these transplantations are presented in Table 2. It can be seen that 11 of 12 such second-set infected grafts underwent accelerated rejection. No evidence of rejection of the autografts was noted in these animals. Possibly the immunization of the animals by the first set of infected grafts prevented spread of the virus to the autografts. The isografts of the non-immune control mice, in these experiments, which also received transplants from infected donors, had clearly started to "take" on the 6th day as

Table 2. Evidence of accelerated rejection of skin grafts from infected donors by recipients previously grafted with infected skin[a]

Status of recipients	No. showing accelerated rejection/ total No. grafted	
	Isografts	Autografts
Previously received infected grafts	11/12	0/12
Previously received non-infected grafts	0/11	0/11
Not previously grafted	0/9	0/10

[a] The mice received 2 grafts: an autograft and an isograft from an infected donor. The condition of the grafts was judged after removal of the bandages on the 6th day after grafting. Necrotic edges and blanching were considered evidence of accelerated rejection. The time intervals between 1st and 2nd transplantations were 8 to 10 weeks.

judged by their pink color and healthy appearance. This observation suggested that mere infection of the graft per se was not sufficient to result in graft rejection. However, such infected grafts were uniformly rejected on the 14th day (Table 1), indicating that a mounting immune response was the effector in the graft rejections. Accelerated rejection of infected isografts was also noted in recipients which previously had been immunized with a subcutaneous injection of LCM virus.

Control transplantations, in addition to those already mentioned, consisted of skin grafts from congenitally infected donors to congenitally infected recipients as well as grafts from uninfected donors to congenitally infected recipients. These grafts were uniformly successful.

The competence of congenitally infected SWR mice with respect to homograft rejection was tested by transfer of skin from either C3H/He (a major histocompatibility barrier) or C57BL/6J (a minor histocompatibility barrier). These grafts were rejected within the same time span by congenitally infected and uninfected SWR mice.

Tumor transplantation. The results of the skin grafting experiments indicate that an immune reaction was involved in the rejection of infected grafts by uninfected recipients. However, the experiments do not conclusively point to the grafted tissue as being the target for this immune reaction. Conceivably, an immune reaction involving extracellular virus and antibody, taking place in the graft bed, might interfere with the survival of grafted skin. Further transplantation studies were, therefore, carried out using a transplantable SWR adenocarcinoma. Two lines of the carcinoma cells were used, one line which was maintained in uninfected animals, and a second line which was maintained in congenitally infected animals. The tumor cells were transplanted in graded numbers in the following types of experiments: 1) uninfected cells - uninfected recipients, 2) uninfected cells - infected recipients, 3) infected cells - infected recipients, and 4) infected cells - uninfected recipients. The animals were observed for appearance of tumors for at least 90 days after transplantation. The results of these experiments are presented in Figure 1 as a graph correlating the numbers of tumor cells transplanted and the time after transplantation at which palpable tumors were noticed. It can be seen from Figure 1 that little or no difference was noticed in the behavior of the tumor cells in the combinations uninfected cells - uninfected recipients and infected cells - infected recipients. Some lag in the time of appearance of tumors was noted in the combination uninfected cells - infected recipients, particularly when low cell inocula were used. In the combination infected cells - uninfected recipients, the tumor cells were rejected unless very high cell numbers (in excess of 10^5) were transplanted. Even at these large cell doses tumors developed in less than 100% of the recipients and only after extended lag periods.

Some observations were made during the tumor transplantation experiments that are not illustrated in Figure 1. A few uninfected animals which received 2×10^6 infected cells or more developed tumors within a lag period comparable to those of the other experimental groups. However, the tumors regressed within 10 days or less. Microscopic examination of these tumors revealed necrosis of the tumor mass and a mononuclear infiltrate.

The tumors which appeared in uninfected recipients of large doses of infected cells after a prolonged lag period were tested for presence of LCM virus. No virus was detected. Some of these delayed tumors were transplanted to other uninfected as well as infected recipients.

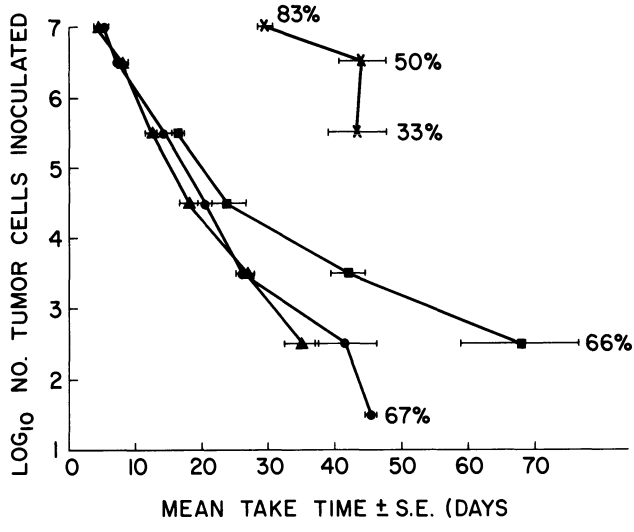

Fig. 1. On day 0, groups of mice were inoculated with the number of infected or uninfected tumor cells indicated on the ordinate. The mean time for appearance of tumors is indicated on the abscissa. The points represent the following tumor cell-recipient combinations: circles, uninfected tumor-uninfected recipients; triangles, infected tumor-infected recipients; squares, uninfected tumor-infected recipients; crosses, infected tumor-uninfected recipients. The percentage of takes is given adjacent to the respective points; undesignated points represent 100% take. Standard errors (SE) are indicated for each point.

In both groups of recipients these cells produced progressively growing tumors. The uninfected recipients of cells from delayed tumors did not show immunity to intracerebral challenge with a lethal dose of virus 2 weeks after tumor cell inoculation. When propagated in vitro, cells from delayed tumors did not release any demonstrable virus; under such conditions tumor cells from chronically infected animals consistently produced virus.

Cytotoxicity of lymph node cells in vitro. Infection of mouse L cells with LCM virus in vitro results in a transient slight reduction in cell viability. However, within a few weeks after infection a chronic virus carrier state is established without detectable change in cell multiplication rate (8). CFW mice were injected intraperitoneally with plasma from a chronic virus carrier of the same strain. Seven days later lymph nodes were harvested from these as well as from normal mice. Suspensions of lymph node cells were co-cultivated with infected L cells at a ratio of 50 lymph node cells to 1 L cell. At 48 and 72 h the L cells were counted. In a series of 3 experiments, the lymph node cells of immunized donors consistently had growth inhibiting and cytotoxic effects on the infected L cells. The results of one of the experiments are presented in Table 3. These findings provide further evidence in support of the view that an immune reaction directed against LCM viral antigens may have cytotoxic effects on cells infected with LCM virus.

Table 3. Destruction of L cells infected with LCM virus by lymph node cells of mice immunized with the virus[a]

Status of L cells	Status of lymph node donors	No. of L cells at hour 0	48	72
Infected for 48 h	Normal	110,000	101,000	133,000
	Immunized	110,000	49,000	45,000
Chronically infected[b]	Normal	125,000	124,000	218,000
	Immunized	125,000	72,000	64,000

[a] Immunized mice were given 0.1 ml of plasma intraperitoneally from a chronic virus carrier mouse 7 days prior to the experiment.

[b] Cells had been carrying the virus for more than 4 months.

DISCUSSION

The experimental results presented above demonstrate that tissues and cells infected with LCM virus are rejected by syngeneic recipients. The rejection mechanism is likely to be related to an immune response directed against viral or virus-directed antigens associated with the cytoplasmic membrane of infected cells.

Skin grafts from mice congenitally infected with LCM virus were consistently rejected by syngeneic uninfected recipients. The accelerated rejection of second-set infected grafts by recipients that previously had rejected infected grafts indicates that an immune response was responsible for the graft rejections. The target for this immune response is not clear from the skin grafting experiments. One possibility to be considered is that the immune response was directed against antigenic determinants on the grafted tissue. However, since the infected grafts most likely were releasing antigenic viral material, an immune reaction involving such antigens taking place in the graft bed conceivably could have interfered with the delicate interaction between graft and host, including vascularization of the transplanted tissue, leading to graft rejection.

The results of the experiments dealing with infected and non-infected cells of a transplantable adenocarcinoma were in agreement with the results of the skin grafting experiments. The difficulties in interpretation of the results of the skin grafting experiments are less likely to pertain to the tumor transplantation experiments. Thus, the rejection of dispersed infected tumor cells by uninfected recipients points more strongly to a host reaction directed towards the infected cells themselves as being the basis for their rejection by uninfected animals.

The in vitro destruction of infected L cells by lymph node cells of mice which previously had been injected with LCM virus points to cell-mediated immunity as part of the mechanisms for rejection of infected cells and tissues by uninfected hosts. These observations confirm and extend similar findings by Lundstedt (9). In addition to cell-mediated immunity, the in vivo elimination of infected cells may also involve specific antibody.

Possibly related to our findings is the observation by Breyere and Williams (1) of rejection of skin grafts of mice infected as neonates with a murine leukemia virus by syngeneic uninfected recipients. However, since leukemic cells were encountered in a number of the grafts, it is not clear whether the graft rejections were due to rejection of these neoplastic cells or due to rejection of infected normal tissue. Probably more closely related to our findings are the observations of Svet-Moldavsky et al. (13) of rejection of syngeneic skin grafts of mice after infection of the donors with an unidentified non-pathogenic virus which had been isolated from a transplantable tumor.

The antigens responsible for the transplant rejections and the in vitro destruction of infected cells have not been defined. Analogous to cells transformed by integrated oncogenic viruses, cells infected with LCM virus may carry virus-directed antigens distinct from viral capsid antigens in or on the cytoplasmic membrane. Alternatively, virus particles budding from the cytoplasmic membrane may represent the target for the immune response involved in the transplant rejections.

An association of virus-specific antigens with the cytoplasmic membrane may be a common occurrence in moderate non-oncogenic virus infections. Such membrane-associated antigens are likely to have been the target in the cytotoxic interaction in vitro between cells infected with influenza or mumps virus and lymphoid cells of animals immunized with the respective viruses as reported by Speel et al. (12).

Mice congenitally infected with LCM virus are considered by several authors (2,7,14) to be immunologically tolerant to the viral antigens. However, recently antibody to the virus has been demonstrated in neonatally infected virus carrier mice (10), and it has been pointed out that such animals are, therefore, not tolerant to the virus. The magnitude of the immune response is related to the time after birth at which the animals were exposed to the virus (4). Our studies indicate that tissue cells of healthy chronically infected animals exhibit viral or virus-directed surface antigens. If an active immune response to these antigens were to exist in such animals, some mechanism curtailing its effect must be present. One such mechanism could be blocking antibody interfering with the expression of cytotoxic activity of specifically sensitized leukocytes or cytotoxic antibody. Alternatively, a situation of split tolerance (3) could be envisioned in which non-cytotoxic antibody is present in the absence of cytotoxic antibody or cytotoxic cell-mediated immunity.

Immunity undoubtedly plays an important role in the pathogenesis of disease in acute as well as chronic infections with LCM virus (7). The glomerulonephritis associated with persistent infection of some strains of mice has been related to immune complexes consisting of viral antigen and specific antibody (10,11). Our experimental results demonstrate that virus-infected counterparts of transplantable tissues and cells are rejected by uninfected syngeneic recipients. The target for the rejection mechanism is considered to be viral or virus-directed antigens in or on the cytoplasmic membranes of infected cells. An immune response directed against such antigens is likely to be a contributing factor in the pathogenesis of disease both in acute and chronic LCM virus infections. A similar mechanism may play a role in the pathogenesis of other diseases of viral etiology.

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Persistent Infection in Vitro and in Vivo, Including Interference Phenomena

Chairman: J. Hotchin

Cyclical Transient Infection, An Explanation of Persistent Virus Infection

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INTRODUCTION

The relationship between LCM virus and a population of persistently infected cells has long been enigmatic. In the LCM virus-infected mouse, immunofluorescent examination of tissue sections has established (1,4, 12,13,24) that a considerable percentage of cells of any tissue type does not show fluorescence. This is explainable either in terms of an advancing but incomplete infection or as a result of partial eradication of the virus by the recovery mechanisms. But in the persistently infected, immunologically tolerant animal these modifying factors are inoperative; the infection has had plenty of time to reach saturation, and there is no detectable cellular or virus-neutralizing immune response. How, then, do significant numbers of cells remain free of antigens?

Traub (17) postulated a special property of tissues of animals persistently infected with LCM virus to explain their resistance to superinfection in the absence of humoral or cellular immune response. Rowe (16) noticed that live, but not heat-killed, viscerotropic LCM virus conferred protection against a more virulent neurotropic strain; and interference (19) was suggested as an explanation for this and the similar resistance of congenitally infected animals. An apparently similar type of specific "immunity" of chronically LCM virus-infected strain L mouse fibroblasts to the usual effects of LCM virus was described by Benson et al. (3). As a result of observations of minimal specific fluorescence in mice with persistent tolerant LCM virus infection (PTI), Mims and Subrahmanyan (13) and Wilsnack and Rowe (24) suggested that the infective process was limited by some form of autointerference. Yet challenge virus did not grow in normally susceptible cells (macrophages, Kupffer or ependymal cells) of LCM virus PTI mice, even though these completely lacked any detectable immunofluorescence (13) or interferon. Other workers have failed to detect interferon in LCM virus PTI mice (14,20) or in persistently LCM virus-infected cultures (10). Virus other than LCM was able to multiply in the LCM virus-resistant cells (13).

These observations suggest that LCM virus can induce a state of specific resistance in cells in which the virus infection seems to be regulated in some way (5). When it was noticed (6,7) that a shutdown of LCM virus antigen synthesis occurred a few days after infection of cells infected in vitro with LCM virus, it seemed likely that the above observations might all be explained by the shutdown mechanism, which was therefore studied in more detail.

MATERIALS AND METHODS

Immunofluorescence. A quantitative indirect immunofluorescent technique (2) for detecting LCM virus antigens, with standardized fluorescent conjugate and standardized human convalescent anti-LCM virus serum, was used to follow the development of LCM viral antigens in infected cells.

Preparation of infected cells. Strain L mouse fibroblasts were infected with LCM virus (UBC variant of the WE strain) using an L cell-grown pool LM_4/L_1 containing 2 x 10^7 PFU/ml of the turbid plaque variant (9). The virus pool was harvested 2 days after infection with a low multiplicity inoculum of a turbid LCM virus plaque, passed once in L cells. This procedure was used to avoid interference from defective virus particles. The cells were seeded by a standard method. A 6 cm plastic Petri dish was inoculated with 4 x 10^5 cells in 8 ml of Eagle's medium containing 10% fetal bovine serum and antibiotics. After 2 days of CO_2 incubation, this monolayer was trypsinized, and 2 x 10^5 cells were used to similarly seed a 2nd Petri dish containing coverslips. After CO_2 incubation for 24 h, the medium was removed and saved; 0.5 ml of LM_4/L_1 LCM virus was added and adsorbed for 2 h at 37°C with gentle agitation of the fluid every 15 min. After adsorption the medium was replaced and the culture incubated as before. Titration of the fluid at this time showed that less than 5% of the virus had been adsorbed by the cells, giving a multiplicity of infection of <1. Coverslips were removed at 8 h intervals and stained for immunofluorescence. When the infected monolayer was almost confluent, it was gently trypsinized and reseeded in fresh medium at 2 x 10^5 cells/dish to maintain logarithmic cell growth. Immunofluorescence of the cells was evaluated on a scale of 0 to 4 degrees of intensity of the typical punctate cytoplasmic LCM virus pattern. Cells showing intensities of >1 fluorescence were counted as positive. A total of at least 500 cells were counted on several widely separated, representative fields for each coverslip.

Harvesting of L cell clones for immunofluorescence. Normal or LCM virus-infected L cells were diluted serially and plated in 0.1 ml amounts onto the surface of agarose monolayers (0.5% agarose in Eagle's minimum essential medium containing 10% fetal bovine calf serum and freshly added antibiotics and glutamine); numerous colonies of L cells appeared a few days later. When a sterile coverslip was dropped onto an area of colonies, the cells were found to transfer and spread out on the glass within a period of 2 h. By this means, colonies could be sampled and stained for virus-specific immunofluorescence.

Suspensions of infected and normal cells for analysis of virus production. Suspensions of infected or normal cells were prepared by the method described above. For comparisons of cloning efficiency, optical counts were made on 6 replicate aliquots of suspended cells, and clone counts were made on 6 to 10 replicate agarose plates. Before cloning, both infected and normal cell populations were brought to the same density in L cell medium. In the later experiments, an aliquot of the cell suspension was seeded onto one or more coverslips, and the percentage of infected cells was determined by immunofluorescence at 12 to 15 h after inoculation. One-step growth curves had shown that the latent period of this virus in L cells (under these conditions) was 12 h. In the final technique, cells were removed from a 6 cm dish with 0.3 ml of trypsin versene salt mixture (0.2% versene and 0.5% trypsin in divalent cation-free, bicarbonate-buffered balanced salt solution diluted 1:2 with the same salt solution) which removed approximately 90% of the cells within 3 min at room temperature. The process was

watched under the microscope, and 5 ml of L cell medium were added as soon as about 95% of the cells were detached.

RESULTS

Production of LCM virus antigen in L cell cultures as revealed by immunofluorescence. The variation in the percentage of fluorescent cells prepared and harvested as described above was plotted with respect to time (Fig. 1). The results confirmed the previous observation that the

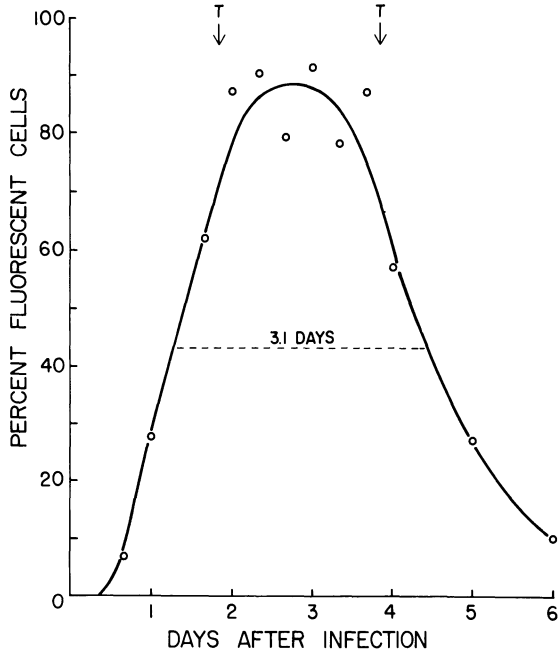

Fig. 1. Percentage of fluorescent L cells at different times after infection with LCM virus. T = trypsinization and transfer of the cells.

percentage of fluorescent cells reached a peak about 72 h after infection and then rapidly declined to a very low level. At this time, the medium contained virus at a titer of approximately 10^7 PFU/ml, and it caused the same rapid development of fluorescence and virus production when placed on a control (uninfected) L cell monolayer of the same age and cell density as the experimental monolayer. The experimental monolayer could not be reinfected (as judged by immunofluorescence) either with fresh LCM virus of turbid or lytic plaque type or with wild type virus. This refractoriness was maintained for at least 50 transfers of the chronically infected culture.

It was concluded that the LCM virus infectious cycle in L cells passes through an initial virus-productive stage in which the cell fills up with viral antigen. This stage is followed by a shutdown of further antigen production and a depletion of intracellular viral antigen, leaving the cell resistant to LCM virus reinfection but indistinguishable from an uninfected cell by microscopical appearance and immunofluorescence.

The antigen-containing period of infected cells lasted for an average of 3.1 days. At this time no significant (<5% of cells) cytopathic effect could be seen; all cells remained adherent to and spread out on the coverslip, and all stained supravitally with neutral red. When fresh, uninfected L cells were added to a persistently infected culture after shutdown, they became infected and immunofluorescence-positive in the usual way. However, in other experiments, shutdown cells could not be rendered fluorescent by superinfection with fresh virus (Fig. 2). When culture fluid from a persistently infected monolayer was added to a normal L cell monolayer (Fig. 3), this developed a

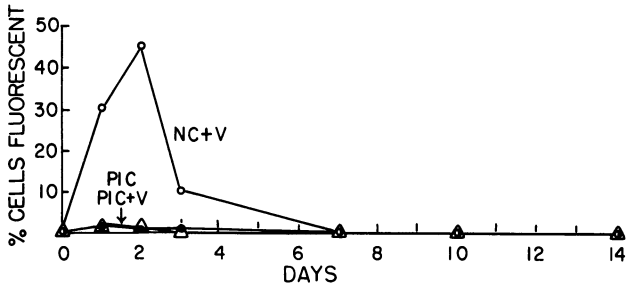

Fig. 2. Development of fluorescence after addition of MB_6L_{11} LCM virus to normal L cells (NC + V) or persistently infected L cells (PIC + V). The level of fluorescence in persistently infected L cells without virus challenge (PIC) is identical to PIC + V, as shown.

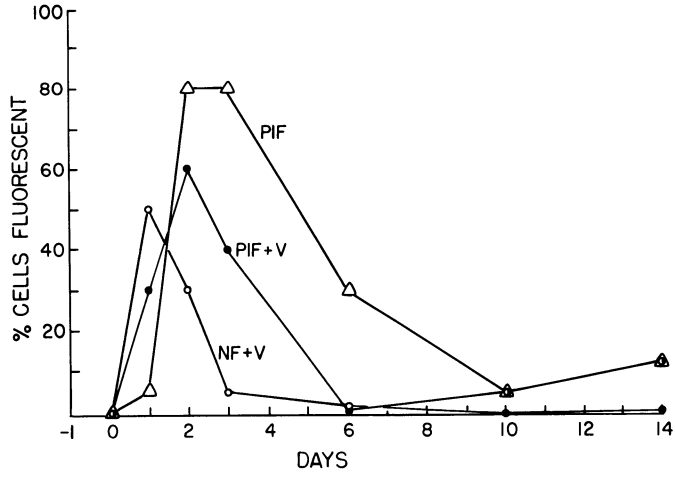

Fig. 3. Development of fluorescence of normal L cells after addition of 1 ml of fluid from a persistently infected culture plus 1 ml of normal fluid (PIF), or 1 ml of fluid from a persistently infected culture plus 1 ml of MB_6L_{11} LCM virus (PIF + V), or 1 ml of normal fluid plus 1 ml MB_6L_{11} LCM virus (NF + V).

greater degree of fluorescence than when persistently infected fluid plus virus - or fluid from normal cells plus virus - was added. Thus the shutdown state appeared to be due to an intracellular phenomenon

resulting from infection (as judged by immunofluorescence) and not due to an extracellular factor such as interferon or non-"infectious" virus. The results also indicated that, surprisingly, the stock mouse liver-passed virus (MB_6L_{11}) of the same strain and plaque type contained more inhibitor (defective virus) than the fluid from persistently infected cultures. Tests for interferon in the medium by vesicular stomatitis virus (VSV) plaque reduction (kindly performed by Dr. Paul Came, Virology Department, Schering Corp., Bloomfield, New Jersey) failed to detect any, and VSV produced plaques of slightly greater size on the LCM virus-shutdown L cells than on LCM virus-free control L cells.

Immunofluorescent study of LCM virus-infected L cell clones. In a series of experiments, log-phase L cell monolayers were grown in 6 cm plastic Petri dishes and infected by the procedure described under Materials and Methods. The cloning efficiencies of control, normal, and LCM virus-infected L cells were found to vary somewhat in different experiments, and the relative efficiency of cloning of infected, compared to uninfected, cells ranged from 43 to 100% (see Table 1).

Table 1. Cloning efficiency of normal and LCM virus-infected L cells

Experiment	% optical count/clone count		% infected/ normal	% fluorescent cells	% infected centers
	Normal cells	Infected cells			
1[a]	n.d.[b]	49		n.d.	n.d.
2[a]	96	73	76	n.d.	n.d.
3	64	27.5	43	n.d.	n.d.
4	76.3	69.4	91	57	n.d.
5	55	69	100	12	n.d.
6	73	76	100	40	32[c]

[a] Liquid overlay (no agarose).

[b] Not done.

[c] Supernatant fluid showed less than 1% plaque count.

The relative cloning efficiency of infected cells tended to improve with successive experiments, reaching 100% of the normal cell value as the trypsinization technique was improved.

The results showed that LM_4 LCM virus-infected cells were not killed by the virus but were as capable of forming colonies as normal control cells. When serial dilutions of infected clones were examined by immunofluorescence microscopy, using the coverslip technique to remove the clones from the agarose base layer, the fluorescent cells were seen to be dividing, and for the first few cell divisions the whole clone was equally fluorescent. The percentage of fluorescent clones was greatest in clones derived from plates with the highest inoculation

cell density; in successive 10-fold dilutions the percentage of fluorescent clones decreased. Different clones varied in the intensity of fluorescence, and this difference became greater after the 2nd or 3rd day following cloning. Thereafter the cells of clones ceased to be of uniform fluorescence; as the fluorescence decreased and shut down in certain clones, it was clearly increasing in others. Infection appeared to spread from the most fluorescent clones to adjacent ones which had shut down and become susceptible again.

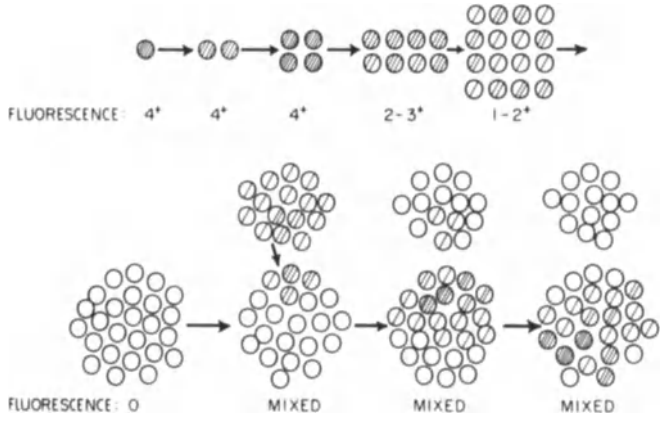

Fig. 4. Diagram of the sequence of changes in virus-specific immunofluorescence of clones of LCM-infected L cells. The intensity of fluorescence of individual cells is indicated by the degree of cross-hatching. The average fluorescence of clones is shown by numbers where this seems meaningful. Horizontal arrows indicate the sequence of events. The oblique arrow shows the presumed transfer of virus from an infected clone to one that has shut down and become susceptible again. The sequence continues cyclically.

The overall impression was that clones go through repeated cycles of fluorescence, shutdown, cell recovery, and reinfection (Fig. 4). This sequence was most rapid in plates with high clone densities and was slower in the more dilute plates. Attempts were made to isolate clones and prevent reinfection; however, this could only be carried out by separating the clones by dilution of the seed cell population. Under these conditions, sampling rates became prohibitively low, with consequent loss of statistical significance of the results. The addition of more agarose or methyl cellulose to the basal medium failed to prevent the apparent cyclical reinfection process; neutralizing antibody was not used, since this factor would have complicated the interpretation of the results. The above conclusions regarding the transient nature of virus antigen production could only be regarded as inferred and not proved. To further substantiate the immunofluorescent evidence for cell recovery, clones were tested for the presence of virus.

Production of infectious virus by clones of LCM virus-infected L cells grown on agarose. Colonies of cells from the previously described immunofluorescence experiments were individually picked and transferred to tubes containing 1 ml of L cell medium. After luxuriant cell growth was established (2 to 4 days), the fluid was tested for virus by mouse inoculation, followed 7 days later by endotoxin challenge (Hotchin, to

be published). This method has been found to simplify LCM virus detection and eliminate false negative results. The results are shown in Table 2. When acutely infected cells were used (prepared as described earlier), only 15% of the clones were found to be producing virus when harvested 22 days after infection. When persistently LCM virus-infected L cells (passage 18 after infection) were used, the proportion of virus-producing cells was usually less than 1% except when cloned shortly after trypsinization and reseeding; at this time the percentage rose. The percentage of virus-producing clones was roughly proportional to the percentage of fluorescent cells in the persistently infected cultures; and in the acutely infected cultures the percentage of virus producers was much less (15.5) than the percentage of fluorescent cells (53.2) at the time of cloning. Older colonies (4 to 6 weeks) were invariably virus-free, although the colonies still transferred to coverslips and stained with neutral red. This suggested that the infection died out in non-replicating cells.

Examination of the colonies by low-power microscopy revealed several different morphological colony types. These were roughly grouped into 9 different categories, and 200 colonies from infected and non-infected cells were classified to determine whether there was any obvious correlation between virus production and colony type. No such correlation was seen. One colony type was noticed only in the infected cell clones, and this colony type had a high incidence of virus persistence, but virus was also found in other colony types. Lack of any significant difference in the distribution of colony types suggested that colony type is no indication of infection or persistence. However, the sampling of colonies was not randomized in any way, and a disproportionate number of "interesting-looking" colonies may have been unconsciously selected, particularly from the infected plates.

The results of these experiments were all consistent with the idea that infection of cells with the turbid plaque variant of LCM virus is a self-limiting process, resulting in a brief period of virus production followed by shutdown of the synthesis of virus antigens and final recovery of the cell. The low proportion of fluorescent cells and virus in persistently infected cultures and the inability to reinfect freshly shutdown cultures with LCM virus indicated that the shutdown process is followed by a refractory, LCM virus-resistant phase. However, the results did not show that cells destined for shutdown and recovery were the same ones which produced infective virus, and it is possible that shutdown and recovery were due to some form of abortive infection, possibly induced by defective virus particles which could not initiate the production of infective progeny. In order to resolve these uncertainties, the virus production of cloned infected cells was examined in greater detail.

The virus production of LCM virus-infected L cell clones isolated in liquid medium. Since it was not possible to grow significant numbers of clones (approximately 100) in isolated batches on agarose in Petri dishes, other methods were examined. A useful method was obtained by planting microdrops of 0.025 ml, delivered by a Microliter pipette dropper (No. 220-5, Cooke Engineering Co., Alexandria, Virginia) into 6 mm cups in a series of disposable sterile plastic trays (No. IS-FB-96-TC, Multi-dish-dispo-trays, Linbro Chemical Co. Inc., New Haven, Connecticut). Each tray contained 96 cups, and 40 trays (3,840 cultures) were used in most experiments. Each cup had previously received a volume of 0.125 ml (sometimes a larger volume) of agarose medium. The trays (with lids) were incubated inside semi-airtight plastic boxes containing wet filter paper, within a humidified CO_2 incubator.

Table 2. Infectivity of agarose colonies from clones of LCM virus-infected L cells

Type	Colony age at harvest (days)	Number of colonies tested	Virus-positive colonies	% virus-positive colonies	% fluorescent cells at cloning	Approx. virus titer at cloning PFU/ml
Acute	23	84	13	15.5	56.7	10^7
Acute	22	46	7	15.2	63.0	10^7
Acute	22	50	8	16.0	40.0	$5x10^6$
	Total	180	28	15.5	53.2	10^7
Persistent	8-11	35	0	0.0	1-3	10^4
Persistent	5[a]	15	4	27.0	10-20	10^5
Persistent	8	16	1	6.0	1-10	10^4
Persistent	21	16	0	0.0	1-10	10^4
	Total	82	5	6.1	1-10	10^4-10^5

[a] Cells harvested 2 days after transfer.

Cell concentrations were chosen on the basis of the Poisson distribution,

$$f(x) = Ne^{-\mu} \frac{\mu^x}{x!}$$

(where x = number of cells per cup, N = proportion of cups receiving x cells, and μ = cell density or mean number of cells per cup). Since it was very important to avoid cyclical reinfection within individual cups, it was imperative to use a cell density which gave a useful yield of cups with single cells but relatively few cups with multiple cells. For cell densities of 0.5 or 1.0/0.025 ml of inoculum the percentage of cups receiving 1 cell only is about the same, namely 30.3% or 36.8%, while the percentage of cups receiving multiple cells ranges from 9.0% to 26.4%, respectively; these 2 values of μ were chosen for the experiments and were equivalent to a cell suspension containing 20 or 40 cells/ml.

The basic experimental procedure allowed the growth of several hundred cultures from single virus-infected cells. Replicate batches of these were then tested for virus production on successive days in order to determine whether productively infected cells underwent shutdown and full recovery from infection. The source of infected cells was the same as in the preceding experiment in which L cells were harvested 40 h after infection with the turbid plaque type LM_4 LCM virus variant. The percentage of infected cells at 40 h post-infection was monitored by immunofluorescence count, and the trypsinized suspension in L cell medium was counted 6 times in separate hemocytometer counts. The average result was used to determine the dilution necessary to bring the cell density to 20 (or 40) cells/ml. The drop size was checked by weighing a known number of drops, and the number of viable cells delivered was checked by dropping a total of 100 drops onto a total of 10 nutrient agarose layers in Petri dishes and counting the resulting colonies.

The virus plaque titer of the final suspension was measured and found to range from 6 to 10 times the cell count. Repeated washing of the cells did not reduce this appreciably, even when performed at 0°C. Repeated assay of infective centers (virus-producing cells) at different times after infection gave the same figures as those for fluorescent cells. However, at the 40 h stage of infection used, many of the cells could release virus when agitated at 0°C, rendering infectious center counts unreliable (greater than 100%).

In order to detect the virus released by the single cells present but not visible in a proportion of the cups after cloning, a method was devised which utilized amplifier cells to enhance the potentially very low virus level in the 0.025 ml volume of fluid surrounding the cell. The amplifier cells were L cells (BHK21 cells worked equally well), added in a volume of 0.05 ml/cup from a pipette dropper using a cell density of 5×10^5 cells/ml. This cell density formed an almost confluent monolayer in each cup, which became confluent within 2 days, making infection of this layer from the clone virtually certain if virus was being produced. The trays were incubated for 2 days for virus amplification to occur, then stored at -80°C until used for mouse inoculation.

Initially 3 mice were used for each cup, but the number was reduced to 1/cup after results from several hundred samples showed that in 99% of cases all 3 mice succumbed to the virus if any one of them did. One or more trays, each with 96 cups, were sampled for virus at suitable intervals after seeding with clones.

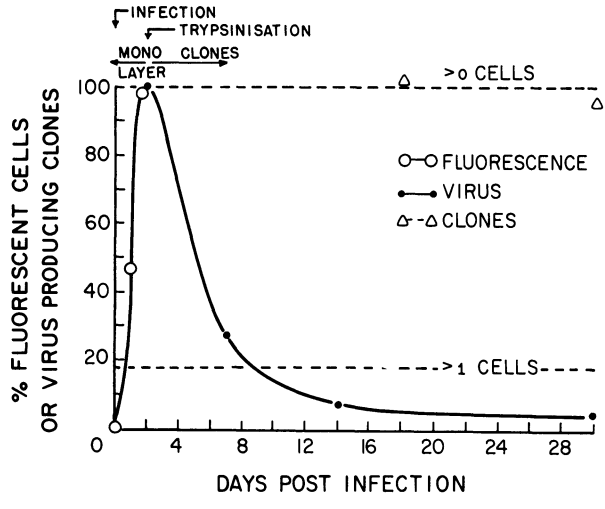

Fig. 5. Changes in % virus-producing clones with respect to time after cloning. The first part of the curve shows the increase in % fluorescent cells after infection (at time 0) of a monolayer of L cells until cloning at 40 h post-infection. Each point represents 96 cups. The original suspension of infected cells showed 98% fluorescent cells and contained 20 cells/ml. Each cup received 0.025 ml (μ = 0.5). For 100% cloning efficiency, Poissonian distribution would give 39.4% of cups with cells; the found value was 32.5%, equivalent to a cloning efficiency of 79% (corrected for cultures arising from multiple cells). The dotted lines show the % cultures arising from >0 and >1 cells, corrected for a 78% cloning efficiency. The values for cell growth were determined by counting the % cups with visible cells after neutral red staining of 2 trays each on days 14 and 30. (Taken from ref. 8 by permission of the editor of Nature New Biology.)

The results of a typical experiment are shown in Figure 5. The curve fits the concept that initially the cells produce virus but after a few days the majority cease to produce virus and give rise to virus-free clones. To strengthen the significance of these results, the data from 3 similar experiments were combined (Fig. 6), representing a total of 22 points from over 2,000 cultures. The cells average 95% immunofluorescent at the time of cloning. A curve of the same shape was obtained. In 2 experiments normal cells were also treated the same way, and cloning efficiencies were only slightly higher (10 to 12%) than the values for infected cells. Cloning efficiency was found to be somewhat higher (about 10%) on agarose Petri dishes than in the cups for both normal and infected cells; however, this may have resulted from the difficulty of seeing very small clones in the cups, even when neutral red vital staining was used.

An additional experiment was performed to eliminate the possibility that some of the virus detected in the early points after cloning was due to free virus in the medium, rather than to virus released from an infected clone. In this experiment (Fig. 7) the supernatant fluid from an aliquot of the cell suspension (after centrifugation at 2,000 rev/min for 15 min) was dispensed into the cups of 3 trays in the same way as the cell suspension. This experiment was performed using a line of L cells developed from a large clone (designated F6) which grew luxu-

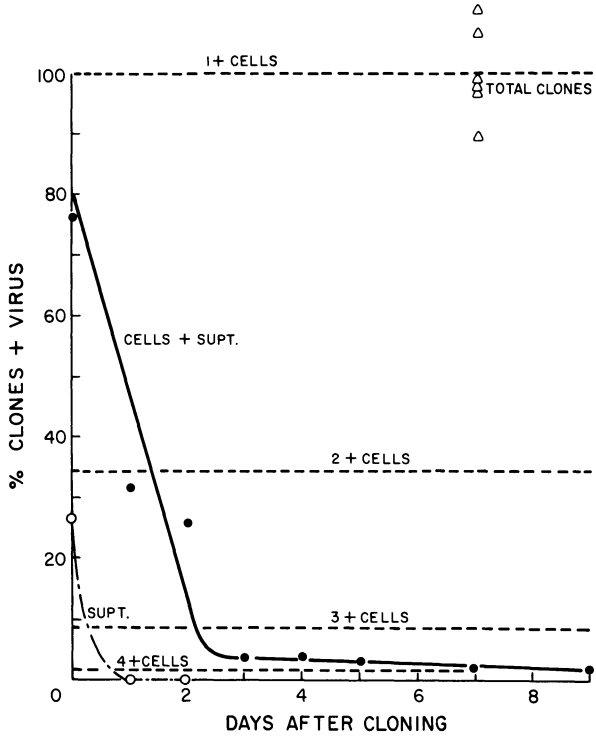

Fig. 6. Results of 3 experiments similar to the one shown in Figure 3. The time scale is relative to the time of trypsinization and cloning. Each point represents 96 cups except those after 10 days, which were each obtained from 192 cups. Virus + ^{ve} = clones shown to produce virus by amplifying the titer with normal L cells and then detecting virus by mouse inoculation.

riantly and produced uniform, easily visible, white colonies with a higher efficiency than the parent L cell culture. The 3 supernatant trays were treated with amplifier cells on days 0, 1, and 2 and were assayed for virus by mouse inoculation in the same way as the trays which contained cells. The count of virus-containing supernatant cups at day 0 was only 26% of the number of cell cups found to have colonies on day 7 of the experiment. By day 1 no free virus was detectable in the supernatant cups, whereas the figure for virus-productive cell cups was still high. The results show that the virus found in cell-containing cups which were virus-positive on day 0 must have originated from virus-producing cells in at least

$$\frac{77-26}{77}, \text{ or } 66\%, \text{of the clones.}$$

Of these only 4 were still producing virus by day 3, and only 2 by day 10;

$$\text{therefore, } \frac{66-4}{66}, \text{ or } 94\%, \text{ and } \frac{66-2}{66}, \text{ or } 97\%,$$

of the virus-producing clones (on day 0) had recovered from infection by days 3 and 10, respectively. The fact that virus continued to be found in 2 to 4% of the clones after day 3 suggests that, under the

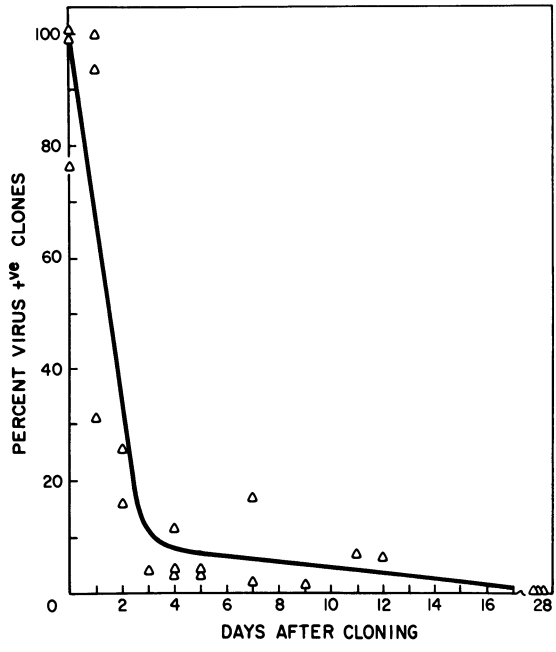

Fig. 7. Comparison between the rate of disappearance of virus from cups seeded with infected cells (closed circles) and cups seeded with the same volume of cell-free supernatant fluid from the cell suspension (open circles). Each point was obtained by testing 96 cups for virus except those after 4 days, for which 192 cups were tested. The horizontal broken lines illustrate the predicted proportions of cups containing cells arising from 1 or more cells (1+), 2 or more cells (2+), 3 or more cells (3+) and 4 or more cells (4+). The cells showed 95% fluorescence at the time of seeding with 0.025 ml to each cup, using a density of 40 cells/ml (μ = 1.0). Cell growth was evaluated on the basis of cups containing cells on day 7 after staining with neutral red. No differentiation was made between single and multiple colonies in a given cup, since in many cases these were confluent and differentiation was unreliable. (Taken from ref. 8 by permission of the editor of Nature New Biology.)

conditions of this experiment, between 3 and 4 clones per cup were needed to maintain cyclical reinfection as they successively passed through productive, shutdown, refractory, and susceptible phases. The tendency for these cyclical persistent infections slowly to die out may be due to a secondary shutdown consequent upon the onset of lag-phase in the cultures.

Twenty randomly selected virus-free clones from virus-infected cultures were tested for their susceptibility to LCM virus in this experiment. All were fully susceptible and produced virus as judged by immunofluorescence, plaque assay, and mouse inoculation. The immunofluorescence tests showed the same shutdown phenomenon to occur. The cells could be used for LCM virus plaque assay, and no difference from normal uninfected L cells could be detected.

DISCUSSION

Although no examples of spontaneous recovery of individual virus-producing cells appear to have been reported, some aspects of the phenomenon described here appear to have been observed by other workers. Diminution or "fading" of immunofluorescent staining a few days after infection of human diploid cells with LCM virus was observed by Wiktor et al. (23). By the 5th day immunofluorescence had disappeared, although infectious virus was still detectable. Mims and Subrahmanyan (13) also noted a marked decrease in the number of LCM virus-positive, immunofluorescent, mouse peritoneal macrophages a week or two after in vitro infection with LCM virus. The non-fluorescent cells could not be reinfected with virus. These results appear to be examples of the same shutdown process described in this paper and lend support to the conclusion that it is a general phenomenon with different LCM virus strains and host cell lines. In a detailed study of the development of specific immunofluorescence in LCM virus-infected L cells, Pedersen and Volkert (15) found a close correlation between specific fluorescence and virus production; no diminution or shutdown was described, but cultures were not followed into the stage of chronic infection. Lehmann-Grube et al. (11) described cyclical changes in the fluorescence of newly established, persistently LCM virus-infected cultures of mouse L cells which exactly parallel the results described here.

The in vitro resistance of LCM virus-infected cells to superinfection by LCM virus strains (but not other viruses) has also been observed previously (10,11,13). The results described above suggest that the same mechanism which causes shutdown of the immunofluorescent antigen may also be responsible for the continued specific resistance to LCM virus antigen synthesis during the refractory period. This in turn suggests that a specific intracellular substance is the cause of both these events and that the refractory state lasts only as long as an adequate level of this substance remains in the cell. If this is so, it is not clear whether the loss of this substance is due to its destruction or to dilution consequent upon cell division. The absence of any detectable soluble interfering agent such as interferon in media from acutely or chronically LCM virus-infected cell cultures is well substantiated. The ability of VSV to produce plaques on LCM virus shutdown cells was clearly shown in these experiments, and similar results on the absence of interfering substances have been recorded previously (11,13,19,20).

The isolation of occasional fully susceptible, non-infected clones from acutely infected cultures may be accounted for on the basis that these have somehow escaped infection, conceivably by protection via defective interfering particles. While there may be some defective or dead interfering particles in the inocula used in these experiments, they cannot account for the results, since such defective particles would also prevent virus production. Interference and protection by defective particles cannot account for the high proportion of virus-free, susceptible clones stemming from productively infected cells. This result can only be explained by the assumption that LCM virus induces no lethal or permanent damage in the cell and that virus production is only a transient event followed by full recovery.

The phenomenon of cyclical transient infection, which in vitro seems to require a minimum of 3 or 4 adjacent single cells, can explain many of the puzzling phenomena of persistent infection of the mouse, particularly the presence of many non-fluorescent LCM virus-resistant cells, as demonstrated so elegantly by Mims and Subrahmanyan (13). This phenomenon also accounts for the cyclical rise and fall of specific im-

munofluorescence and virus titer in newly established, persistently infected cell cultures (11,18). While the mechanism of transient productive infection is at present obscure, it clearly originates in productively infected cells and is not due to defective virus particles, although defective virions have been demonstrated in LCM virus infection by Welsh and Pfau (22). It is conceivable that the intracellular accumulation of defective particles or their derivatives in sufficient numbers at some point during the productive infection of every cell could induce a blockage of further virus synthesis; but it seems more likely that shutdown constitutes a more subtle, natural intracellular defense mechanism. Superficially, persistent LCM virus infection qualifies as regulated infection (21); however, the transient nature of the process in individual cells makes this description inapplicable, since the infection is not regulated but is actually suppressed.

It remains to be seen whether transient infection is exhibited by other persistent viruses, particularly the remaining arenaviruses, and whether the molecular components responsible for shutdown and termination of the infection have any therapeutic application.

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Plaque Assays and Current Concepts of Regulation in Arenavirus Infections

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SUMMARY

Use of recently developed plaque assays for LCM virus-infected cells and free virus has led to a better understanding of the in vitro host-virus interactions. Many LCM virus stocks, even at high input multiplicities of infection, have very low efficiencies of infecting cells. Under these conditions where multiple cycles of infection are necessary to achieve total infectivity of a BHK21/13 S monolayer there appears to be a preferential cell to cell transfer of the viral genome. The low efficiency of virus stocks in forming infective centers is due to an interference phenomenon that can be readily demonstrated using an L cell monolayer plaque assay. The properties of the interfering agent in recently infected cultures as well as in long-term infections are those of defective interfering virus. Further studies to elucidate the host-virus relationship in the regulation of virus production have shown that conditions favoring cell replication enhance virus replication or maturation.

INTRODUCTION

Quantitative and molecularly oriented animal virology had its beginnings in 1949 with the report of Enders, Weller, and Robbins (8) that poliovirus could be grown under precise conditions in tissue culture. This, shortly followed by Dulbecco's (6) adaptation of the plaque assay to animal cell systems, opened up almost overnight the possibility of exploiting the concepts developed from the bacteriophage studies. The rapid advances seen with the picornaviruses and poxviruses, to name a few, were not forthcoming with LCM virus. This can, in part, be traced to lack of a quantitative and reproducible plaque assay. Although cytopathic effects in various cell lines were observed as early as 1956 (7,13,19) and several plaque assays were reported shortly thereafter (1,2,4), a reproducible and reliable plaque assay was not developed until 1967 (17). This communication illustrates the vistas coming into focus for us by use of this BHK21/13 S agarose suspension assay in attempting to answer several questions concerning basic arenavirus-cell interactions.

MATERIALS AND METHODS

Viruses. The origins and passage histories of the various types of viruses may be found in previous publications (14,15,18).

Cells. BHK21/13 S and L-929 cell propagation techniques have been described (15,24). The HEp-2, FL, Detroit-98, MDBK, Vero, and 3T3 cell lines were grown as monolayers using Eagle's minimum essential medium supplemented with 10% heat-inactivated calf serum. The growth medium for the HeLa, JLSV-9, and Vole cells was that used for the BHK21/13 S monolayer cultures (15).

Plaque assay. The general techniques used were those employed for either the BHK21/13 S suspension assay (15) or the L cell monolayer assay (24).

Measurement of the spread of infection. Confluent BHK21/13 S monolayers (1.2×10^7 cells/25 cm^2 plastic flask) were infected with LCM virus, strain CA1371, at an input multiplicity of 0.5. After 1 h at 37°C the inoculum was decanted from each flask and the monolayers were washed 3 times with 2 ml of Eagle's medium. Medium was replaced to the original volume (5 ml) and incubation was continued at 37°C. At various times after infection the medium was withdrawn, the cells were dispersed with trypsin and counted in a hemacytometer, and then washed (23). The infectivities of the original medium, the combined washes and the washed cells (before and after sonic disruption) were determined.

RESULTS

Intercellular spread of infection after low efficiency primary infection. With delineation of an assay capable of distinguishing free virus from infected cells (i.e., infective centers), an experiment was designed to determine if the Poisson series could be used to predict the number of cells infected with a given input multiplicity of infection (MOI). Table 1 shows the results of exposing a virus stock, titering 6×10^6 PFU/ml, to a monolayer containing 4×10^6 L cells.

Table 1. Infective center-forming ability of a standard LCM virus-UBC stock (6×10^6 PFU/ml)[a]

Virus dilution	10^0	10^1	10^2	10^3	10^4
Exp. A	11.0[b]	10.0	0.5	0.04	<0.01
Exp. B	12.0	7.6	0.9	0.04	0.006

[a] One ml of virus (the supernatant from an L cell monolayer culture infected 72 h previously) was used to overlay an L cell monolayer, in a 25 cm^2 plastic flask, for 1 h. The cells were washed 3 times, dispersed with trypsin, and centrifuge-washed 5 times prior to titration.

[b] Percentage of infective centers formed.

Under ideal bacteriophage-like conditions with an MOI of 1.5 up to 80% of the cells should become infected. In duplicate experiments, however, only 11 and 12% of the cell population scored as infective centers. Nearly as many infective centers, 10 and 7.6%, respectively, were scored when the stock was diluted 10-fold before infection. Successive 10-fold dilutions of the infecting stock paralleled a decrease in infective center numbers in a relatively linear manner. To determine if at any time during the course of infection 100% of the cells could be scored as infective centers, the infectivity in 1 of a set of identically infected monolayer cultures was measured at various intervals. The results, presented in Figure 1, show that: 1) at an in-

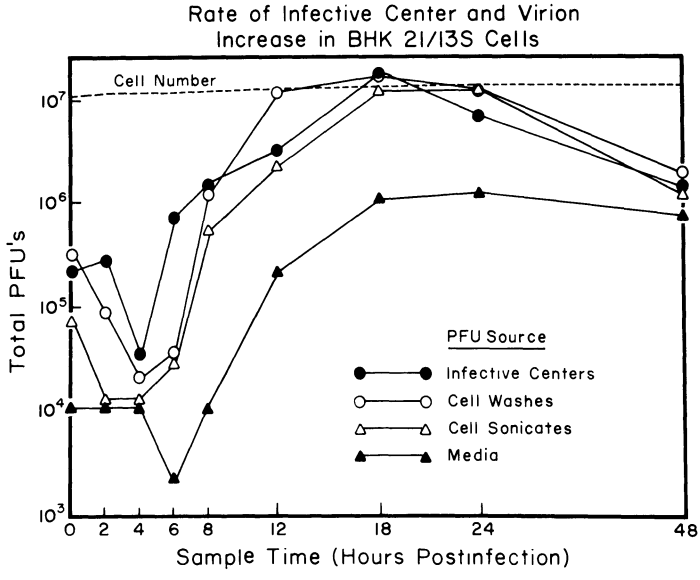

Fig. 1. Ten identical BHK21/13 S monolayer cultures were infected with LCM virus CA1371, and at the indicated times after infection 1 flask was treated to determine the free virus, the virus released by washing the cells, the number of infective centers, and the virus present in sonic disrupted washed cells. The cell numbers at 0, 24, and 48 h after infection were 1.2, 2.2, and 2.2 x 10^7/culture, respectively.

put MOI of 0.5 less than 10% of the cell population was initially infected; 2) the infective center number at 18 h post-infection was equal to or slightly higher than the cell number and gradually declined thereafter; 3) throughout the first 24 h post-infection greater than 90% of the infectivity was cell associated; and 4) between 2 and 4 h after infection there was a marked decrease in cell-associated infectivity followed by a similar decrease in free virus between the 4th and 6th h. Thus 100% infective centers could be detected by the plaque assay. Attention was also focused on the relative paucity of extracellular virus throughout the growth curve indicating a possible direct cell to cell transmission of the infection without an extracellular phase. The apparent lack of free virus could be due to a low EOP, compared to that of the infective centers, or to a rapid readsorption after release into the medium. The widely accepted way to demonstrate

direct cell to cell transfer is to show unimpeded spread of infection in the presence of specific anti-viral serum in the medium. Since high titer neutralizing antibody against LCM virus was not immediately available, an attempt was made to resolve the problem through use of a compound that would block an early stage in free virus-host cell interactions. Amantadine hydrochloride was found to be effective in blocking penetration of LCM virus into BHK21/13 S cells (23) as well as inhibiting the synthesis of all arenaviruses examined (14). An experiment designed to follow the spread of infection, as shown in Figure 1, was carried out in the presence and absence of amantadine. Twenty-four h after infection and addition of the drug, free virus was reduced by 93% compared to the culture without drug. The infectivity from the centrifuge washes was reduced by 85%, the cell-associated PFU were down 83%, but the infective center number was reduced by only 33%. Thus the spread of infection was impaired somewhat by amantadine but not as much as virion synthesis.

Permissive cell lines for LCM virus plaque formation and interference. Cell lines, other than BHK21/13 S, were screened for their ability to allow plaque formation when infected with LCM virus. Using conditions similar to those already described (15), agarose suspension assays were successful with JLSV-9, L-929, and Vero cells. Quantitation of virus could also be reliably accomplished using monolayer cultures of Detroit-98, HeLa, HEp-2, JLSV-9, L-929, MDBK, Vero, Vole, and 3T3 cells. The L cell monolayers were of immediate interest because cytolytic activity was observed at low but not at high concentrations of infecting virus. Figure 2 shows that an undiluted and 10-fold diluted virus stock

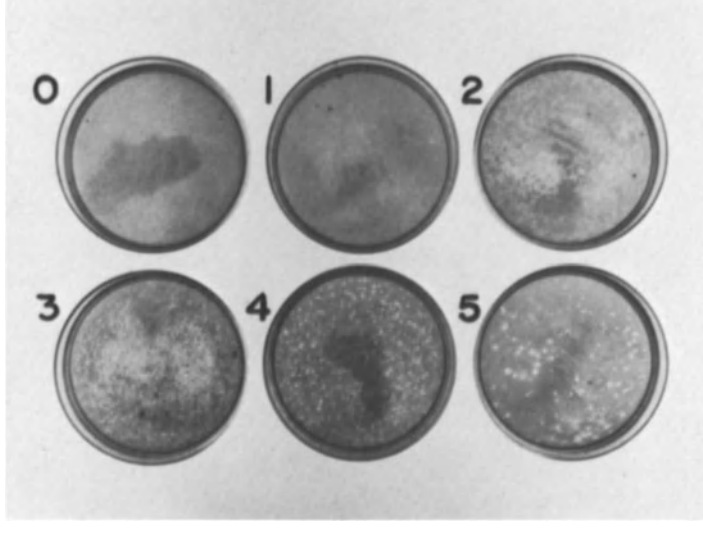

Fig. 2. Dilution series of LCM virus, strain UBC, on L cell plaque assay plates. The numbers on the upper left side of each plate refer to the $\log_{10}$ of the virus dilution.

did not produce any change in the cell's ability to take up neutral red. When diluted 100-fold, plaques first appeared and reached a maximum at the 1,000-fold dilution before starting to decline in number with suc-

cessive dilutions. All cells were lysed in the plaque areas when plates were incubated at CO_2 concentrations between 2 and 4%. With CO_2 concentrations between 4 and 8%, however, concentric rings of lysed and intact cells developed. This characteristic morphology was sometimes evident shortly after staining (at the 4th day post-infection) but frequently took several days to develop. Figure 3 shows a portion of a

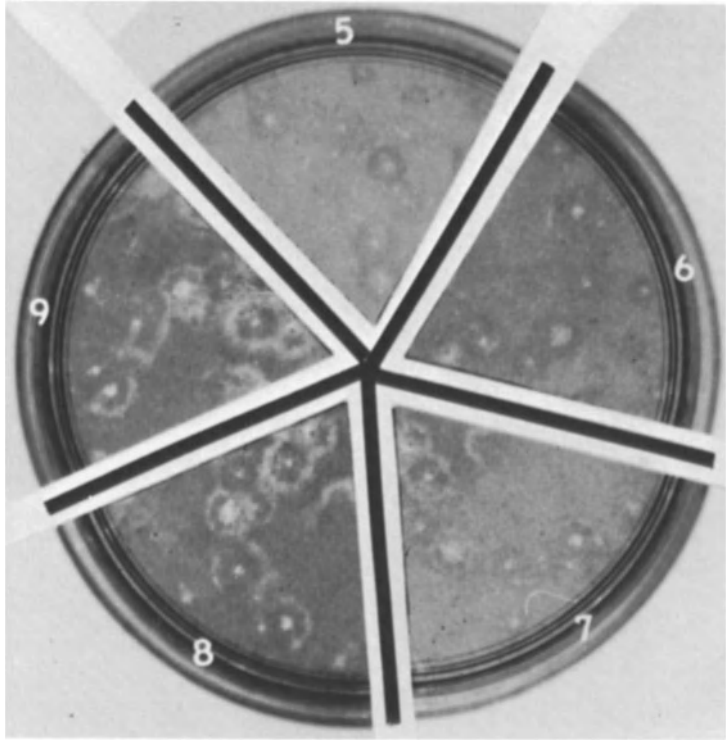

Fig. 3. Sequential development of ringed LCM virus-UBC plaques on L cell monolayers. The plate was stained on the 4th day after infection and photographed on each of the 5 succeeding days. Numbers on the sector refer to the days post-infection.

plate infected with LCM virus, strain UBC, photographed on days 5, 6, 7, 8, and 9 post-infection. Because of this observed autointerference in cytolytic activity and low efficiency of forming infective centers in L cells with high concentrations of LCM virus (Table 1), virus growth curves were initiated with different input MOI. Again an interference phenomenon was observed (Fig. 4). Maximal titers of LCM virus, strain UBC, were obtained in Vero cells with input MOI of 3×10^{-2} to 3×10^{-4}. The interference using high concentrations of virus in the inoculum was also observed in L cells using the UBC, Traub and CA1371 strains of LCM virus (24).

Conditions for optimal virus synthesis. It was noted in the experiment presented in Figure 1 that the infective center number as well as the cell-associated and free virus number declined between 24 and 48 h after infection. The possibilities considered to cause this decrease

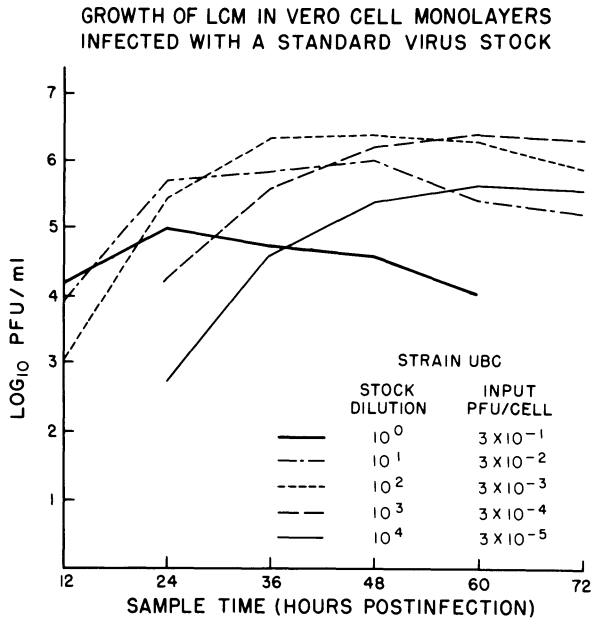

Fig. 4. Confluent (3 x 10^6 cells/plate) monolayers of Vero cells were infected with 1 ml of 10-fold dilutions of a standard LCM virus-UBC stock. The monolayers were then washed 3 times with 1 ml amounts of Eagle's minimum essential medium, overlaid with fresh medium, and incubated at 37°C. Samples of 0.1 ml were withdrawn from the medium at 12 h intervals and frozen at -70°C until use.

were: 1) the medium in the culture became virucidal between 24 and 48 h after infection; 2) the nutrients necessary for the cell to maintain viral replication became exhausted; 3) the infected culture was entering a state of persistent infection whereby infectious virus synthesis was regulated by either a cell-mediated or a virus-mediated mechanism; or 4) a composite of the above possibilities. Free virus was found to be inactivated, over a period of 24 h at 37°C, significantly slower in 24 and 48 h conditioned medium (fresh medium used to maintain uninfected BHK21/13 S monolayers) than in fresh medium. However, 48 h conditioned medium, when added to a culture immediately after infection, reduced the 24 h infectious virus yield to 25% of that found in the fresh medium control. Using BHK21/13 S cells it was also found that the yields of virus 24 h after infection decreased as the interval between a monolayer becoming confluent and the time of infection increased. The same types of phenomena appeared to operate in L cells. To define more fully the role of the host in infectious virus synthesis, and as a preliminary to establishing a persistent in vitro LCM virus infection, studies were performed to determine what cultural conditions allowed maximal virus synthesis in BHK21/13 S suspension cultures. The 1st experiment employed BHK21/13 S cells that had just entered the plateau growth phase (2 x 10^6 cells/ml) when infected with LCM virus CA1371. As observed in BHK monolayer cultures (Fig. 1) there was a lag of 6 h before free and cell-associated PFU increased. Peak infectivities were observed 18 h post-infection with a PFU to infective center ratio of 1 with only 10% of the cells scoring as infective centers (a 3-fold increase since initial infection). Between 24 and 72 h after infection infectivity declined. In the next experiment (Fig. 5) BHK suspension cells were in-

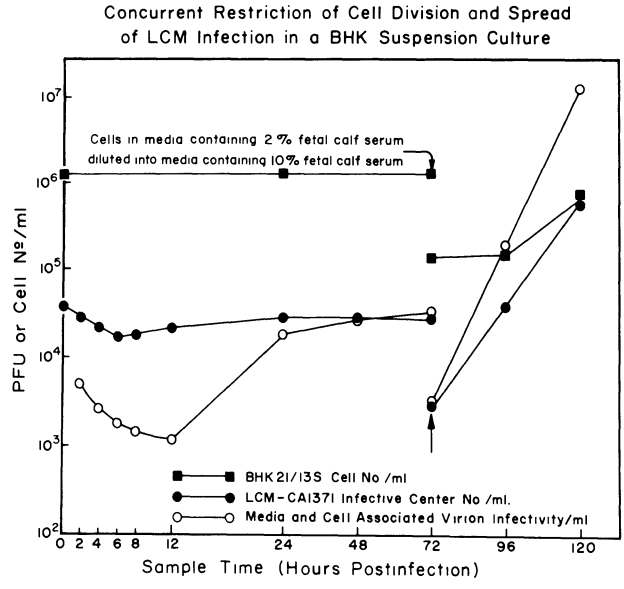

Fig. 5. From a spinner culture in the log phase of growth 4.7 x 10^7 cells were removed and pelleted at 225 x G for 5 min. These cells were then suspended in 10 ml of an LCM virus CA1371 stock titering 2 x 10^6 PFU/ml and incubated for 1 h at 37°C on a roller drum at 50 rev/min. These cells were pelleted and washed 3 times with 20 ml volumes of spinner medium (15) plus 2% fetal calf serum, and finally resuspended in 25 ml of medium and transferred to a spinner flask. At various times during the incubation period at 37°C infectivities were determined as outlined in Figure 1 and in the section on Materials and Methods. The culture was diluted 10-fold 72 h after infection by placing 2.5 ml of the culture in 22.5 ml of spinner medium plus 10% fetal calf serum.

fected during log phase growth and then shifted to stationary phase by lowering the fetal calf serum concentration from the standard 10% to 2%. Free and cell-associated virus did not increase until 12 h postinfection and at no time was the PFU to infective center ratio more than 1. Only a constant 3% of the cell population scored as infective centers. However, when the culture was shifted into log phase growth by dilution into medium containing 10% fetal calf serum the virus yield quickly increased. Within 2 to 3 cell generations the culture produced 18 PFU/infective center and the entire cell population scored as infective centers. Results of a similar nature are seen in cell culture infections with Rous sarcoma virus (21). Thus when long-term infections were established with arenaviruses (18), BHK suspension cultures were maintained in the log phase of growth for as much of the time as possible.

DISCUSSION

The studies presented in Figure 1 and those previously reported (23) have led to a partial understanding of the time sequence of the early events in the virus-BHK21/13 S interaction. Maximal irreversible adsorption of the virus to the cell requires 45 to 60 min as does the ensuing penetration step (23). Assuming that a drop in sonic resis-

tant intracellular infectivity is an indication of uncoating, this process is complete (except for a very small fraction of resistant virus) within 2 h (Fig. 1). Infectivity in the cell washes, an indication of reversibly bound virus, declines through the 4th h post-infection. This is possibly due to inactivation of the virus at the cell surface or to a continuance at a slower rate of earlier events, i.e., irreversible adsorption and penetration. Quantitation of infective centers indicates a sharp drop in PFU between the 2nd and 4th h after infection with complete recovery at the 6th h. This quite reproducible observation may indicate that this transient disappearance of infective centers is due to a temporary susceptibility to physical shock in the cell washing procedure. With this in mind, it was also considered that the infective centers at the 4th h post-infection were slow in developing in the plaque assay plates. Duplicate results, however, were obtained when the experiment was repeated and plates were stained on the 5th or 6th day post-infection (instead of the 4th day as is the standard practice). Another indication of this critical time period was shown by adding standard virus to L cells at various times after infection with defective interfering (DI) virus (see below). A 4-fold increase in ability to inhibit infective center formation was observed between the 4th and 5th h after DI virus infection (22). Further work is obviously necessary to explain these observations. Newly synthesized virus, first associated with the cell, accumulates in significant quantities between 6 and 8 h post-infection. A minimum of 3 to 4 h appears to be necessary for this sonic resistant virus to be released from the cell. The explanation for the transient and reproducible drop in free virus at the 6th h post-infection, also observed in LCM virus-infected L cells, is still lacking. One might consider that just prior to or concomitant with rearrangement of the cell surface for newly synthesized budding virus there is a second wave of virus adsorption from the medium.

The low efficiency of a virus stock in forming infective centers, even at high input MOI (Table 1) plus the L cell interference assay led to speculation that defective interfering virus might play a role in our system. This was further strengthened by the similarity of LCM virus growth curves initiated by differing MOI inocula to those observed with influenza virus (11). An interferon-mediated phenomenon has been ruled out for several reasons previously discussed, and the data presented with Vero cells (Fig. 4) add further support since these cells are reported to be deficient in their ability to produce interferon (5).

The biology of the DI virus has come through use of long-term infections of BHK21/13 S cells with both LCM and Parana viruses (18). The early parts of this work, mimicking many of the results seen by Lehmann-Grube and co-workers (10), showed that about 50 generations after infection both types of cultures ceased producing detectable quantities of PFU. Instead, DI virus was produced in quantities similar to those one would expect from a normal type of infection. Our stochiometric data indicate that only 1 defective virus is necessary to prevent a cell from scoring as an infective center if added before infection with standard virus (25). We have no evidence that the LCM-DI virus can complete its own replication cycle in a cell (possibly a unique source of vaccines especially if other arenavirus DI particles behave similarly), yet over 90% of the cells in a persistently infected culture, which produces the DI virus, contain immunofluorescent viral antigen. This implies that the majority of these cells harbor the original viral genome. Indeed, if the LCM virus persistently infected cells are frozen in liquid nitrogen and then thawed, plaque forming virus will be produced for a brief period (18). This demonstrates that the original virus genome has been preserved by transmission from mother to

daughter cell, and indicates that the viral genome has not yielded to a selective pressure for fast replication. This condition could hypothetically result in a loss of a portion of the viral genome as has been found to occur in vitro with the replication of Qβ RNA (12). This restricts the number of possible models of the LCM virus-host cell relationship which could adequately explain the stability of the association. The viral genome must multiply independently of the host's DNA or its replication and distribution to daughter cells is integrated in some way with the replication and distribution of the host's genetic material. Determination of the location of the LCM virus genetic information in persistently infected cells must await the results of appropriate genetic and biochemical experiments. It is tempting to speculate about the existence of a provirus state (20) as seen in Rous sarcoma virus infections.

We have documented that the LCM virus infection in cell culture is highly regulated by both the host cell (Fig. 5) and the virus itself. It is neither surprising nor unprecedented (21) that viral synthesis is dependent on the nutritional state of the cell. The data presented here complement reports that the replication of LCM virus, an RNA virus lacking detectable DNA, and detectable (thus far) reverse transcriptase (16) is inhibited by relatively low concentrations of actinomycin D (3). Here the role of some type of cellular template in viral synthesis is implicated.

The viral role in regulation of the infection has not been entirely resolved, but the data presented here and elsewhere (24,25) certainly indicate that DI virus particles are involved in the regulation. The magnitude of the interference phenomenon appears to vary between strains, with the UBC strain more prone to produce DI virus during short-term infection than the CA1371 strain (and thus one should not strictly compare the data in Table 1 with those in Figure 1). The initial infection of a cell population by stocks of LCM virus, strain UBC, results in 4 different types of cells: 1) those that are infected only with standard LCM virus; 2) those that are infected only with LCM-DI virus; 3) those that are not infected; and 4) those that are infected with both standard and DI virus. The uninfected cells and those infected with DI virus alone, do not score as infective centers. Most of those infected with standard virus alone probably do score as infective centers since there is an almost linear relationship between virus dilution and infective center formation during infection with a low multiplicity (Table 1). Many of the cells receiving both standard and DI virus do not score as infective centers and, if data derived from other virus systems (9) are examined, it seems probable that those cells that do score as infective centers release a reduced number of standard virions. The final virus yield in a culture depends to a large extent on the degree of pre-conditioning of the cells at the time of infection with DI virus. DI virus cannot replicate by itself, but in the presence of the standard virus replication of DI virus is favored. Results in other systems suggest that this is due to preferential synthesis of a smaller defective virus genome (9). This results in lower standard virus yields and reduction of cytolytic activity. The preferential synthesis of DI virus is best shown in the long-term persistent infections in cell culture (18). In any given culture infected with DI and standard virus, the DI virus eventually dominates the synthesis. Stocks containing high quantities of DI virus have poor efficiencies in initiating standard virus infections. Our results here and elsewhere (24) show that about the same number of infective centers are produced whether cells are infected with undiluted or slightly diluted virus stocks suggesting that the percentage of cells that score as infective centers reflect the proportion of standard to DI virus

particles in the preparation. The characteristic bull's-eye plaque formation by LCM virus (and Parana virus) suggests that even when one standard virus particle initiates an infection, there is a probable eventual production of DI virus which inhibits cytolytic activity. The cycling pattern of lysis in the bull's-eye plaques may be analogous to the cycling PFU titer seen during initial stages of long-term persistent tissue culture infections (18). The influence of CO_2 tensions on bull's-eye plaque morphologies may be another indication that the metabolic state of the cell plays a role in manifestation of the interference phenomenon.

ACKNOWLEDGMENTS

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Inapparent Infection of Syrian Hamsters with the Virus of Lymphocytic Choriomeningitis

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SUMMARY

Employing complement fixation tests and virus isolation procedures, 598 Syrian hamsters from 11 colonies were examined for LCM virus; 6 colonies were found to be infected. A new colony was started with adult hamsters from a heavily infected colony as breeding stock. LCM virus could not be detected among the progeny.

INTRODUCTION

In 1971 it became known that a number of human cases of lymphocytic choriomeningitis in the Federal Republic of Germany could be traced to recently purchased Syrian hamsters (Mesocricetus auratus) (1,2,3), and these reports received broad and dramatic coverage by the press. This adverse publicity caused a severe drop in the sales figures for Syrian hamsters and, hoping to obtain clean bills of health for their colonies, breeders submitted samples of their colonies for examination. This was done on a strictly voluntary basis as LCM is not among the communicable diseases for which public health regulations exist.

MATERIALS AND METHODS

Since in previous investigations of this kind the infection rates were never lower than 20% (1), high proportions of infected animals within colonies were expected to be found also in this survey. The number of animals kept in one colony ranged from several 100 to about 2,000. In order to detect the virus with a margin of error of 1%, a sample size of 45 animals is sufficient, provided the rate of infection is 10% (5). Thus we strove to obtain 45 young and 45 adult animals from each colony. Eleven breeders in 4 states sent to our laboratory a total of 598 hamsters, of which 416 were juveniles and 182 adults. The sample size per colony ranged from 10 to 110. Nine runts of a colony, known to be heavily infected (colony 3), were killed by the breeder and kept at -20°C until examination. The other animals were alive. They appeared to be perfectly healthy with the exception of 22 runts which we collected in colony 2. All hamsters were bled under ether anesthesia. Blood clots and sera were separated and the sera were individually stored at -20°C until examination. Since the virus seems to be detectable in the brain for a longer period than in the blood (20), blood clots and brains were kept separate. Specimens from 5 juvenile hamsters were combined. They

were ground and diluted 5-fold with phosphate-buffered saline containing 1,000 units of penicillin/ml, 2 mg of streptomycin/ml, and 10% inactivated calf serum shown previously to be free of LCM virus-inhibiting substances. Pools were centrifuged at low speed and the supernatants inoculated into groups of 5 mice, each mouse receiving 0.03 ml intracerebrally. The period of observation was 21 days, in 1 instance 28 days (colony 4). One isolate per colony was passaged 10 to 12 times in white mice before it was identified by a serum neutralization test which was carried out by the constant serum-varying virus method (10). Six mice per dilution were employed. The neutralization end-points were calculated by the Kärber method (7). One human serum (for colony 2) and a hyperimmune serum pool of guinea-pig origin were employed. The guinea-pigs had been immunized by serial injections, beginning with a strain of low pathogenicity ("E-350" of Armstrong), followed by the highly virulent strain WE (18). Randomly bred white mice of NMRI origin weighing 10 to 12 g were supplied by the Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen. The guinea-pigs were raised in this institute.

CF tests were performed by the micro-technique (10). The antigen was prepared from consolidated lungs of guinea-pigs (11,12), which had succumbed to the infection with strain WE or were killed when moribund. A 10% homogenate of lung tissue in physiological saline was centrifuged twice. After low speed centrifugation the supernatant was centrifuged again for 20 min at 30,000 rev/min. The remaining liquid contained the soluble CF antigen (18,19).

Control serum was prepared in guinea-pigs immunized as described above by infection with LCM virus E-350, followed by 2 injections with strain WE.

To see whether hamsters free of LCM virus could be bred from animals of an infected colony, a subcolony (colony 3a) was established with about 30 hamsters, at least 6 months old, from colony 3. These animals' offspring were pooled and 2 weeks later 20 of them were examined for viral infection.

RESULTS

LCM virus was isolated from animals of 6 colonies in 3 states of the Federal Republic of Germany. Of 172 brain and blood pools, from which isolations were attempted, 25 (15%) were positive. In 1 series (colony 4) 12 pools were examined and only 1 yielded LCM virus. In this particular case 1 recipient mouse died, and the incubation period was 28 days. Table 1 shows the results of the isolation attempts.

In the CF test 17 out of 445 individual sera were positive (4%) with titers ranging from 2 to 8. The positive sera originated from 3 colonies. Nine sera from 4 colonies (2%) gave inconclusive results. All sera of the animals from 5 further colonies were negative. Because of anti-complementary activity 54 of the sera (12%) could not be evaluated.

Neither virus nor CF antibody could be detected in the animals of subcolony 3a. The CF results are presented in Table 2.

Table 3 summarizes the epizootiological status of the colonies as revealed by CF test and compared with the results of the isolation attempts.

Table 1. Detection of LCM virus in hamster colonies

Colony	Isolation attempts			Identification of isolate (Neutralization index)
	Negative pools	Positive pools		
		Brain	Blood	
1	11	4	1	3.83
2	26	4	0	2.35
3	0	4	4	3.50
3a	8	0	0	n.d.[a]
4	11	0	1	4.00
5	10	2	0	3.16
6	3	2	3	3.32
7	14	0	0	n.d.
8	20	0	0	n.d.
9	20	0	0	n.d.
10	20	0	0	n.d.
11	4	0	0	n.d.

[a] Not done.

Table 2. Results of complement fixation tests

Colony	Number of sera		Negative		Positive		Inconclusive		Anti-complementary
	juv.	ad.	juv.	ad.	juv.	ad.	juv.	ad.	
1	36	27	31	25	1	1	0	0	5
2	48	8	44	7	0	1	1	0	3
3	0	29	0	5	0	14	0	0	10
3a	14	0	12	0	0	0	0	0	2
4	18	22	18	15	0	0	0	6	1
5	13	0	11	0	0	0	1	0	1
6	17	0	14	0	0	0	0	0	3
7	32	27	16	22	0	0	1	0	20
8	41	53	41	49	0	0	0	0	4
9	26	0	24	0	0	0	0	0	2
10	24	0	21	0	0	0	0	0	3
11	10	0	10	0	0	0	0	0	0
Sub-totals	279	166	242	123	1	16	3	6	54
Totals	445 (100)%		365 (82)%		17 (4)%		9 (2)%		54 (12)%

Table 3. Comparison of the results obtained with either CF test or virus isolation

Colony	CF test	Virus
1	+	+
2	+	+
3	+	+
3a	-	-
4	?	+
5	?	+
6	-	+
7	?	-
8	-	-
9	-	-
10	-	-
11	-	-

DISCUSSION

Inapparent infections of Syrian hamsters with the LCM virus with (4, 13) or without (14) spread to humans is not a new observation. The results of this survey leave no doubt that the occurrence of LCM virus among hamster colonies is not a rare event: in 6 out of 11 colonies LCM virus could be detected. The 6 infected colonies are located in 3 different states. This finding is of great concern for the public health authorities, considering that in the Federal Republic each year approximately 1 million hamsters are sold as pets. Yet it will be an extremely difficult task to improve this situation. Several facts have to be taken into account.

One set of problems concerns the laboratory diagnosis. The request for 45 juvenile and 45 adult animals per colony was based on the assumption that if a colony is infected the rate is 10% or more. But lower infection rates have to be expected and in order to detect, for instance, 1 infected hamster out of 100, the sample from a given colony would have to be increased to at least 300 animals (5).

The different laboratory techniques have their advantages and disadvantages. The CF test gives quick results, and a large number of sera can be tested with a minimum of effort. Inasmuch as CF antibody appears in the blood about 1 week after the virus and persists for many months (20), sera of adults should be tested. Naturally, a recently introduced infection would be missed by this method. During this survey, 3 colonies were shown to be infected by CF tests, although in no case the desired number of 45 sera of adult hamsters was available. In 2 of the 3 cases in which the CF test failed to reveal an existing infection no sera of adult animals were tested. In the 3rd case 22 adult sera were available. Only 1 serum out of 132 from young hamsters of infected colonies was positive. Furthermore, 12% of all sera were anti-complementary and 2% gave inconclusive results.

Isolation of the virus with subsequent serological identification detected more infected colonies than the CF test and, furthermore, led to unequivocal results. However, this method is cumbersome and time-consuming, which is of relevance if a large number of colonies have to be checked. A continual surveillance with this technique would be impractical.

The identification of an isolate by cross-immunization test is not as widely used and generally accepted as by neutralization test (8). Therefore it was not employed during this survey, but it has been successfully used in our laboratory for the identification of other field strains of LCM. It has the advantage that it can be carried out after fewer passages than the neutralization test. In addition, it is easier to perform.

A promising method for the routine diagnosis is the fluorescent antibody technique. It remains to be seen whether this method gives more reliable results when applied directly to hamster tissue (15) than to mouse brain after intracerebral passage in mice (6).

Another problem is the commercial situation. An unknown number of small breeders are not registered with the local authorities. Larger breeders often cooperate in the way that animals are exchanged when large orders have to be filled. Until shipment the animals stay together in large containers and unsold ones usually are retained in the colony. It is in these pools with rapid population turnovers where the hamsters probably become infected. Furthermore, since these animals may harbor the virus for up to 3 months (21), the possibility that a female infects her offspring after having reached sexual maturity at the age of 6 weeks has to be considered.

Since hamsters are not identified individually, their source as declared by the breeder cannot be checked. Obviously, breeders could easily obtain animals which they intend to submit for examination from an LCM virus-free colony.

An important point is the sampling procedure. From colony 2, 50 young hamsters were sent to us, all of which were in perfect health. Isolation attempts were negative. We then collected ourselves 22 runts on the premises and virus was isolated in 4 instances from 12 tissue pools. Whether the infection caused retardation of development or whether the runts which occur in any colony are more likely to become infected is an open question. Reports on the effect of LCM virus on hamsters are conflicting. Often no signs of illness are seen (13,14, 22); other investigators observed illness and even death in LCM virus-infected hamsters (16,17,20,21). We were able to produce by intraperitoneal injection of strain WE virus in adult hamsters a disease which was characterized by loss of weight, drowsiness, jerkiness, and change of voice. Probably these contradicting observations are due to differences in route of infection, virus dose, and strain of virus.

The outlook for an effective control is not bright. Since there are no legal grounds, no steps have been taken yet to stop the sale even from infected colonies. Active immunization has been tried in different species with variable results (9), but no vaccine for mass immunization is available.

A way of eradicating infected hamsters would be to establish new colonies with virus-free breeding stock. This measure would require not much more than good will and the application of basic hygiene in the

care of the animals. Of course, a single animal which sheds virus would frustrate all efforts.

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Congenital LCM Virus Infection in "Germ-Free" Haas Strain Mice

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SUMMARY

"Germ-free" Haas strain mice are congenitally and persistently infected with LCM virus which is associated with a life-limiting immunoproliferative syndrome. The principal lesions are lymphoid infiltrations of visceral organs, elevated levels of serum globulins, and glomerulonephritis. The germ-free mice tolerate maximum subtoxic doses of cyclophosphamide, from which conventional counterpart mice die of bacterial infections. Cyclophosphamide treatments have prevented the development of and have reversed the lesions in young and old Haas strain mice, respectively. Intermittent cyclophosphamide therapy has elicited new reticulum cell sarcomas in Haas strain mice. Thus far, adoptive immunization procedures have not interrupted the congenital passage of LCM virus, and the results with radiation-induced allogeneic chimeras are incomplete. The protean manifestations of disease in Haas mice provide model experimental systems for congenital and persistent infection, immunoproliferative disease, immune complex disease, immunological tolerance, viral and cancer chemotherapy, and tissue transplantation procedures.

INTRODUCTION

During the past 35 years, a new technological development, gnotobiology, has resulted in controlled, defined environments in which animals live, propagate, and are used in the absence of a parasitic and bacterial flora. The animals have been referred to as axenic and germ-free, but the term gnotobiotic (of known content) is widely used and appropriate, because in one animal species (mice) some viral agents are transmitted to progeny by congenital routes. For convenience in this report they are called germ-free (GF). Of the many species of domestic and laboratory animals which have been derived, propagated, and studied under GF conditions, rats and mice have been subjected to the most extensive examinations for microbial flora and for applications to experimental procedures (13,14,15,17).

GF animals are delivered from full-term pregnant mothers by cesarean procedure directly into a sterile steel or plastic enclosure called an isolator (23,31). In most instances, the uterine contents are free of bacteria, fungi, and parasites; however, there is always the possibility of contamination from maternal blood during surgery. The babies are fed a sterile liquid diet by stomach tube up to weaning age, at which time they are provided a balanced solid diet on which they thrive. All

food, water, and bedding are heat-sterilized, and filter-sterilized air is provided at increased pressure. The animals propagate in the GF environment by natural means, and time-bred females thereof serve as foster mothers for the newborn of other strains that are delivered into the isolator system by cesarean procedures. At present, 8 strains of mice and 3 of rats have been propagated in this laboratory under GF conditions through 30 successive generations.

Infectious agents are transmitted from animal to animal by "horizontal" and/or by "vertical" routes. The former are usually acquired postnatally, whereas by the latter route the animals are born with specific organisms, either by transovarial or by transplacental passage. The animals are monitored for microbial flora by standard bacteriological tests at weekly intervals (33), and on this basis they are designated GF. They have been examined extensively for apparent, latent, or occult viruses by procedures which have been described (13,14,15). No viral agent has as yet been detected in GF rats (17); however, all strains of GF mice and conventional mice thus far examined have been found infected with leukemogenic virus (8). "B"-type virus particles have been observed in 3 strains of GF mice in association with mammary carcinomas (7). In a third mouse strain (Haas strain), in addition to leukemogenic virus, all of the progeny have been persistent carriers of LCM virus (22). In specific instances, congenitally transmitted viruses have been associated with disease syndromes, i.e. lymphatic leukemia in AKR mice (20) and immunoproliferative lesions in Haas mice (19). The diseases are clearly defined, occur in high incidence, and are predictable and thus provide excellent experimental instruments for biomedical research.

GF animals offer the investigator improved and controlled experimental conditions through better defined animals and their environments, from which irrelevant and complicating microbial, physical, and chemical pollutants have been excluded. By this technology, animals have been cleared of "horizontally"-transmitted agents, leaving only those which are transmitted "vertically". Experimental procedures, such as neonatal thymectomy, whole-body irradiation, and other immunosuppressive procedures, can be performed without the infectious sequelae which result in more clearly defined disease syndromes.

MATERIALS AND METHODS

In 1941, Haas (2) described a mouse strain with a lifelong LCM viremia, which was transmitted to progeny by congenital route(s). A colony of the Haas mice was maintained and propagated for several years by Dr. Wallace P. Rowe of the U.S. National Institutes of Health, and in 1967, several of them were sent to Lobund Laboratory in order that they be entered into GF status. This was done by standard cesarean procedures (23). The babies were foster-nursed on GF C3H/f mothers up to weaning age, and since then they have been propagated through 26 successive generations. During this entire period they have been free of bacterial flora, they propagated abundantly, weaned high percentages of litters, and survived longer than their conventional counterparts. They provided the material on which this report is based.

Groups of Haas mice were selected at various age levels for thorough examinations which included blood counts, serum electrophoretic patterns as well as gross, microscopic, and ultrastructural characteristics. LCM viremia was determined by inoculating susceptible weanling Swiss-Webster mice intracerebrally with 0.03 ml of blood which induced acute convulsive deaths at 7 to 9 days later. In an alternate sensitive virus detection procedure, blood or tissue extracts were inocu-

lated onto monolayer tissue cultures of "L" cells on coverslips which, after incubation at 37°C for 48 to 72 h, were stained with specific fluorescein-tagged antibody and examined for virus-infected cells by ultraviolet microscopy.

The GF Haas mice have been subjected to several study programs: (a) their gross and microscopic manifestations of disease; (b) applications to cancer and viral chemotherapeutic trials; and (c) attempts to interrupt congenital passage of LCM virus to progeny. The protocols required that the Haas mice be maintained GF for periods up to 1 year.

RESULTS

(a) GF mice of disease-free strains (such as Swiss-Webster and CFW) have unique anatomical and physiological characteristics which distinguish them from conventional counterpart animals. They have provided baseline and comparative information for studies on GF Haas mice. The intestines and cecums are thin-walled and dilated, and the laminae propriae are small, thin, and contain relatively few cells. The Peyer's patches, lymph nodes, and spleens are small, contain rare germinal zones and few plasma cells. The levels of serum globulins, white blood cell counts, and hematocrit levels are low. The pattern of enlargement and involution of the thymus glands resembles that of conventional mice. The visceral organs are unique in the absence of perivascular and peribronchial accumulations of lymphoid cells. In general, GF mice are as good or better than conventional mice in reproduction patterns, growth rates, and appearance. The only spontaneous disease observed by us only in GF mice occurred in AKR strain mice in the form of a lethal "wasting" syndrome which we have called "puny" disease (16). This syndrome has not been observed in conventional AKR mice. Other spontaneous diseases observed in GF mice were the same as those observed in their conventional counterparts: they included hemolytic anemia in NZB mice (1), reticulum cell sarcomas in SJL/J mice (18), lymphatic leukemia in AKR mice (20), mammary carcinoma in C3H mice (7), and the immunoproliferative syndrome of LCM virus-infected Haas mice (22).

All Haas strain mice show a lifelong LCM viremia (10^5 LD50/ml blood). They manifest no clinical evidence of disease until about 10 months of age and then they appear ill and few of them survive up to age 1 year. In mice over 1 month of age, the lymph nodes and spleens are enlarged with prominent germinal zones and extensive aggregations of lymphoid cells, many of them plasma cells. The Peyer's patches are swollen with large germinal zones and globulin-staining cells. Segments of the thymic cortex are depleted of cells, the depleted areas expand, and the organ appears prematurely involuted. More distinct microscopic evidence of disease appears at age 5 months in the form of expanding aggregations of lymphoid cells in the visceral organs. Serum globulin levels become markedly elevated and degenerative lesions appear in the glomeruli which are actually due to vascular occlusions by fibrinoid, globulin-staining material (9). Eventually the glomeruli become hyalinized, sclerotic, and obliterated. At age 9 to 12 months, the visceral organs (including the thymus) are distorted and infiltrated extensively with lymphoid cells, many of the latter are amassed in the form of plasmacytomas. Imprint smears of lymph nodes, spleens, thymus, and enlarged organs, stained with fluorescein-tagged anti-mouse globulin have revealed numerous fluorescing cells by ultraviolet microscopy. The chronic disease manifestations in all of the mice have been progressive and uniform, but they did not develop in Haas GF mice which had been thymectomized within 24 h of birth (11).

(b) The Haas mice have been subjected to extensive chemotherapeutic trials, aimed at the proliferative lesions and at the LCM virus associated with them. In preliminary trials it was found that GF mice could tolerate larger subtoxic doses of cyclophosphamide (CPA) than conventional mice: the GF mice survived 5 intraperitoneal injections of 250 mg CPA/kg body weight at weekly intervals, while the conventional mice died of respiratory disease during and after the intraperitoneal administration of 75 mg CPA/kg body weight/week for 5 weeks. It was decided to administer, at weekly intervals, 100 mg CPA/kg body weight intraperitoneally to groups of Haas mice at age levels of 3, 5, and 11 months. This involved mice which had not yet developed lesions (3 months), mice which had initial stages of disease (5 months), and mice which already had developed extensive lesions (11 months). Except for becoming depilated they survived in excellent condition, and at age 12 months they were killed and examined. Mice in the first 2 groups were viremic, but they were free of LCM-related lesions, and their serum globulin levels were within normal limits. The lymphoreticular organs appeared depleted of lymphoid cells. After 3 inoculations of CPA, the 3rd group of mice were viremic, their visceral organs showed marginal lesions only in the kidneys and thymus, and their globulin levels were elevated. All of the saline-inoculated control Haas mice showed the entire spectrum of tissue lesions described above (26). The weekly CPA regime was discontinued in a group of Haas mice, and they were examined periodically during the following 6 months. Characteristic immunoproliferative lesions reappeared in their organs after an interval of 5 months (27). Groups of Haas mice were administered intraperitoneally 100 mg CPA/kg body weight periodically for 5 successive days, at intervals of 3 months. They were killed and examined 3 months after the 3rd series of CPA treatments. The predominant lesion observed in them was reticulum cell sarcomas which were distinct from the lesions observed in untreated mice and in continuously treated mice (28). Haas mice were highly sensitive to whole-body irradiation with ordinarily sublethal doses of x-rays (4 x 150 r, each at weekly intervals). Smaller doses of x-rays were tolerated, and the mice developed lesions of lymphatic leukemia which were accompanied by the LCM-associated lesions described in this mouse strain (29).

(c) Two protocols were developed as attempts to interrupt congenital passage of LCM virus. Earlier tests showed that the virus was actually in the ovary and the ova of Haas mice. This was demonstrated by direct immunofluorescence of frozen tissue sections and by intracerebral inoculations of tissue extracts into LCM-susceptible mice. In the first trial, newborn Haas mice were each inoculated intravenously, from 1 to 5 times, with spleen, lymph node, or thymus cells from LCM virus-immunized Swiss-Webster mice. At 5 to 22 days later, LCM virus was demonstrated in the blood from all of them. Thus, this procedure was unsuccessful. The second trial was based on the successful induction of long-term allogeneic bone marrow chimeras in lethally-irradiated GF mice (6). Forty GF Haas mice, at age 11 weeks, were administered whole-body exposure to 1,000 r x-rays and 24 h later they were each inoculated intravenously with bone marrow cells from 2 femurs ($>10^7$ viable cells) from normal GF C3H mice. All of the mice have now survived over 3 months without any clinical evidence of graft versus host disease. Three of the mice which have thus far been killed and examined were LCM viremic as detected by specific immunofluorescence in "L" cells which had been inoculated with their blood 48 h previously. Forty lethally-irradiated GF Haas mice were inoculated intravenously with bone marrow cells from C3H mice which had been immunized against LCM virus. They have not yet been tested for LCM virus.

DISCUSSION

LCM virus has been associated for many years with chronic persistent disease in mice (5,10,30). Chronic LCM virus infections have been initiated by injection of virus into newborn or into immunologically handicapped mice. The acute convulsive lethal LCM disease has been modified by prior immune suppressive measures, such as thymectomy, x-irradiation, and immunosuppressive drugs and serums (3,4,24,25). The lesions in mice infected persistently with LCM virus vary among mouse strains (12). The disease manifestations in Haas mice, and especially in GF stock, have been the most extensive, uniform, and clearly defined of all mouse strains thus far examined by us. The disease is self-perpetuating and predictable in all progeny, as regards onset and type of lesions, and life expectancy.

The Haas mouse should be considered a distinct strain with virus-related disease characteristics. As with NZB and SJL/J mice, we do not have unaffected controls, so we must compare them with mice of other strains. Haas strain mice have served as model experimental systems for studies on chronic, persistent, congenital viral infection, and for immunoproliferative diseases. There are experimental advantages in this mouse strain: 1) all progeny are infected for life; 2) the assay for LCM virus is rapid and accurate; 3) the development of chronic lesions is predictable; 4) the syndrome appears to result from an immunological dyscrasia; 5) the lesions respond to immunosuppressive therapy with continuing viremia; 6) the virus is associated with tumor-like lesions; and 7) they live in a defined GF environment. The lesions in Haas mice manifest features of some human diseases in which causes and mechanisms have not yet been determined.

It appears thus far that the Haas mice have not been "cured" of their disease complex by treatments with maximum subtoxic doses of cyclophosphamide. However, they survived because of their bacteria-free status, and they were free of demonstrable lesions so long as CPA treatment was continued. Because of their bacteria-free status, the GF animal provides an excellent experimental system for assessment of therapeutic drugs, especially the immunosuppressive agents. This has been demonstrated here with Haas strain mice and also in therapeutic trials with AKR mice (21) and with SJL/J mice (18).

The interruption of congenitally transmitted viruses is of great concern in the practice of preventive medicine. Specific virucidal agents have not yet been found for this purpose. Adoptive immunization procedures may be instrumental towards that goal, but thus far the published results have been indecisive (32). It is anticipated that the induction of allogeneic bone marrow chimeras should provide effective therapeutic and prophylactic measures for congenitally acquired and for neoplastic diseases; however, this has not yet been substantiated.

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LCM Virus-Induced Hemopoietic Disorder in Mice and its Role in the Induction of Tolerance

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SUMMARY

It was observed that doses of x-rays which were sublethal to normal mice could induce fatal diseases in mice infected with LCM virus. The enhanced sensitivity to x-irradiation was seen in persistent, tolerant virus carriers and also in acutely infected mice. The findings indicated that both types of LCM virus infection were associated with hemopoietic disorders. By means of the spleen colony methods it was shown that the function of hemopoietic stem cells is strongly inhibited during acute LCM virus infection. In the virus carriers no inhibition of the hemopoietic stem cells was observed, but the numbers of these cells were significantly decreased. It is suggested that the viral influence upon the stem cells may play an important role for the induction of immunological tolerance by the LCM virus.

INTRODUCTION

For many years it has been well known that sublethal doses of x-rays may prevent deaths and pathological lesions caused by the LCM virus (3,4,5,7). This protective effect is seen when mice are treated with x-rays prior to virus inoculation and is, undoubtedly, related to a suppression of the immune response against the viral antigen. However, in our laboratory it has been demonstrated that the same doses of x-rays could also have the opposite effect. They could cause a fatal disease in mice with inapparent LCM virus infection. This striking harmful influence of irradiation was observed in persistent, tolerant virus carriers and also in acutely infected adult mice which had received the virus by the intraperitoneal route a few days prior to the x-ray treatment. Further studies indicated that the phenomenon observed was a consequence of some virus-induced hemopoietic disorder in the infected mice.

MATERIALS AND METHODS

Virus. The LCM virus strain used for all experiments was obtained from Dr. E. Traub. The virus preparations employed were 10% suspensions of spleens from infected mice. Virus titrations were carried out by intracerebral inoculation of 10-fold dilutions into groups of 12 to 14 g random-bred albino mice.

Mice. For the experiments, highly inbred C3H strain mice were employed.

Persistent, tolerant virus carriers were produced by inoculating newborn mice with LCM virus within the first 18 h of life. Acutely infected mice were given intraperitoneal injections of virus which caused inapparent and transient infections. Immunized mice were mothers of infected babies. These mothers uniformly develop inapparent infections and are highly immune when the babies are taken from them at the age of 4 weeks.

Preparation of cells. Cellular suspensions from livers of near term fetuses and from femoral bone marrows were prepared in Hanks' balanced salt solution as described previously (11).

X-irradiation. A Siemens Stabilipan therapy machine was used and operated at 200 kV and 16 mA with 0.9 mm Cu and 0.5 mm Al filtration.

Hemopoietic colony assays. The recipient mice which were used for the study of exogenous colony formation were given 800 r of x-rays. Within 3 h of this treatment pooled bone marrow cells were injected intravenously. In all the experiments recipients and bone marrow donors were of the same sex. Five recipients were injected per cell pool, and a minimum of 5 donors were used for the preparation of each pool. The endogenous colony formation was studied in mice given 495, 585 or 675 r of x-rays.

Six days after irradiation spleens were removed and fixed in Bouin's solution. The spleens were examined by microscopy of a single longitudinal midline section which was stained with hematoxylin-eosin. The hemopoietic colonies were scored using criteria similar to those employed by Curry et al. (2).

RESULTS

From Figure 1 it can be seen that no mortality was observed in groups of unirradiated virus carriers or acutely intraperitoneally infected

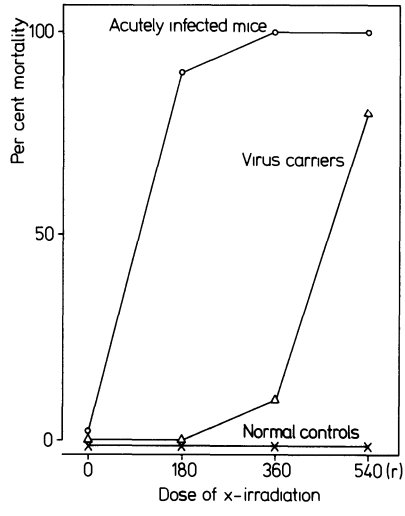

Fig. 1. Mortality of acutely infected mice, virus carriers, and normal controls after receiving various doses of x-rays. The acutely infected mice were inoculated with virus intraperitoneally 6 days prior to irradiation. Mortality was recorded during a period of 30 days following the x-ray treatment. Ten mice per group.

mice. However, when these mice were exposed to doses of x-rays which were sublethal for normal mice, they died. It is obvious that the increase in sensitivity to x-irradiation was most pronounced in the acutely infected mice which had been inoculated with the virus 6 days prior to the x-ray treatment.

It seemed reasonable to suspect that the mortality in the irradiated, infected mice could be related to an x-ray-induced enhancement of the growth of the virus. However, examination of the blood virus titers in the irradiated mice gave no support to this supposition (1). Moreover, in the mice which died during the experiments the clinical signs were not typical of fatal LCM disease. The mice showed progressive loss of weight, severe anemia, and pronounced hemorrhages in tissues and body cavities. This picture suggested that death was caused by a severe hemopoietic disease. This suggestion was strongly emphasized by the findings in the following experiments, in which attempts were made to reconstitute the animals with hemopoietic tissue. Groups of acutely infected mice and virus carriers were treated with doses of x-rays which were sublethal for normal mice, and after irradiation one half of each group was injected with hemopoietic cells from fetal livers. As can be seen from Table 1, a beneficial effect of this transplantation treatment

Table 1. Effect of reconstitution with fetal liver cells on mortality of irradiated LCM virus-infected mice

	Dose of x-rays	Proportion of survivors after 30 days	
		Non-transplanted	Transplanted[a]
Acutely infected mice[b]	270 r	4/20	12/20
Virus carriers	400 r	2/10	10/10

[a] Injected with 10 to 20 x 10^6 fetal liver cells within 3 h after x-irradiation.

[b] Inoculated intraperitoneally with virus 6 days prior to x-ray treatment.

was recorded. The survival rate of the irradiated, acutely infected mice was significantly increased ($P < 0.02$), and treatment of the irradiated virus carriers was even 100% curative. These results suggested to us that the deaths induced by irradiation were caused by hemopoietic failure and, therefore, indicated some abnormal sensitivity or disorder in the hemopoietic system of LCM virus-infected mice.

As a consequence of our observations, histological examinations of the bone marrows of virus carriers and acutely infected mice were carried out. These morphological studies were, however, not very informative as regards the nature of the hemopoietic defect (1). In order to elucidate the function of the hemopoietic system in LCM virus-infected mice, the spleen colony method of Till and McCulloch (8) was employed. By this assay, hemopoietic stem cells can be studied and enumerated by means of their ability to produce distinct colonies of hemopoietic cells in the spleens of lethally irradiated recipients.

In the first series of experiments irradiated normal mice were used as recipients and injections were made with bone marrow cells from acutely infected mice, virus carriers, and normal controls. As is apparent from Table 2, a striking difference was found between the donor cells

Table 2. Spleen colonies in 800 r irradiated normal mice injected with bone marrow cells from normal or infected donors

Marrow donors	Recipients	Mean no. of colonies per 0.25×10^6 donor cells[a]
Normal	Normal	8.2
Virus carriers	Normal	0
Acutely infected[b]	Normal	0

[a] Calculated from numbers of colonies per mid-section of recipient spleens.

[b] Inoculated intraperitoneally with virus 6 days before transplantation.

from the normal and the infected mice. A quarter of a million normal bone marrow cells gave rise to about 8 hemopoietic colonies per spleen section. The cells from the virus carriers and the acutely infected mice, on the other hand, did not produce colonies. Not a single colony was found, even when doses were increased to several million cells. However, in evaluating these results it has to be borne in mind that

Table 3. Spleen colonies in 800 r irradiated normal and immunized mice injected with various preparations of bone marrow cells

Marrow donors	Addition of virus to donor cells[a]	Recipients	Mean no. of colonies per 0.25×10^6 donor cells[b]
Normal	+	Normal	0
Normal	+	Immunized	9.7
Virus carriers	-	Immunized	4.5
Acutely infected	-	Immunized	4.6

[a] 10^5 LD50 of virus in 0.5 ml were added to the suspended donor cells.

[b] Calculated from numbers of colonies per mid-section of recipient spleens.

during transplantation of cells from virus carriers and from acutely infected mice it is unavoidable that infectious virus is also transferred to the recipients; that is, the recipients of bone marrow cell suspensions from infected animals will be acutely infected themselves. In order to rule out the possibility that this acute infection of the recipient mice could interfere with the stem cell assay, the following experiment was performed. A suspension of normal bone marrow cells was mixed with infectious virus in vitro. After an incubation period of about 30 min, the mixture was injected into irradiated normal mice and also into a group of irradiated mice which had previously been immunized against LCM virus. As can be seen from Table 3, the numbers of colonies observed in the immunized recipients were at normal levels and, therefore, the stem cells could not have been damaged during the in vitro incubation with the virus. However, it is apparent that hemopoietic colonies were not produced in the normal recipients injected with the virus-cell mixture. Obviously, some severe interference with the stem cell proliferation had occurred during the first 6 days of the acute infections which were induced in these non-immunized mice.

The ability of immunized and irradiated mice to support the growth of exogenous stem cells in spite of contaminating virus was used in the following experiments to carry out a further evaluation of the stem cells in the virus-infected animals. Bone marrow cells from virus carriers and from mice which had been infected 6 days previously were injected into groups of irradiated immunized recipients. From Table 3 it is apparent that these bone marrows in fact contained certain numbers of stem cells. The hemopoietic colonies which were produced had a normal appearance; their numbers, however, were somewhat smaller than those obtained with the same doses of normal bone marrow cells.

From the spleen colony data which have been described so far, it is obvious that stem cells with colony-forming potentials are present in considerable numbers both in virus carriers and in acutely infected mice. The proliferation of the stem cells is, however, strongly inhibited in the acutely infected mice. In the following experiments the proliferative response of stem cells which were injected into irradiated virus carriers was studied. The results from 4 independent experiments are shown in Table 4. It can be seen that the virus carriers, in spite of their pronounced virus titers in blood and organs, supported the growth of the colony-forming stem cells. In fact, it was very characteristic that the spleen colonies seen in the virus carriers were larger than those which were produced in normal recipients. As was also noticed in the foregoing experiments with the immunized recipients, fewer colonies were seen in mice injected with cells from virus carriers than in those injected with normal bone marrow cells. This decrease, which amounted to about 50%, was found to be significant at the 0.02 level.

The hemopoietic stem cell activity in LCM virus-infected mice was also investigated by means of the endogenous spleen colony assay. By this method mice are given different doses of x-rays which should lead to the survival of a few endogenous stem cells. Six days later the numbers of surviving stem cells can be scored as endogenous hemopoietic spleen colonies. In Table 5 are shown the numbers of colonies which were produced after treatment with various doses of x-rays in controls, in virus carriers, and in acutely infected mice injected with virus 6 days previously. It can be seen that the normal mice and the virus carriers showed a dose-dependent reduction in colony counts similar to what has been reported by others (9). The virus carriers were clearly inferior to the normal controls, and the counts again suggested a 50% reduction of the stem cells in these animals. As might have been ex-

Table 4. Spleen colonies in 800 r irradiated virus carrier mice injected with normal and virus carrier bone marrow cells

Marrow donors	Recipients	Mean no. of colonies per 0.25×10^6 donor cells[a]			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4
Normal	Virus carrier	9.3	10.5	8.2	8.7
Virus carrier	Virus carrier	5.5	3.0	3.9	5.2

[a] Calculated from numbers of colonies per mid-section of recipient spleens.

Table 5. Endogenous spleen colonies in normal and virus-infected mice given various doses of x-rays

Mice	Mean no. of endogenous colonies[a] 495 r	585 r	675 r
Normal	9.0	4.0	1.2
Virus carriers	4.7	1.6	0.5
Acutely infected	0	0	0.2

[a] Calculated from number of colonies per mid-section of spleens.

pected, the most striking results were recorded with the acutely infected mice. In these animals hemopoietic spleen colonies were not seen when they were killed 6 days after the x-ray treatment, that is, 12 days after initiation of the infection.

DISCUSSION

The spleen colony data which are presented above, as well as the observations which were made on the x-ray-induced mortality, provide convincing evidence for the occurrence of hemopoietic disorders in mice infected with LCM virus. From the spleen colony data it is apparent that the defects in virus carriers and acutely infected mice were not the same. In the adult virus carriers the numbers of hemopoietic stem cells were decreased by about 50%, but the colony-forming response of these cells was not suppressed. In the acutely infected mice there was also a suggestion of some reduction of the stem cell pool; however, in these mice the most striking finding by far was that of a profound inhibition of the proliferative response of the stem cells. These observations, based upon the spleen colony assays, seem to agree very well with the mortality rates which were recorded in the virus carriers and in the acutely infected mice after exposure to x-irradiation.

The mechanism by which the LCM virus inhibits hemopoietic colony formation during the acute infection is obscure. However, the findings of considerable numbers of stem cells with colony-forming potentials in the acutely infected mice indicate that the phenomenon is not caused by any destruction of or irreversible damage to the stem cells. Moreover, the fact that colony formation was unimpaired in the virus carriers in spite of their high titers of infectious virus makes it very improbable that the LCM virus has any direct inhibitory influence upon the stem cell function. It would be more in accordance with our observations to suggest some virus-induced interference during the acute LCM virus infection with humoral or micro-environmental factors which are known to provide stimuli for the stem cells (6).

In normal adult mice the immunocompetent cells are available in sufficient numbers to afford a prompt and effective response to the LCM virus. In such animals it is extremely difficult to induce a state of tolerance to the virus. Newborn mice, however, have only very few immunologically reactive cells and their immune response is strongly de-

pendent upon the differentiation of immature precursor cells to mature immunocompetent cells. This situation delays the immune response to any antigen and, furthermore, if the antigen is a living virus, it allows the virus to grow relatively unchecked for some time, resulting in the production of large amounts of viral antigens. During infection with a non-cytopathogenic virus, both the impact of this antigenic mass on the immune system and the fact that few immunocompetent cells are present will probably facilitate the induction of a state of tolerance to the virus. Nevertheless, only very few viruses are known to be able to induce tolerance in newborn animals. During infections with most viruses, immunocompetent cells will develop and an immune response will be produced. Thus, the antigenic influence during the first few days of life has in these cases not been sufficient to cause a lasting immunosuppression to the virus. In contrast, as far as the LCM virus is concerned, our findings have shown that the acute viral infection causes a decrease in the number of colony-forming stem cells as well as a strong inhibition of their function. Since these colony-forming stem cells seem to be identical with the hemopoietic precursor cells (10,12), it is reasonable to assume that neonatal infection with LCM virus causes serious impairment of the development of immunocompetent cells. When this viral effect is added on top of the other factors which influence the immune response of the newborn, it might very well be decisive in inducing a state of tolerance to the virus.

It is well known that tolerance to the LCM virus can also be produced in adult mice but, with rare exceptions, only in animals deprived of the main part of their immunocompetent cells, such as by treatment with anti-lymphocytic serum. In order to mount an immune response, these animals are in the same urgent need of hemopoietic precursor cells as are newborn mice. Therefore, it seems reasonable to suggest that the LCM viral effect on the stem cells is also an important determinant in the induction of tolerance in immunosuppressed adult mice.

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Murine LCM Virus Infection: Tolerance and Immunity

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SUMMARY

Evaluation of experimental results obtained during work with the murine LCM model is difficult because of the great number of variables which have to be taken into consideration. In this paper, an attempt is made at interpreting comprehensively the various patterns of reactions of the mouse towards the virus. Experiments were performed in an attempt to elucidate the role which a soluble virus antigen may play in inducing LCM virus-specific immunological tolerance. Its chemical composition is unknown. Though it is an effective antigen in serological assays, e.g. complement fixation, its immunogenic potential in mice is low. Tolerant virus carrier mice have more antigen in their organs than have mice infected intraperitoneally, although the virus concentrations do not differ significantly. However, this phenomenon does not seem to be causally related to the state of tolerance since the soluble antigen per se is non-tolerogenic.

The results suggest that my previous hypothesis accounting for LCM virus tolerance should be extended so that specific continuous immunosuppression is due to replication of the viral genome in the LCM virus-reactive cells of the immune apparatus of the host.

INTRODUCTION

The question of immunological tolerance in mice to LCM virus has received much attention during previous years. The original concept of Burnet and Fenner (2), according to which the viral antigens are integrated by the immunologically immature host as "self", has been questioned, especially by Oldstone and Dixon (16,17) who use a definition of the state of tolerance which is different from the one proposed by myself (8,11). In particular, these latter authors neglect the role of cell-mediated immunity which has been shown to be an integral part of the state of LCM virus-specific immunological tolerance (8,10,11).

Since we do not know how immunological tolerance is brought about in general, in order to avoid confusion we must begin with an exact definition. Bearing in mind that immunological tolerance has quantitative elements besides a qualitative one, we may tentatively define complete immunological tolerance as a state which is characterized by an animal's continuous inability to give a specific immune response - humoral as well as cell-mediated - after antigenic stimulation. In theory, two central mechanisms may be visualized as being responsible for this state: 1) according to the classical Burnet theory, the immunological stem cells do not exist in a tolerant animal; 2) alterna-

tively, the stem cells are present but are continually suppressed, perhaps being in a state of low activity. The latter implies that complete immunological tolerance may be considered a state of continuous, complete, specific immunosuppression. I have previously presented arguments which favor the latter assumption as regards the LCM virus tolerance in mice (8) and, furthermore, find this notion to have a better physiological basis. Also it explains findings which otherwise would strengthen theories according to which specific immunosuppression is due to "peripheral" rather than "central" mechanisms.

A comprehensive classification of the various ways in which a mouse may react towards the LCM virus is presented in Table 1. In this scheme, the many variables in the murine LCM virus model have been

Table 1. Classification of the immunological reaction patterns of the mouse towards LCM virus

Type I.	Complete immunological tolerance, characterized by: a) continual absence of detectable circulating antibodies and of cell-mediated immunity to the virus; b) continual presence of viremia; c) absence of specific disease; d) abolition by adoptive immunization.
Type II.	Split tolerance, characterized by: complete suppression of humoral immunity without suppression of the cell-mediated immunity or complete suppression of cell-mediated immunity without suppression of humoral immunity.
Type III.	Incomplete tolerance, characterized by: various degrees of suppression of the humoral and/or cell-mediated immunity to the virus.
Type IV.	Normal immunological response, characterized by: a) formation of circulating antibodies; b) development of cell-mediated immunity.

taken into account, such as strain and age of the mouse, strain and dose of the virus, route of inoculation, results from virus and antibody titrations, state of cell-mediated immunity. Types II and III (Table 1) are temporary states and usually result in the development of the disease. The evidence presented by Oldstone and Dixon clearly shows that they are dealing with types II and III. Type IV often leads to the development of acute fatal disease. If the animal survives, solid resistance to reinfection ensues.

One of the basic questions in connection with tolerance of the mouse towards the LCM virus is: what part of the virus is responsible for

the specific unresponsiveness of the host? Is it the virion as a whole or only the viral genome; are structural components of the virus or are virus-associated or virus-induced "new" antigens involved; could it be a mixture of all these? Or, even, is LCM virus-specific "tolerance" in the mouse caused by excessive amounts of viral proteins, being essentially a protein-overloading phenomenon (3)? Experiments were performed to define the effects viral antigens may have on the immune reactions of the mouse.

MATERIALS AND METHODS

In these experiments highly inbred C3H mice were employed. The strain of virus and virus titrations were described previously (9). The antiserum against LCM virus was a pool of hyperimmune serum obtained from C3H mice.

CF tests were performed with LCM virus s-antigen, using 2-fold dilutions of serum and 4 units of antigen in 0.1 ml volumes; alternatively, box titrations were done in which 2-fold dilutions of antiserum or normal serum were combined with 2-fold dilutions of s-antigen. All tests were performed on perspex plates. Further details may be found in a previous publication (20).

The soluble antigen was prepared from murine tissues, following a procedure slightly modified from the one originally described by Clarke and Casals (5) and by Grešíková and Casals (7). Working at 4°C throughout, the organs were removed aseptically from mice, weighed, and homogenized for 3 min in an "Omni-mixer" (Sorvall, Norwalk, Connecticut) with 4 volumes of an 8.5% sucrose solution. This homogenate was added with continuous stirring to 20 volumes of cold, chemically pure acetone, agitated for a few minutes and then centrifuged at 1,200 x G for 5 min; the supernatant was discarded. The pellet was resuspended in 20 x the volume of acetone, stirred, and incubated in an ice bath for 1 h. The mixture was centrifuged, the supernatant again discarded, and the sediment extracted a 2nd time with acetone. After the final centrifugation, the acetone was removed by evaporation for 4 to 5 h in an ice bath, and the dried, powdery material was incubated overnight with 0.4 x its volume of saline made up with redistilled water. The mixture was centrifuged at 10,000 rev/min (Spinco L, rotor 30) for 1 h, and the supernatant, which was used as the antigen, was stored frozen in ampules at -70°C. Batches of antigen produced in this way have been kept for years at -70°C without loss of activity.

Precipitating antisera were prepared in rabbits by biweekly intradermal injections over a period of 4 months of either 1 ml CF antigen or 1 ml virus suspension emulsified with equal amounts of incomplete Freund's adjuvant.

Immunoelectrophoresis was performed as crossed procedure, according to Laurell (13) and Clarke and Freeman (6). Absorption of the sera was performed by adding equal amounts of antigen (control or viral) or virus suspension to the antiserum and incubating overnight in a 37°C water bath, followed by centrifugation.

RESULTS

The soluble antigen. The soluble LCM virus antigen investigated in the experiments to be described is non-infectious. It is preserved by sucrose; if homogenization is done without sucrose, no antigen can

be extracted. The antigen is soluble in water and insoluble in acetone. It reacts with sera from mice after LCM virus infection by complement fixation but not by precipitation or mixed hemagglutination. It does not react with sera from normal mice or from mice which had undergone infection with vesicular stomatitis virus, Gross virus, or yellow fever virus.

Analysis of the precipitating rabbit antisera by crossed immunoelectrophoresis against s-antigen and virus suspension together with the appropriate controls gave consistently 15 to 20 lines of precipitation. After removal of C3H antigens by absorption, in repeated experiments only 1 or 2 lines formed. Similar observations were made by Bro-Jørgensen (1). Chastel (4) reported the occurrence of at least 4 lines of precipitation and Simon (18) saw even more; however, the sera used by these authors had not been absorbed. At present we do not know whether these soluble antigens are capsid material or whether they are virus-induced "new" antigens; one of them seems to be a protein (1).

The antigenic effect. In this laboratory, soluble antigen, extracted as described from the livers and spleens of tolerant C3H virus carrier mice, has for several years been used in routine tests as LCM virus CF antigen. In numerous CF tests it has proved to be very specific, and non-specific reactions on account of the antigen have never been encountered with murine sera.

Sera from mice infected with LCM virus contain CF antibody after 1 week. In the following week the titers increase and reach constant levels usually at 128. Hyperimmunization does not result in further increases, and antibody levels remain constant for many months; apparently, they never disappear (10). In adoptively immunized virus carriers, CF antibody becomes demonstrable after 1 week and reaches maximum titers of 8,000 to 64,000. More details may be found in a previous report (20).

The concentration of s-antigen in the organs of acutely and persistently infected adult mice. In order to investigate the contents of s-antigen and virus in organs of adult mice either tolerant to the LCM virus or undergoing an acute infection, the following experiments were performed. Six groups of 45 3-month-old C3H mice were infected intraperitoneally with LCM virus, each mouse receiving 3,000 LD50 (day 0). This dose, administered peripherally, is never lethal and always induces solid immunity (10). On days 0, 3, 6, 9, 15, 30, and 60 the mice of one group were killed and their spleens, livers, brains, and kidneys were pooled separately and homogenized. Samples of the resulting homogenates were used for virus titrations, and from the bulk s-antigen was prepared as described in Materials and Methods. The same procedure was followed with a group of 45 3-month-old LCM virus-tolerant C3H virus carrier mice.

Antigen contents were determined in box titrations against either a pool of hyperimmune serum or a pool of normal serum, both obtained from C3H mice. The highest dilution of the immune serum which gave a positive reaction was 1:128. Consequently, this dilution was chosen as reference and the antigen titer was defined as the reciprocal of that dilution which reacted (= 50% hemolysis) with the 128-fold diluted reference serum.

The results of these experiments are shown graphically in Figures 1 to 4. Virus and antigen titers at various times after intraperitoneal infection are recorded, and the results from the tolerant virus carrier mice are shown to the right in the Figures.

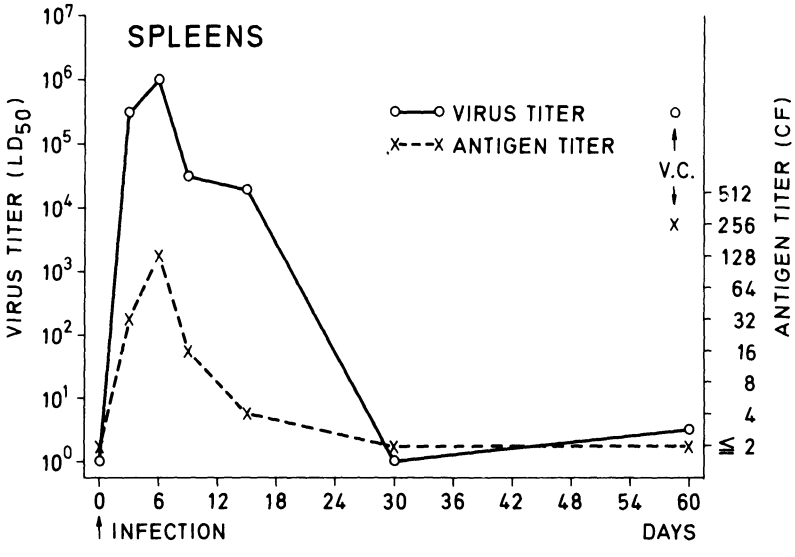

Fig. 1. Titers of virus and s-antigen in the spleens of C3H mice, infected intraperitoneally with 3,000 LD50 LCM virus. To the right in the Figure are shown the corresponding results from tolerant LCM virus carrier mice.

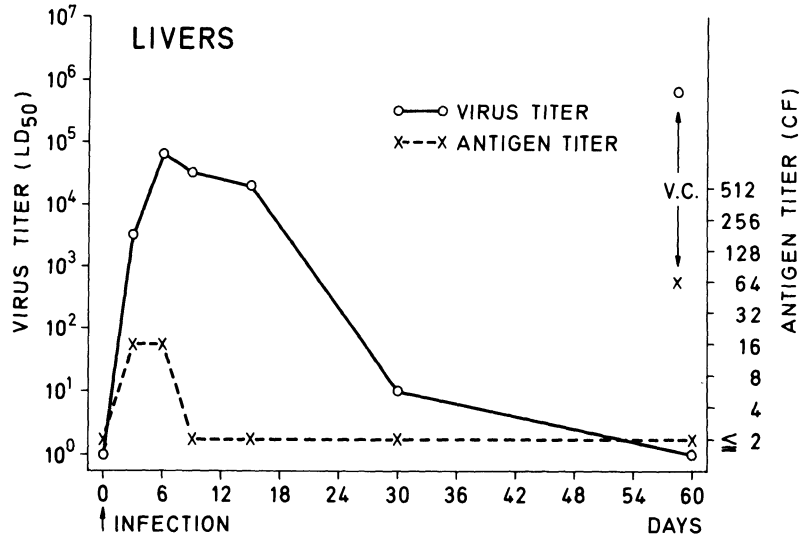

Fig. 2. Titers of virus and s-antigen in the livers of the mice shown in Figure 1.

The development of virus titers in the organs was usually identical with that which was observed previously (8). Maxima of $10^{3.3}$ to $10^{6.0}$ were found on day 6. Virus contents then decreased and by day 30 in-

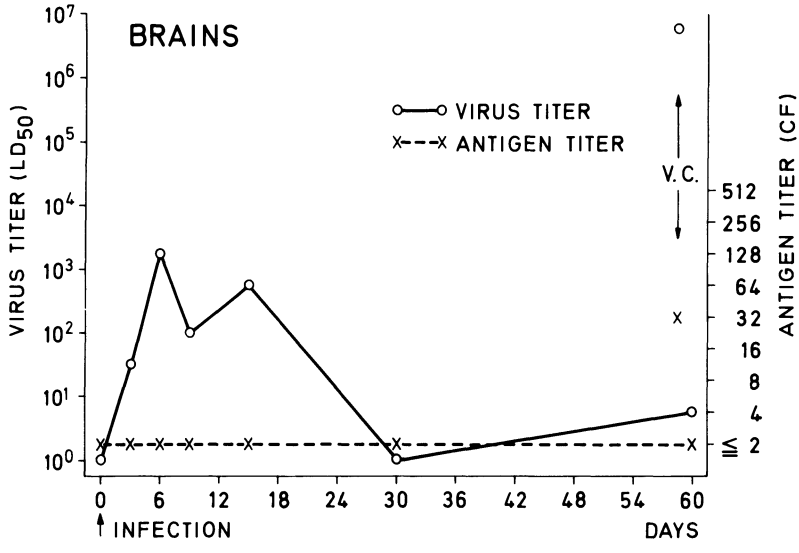

Fig. 3. Titers of virus and s-antigen in the brains of the mice as in the foregoing Figures.

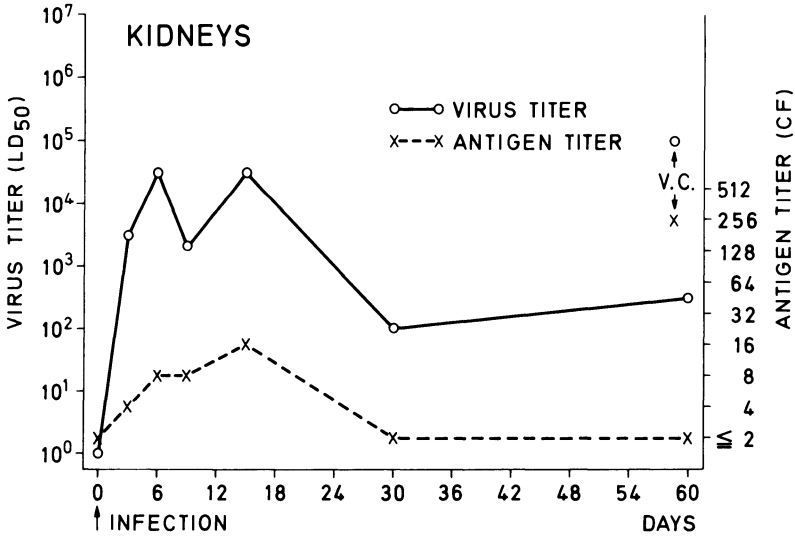

Fig. 4. Titers of virus and s-antigen in the kidneys of the mice as in the foregoing Figures.

fectivity had all but disappeared from most organs with the exception of the kidneys (Fig. 4) where it remained at a level of about $10^{2.5}$, a phenomenon which has been observed and discussed previously (8,11,12). In the carrier mice, virus concentrations were high in all organs.

The s-antigen titers followed the same overall course as the virus titers except in the brain from which no s-antigen could ever be extracted (Fig. 3). Relatively large amounts of antigen were formed in the

spleens (Fig. 1) where peak values in mice infected intraperitoneally were almost as high as in tolerant virus carriers. In the livers (Fig. 2) of intraperitoneally infected mice less antigen was formed and this amount was formed only during a short initial period. More antigen was found in the livers of virus carriers despite the fact that at the same time virus titers were at equivalent levels in both types of virus infection. In the kidneys of intraperitoneally infected animals only small amounts of antigen were detected, which contrasts with the higher concentrations found in virus carriers. Though the virus had not disappeared from these organs 30 days after an acute infection, s-antigen could not be demonstrated at this time or later (Fig. 4).

The immunogenic effect. The immunogenic effect of the soluble LCM virus antigen was investigated in mice. Two groups of adult C3H mice, one consisting of 10 normal mice, the other of 10 virus carriers, were inoculated subcutaneously with 0.2 ml of an s-antigen solution having a titer of 32. Blood samples were obtained from the inner canthus of the eye at weekly intervals for the following 2 months. CF titrations of the individual sera revealed that none of the mice in either group had responded with antibody formation (Table 2). Also, the virus titers in the carriers were unchanged.

An attempt was made to immunize more intensively. Groups of mice as before were inoculated subcutaneously every other day, altogether 3 times, with an emulsion consisting of 0.1 ml s-antigen and 0.1 ml complete Freund's adjuvant. With this immunization regimen, all mice in both groups were severely affected, and a few of them even died during week 2 and 3 after immunization. Notwithstanding this adverse effect, all uninfected mice formed CF antibody during the first 2 weeks with peak titers of 8 to 32. In contrast to mice which survived an infection, however, this antibody soon had disappeared again. Virus carriers also formed CF antibody during the same period of time with peak titers of 16 to 64 (Table 2) which soon disappeared. Virus titers in these animals were unaffected.

The tolerogenic effect. Attempts to induce LCM virus-specific immunological tolerance by administration of CF antigen were made by a method similar to the one used for the induction of complete and permanent tolerance for virus (8,11). Newborn C3H mice were inoculated intraperitoneally on days 1, 3, and 5 after birth with 0.03 ml of an s-antigen solution with a titer of 32. Control animals were left untreated. Other mice were inoculated with a single dose of 1,000 LD50 of LCM virus on the 1st day of their life. These latter animals all became typical tolerant virus carriers with blood virus titers of $10^{2.3}$ or higher and CF titers of less than 4.

The antigen-inoculated and the untreated mice were further investigated at the age of 3 weeks, at which time the CF titers in all these animals were below 4. They were divided into 6 groups, each consisting of 8 to 10 mice, as indicated in Table 3, and at the age of 3.5 weeks these mice were challenged in 3 different ways and their fates were recorded individually. One group of untreated and one group of s-antigen-inoculated mice were challenged with s-antigen, each mouse receiving 0.3 ml intraperitoneally. None of these animals formed CF antibody during the period of observation, as indicated in Table 3. Mice from 2 similar groups were challenged intracerebrally with 1,000 LD50 of LCM virus. All mice died with a typical disease 8 to 12 days later. Mice from the remaining groups were infected intraperitoneally with 3,000 LD50 of LCM virus. Again, the responses in the 2 groups were essentially identical; viremia was of short duration and CF antibody reached approximately the same titers.

Table 2. The immunogenic effect of LCM virus s-antigen in adult C3H mice

No. mice	Category	Freund's adjuvant	Dose	Result	Titer
10	Normal	-	0.2 ml subc.	No antibody formation	<4
10	Virus carrier	-	0.2 ml subc.	No antibody formation; virus titers unchanged	<4
10	Normal	+	0.1 ml x 3 subc.	Antibody formation	8-32
10	Virus carrier	+	0.1 ml x 3 subc.	Antibody formation; virus titers unchanged	16-64

Table 3. The effect of challenge with s-antigen or virus on 3-week-old C3H mice, untreated or neonatally inoculated with s-antigen[a]

No. mice	Category	CF titer before challenge	Challenge	Virus and/or CF titers after challenge				
				8 days	12 days	24 days	45 days	129 days
8	Untreated	< 4	s-antigen 0.3 ml i.p.	<4	<4	<4	<4	-
9	s-antigen, neonatally	<4	s-antigen 0.3 ml i.p.	<4	<4	<4	<4	-
10	Untreated	<4	1,000 LD50 i.c.	All died				
8	s-antigen, neonatally	<4	1,000 LD50 i.c.	All died				
8	Untreated	<4	3,000 LD50 i.p.	$10^{1.8}$; <4	$<10^{0.5}$; 16	$<10^{0.5}$; 155	$<10^{0.5}$; 238	$<10^{0.5}$; 101
8	Neonatally infected	<4	3,000 LD50 i.p.	$10^{1.5}$; <4	$<10^{0.5}$; 10	$<10^{0.5}$; 137	$<10^{0.5}$; 384	$<10^{0.5}$; 146

[a] Inoculated on the 1st, 3rd, and 5th day of life with 0.03 ml s-antigen.

DISCUSSION

The soluble LCM virus antigen has been found by CF testing to be both specific and potent. Its chemical nature is virtually obscure. The little we know of its properties can be summarized as follows. It can only be isolated from LCM virus-infected tissues. It is stabilized by sucrose. The antigen is soluble in water, insoluble in acetone. It is non-infectious, strongly antigenic, weakly immunogenic, and non-tolerogenic. It consists of 2, possibly more, components, of which one is probably a protein.

The correlation which seems to exist between s-antigen and infectious virus is similar in the spleens of intraperitoneally infected mice and in tolerant virus carriers. The pattern is different in liver and kidneys; in carrier mice concentrations of both infectious virus and s-antigen are high, while in the adult infected mice high virus titers (day 6) contrast with low s-antigen titers. In the brain, no s-antigen at all can be demonstrated, although considerable quantities of infectious virus are present, less in intraperitoneally infected mice than in carriers.

Thus in adult mice, infected intraperitoneally, maximum production of infectious virus is not necessarily accompanied by maximum production of s-antigen as seems to be the rule in tolerant carrier mice. During this early period of infection, immune elimination, either humoral or cellular, can hardly be assumed to be working efficiently, and rather than assuming low s-antigen concentrations in the organs of mice undergoing acute intraperitoneal infections, we conclude that in virus carriers an excess of antigen is produced. The question then arises whether this massive accumulation of s-antigen is cause or consequence of virus persistence. The experiments recorded in Table 2 would seem to indicate that the latter is the case; the s-antigen per se induced neither tolerance nor resistance to the virus. This contrasts with live virus which is highly tolerogenic as well as immunogenic when injected into newborn mice, affecting both the cell-mediated (virus elimination) and the humoral (CF antibody) components of immunity (10,11). It is, however, possible that viral antigens other than the soluble material investigated here are responsible for the induction of tolerance. During infection with LCM virus, the mouse forms neutralizing antibody at low concentrations (14). After adoptive immunization of tolerant virus carrier mice with sensitized lymphoid cells, high titers of this antibody are attained (20). Lehmann-Grube (15) found that CF antigen with high specific titer, prepared from infected L cells or guinea-pig organs, has no blocking effect on the neutralization of infectivity by rabbit immune serum and, hence, soluble CF antigen(s) and antigen(s) inducing neutralizing antibody presumably are different. Since both the CF antibody and the neutralizing antibody seem to play only a minor role, if any, in the elimination of the virus and the abolition of tolerance (8), it seems likely that the respective antigens are not responsible for LCM virus tolerance in the mouse. This supposition is strengthened by our failure to induce tolerance by means of the soluble antigen(s).

Thus my assumption that LCM virus tolerance is a specific, continuous immunosuppression (8) is still tenable with the modification that immunosuppression is not caused by viral antigen(s). I propose that it is the viral genome which replicates and persists in the LCM virus-reactive cells of the immune apparatus of the host, thereby rendering these cells incapable of responding immunologically.

ACKNOWLEDGMENTS

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Inhibition of Mouse Tumors and Viruses by the M-P Strain of LCM Virus

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SUMMARY

The inhibitory action of the M-P strain of LCM virus against mouse tumors and oncogenic viruses appears to be mediated via induced serum interferon during 2 to 5 days, followed by an immune action.

These mechanisms are more clearly elucidated in the inhibition of pseudorabies virus disease in mice by LCM(M-P) virus. Here host resistance is caused by interferon, followed by virus host-specific immunity directed against host antigens incorporated on both the LCM(M-P) virus and the pseudorabies virus, acquired during replication in mice.

INTRODUCTION

The M-P strain of LCM virus, isolated from a cell culture of Ehrlich carcinoma, has lymphocytopenic properties. These properties stimulated a series of studies on tumor inhibition by ourselves and others, demonstrating an inhibition of transplantable and spontaneous murine leukemia and carcinoma as well as virus-induced tumors (4,5,13). In 1966, LCM(M-P) virus from the serum of BALB/c mice was grown on HeLa S3 cells and subcultured on L929 mouse fibroblasts, as well as many other cell lines of different host origin. The HeLa cell-grown virus has a broad spectrum of infectivity in mice, rats, dogs, swine, and man, where parenteral injection produces a transient viremia.

The electron microscopic morphology of the LCM(M-P) virus (Fig. 1) is indistinguishable from other members of the arenavirus group, and LCM (M-P) virus shares the biological and chemical properties of this group (7). It is antigenically a strain of the LCM virus (4,7). Differences with all other strains of LCM virus are apparent in the immune responsiveness of the mouse host principally by the early production of neutralizing antibody, following a single intraperitoneal injection, which is titratable during the acute viremia and levels off at a titer of 256 between 30 and 70 days (Fig. 2), decreasing to less than 4 at 195 days post-infection. Other parameters of infection are: a serum virus titer which reaches 4×10^5 ID50/ml 7 to 10 days post-infection and is cleared 21 days post-infection; a brain virus titer which reaches a peak of 4×10^6 ID50/ml and is cleared in the same period; kidney virus which peaks at 4×10^5 ID50/ml, persists in the renal circulation and coexists in the presence of high titers of neutralizing antibody. Similarly, Machupo virus has been reported by Mackenzie to induce high titer neutralizing antibody coexistent in vivo with infectious virus in hamster kidney (3). Kidney virus decreases at approximately 1 $\log_{10}$/month and

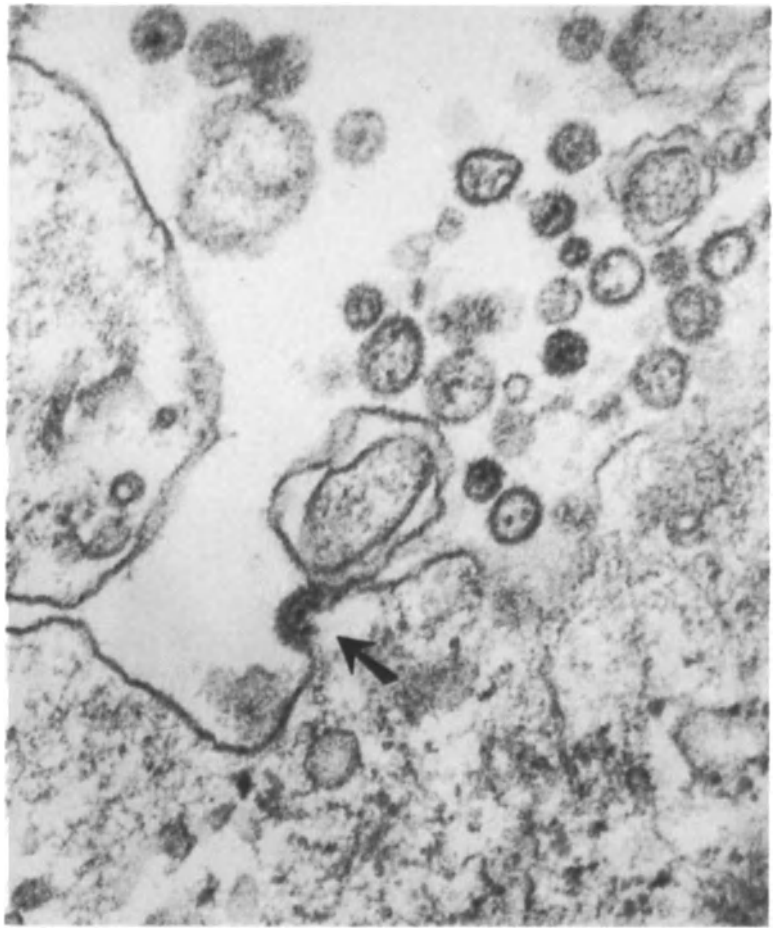

Fig. 1. LCM(M-P) virus budding from plasma membrane of HeLa cell. Magnification x75,000. (Kindly supplied by Dr. J.L. Duffy.)

is not detectable 5 months post-infection. Serum interferon levels (11) develop from day 1 through day 5, reaching peak titers on day 3 in all mouse strains, varying in titer among different mouse strains (4,500 interferon units/ml for Swiss mice and 2,500 interferon units/ml for BALB/c mice on day 3) (Fig. 2).

We present studies on the inhibition of a transplantable lymphatic leukemia, Rauscher virus leukemia and pseudorabies pathogenesis in BALB/c mice during varying phases of LCM(M-P) virus infection. These are examined for their relative quantitative effectiveness and to elucidate the mechanisms operative in this inhibition, viewed against life-span studies of LCM(M-P) virus inhibition of spontaneous tumors in C3H and AKR mice.

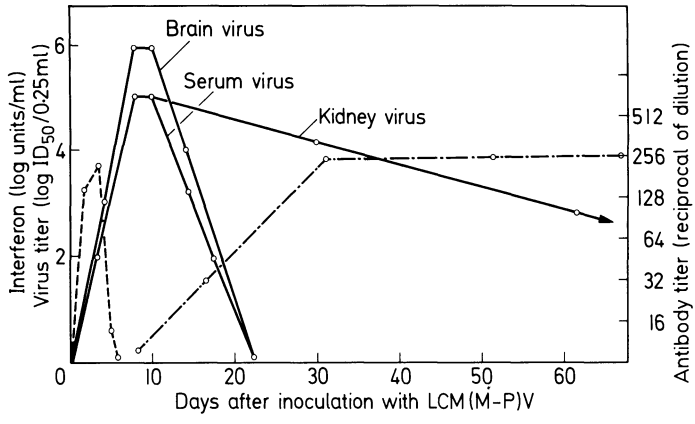

Fig. 2. Parameters of LCM(M-P) virus infection in BALB/c mice.
———— Virus in brain: Each point represents the titer of a pooled brain homogenate from 3 mice. Virus in serum: Each point represents the titer of pooled sera from 6 mice. Virus in kidney: Each point represents the titer of pooled homogenate of kidneys from 3 mice.
— — — — — Interferon titer: Each point represents pooled sera of 6 mice.
—•—•—•— Neutralizing antibody: Each point represents the titer of pooled sera from 12 mice, except on days 30, 50, and 70, when sera from 24 mice were pooled.

MATERIALS AND METHODS

Strains of mice. C3H/An and BALB/c inbred mouse strains have been bred in our laboratories since 1958 from breeders obtained from the National Institutes of Health, Bethesda, Maryland. Swiss/Inness mice were obtained as a closed colony from the Brookhaven National Laboratory in 1962 and AKR mice from Jackson Laboratories, Bar Harbor, Maine, in 1964.

Transplantable lymphatic leukemia (TLL). A transplantable tumor was induced by methylcholanthrene in BALB/c mice in 1960 at this laboratory and was subsequently propagated by mouse to mouse passage in mature BALB/c mice, both sexes (15). A dose of 500 cells administered intraperitoneally kills all mice in 11 to 13 days (5).

Virus stocks. LCM(M-P) virus was propagated on HeLa S3 cell monolayers in Eagle's basal medium with Hanks' salts, supplemented with 10% fetal calf serum and antibiotics. After LCM(M-P) virus inoculation, the culture was maintained on Eagle's medium supplemented with 5% fetal calf serum. Five days post-inoculation the cells were scraped from the glass and the tissue culture fluid, including suspended cells, was centrifuged at 1,200 x G at 4°C. The cell-free supernatant containing LCM (M-P) virus was sterile-filtered and stored at -85°C or -196°C.

LCM(M-P) virus was diluted 10-fold serially in phosphate-buffered saline, pH 7.4, and was assayed in vivo by intraperitoneal injection into 6 adult (5 to 8 weeks of age) Swiss mice for each dilution. Each mouse received 0.25 ml of the respective dilution.

The LCM(M-P) virus titer was calculated on the basis of mice whose circulating lymphocytes (differential white blood cell count of a stained blood smear) had decreased by 10% 3 to 4 days after injection or of mice in which pleural and/or peritoneal fluid had appeared 8 to 10 days post-injection. The ID50 end-point was determined by the method of Reed and Muench (12).

Virus neutralization. The neutralization procedure employed the constant virus-serum dilution method. Pooled sera from 12 or 24 BALB/c mice at varying times post-infection following a single intraperitoneal injection of 10^3 ID50 of HeLa cell-grown LCM(M-P) virus were sterile-filtered, heat-inactivated and serially diluted with 0.1% bovine serum albumin in water. A 10% suspension of virus-infected mouse brain was homogenated and diluted in the diluent to contain 500 ID50 LCM(M-P) virus in the incubation mixture. After incubation at 37°C for 90 min, the incubation mixtures were injected intraperitoneally into groups of 6 adult Swiss mice per dilution for infectivity assay. Control incubation mixtures were combined with normal BALB/c serum treated in the same manner as the immune serum.

Rauscher leukemia virus (RLV), obtained from Dr. Frank J. Rauscher, Jr., was isolated from plasma of BALB/c mice and supplied as a frozen suspension in 0.05 M sodium citrate, pH 6.7. RLV was diluted 200-fold in phosphate-buffered saline, pH 7.4, and inoculated intraperitoneally into BALB/c mice (0.01 ml/g).

Pseudorabies virus (PrV), Sullivan strain, obtained from Dr. D.P. Gustafson, was grown on porcine kidney cells (PK 15) in Eagle's minimum essential medium prepared with Earle's balanced salt solution with reduced bicarbonate (0.85 g/l), supplemented with sodium pyruvate, 5% fetal calf serum and antibiotics. PrV was harvested 72 h post-infection, centrifuged free of cell debris and stored at -85°C in 1 to 5 ml volumes. Each harvest of PrV was titrated in vivo in adult mice to determine the LD50 and assayed on L929 cells to determine the number of PFU. The minimal dose that killed 100% of mice (MLD100) when administered intraperitoneally was found to contain 25 PFU.

RESULTS

In life-span studies on spontaneous tumor inhibition by LCM(M-P) virus in mice, we have found the following: Virgin C3H/An female mice, treated at 6 to 9 weeks of age with a single intraperitoneal injection of 500 ID50 of HeLa cell-grown LCM(M-P) virus, demonstrate a 50% lower incidence of mammary tumor than comparable controls after 2 years observation. C3H/An females which had borne 3 litters prior to the test described, treated at the time when mammary tumor was palpable, did not exhibit significant inhibition (Table 1) from HeLa LCM(M-P) virus treatment.

AKR mice treated at 6 months of age, at a time concurrent with malignant conversion of the spontaneous leukemia, have a marked delay of leukemogenesis in the first 10 months of life, an increased incidence by 24 months, and a significantly prolonged survival time over control untreated mice (Table 2).

To elucidate the mechanisms involved in tumor inhibition by LCM(M-P) virus, we quantitated splenomegaly induced by Rauscher leukemia virus (RLV) and death by a transplantable lymphatic leukemia (TLL) in separate experiments which involved challenging adult BALB/c mice with RLV or TLL during times after LCM(M-P) virus up to 30 days post-infection

Table 1. Inhibition of spontaneous mammary carcinoma in C3H mice treated with LCM(M-P) virus: life-span study

Group	No. of mice	Mean age[a] when tumor palpable	Mean age[a] when dead with tumor	No. dead from tumor	No. dead without tumor
Control	75[b]	11.2	14.2	64	11
LCM(M-P)V-treated[d]	75[b]	13.5	15.3	60	15
Control	45[c]	18.5	21.2	30	15
LCM(M-P)V-treated[d]	45[c]	18.7	21.8	15	30

[a] Age in months.

[b] Females that had delivered 3 litters prior to treatment.

[c] Virgin females (treated at 2 months of age).

[d] 500 ID50 LCM(M-P) virus inoculated intraperitoneally.

Table 2. Inhibition by LCM(M-P) virus of spontaneous leukemia in AKR mice

Group and treatment	No. of mice	Sex	Mice dead with leukemia (%) at 10 months	Mice dead with leukemia (%) at 24 months	No. without tumor
Control (no treatment)	28	F	61	89	1
	23	M	17	91	2
LCM(M-P)V at 6 months[a]	21	F	0	65	6
	18	M	6	50	9

[a] 500 ID50 LCM(M-P) virus inoculated intraperitoneally.

when immunity to LCM(M-P) virus had developed. LCM(M-P) virus-tolerant adult mice were also challenged. LCM(M-P) virus-tolerant mice are mice infected intraperitoneally with the virus within 24 h post-parturition. Such mice exhibit a continuous viremia throughout their life-span but do not develop anti-viral antibody (9).

In the Rauscher virus trial, a dose of RLV was injected intraperitoneally, which produced a mean splenomegaly of 1,000 mg 28 days post-infection. We found inhibition of the splenomegaly when RLV was injected 2 or 4 days (separate groups) after the acute LCM(M-P) virus infection. No inhibition of splenomegaly occurred in LCM(M-P) virus-tolerant or immune mice with the same challenge dose of RLV (Fig. 3).

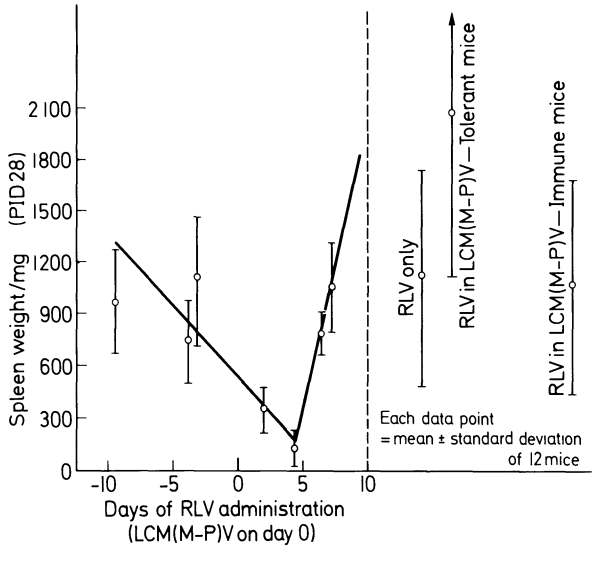

Fig. 3. Inhibition of splenomegaly induced by Rauscher leukemia virus (RLV) in BALB/c mice (8 to 10 weeks of age) by LCM(M-P) virus infection. LCM(M-P) virus dose: 10^3 ID50 inoculated intraperitoneally; RLV dose: 0.01 ml/g of 1:200 dilution of seed virus given intraperitoneally. Significance (Student t): Day 2, $P < 0.03$; day 4, $P < 0.01$. LCM(M-P) virus-tolerant mice, $P \sim 0.07$.

In studies with the transplantable lymphatic leukemia, employing a tumor cell dose which resulted in death of 100% of control untreated mice in 11 to 13 days post-infection, there was prolongation of survival during the acute LCM(M-P) viremia and also in mice immune to LCM(M-P) virus, with significant numbers surviving without leukemia (Table 3). An enhancement of TLL with earlier death occurred in mice tolerant to LCM(M-P) virus (Table 3).

In groups of 36 BALB/c mice 30 days after LCM(M-P) viremia, with demonstrable neutralizing antibody, a challenge dose of 500 TLL cells administered intraperitoneally resulted in delayed deaths as compared with non-immune control mice, with a statistically significant P value of less than 0.001 (Table 3).

Additional experiments were conducted to determine the effect of combined infections with LCM(M-P) virus and pseudorabies virus (PrV). Groups of 12 BALB/c mice, 2 months old, were injected with 10^3 ID50 LCM(M-P) virus grown in HeLa cells. At various times in relation to LCM(M-P) virus infection (Fig. 4), a single dose of 25 PFU (equivalent to 1 MLD100 of PrV) was also injected intraperitoneally. Control groups of mice infected with PrV only were included for each time period employed. The findings were as follows.

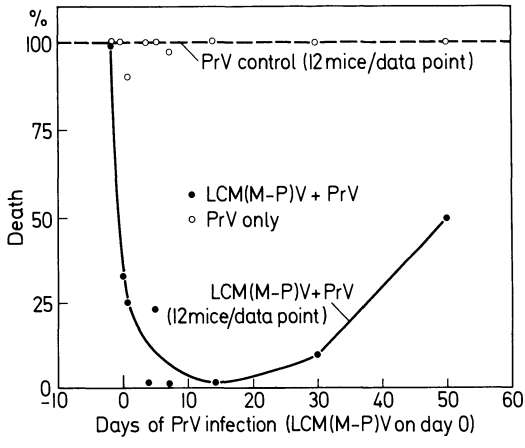

Fig. 4. Effect on pseudorabies lethality in BALB/c mice by infection with LCM(M-P) virus. LCM(M-P) virus dose: 10^3 ID50 intraperitoneally; PrV dose: 1 MLD100 intraperitoneally.

When PrV was injected in separate groups of mice on days 0, 1, 3, 5, 7, 14, 30 and 50 with respect to the time of LCM(M-P) virus infection, there resulted a significant diminution in the pathogenesis and lethality of pseudorabies as compared with parallel control mice; control mice were all dead by the 5th day while previously infected LCM(M-P) virus mice exhibited significant protection and survival during the acute viremia and up to the 50th day after the period of viremia (total time studied) (Fig. 4). Significant interference with pseudorabies pathogenesis was noted also when infection occurred with PrV 1 day before and, in a separate group, simultaneously with LCM(M-P) virus infection; 75% and 60% of mice, respectively, survived for a 120-day period of observation with no overt signs of pseudorabies disease as compared with 90% and 100% death in control mice (Fig. 4). It is clear from these

Table 3. Survival of adult BALB/c mice[a] challenged with 500 cells of transplantable lymphatic leukemia (TLL) at different phases of LCM(M-P) viremia

Status of mice	No. dead from leukemia on day 11	12	13	14	15	16	17	18	19	MST[b]	Significance (Student t)	No. dead over total	Free of leukemia (%)
Control (TLL only)	7	3	38							12.65±0.73		48/48	0
											P <.001		
Tolerant to LCM(M-P)V	42	6								11.13±0.33		48/48	0
Control (TLL only)		4	8							12.67±0.52		12/12	0
LCM(M-P)V acute infection[c]						3	2	2	1	17.13±0.95		8/12	33
LCM(M-P)V acute infection[d]						1	2			16.67±0.61		3/12	75
Control (TLL only)		20	16							12.44±0.50		36/36	0
											P <.001		
Immune to LCM(M-P)V[e]		4	18	8		3	1	1		13.25±1.94		35/36	2.5

[a] Age 8 to 10 weeks. [b] Mean survival time in days ± standard deviation. [c] Infected with 10^3 ID50 HeLa cell-grown LCM(M-P) virus. [d] Infected with 10^3 ID50 L929 fibroblast-grown LCM(M-P) virus. [e] Thirty days after infection.

data that infection with LCM(M-P) virus significantly prevents and/or aborts the in vivo pathogenesis of pseudorabies as evidenced by survival and absence of overt signs.

A similar experiment with the same virus doses employed was conducted in a group of 12 2-month-old BALB/c mice rendered tolerant to LCM(M-P) virus by neonatal infection. A control group of normal mice received PrV only. All of the control mice were dead by the 5th day post-infection. Of the tolerant mice, 11 were still alive at 125 days (time of termination of observation). These 11 mice did not exhibit any overt signs of PrV infection at any time.

In this study we found that inhibition of pseudorabies pathogenesis by LCM(M-P) virus occurred in mice during the acute viremia, in mice convalescent from LCM(M-P) virus and also in mice tolerant to LCM(M-P) virus.

To determine the maximum inhibition afforded at the above stated periods relative to LCM(M-P) virus infection (Fig. 4), groups of mice were challenged with doses of PrV in excess of the minimum lethal dose. On day 3 after LCM(M-P) virus infection, at a time coincident with the peak serum interferon titer, mice survived a dose 450 times greater than the MLD100, which corresponds to 11,250 PFU. In contrast, LCM (M-P) virus-convalescent mice 30 and 50 days post-infection did not survive 10 times the MLD100 (250 PFU) of PrV. Adult mice tolerant to LCM(M-P) virus survived 40 times the MLD100 (1,000 PFU) of PrV.

These data demonstrate that the pathogenesis of PrV disease is modified by co-infection with LCM(M-P) virus. It was deemed necessary to obtain data relative to the role that LCM(M-P) virus-induced interferon may play in this observed interference. A pilot trial was carried out on a group of 12 BALB/c mice which received PrV and serum from syngeneic donor BALB/c mice containing interferon induced by prior infection with LCM(M-P) virus. These sera were titrated and contained 2,500 interferon units/ml, determined by plaque inhibition assay described previously (11). A comparable control group of 12 mice were injected with PrV only. Each of the 12 treated mice were injected intraperitoneally on 3 successive days with a total of 4,000 interferon units. On day 3, 2 h after the 3rd interferon dose, each was injected with 25 PFU of PrV. Control mice were simultaneously injected with an equal dose.

The group of 12 interferon-treated mice was separated into 2 subgroups of 6 mice each; one subgroup received no further treatment; the other subgroup was injected for an additional 3 days with a total of 2,000 interferon units/mouse. All the untreated, infected control mice died by the 5th day; 1 of 6 mice pre-treated with 4,000 interferon units survived and 4 of 6 mice receiving the 2,000 additional units of interferon survived. Survival was observed for 125 days post-virus and no overt signs of PrV infection developed.

DISCUSSION

In the studies described above, we have used rapidly growing tumors, a transplantable lymphatic leukemia and Rauscher virus-induced leukemia, as well as the rapidly lethal pseudorabies virus, to probe the status of mouse host resistance altered by infection with a single intraperitoneal injection of LCM(M-P) virus. These studies are a basis for interpreting the inhibition observed in the C3H spontaneous mammary carcinoma and AKR spontaneous lymphatic leukemia during a 2-year lifespan study.

These data demonstrate that host resistance to pseudorabies pathogenesis is aborted by LCM(M-P) virus-induced serum interferon. In the transplantable lymphatic leukemia (TLL) and Rauscher leukemia virus (RLV) tumor studies, resistance to tumor progression is most apparent during the time of inhibition of RLV splenomegaly, 2 to 4 days after LCM(M-P) virus infection. This is the period coincident with peak interferon induction (11). The Armstrong, E-350, strain of LCM virus has similarly been shown to inhibit RLV in BALB/c mice by Youn and Barski (21) who also proposed an interferon mechanism.

Beyond the 5th day after LCM(M-P) virus infection, serum interferon is not detectable and the host resistance observed in LCM(M-P) virus-convalescent mice challenged with tumor or pseudorabies virus is associated with the induced host-immune response. Suggestive evidence is contained in our studies and the studies of others that the observed inhibition is mediated by an immune response in the host to the LCM (M-P) virus. Such an immune reaction may have its source in a common moiety to mouse antigens which are incorporated in the LCM(M-P) virion. This mechanism is well discussed in a recent review by H. Smith (14), and he states: "It is now clearly established that some viruses incorporate host-cell constituents, especially membrane constituents, into their structure. Hence, antibodies against these virus-host complexes could react with the membrane constituents of infected and normal cells. Also, virus infection may change the host-cell membrane constituents and form neoantigens, the antibodies against which could react with infected and normal cells." The LCM(M-P) virus is characterized by this description as a membrane enveloped virus (Fig. 1).

In vitro serologic studies support this contention that cell membrane antigens are incorporated in the LCM(M-P) viral envelope as determined by selective neutralization of HeLa cell-grown LCM(M-P) virus by rabbit anti-HeLa cell serum, which fails to neutralize L cell-propagated LCM (M-P) virus or mouse-propagated LCM(M-P) virus (8). The M-P strain induces neutralizing antibody (Fig. 2) as well as fluorescent and CF antibodies (7). An immune action against the LCM(M-P) virus-host complex, which involves humoral as well as cell-bound factors, could react with membrane components of infected and normal cells.

In the TLL study, there is a syngeneic relationship between a BALB/c tumor and the BALB/c host. The enhancement of this leukemia in mice tolerant to LCM(M-P) virus may result from replication of the M-P strain in the tumor cells, producing virus-modified cell membranes which now become tolerogenic, resulting in a more rapid progression of the leukemia. In BALB/c mice immune to LCM(M-P) virus, inhibition of TLL may result from an immune reaction directed against normal host membrane components present on the tumor cell membrane. It is clear that immunopathology responsible for host damage in a variety of pathogenic processes is more likely to occur in virus diseases precisely because virions do incorporate host antigens linked as haptenes and, therefore, may elicit host reactions to the complex as well as to the several antigenic components of such complexes which include the host's own antigens. Damage to host cells and host reaction mechanisms could therefore result from any of the 4 types of allergic reaction described by Coombs and Gell (1).

We have found in repeated trials that with the same infecting dose an acute infection following administration of L929 cell-grown LCM(M-P) virus produced greater tumor inhibition than the HeLa cell-grown variant against the TLL tumor in BALB/c mice, producing 75% and 33% survival, respectively (Table 3).

We have reported that LCM(M-P) virus, administered simultaneously with or 48 h prior to sheep red blood cells, had an adjuvant-like effect on the primary antibody response; the hemolysin titer was increased 5-fold (10). By analogy, it would appear that the cell membrane antigens accompanying the initial dose of LCM(M-P) virus result in increased antibody to the accompanying antigen. Since L929 cell membrane antigens are more closely related to the TLL leukemia antigens than are the human antigens on the HeLa cell membrane, a greater immune response is elicited, resulting in more specific inhibition of TLL cells.

These observations led us to believe that LCM(M-P) virus, grown in cells from a particular tumor, would produce a greater degree of inhibition against this tumor than the virus variant adapted to another host cell. A similar view has been expressed by Webb and Gorden Smith in 1970 as a theoretical approach to virus therapy of cancer (20).

Studies on the interference of arenavirus-infected hosts to superinfection by other heterologous viruses undertaken in other laboratories with different strains of LCM virus are pertinent to our study with pseudorabies virus. These reports describe interference with arbovirus infection. In the studies of Wagner and Snyder on the interference against eastern equine encephalitis (EEE) virus in mice acutely and persistently infected with LCM virus (18), an appreciable resistance in the acutely infected mice by the 2nd day post-infection was noted, with no interferon detectable in the acute and persistently infected tissue extracts of lung and brain. Traub found that mice persistently infected with LCM virus, as well as mice immune to LCM virus, showed increased resistance to EEE virus (16,17). LCM virus carrier mice also have been reported to be protected against western equine encephalitis and St. Louis encephalitis viruses (2). Our studies on the inhibition of pseudorabies virus in LCM(M-P) virus-convalescent mice in the absence of serum interferon may reside in a similar host immune response to LCM(M-P) virus which consists of host cell and viral antigens and may also be directed against common host membrane antigens incorporated in the pseudorabies virus envelope (6,19) acquired during replication in this same mouse host.

These data point in the direction that the property of LCM(M-P) virus of incorporating host antigens in the virion is the source of the antigenic stimulus to immune responses, resulting in the interference with the progression of both the neoplasias and the pseudorabies virus disease.

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Acute and Chronic LCM Disease in Man and Animals

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Acute and Chronic LCM Disease

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SUMMARY

Cell-mediated immunity plays an important part in the pathogenesis of the acute disease in mice infected with LCM virus. Sensitized lymphocytes are present in mice 6 days after primary infection with LCM virus; these cells have an anti-viral and at the same time a pathogenic action.

The effects of chronic LCM virus carriage in laboratory mice include glomerulonephritis, decreased growth rate, and reproductive inefficiency. These are discussed in relation to the evolution of LCM virus and its maintenance in nature.

INTRODUCTION

LCM virus infection in mice has become a unique model system where some of the most interesting aspects of infectious processes all meet and play an important part in the same host-virus system. In this system we have vertical transmission of a non-cytopathic virus, immune tolerance, persistent infection, and all types of immunopathology. I shall talk briefly about the acute disease in infected adult mice and then about the chronic carrier state.

ACUTE DISEASE

We accept that LCM virus in mouse cells causes, at least over a short period of time, a completely non-cytopathic infection in the sense that the various types of infected cells in the body appear to function perfectly normally. We also accept that it is the immune response that causes all the trouble. What of the relative roles of antibody, cell-mediated immunity, and complement? Certainly antibody and complement can destroy infected cells in vitro and, presumably, if enough antibody of this type and complement were present in the right place at the right time, they could cause acute disease and death in infected mice. Yet, so far there is no convincing evidence that this does in fact occur. Cell-mediated immunity, however, appears to be a powerful disease-producing force (6). Immune lymphoid cells taken from donors only 6 days after primary infection produced disease and death when transferred to persistently infected recipients. Serum was ineffective. Anti-θ treatment of the cells abolished the effect, indicating that thymus-derived lymphocytes were responsible. Presumably, virus-induced antigens on the surface of infected cells make them targets for the cell-

mediated immune response, which leads to severe pathological results. The host's own cells, non-cytopathically infected by LCM virus, are destroyed by the host's own immune armory. John Hotchin has pioneered the idea that acute LCM in mice is a type of virus-induced autoimmune disease. It is interesting to note that there is another condition in which host cells are the target for a large-scale cell-mediated immunity attack. This is the graft versus host reaction, where by a variety of tricks transfused lymphocytes, which are themselves tolerated, carry out a destructive immunological attack on normal host tissues. I have tabulated the similarities between acute LCM disease and the graft versus host reaction (Table 1). I am not sure how much it means, but at least it is interesting to compare the two.

Table 1. Comparison of primary LCM virus infection and graft versus host reaction in mice

	LCM virus infection	Graft versus host reaction
Perifollicular eosinophilic spleen lesions	+ (13)	+
Eosinophilic liver necrosis	+ (13)	+
Mononuclear cells in central veins in liver	+ (13)	+ (7)
Eosinophilic necrosis in foot after foot pad injection of virus or cells	+ (11)	?
Foot pad swelling after foot pad injection of virus or cells	+	+ (8)
Popliteal lymph node swelling after foot pad injection of virus or cells	+	+ (8)
Increased macrophage-mediated resistance to Listeria infection	+ (4)	+ (3)
Immunosuppression	+ (14)	+
Late neoplastic sequelae	? (17)	+ (1)
Runting in suckling mice	+	+ (2)

When we list the many ways in which immune responses can be harmful, it is easy to get the impression that the immune response does more harm than good, and it is then that we need to remember its origin. The immune response arose in evolution because of its survival value, because it was an exceedingly precise and powerful piece of machinery for the destruction of invading micro-organisms, parasites, and tumor cells. If, as the paleolithic hunter stood in the long grass, spear in hand, sniffing the air, he was ever overcome with an attack of hay fever, then it was because this allergic immune response was so vitally useful in

other ways that it had survived the rigorous processes of evolutionary selection. There is indeed evidence that those with allergic respiratory conditions are less likely to develop cancer.

Therefore, it has been a relief to me to find that the immune response has a clear anti-viral role in mice infected with LCM virus. Bob Blanden and I transfused immune lymphocytes into mice infected with LCM virus 2 days earlier and tested for a reduction in titer in target organs (12). It can be seen (Table 2) that recipients of immune cells showed striking falls in spleen and liver titers within 24 h of the transfer. Brain titers were not significantly affected in these experiments. The donor spleen cells were effective when taken from mice as early as 6 days after primary infection, and the activity of cells was largely, but by no means completely, abolished by potent anti-θ serum. It is important to notice that the anti-viral activity, like the pathogenic activity (6), is present in donor cells as early as 6 days after primary infection. At this time we cannot detect neutralizing antibody, but delayed hypersensitivity foot pad responses can readily be elicited (16) and spleen cells exhibit cytotoxic activity in vitro (9).

Table 2. Effect of immune and non-immune spleen cells on target organ titers in CBA mice infected with LCM virus; 1.3×10^8 viable cells/mouse given 2 days after infection

Time after infection	Treatment	Liver	Spleen	Brain
2 Days	Nil	3.2 ± 0.2[a]	5.5 ± 0.2	5.7 ± 0.07
	Nil	4.3 ± 0.2	5.9 ± 0.2	6.9 ± 0.1
3 Days	Immune cells	2.8 ± 0.1	3.0 ± 0.2	6.8 ± 0.04
	Normal cells	4.2 ± 0.3	5.8 ± 0.2	6.8 ± 0.05

[a] Titers expressed as mean log PFU/g tissue ± standard deviation in groups of 4 mice.

The immune response, therefore, is clearly a two-edged sword. A cellular infiltration into the liver or skin can play a useful anti-viral role, even if there is a good deal of inflammation, edema, and necrosis, but the same infiltration into the infected leptomeninges leads to inflammation, cerebral edema, sickness, and death. So much for the acute disease process. It is important to recall that our favorite acute LCM disease is completely artificial and is not seen under natural circumstances in infected mice. If an adult mouse is infected in nature, it would be by horizontal transmission, and with a mouse-adapted strain of low extraneural pathogenicity - never, of course, by the highly artificial intracerebral route which is our standard model for LCM virus immunopathology.

CHRONIC LCM VIRUS CARRIAGE

I will now refer briefly to the other type of LCM virus-mouse interaction and discuss the long-term effects of infection in mice.

Immune complex-mediated glomerulonephritis

Thanks to the work of Oldstone and Dixon, this syndrome is now more clearly understood.

Decreased growth rate

Depending on the strain of mice, adult carriers weigh less than uninfected controls. In some carrier colonies these differences are striking, with severe runting in carrier mice, sometimes together with anemia and death (10). Nothing is known of the pathogenesis of the condition. Perhaps the antigenic load represented by the persistent widespread infection has some growth-inhibiting action analogous to the runting produced in suckling mice by the administration of gram-negative bacteria (5). Certainly the runting is less severe as the intestinal microbial antigen load is lightened. Specific pathogen-free carrier mice tend to runt less than conventional "dirty" mice, and germ-free carrier mice less than specific pathogen-free mice. A similar picture is seen in the runting that follows neonatal thymectomy.

Reproductive inefficiency

This may be a striking feature in some colonies of carrier mice (10) and is present, although less marked in other colonies (H.H. Skinner, personal communication), and is presumably to some extent determined by the genetic constitution of the host strain. The mechanism is unknown, but average litter sizes are low as a result of fetal death and resorption, with some neonatal mortality.

Shortened life span

Expectancy of life is shortened in carrier mice, a feature which is probably only partly due to glomerulonephritis.

EVOLUTIONARY ASPECTS OF LCM VIRUS

I would now like to consider the evolutionary aspects of LCM virus and see what relevance this has for the chronic "disease" conditions I have mentioned. If LCM virus is to maintain itself in wild (i.e., non-laboratory) Mus musculus populations (and this it does largely by persisting in carrier animals with vertical transmission), then the chronic infection cannot afford to be too disadvantageous. If carrier mice show shortened survival or reproductive inadequacy, there will be selection against such mice and the carrier state will disappear. Yet it exists in wild mice. It goes without saying that there will be strong selective forces favoring the emergence of a carrier state with minimal side effects. First, wild carrier mice will tend to carry virus variants which induce in them, at the most, mild pathogenic effects. We do in fact know that carrier mouse strains may show poor ability to infect the viscera of non-carrier mice (16). Second, wild naturally infected carrier mice will tend to be those with a genetically determined insusceptibility to the harmful consequences of viral carriage. That is to say they will tend to have less severe kidney lesions, for instance, just as mice successfully carrying leukemia viruses would not be expected to have severe kidney lesions caused by immune complexes.

They are also unlikely to have the severe reproductive inefficiency or runting that we observed in our laboratory strain of carrier mice (10). If the harmful consequences of viral carriage had an immunopathological basis, then possible mechanisms would include first, a genetically determined immune hyporesponsiveness to LCM virus antigens and second, a genetically determined host-virus interaction such that less viral antigen was shed or appeared on the surface of infected cells. There is suggestive evidence for genetically determined differences in the specific immune responsiveness of mice to LCM viral antigens (16). Is there any evidence for differences in the expression of viral antigen on the surface of infected cells, or in the yields of infectious virus? We have observed that there is a low ratio of infectious virus to number of infected cells (immunofluorescent staining) in the brains of carrier mice as compared with acutely infected brains (10). There are differences in virus titers of tissues from different strains of carrier mice (15), but there is no information about strain differences in the ratio of infectivity titers to infected cell numbers. Is there a decreased density of viral antigen on the surface of cells from congenitally or neonatally infected carrier mice? If so, this might help explain why transfused immune lymphocytes fail to induce disease in many strains of carrier mice, yet cause sickness and death in cyclophosphamide-induced persistently infected adult mice. I have found a similar distribution of LCM virus surface antigen on spleen cells from carrier and from acutely infected mice, but this was not a careful quantitative study, and no other cells or tissues were tested. There have been no studies of differences in surface antigen density in different strains of carrier mice.

Ultimately in the evolution of a vertically transmitted virus there is no need for infectious virus, or even antigen, since virus can be effectively transmitted via the egg or sperm. Infectivity then tends to disappear because it is no longer required, like vision in cave dwelling animals. This is the state of affairs seen in naturally occurring leukemia viruses in wild mice, and it is possible that LCM virus in mice has gone some way in this direction.

Finally, to develop even more generalized ideas before we turn to the hard facts of the succeeding papers, I would like to speculate further about the evolutionary history of arenaviruses. One can imagine that originally there was a well-balanced rodent virus reservoir, say in Africa, with a persistent non-pathogenic infection and some virus shedding to the exterior - perhaps a Lassa-type virus, whose pathogenicity for an accidental host like man is irrelevant for its evolution. The virus may have spread into different rodents, especially Mus musculus in Europe and possibly Asia, reaching a similar type of balance. Virus later reached the Americas, not inconceivably in the form of infected rodents on the slave ships which also, during the 16th century, carried African yellow fever to the Americas. Here, the basic colonizing virus split up into various strains as it established relationships with different native rodents, and some of the host-virus relationships show a similar imperfection to that seen with LCM virus in laboratory strains of mice. Machupo virus in its natural host Calomys callosus causes considerable reproductive inefficiency, suggesting that there could be cycles of infection in local Calomys populations which wax and wane as reproductive inefficiency induced in the host has its effect. Perhaps there are changes in host-virus balance in association with the great fluctuations in numbers of rodent populations. Marked seasonal variations in the proportion of Neacomys rodents carrying infectious Amapari virus have been described (Belem Laboratory Annual Report 1967).

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On the Pathogenesis of LCM Disease: Effects of Immunosuppression with Anti-Lymphocyte Serum

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SUMMARY

Experiments with mice infected with LCM virus and immunosuppressed by anti-lymphocytic serum (ALS) are reviewed. The results show that 1) ALS given for a short time period to adult animals together with 1 injection of LCM virus results in virus-specific tolerance; 2) ALS diminishes the lethality following intracerebral inoculation of the virus into adult mice; 3) immune animals treated for at least 3 weeks with ALS develop viremia, although complement-fixing antibody is still produced; however, after termination of ALS treatment the animals return to the immune state; 4) treatment of immune spleen cells with ALS for 1 h in vitro or for 3 weeks in vivo selectively abolishes these cells' capacity to confer adoptive immunity to syngeneic virus carriers as judged by virus elimination; in contrast, the antibody-producing capacity is left intact. These results support the already well-founded opinion that virus elimination is cell-mediated; they furthermore strengthen the evidence that humoral antibody (complement-fixing) plays no role in the pathogenesis of LCM disease except perhaps in certain combinations of mouse and virus strains. It is argued that B and T cell tolerance to the virus must be looked upon separately.

INTRODUCTION

In this presentation, I should like to review our experiments on immunosuppression with anti-lymphocyte serum (ALS) or anti-lymphocyte globulin (ALG) in mice infected with LCM virus. Since all participants of this symposium are familiar with the events following inoculation of the virus into the adult animal, there is no need to begin with a lengthy description. C3H mice and the Traub strain of virus were used throughout. These appear to be well adapted to each other, and late disease and related phenomena, which have been described to occur with other combinations of mouse and virus strains (16,31,32), were not observed.

Several investigators have studied the effects of a variety of immunosuppressive treatments, such as methotrexate, cortisone, x-rays, cyclophosphamide, and thymectomy, on LCM virus infections (6,10,11,16,33) and have also attempted to induce tolerance in adult mice by inoculating high doses of virus (6). With both types of treatment tolerance to the virus is only rarely established, and most animals are immunized, although the course of the infection may differ from the one seen in unsuppressed intraperitoneally infected adult mice.

Since ALS and ALG have proved to be markedly effective as immunosuppressive agents, affecting predominantly cell-mediated immunity (17, 19,20), and since circumstantial evidence indicates that many virus infections, including infection with LCM virus, are terminated by cell-mediated immunity (3,12,14,34), it appeared relevant to investigate the effect of treatment with ALS on LCM, especially because this drug is being used clinically.

RESULTS

Mice were treated with ALS intraperitoneally for varying periods of time and virus was injected intracerebrally or intraperitoneally (24). When employing the intracerebral route, we found, as have others (8, 13), that most animals survived, although without treatment all would have died. After intraperitoneal injections, in several experiments all animals became tolerant, probably for the rest of their lives. The blood virus titers were as high as in neonatally infected animals and low concentrations of CF antibody appeared for short periods of time in some of them. These experiments have shown that even a short treatment with ALS (5 days) facilitates the induction of a lifelong tolerant state to a self-reproducing antigen (Fig. 1). In fact, this phenomenon

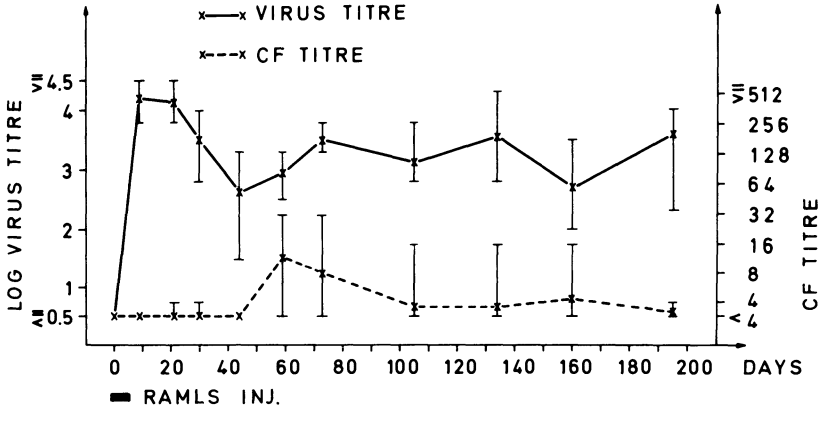

Fig. 1. Average virus titers and CF antibody titers of 10 mice which had received 1,000 LD50 LCM virus on day 0 and 0.25 ml ALS on days -1 to +5. (With permission of Munksgaard, Copenhagen.)

is so regularly observed that I use it routinely for testing the efficacy of ALS. Other investigators obtained tolerance to skin and kidney grafts only by prolonged treatment with ALS (1,26) or a combination of ALS inoculation with thoracic duct drainage (39) or thymectomy (27). The rapid universal distribution of large amounts of viral antigen in the ALS-treated mice may be the cause for the difference between our results and those just mentioned.

Some experimental animals (dogs, cats) have died during ALS treatment with signs pointing to infection with viruses against which they had previously been actively immunized (1,2). Since we knew that our LCM virus-immune animals had small depots of virus in, for instance, the kidneys (9,11) - which is probably the reason for their lifelong im-

munity - we tried to provoke dissemination of the virus with ALS. Mice were inoculated intraperitoneally with LCM virus and after having determined that antibody was present, ALS treatment was begun (37). A typical course is shown in Figure 2, in which a steep rise of virus titer contrasts with no or little change of CF titer. Characteristically, a rather long time had to elapse before the effect became noticeable, which is probably a reflection of the well-known fact that it is difficult to reach organ-localized lymphoid cells with ALS. After termination of the treatment, the immune state in all animals was reestablished rather quickly. If virus per se had been harmful, titers of this magnitude should have had serious consequences for the animals.

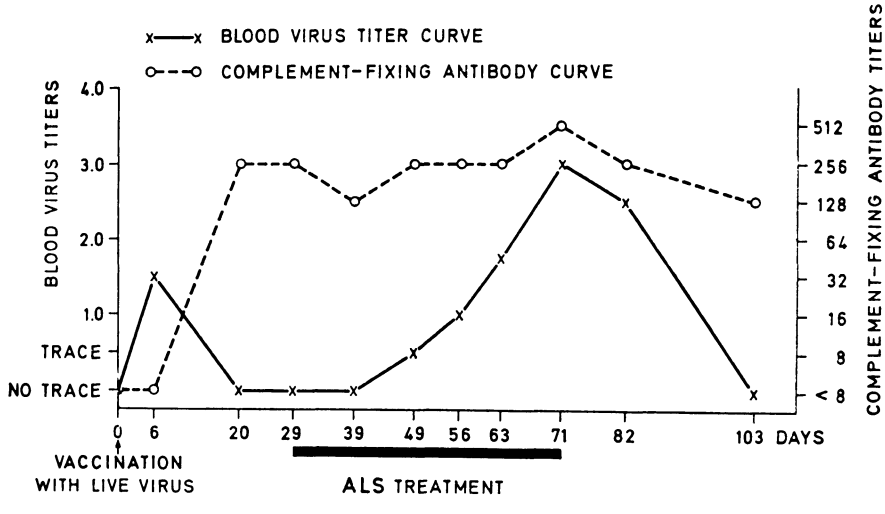

Fig. 2. The effect of ALS treatment on a mouse which had been infected previously with LCM virus. (With permission of The Rockefeller University Press.)

As already mentioned, the available evidence favors cell-mediated immunity as being responsible for elimination of the virus; humoral antibody seems to play a minor role. To add further proof to this supposition, use was made of the different effects of ALS on the 2 components of immunity, being predominantly directed against cell-mediated functions, and the direct effect of ALS on immune cells was investigated.

First, spleens were removed from LCM virus-immune animals, made into cell suspensions, and incubated with ALS for 60 min at 37°C. Thereafter, LCM virus-tolerant syngeneic recipients received 30 x 10^6 living cells intraperitoneally and their virus and CF titers were checked periodically for 6 months (Fig. 3) (23). It turned out that all mice developed CF antibody, 3 more so than the rest. During the next 2 months these 3 animals also eliminated slowly the circulating virus, whereas the remaining 7 mice remained viremic and at the same time produced moderate amounts of antibody. The mice from the 2 control groups which had received spleen cells from the same suspension, treated with normal rabbit serum or Hanks' balanced salt solution, were quickly and completely adoptively immunized with very high CF titers (Fig. 4). Thus a differential effect was obtained: while the capacity of the immune spleen cells to eliminate the virus in the recipients was abolished by

a very short contact with ALS in vitro, the capacity to produce antibody was retained, although perhaps to a slightly lower degree than in untreated animals.

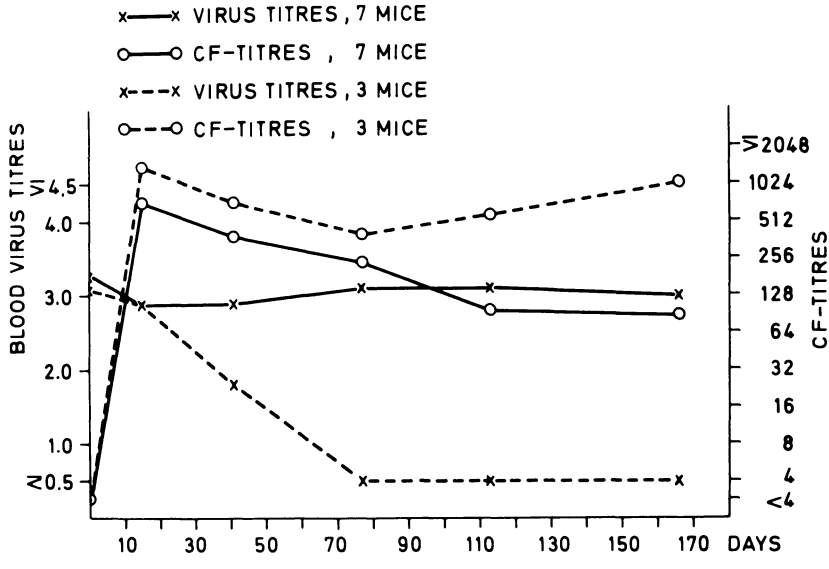

Fig. 3. The effect of transplantation of immune ALS-treated lymphoid cells to 10 syngeneic virus carriers. The 2 alternative courses are shown. (With permission of Munksgaard, Copenhagen.)

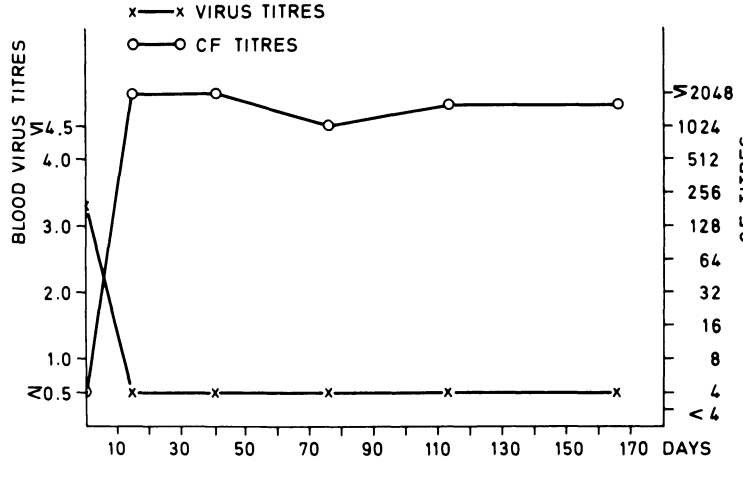

Fig. 4. The effect of transplantation of immune normal rabbit serum-treated lymphoid cells to 5 syngeneic virus carriers. (With permission of Munksgaard, Copenhagen.)

Next, the effect of ALS on immune donors given before transplantation was investigated. The animals received daily intraperitoneal injections of ALS, normal rabbit serum or Hanks' saline. After 1, 8, 15 or

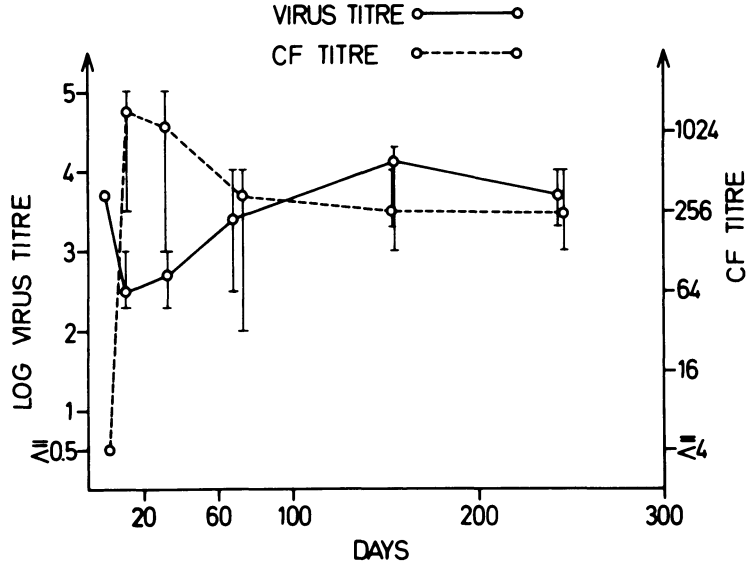

Fig. 5. The effect of transplantation of immune lymphoid cells to 6 syngeneic virus carriers. The donors had been treated with daily injections of 0.25 ml ALS from days -21 to -1.

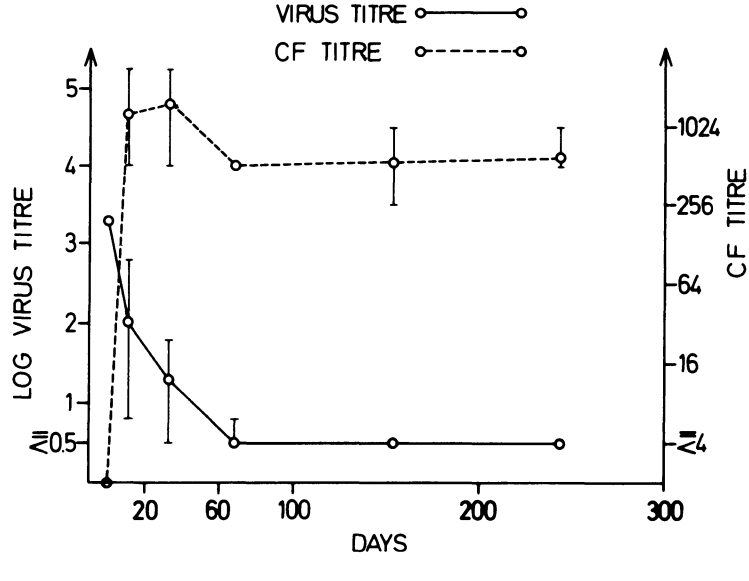

Fig. 6. The effect of transplantation of immune lymphoid cells to 4 syngeneic virus carriers. The donors had been treated with daily injections of 0.25 ml normal rabbit serum from days -21 to -1.

21 days their spleens were removed, made into suspensions, and transplanted directly into virus carriers. After 1 day of treatment the cells from the 3 types of donors acted identically in the recipients by conferring complete adoptive immunity in all animals. In contrast, after 3 weeks of treatment of donors, the spleen cells from the ALS-treated animals were not able to eliminate virus from the blood of the tolerant mice, although at the same time they produced antibody in moderate amounts (Fig. 5). Cells from the control animals led to complete adoptive immunity in all cases (Fig. 6).

Treatment of immune prospective donors with ALS for 8 or 15 days led to varying but small numbers of mice with reduced capacities for transferring immunity.

These experiments were repeated twice with essentially the same results.

DISCUSSION

The experiments presented here aid in understanding the effects of ALS or ALG on LCM virus infection and thereby elucidate the role the different types of immunity play in the pathogenesis of the disease. Our results may be summarized as follows: 1) ALS given for a short period of time provides the basis for the induction of tolerance to LCM virus in the adult mouse. The transient appearance of small amounts of CF antibody is not unexpected and agrees with findings in almost all instances of tolerance induced in adult animals (M. Simonsen, personal communication). 2) ALS reduces the lethality following intracerebral inoculation of the virus into adult animals which is in accord with the observation of others (8,13). 3) Immune animals have small depots of virus, e.g. in the kidneys, which can be activated by treatment with ALS, thus leading to viremia but without affecting significantly CF antibody. 4) Treatment of immune spleen cells with ALS in vitro has a differential effect on the capacity of these cells to confer adoptive immunity; virus is not eliminated in the recipients, but antibody production is left intact. Essentially the same effect is obtained by treatment of immune spleen cell donors with ALS for at least 3 weeks prior to transfer of lymphoid cells.

Additional observations are worth mentioning: 1) As noted previously, mice may harbor virus and CF antibody concurrently at high concentrations for long periods of time (in our experience at least 1.5 years); yet no signs of disease become apparent. 2) On several occasions normal rabbit serum was observed to exert an effect which was similar to the one obtained with ALS, although to a lower degree. Probably this phenomenon results from non-specific immunosuppression by rabbit serum or is due to antigenic competition (18,21,35).

All our results add further evidence to the already well-established notion that the virus elimination mechanism is cell-mediated. They also emphasize that humoral antibody (CF) is of little relevance for the pathogenesis of LCM disease except perhaps in special situations in particular combinations of mouse and virus strains (29).

Apparently, small amounts of antibody may accumulate in the kidneys (28,32) as well as the serum (24,31) of mice after congenital, neonatal or adult induction of tolerance. When trying to evaluate this phenomenon, we may consider separately B cell tolerance and T cell tolerance, and I am not aware that experiments have been performed showing that tolerance of T cells is incomplete as seems to be the case with B cells

which - at least in certain instances - produce antibody even though in low quantities.

We may now ask the question: how does a mouse get rid of LCM virus? Neither interferon (36) nor neutralizing antibody (9,36,38) are of importance, and we also know from this and other investigations that CF antibody plays no role. On the other hand, there is no doubt that immunological mechanisms are involved and hence we are bound to conclude that virus elimination is a cell-mediated process. ALS preferentially acts upon this part of immunity and abolishes preexisting cell-mediated immunity.

Cell-mediated immune functions usually demand close contact between activated cells and their targets and this could also occur in LCM virus infection. In support of this view are the findings of myself (22) and others (15,25,30) which demonstrate that LCM virus-immune spleen and lymph node cells can destroy LCM virus-infected target cells in vitro. The mechanism, which is independent of complement, is known to be exerted by T cells (4,5). Thus one may speculate that virus elimination is accomplished by a direct action of LCM virus-immune T cells on LCM virus-infected cells in the body, possibly causing the occasional death of such cells. Though this hypothesis is attractive, it cannot be true in its simplest form. Firstly, it is common experience that intraperitoneal infection and adoptive immunization of virus carriers are usually not followed by disease, while intracerebral application of the virus to adult animals causes close to 100% lethality. Secondly, aside from choriomeningitis, pathology of the central nervous system is not a feature of LCM (7) and even the choriomeningitis may be of moderate degree (Lundstedt, unpublished observations). Thus, a theory implying cellular destruction by contact in vivo requires modifications, such as the assumption that only certain structures of the central nervous system are susceptible to this mechanism. But, perhaps, immunological destruction of infected cells is not at all relevant for the pathogenesis of the LCM disease of the mouse, and the clue may eventually be found at the subcellular level.

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Thymus-Dependent (T) Cell Competence in Chronic LCM Virus Infection

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INTRODUCTION AND PERSPECTIVE

Once an animal has been infected in utero or neonatally with LCM virus, that virus can persist in most tissues throughout the animal's life (11,29,30). This finding along with the inability to find free circulating antibody to LCM virus in such mice led Burnet to postulate in the late 1940's that mice infected with LCM virus were immunologically tolerant to this agent (3). Doubts about the validity of this concept were first raised in the late 1960's in our laboratory (16). While observing immunopathological and virological events in chronically infected mice, we found host immunoglobulin G (IgG), complement (C3, 3rd complement component) and LCM viral antigen(s) deposited in the renal glomeruli in a granular pattern (16). This picture of antigen, IgG and C3 deposits had been seen many times before in different diseases and is pathognomonic of immune complex deposits (5,6). This suggested that these mice, in spite of carrying persistent titers of LCM virus, were also mounting an anti-viral antibody response, the antibody complexing with virus in the circulation to form virus-anti-viral antibody (V-Ab) complexes which subsequently became trapped in the renal glomeruli. Later experimental work indeed showed both the presence of circulating V-Ab complexes (17,18) and of specific antibody to LCM virus (16,17,18). Later observations indicated that in some strains, i.e., NZB,(NZB x W)F_1, about 15 to 20% of infected mice made detectable free circulating antibody by 6 to 9 months of age.

We extended these observations to lactic dehydrogenase virus infection (21), spontaneous Gross leukemia virus infections of AKR mice (15), and others did in Aleutian disease of mink (8,24,25), Moloney sarcoma virus (9), lactic dehydrogenase virus infection (14,26) and equine infectious anemia (1,13). In all these persistent infections, too, the host mounted a continuous anti-viral immune response which resulted in circulating V-Ab complexes and deposition of specific anti-viral antibody in the glomeruli. Hence, in chronic infections the Burnet hypothesis of tolerance does not fit the scientific data and is no longer tenable in any known natural or experimentally induced in utero or neonatal infection.

Despite an animal's continuous anti-viral immune response, infectious virus persists, and "split tolerance" was hypothesized as an explanation. Split tolerance basically suggests a deficiency of cellular immunity (T cell dysfunction) with a normal humoral response (B cell

function). The foundations for this hypothesis were 1) the observations of Hotchin that mice persistently infected with LCM virus were runts (11), a condition which had been reported in neonatally thymectomized mice, 2) findings that intravenous injection of a peculiar strain of LCM virus into adult mice caused injury to thymus-dependent areas (7), and presumably such a mechanism might occur in mice infected in utero or at birth causing a "viral thymectomy", and 3) Volkert's observation that transfer of normal or immune lymphoid cells to persistently infected isologous recipients caused a reduction in virus titers (32,33). This paper examines the concept of split tolerance to chronic LCM virus infection.

EXPERIMENTAL QUESTIONS AND RESULTS

Is there evidence of T cell destruction? The thymus-dependent areas in the lymphoid organs of mice are within the lymphoid follicles of the spleen immediately surrounding the central arterioles and the mid and deep cortical zones of lymph nodes (23). Histologic examination of thymus, lymph nodes, and spleens from several strains of mice (SWR/J, BlO.D2-old, BlO.D2-new, BALB/c, CBA, C3H/J) infected with LCM virus showed no histologic evidence of selective small lymphocyte destruc-

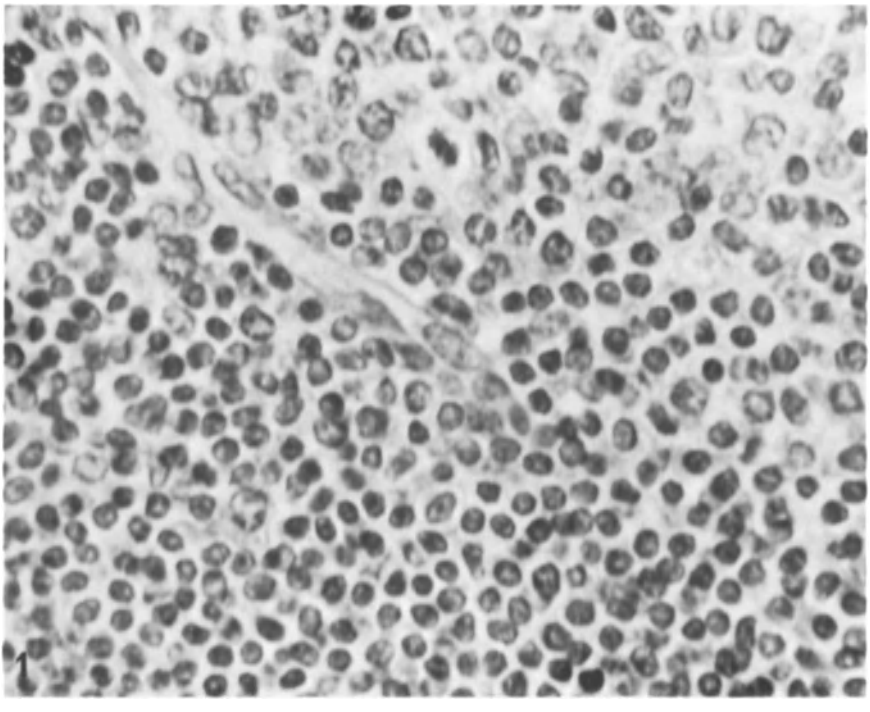

Fig. 1. Photomicrograph of thymus-dependent area surrounding a central arteriole in a 6-week-old SWR/J mouse chronically infected with LCM virus. No depletion in thymus-dependent lymphocytes is seen.

tion in the thymus or in these thymus-dependent areas. Figure 1 shows normal lymphoid tissue from an infected SWR/J mouse surrounding a central arteriole of the spleen, whereas Figure 2 shows a normal cortical

zone of a lymph node taken from a 6-week-old SWR/J mouse chronically infected with LCM virus. Tissues from the other infected mice of different strains all showed normal thymus or thymus-dependent lymphoid

Fig. 2. Photomicrograph of part of a lymph node from a 6-week-old SWR/J mouse chronically infected with LCM virus. The thymus-dependent areas in the mid and deep cortex are normal.

areas. Over 20 mice from each strain were studied, and thymus, spleens, and lymph nodes of SWR/J and C3H/J mice examined from the 1st day of birth through 6 months of life remained normal. In contrast, lymphoid depletion in thymus-dependent lymphoid areas was consistently seen in neonatally thymectomized SWR/J mice. Neither neonatally thymectomized nor LCM virus-infected mice became runted. It is now generally accepted that runting is not a feature of thymectomization, but a condition seen when mice are overcrowded and secondarily infected.

Is there a deficiency in the number of T cells? Theta (Θ) isoantigen serves as a marker of thymus-derived lymphocytes (27), and Θ-bearing lymphocytes are markedly depleted in lymph nodes and spleens of neonatally thymectomized mice and mice with congenital absence (nude mice) of the thymus (28). Antibody to Θ was made by immunizing AKR mice with CBA mouse thymocytes. With either the trypan blue dye exclusion test or a ^{51}Cr assay it was clear that the marked T cell depletion seen in neonatally thymectomized or nude mice was not present in mice chronically infected with LCM virus (Table 1). In several experiments an anti-Θ C3H serum supplied by Dr. Mitchison and Dr. Raff and a congenic anti-Θ serum from Dr. T. Boyse were compared to our anti-Θ sera. All

Table 1. θ-bearing lymphoid cells in 2 to 3-month-old LCM virus-infected mice[a]

Strain	Infected		Non-infected	
	LN (%)	SPL (%)	LN (%)	SPL (%)
SWR/J	67	32	70	33
B10.D2-old/Sn	69	25	74	27
B10.D2-new/Sn	71	34	65	35
NZB	70	29	74	33
NZW	n.d.[b]	26	n.d.	27
(NZB x NZW)F_1	n.d.	30	n.d.	30
BALB/c	63	32	58	37
CBA/J	62	29	66	26
C3H/HeJ	65	32	67	35
SWR/J neonatal thymectomy	6	1	8	2

[a] Mice were infected with LCM virus either by natural infection in utero (18) or experimentally as newborns (17). There were 3 to 5 mice per group, and the test was performed as outlined by Raff and Wortis (28). Numbers given represent the average from at least 4 experiments. LN - lymph node cells; SPL - spleen cells.

[b] Not done.

sera gave similar results. Hence, there is no measurable deficiency in numbers of thymus-dependent θ-bearing cells in the peripheral lymphoid tissues of LCM virus-infected mice.

In addition, Lehmann-Grube and Raff (personal communication) studied T cells in the peripheral lymphoid tissues of mice with acute LCM virus infection and found no deficiency of T cells.

Thus, while there is no evidence that LCM virus causes a "viral thymectomy", there are data to indicate that it does not.

Is there immunologic dysfunction of the T cells? Of lymphoid cells in culture, only T cells are believed to react to phytohemagglutinin (PHA) stimulation (2). Experiments were done in which 2 x 10^6 single splenocytes from mice chronically infected with LCM virus were treated with PHA-P. Four to 18 h prior to harvest, cells were pulsed with H3 thymidine (H3T). The cell pellet was collected, precipitated with TCA, and counted in a liquid scintillation counter. Table 2 shows that cultured lymphoid cells from mice chronically infected with LCM virus were able to incorporate H3T and synthesize DNA after PHA stimulation. In all

Table 2. Phytohemagglutinin stimulation of lymphoid cells from mice chronically infected with LCM virus[a]

Strain	Exp. No.	CPM H3 thymidine -PHA	CPM H3 thymidine +PHA	MI[b]
SWR/J	1	1580	4514	2.9
	2	592	3166	5.3
	3	1571	4856	3.0
	4	668	7560	11.3
B10.D2-old/Sn	1	316	3636	11.5
	2	1792	9440	5.3
NZW	1	756	4045	5.4
	2	464	3321	7.2
	3	565	4228	7.5
(NZB x NZW)F_1	1	1081	4364	4.0
	2	326	2584	7.9
	3	876	4524	5.2
BALB/c	1	613	3501	5.7
	2	955	1944	2.0
	3	1452	2782	1.9
C3H/HeJ	1	518	1119	2.2
	2	592	2410	4.1
	3	419	2945	7.0
	4	1080	9849	9.1

[a] Lymphoid cells were pooled from 3 to 5 mice, 2 to 3 months old, and cultured in medium consisting of RMPI-1640 with 7.5% of fresh, selected, human serum (heated 56° for 30 min), glutamine and antibiotics. In 2 ml of medium, 10 x 10^6 lymphoid cells were cultured with PHA-P (50 µg/ml) for 3 days. Four h before harvest, 1 µCi of H3 thymidine (specific activity 6.7 mCi/mM) was added to each culture. At time of harvest the cells were washed thrice and a TCA precipitate collected on GF/A Whatman filters.

[b] MI = mitogenic index: ratio of $\frac{\text{+PHA CPM}}{\text{-PHA CPM}}$.

experiments done these splenocytes responded to PHA. In many experiments, the response was 1/2 to 1/3 as much as that seen with non-infected litter mates.

The antibody response to sheep red blood cells (SRBC) is strongly thymus-dependent. Table 3 shows that thymus cells taken from mice chronically infected with LCM virus and transferred to thymectomized lethally irradiated mice were able to reconstitute an immunologic re-

Table 3. Anti-sheep red blood cell plaque-forming cells in thymectomized lethally irradiated mice immunologically reconstituted with thymus cells from LCM virus-infected mice[a]

Source of cells		Plaque-forming cells/ 10^6 spleen cells	
Thymus	Bone marrow	Direct	Indirect
LCM mice	Non-infected mice	76	166
Non-infected mice	Non-infected mice	72	239
---	Non-infected mice	1	5
Non-infected mice	---	2	4

[a] Mice were sacrificed by exsanguination; thymuses were excised from the thoracic cavity and the bone marrow expressed from femurs and tibiae by hydrostatic pressure. Single cell preparations were made and the cells washed thrice. Recipient mice were subjected to 900r whole body irradiation and within 3 h were injected with thymus (1.7×10^8) and/or bone marrow (4×10^6) cells. 5×10^8 sheep red blood cells were injected intravenously at the time of cell transfer. Seven days later the mice were sacrificed and the spleens assayed for both immunospecific direct and indirect plaque-forming cells by a modified Jerne plaque assay. Experimental techniques used have been reported (4).

sponse to SRBC. The numbers of plaque-forming cells following reconstitution with thymus cells from LCM virus-infected animals were similar to those seen when thymus cells were transferred from non-infected controls.

Is there evidence of T cell response to LCM viral antigens in mice chronically infected with LCM virus? A specific cellular response to LCM virus occurs in mice chronically infected with LCM virus. When spleen cells from SWR/J mice infected with LCM virus are mixed with isologous LCM virus-infected fibroblasts labeled with ^{51}Cr, cellular release of ^{51}Cr occurs (20,22). In most experiments, the amount of ^{51}Cr released is 1/3 to 1/2 as much as that occurring with spleen cells from mice immunized or infected as adults. These results are in agreement with those of Lundstedt who used lymphoid cells from chronically infected C3H/J mice and L cells as a target (12). Other examples of specific T cell response in LCM virus-infected mice are 1) the release of macrophage inhibitory factor (31) and 2) the release of lymphotoxin or cytotoxin (19) following incubation of spleen or lymph node cells from infected mice with virus or viral antigens.

CONCLUSIONS

The above data clearly indicate that mice chronically infected with LCM virus are capable of making a cellular response against LCM virus as well as a humoral response, and neither tolerance nor split tolerance occurs. In addition, our results suggest that the cellular responses in mice chronically infected with LCM virus may be only 1/3 to

1/2 as much as those seen with similar numbers of lymphoid cells taken from immunized adults, although a definitive quantitation in the face of antigen (viral) excess is at this time impossible. While the immune response of chronically infected mice is not sufficient to clear virus from the animal's tissues, it is sufficient to interact with virus or virus-infected cells to induce injury. A similar cellular response occurs in spontaneous leukemia infections of AKR mice (34).

Finally, the possibility remains that the titer of virus carried by chronically infected mice may be controlled, in part, by mechanisms other than the host's immune responses. Preliminary data from Dr. Frank Dixon's laboratory are suggestive that despite massive and prolonged immunosuppression, titers of virus may not change in chronically infected mice (10).

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Pathogenesis of LCM Disease in the Rat

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SUMMARY

Intracerebral inoculation of neonatal rats with LCM virus resulted in varying extents of central nervous system parenchymal pathology dependent upon host age at infection and upon virus strain. Lesions were confined to brain areas undergoing postnatal proliferation and migration, with the most striking involvement being a profound necrosis of most of the cerebellar cortex following infection of rats at 4 days of age. The immunopathological basis of this LCM virus-induced cerebellar pathology has been demonstrated both by preventing it with immunosuppression of normally susceptible animals and by eliciting it by adoptive immunization, with LCM virus-immune spleen cells, of infected animals not normally susceptible.

INTRODUCTION

The consequences of LCM virus infection in mice have been well established as a prototype of virus-induced immunopathology (4,8,9,18). Intracerebral inoculation of newborn animals, or vertical transmission from infected mother to fetus, is characterized by an essentially inapparent lifelong infection with high levels of virus found in the brain, blood, and other tissues, and with no detectable pathological changes within the central nervous system (CNS). Intracerebral inoculation of the adult mouse leads to a fatal choriomeningitis with no evidence of neuronal destruction.

In contrast to the well-defined outcome of LCM virus infection in the mouse, studies using the rat have been equivocal. Intracerebral inoculation of LCM virus into adult rats has been reported as leading to fatal choriomeningitis (6,15) as well as being without clinical effect (2,10), while intraperitoneal infection of the newborn has been reported as leading to death apparently not immunopathological in origin (17). Recently we have reported the occurrence of a profound cerebellar necrosis which occurs following intracerebral inoculation of the 4-day-old rat with the Armstrong E-350 strain of LCM virus (11,12).

In order to elucidate the critical variables involved in the production of CNS pathology in the rat following LCM virus infection, a series of studies were undertaken and will be reviewed in this paper.

MATERIALS AND METHODS

Animals. Pregnant outbred Wistar and inbred Fischer (F344) rats, known to be free of LCM virus (Microbiological Associates, Walkersville, Maryland), were observed to establish day and time of litter birth. Virus titrations were conducted in Swiss mice, 4 to 8 weeks old.

Virus. The E-350 strain of LCM virus (American Type Culture Collection VR 134) in the form of a clarified suspension, in buffered saline, of brain tissue from infected mice, was used throughout most of the experiments. Additional strains of LCM virus used were CA 1371 (provided by Dr. J. Parker), WE/UBC-A351 (provided by Dr. J. Hotchin), and Traub (provided by Dr. M. Volkert).

Anti-lymphoid serum (ALS). Thymus cells from rats, 6 to 10 weeks of age, were suspended in Eagle's minimum essential medium (MEM) containing penicillin and streptomycin. Four ml of this suspension, or about 4×10^9 cells, were injected intravenously into rabbits, weighing about 2.5 kg. Two weeks later, a similar booster dose of cells was administered, and the rabbits were bled the following week.

Serum was heat inactivated at 56°C for 30 min and absorbed on rat erythrocytes in a ratio of 4 parts serum to 1 part red blood cells. The hemagglutinating titer for the rat erythrocytes was no more than 16. Normal rabbit serum (NRS) was similarly prepared. All sera were stored at -20°C until used.

Experimental design. At the appropriate ages, litters were equalized at 8 pups and each animal received 0.03 ml of either 1,000 adult mouse LD50 of LCM virus or of diluent, 0.75% bovine plasma albumin in pH 7.3 phosphate-buffered saline free of calcium and magnesium, injected into the right cerebral hemisphere.

ALS or NRS, when administered, was given intraperitoneally, 0.1 ml, 1 day prior to or on the day of infection. Treatment continued, on alternate days, for 2 to 3 weeks after injection of virus.

At regular intervals after inoculation, animals were sacrificed for virus and antibody determinations, immunofluorescent staining, and histopathological analyses, details of which have been described elsewhere (3,13).

RESULTS

Cerebellar hypoplasia. Intracerebral inoculation of the E-350 strain of LCM virus into neonatal rats during the first week of life resulted in varying degrees of cerebellar hypoplasia, depending upon the age of infection (11). The lesion was maximal in animals inoculated at 4 days of age, less so at 7 and 1 day, and with no observable cerebellar pathology seen in animals infected as newborns. No signs of illness were observed in animals given intracerebral injections of virus at 14 days of age and older. We will now describe the development of the lesion in animals intracerebrally inoculated at 4 days of age, details of which are given elsewhere (13).

Following infection, virus was initially seen, using immunofluorescent procedures, in the leptomeninges and the choroid plexuses. Typically, first parenchymal involvement of the brain became apparent 5 days after inoculation, in the external granular layer of the cerebellum. Migration of these cells inwards produced an inner granular cell layer which

was also infected (Fig. 1). Focal areas of necrosis in the external and inner granular cell layers were first evident about 7 days after inoculation. Purkinje cell infection and necrosis and the loss of myelin appeared to follow the granule cell pathology.

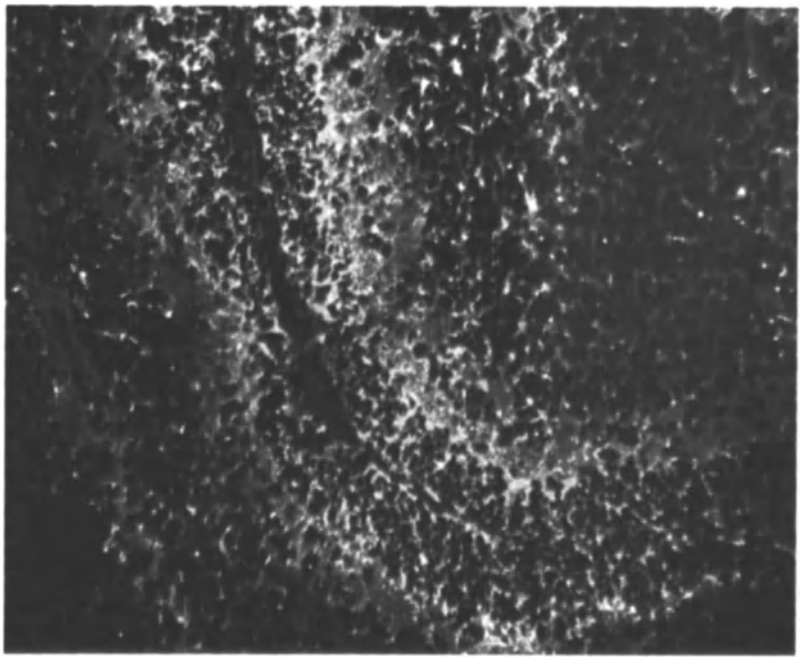


Fig. 1. Early localization of infection following intracerebral inoculation of LCM virus into 4-day-old rats. Fluorescent antibody stain of LCM viral antigen in the external and internal granule cells of a cerebellar folium and the absence of fluorescence in the Purkinje cells, 5 days after infection. Magnification x235.

Ataxia could be observed 9 days after infection, when there was extensive necrosis in all folia of the cerebellum. The evolution of the lesion continued until 3 weeks after infection when its development was essentially complete, with only a few residual folia remaining (Fig. 2A,B).

Coincident with the developing cerebellar pathology, there was an inflammatory response in the leptomeninges and choroid plexuses. This was first seen as a minimal infiltrate of large and small mononuclear cells in the leptomeninges 5 days after inoculation. By the end of the first week, there was a marked inflammation of the choroid plexuses and leptomeninges which included a large number of macrophages containing intracellular vacuoles, often filled with what appeared to be cellular debris. The inflammatory response was effectively resolved by the 3rd week following infection. At no time was there any significant inflammation evident in the cerebellum, as seen by the light microscope.

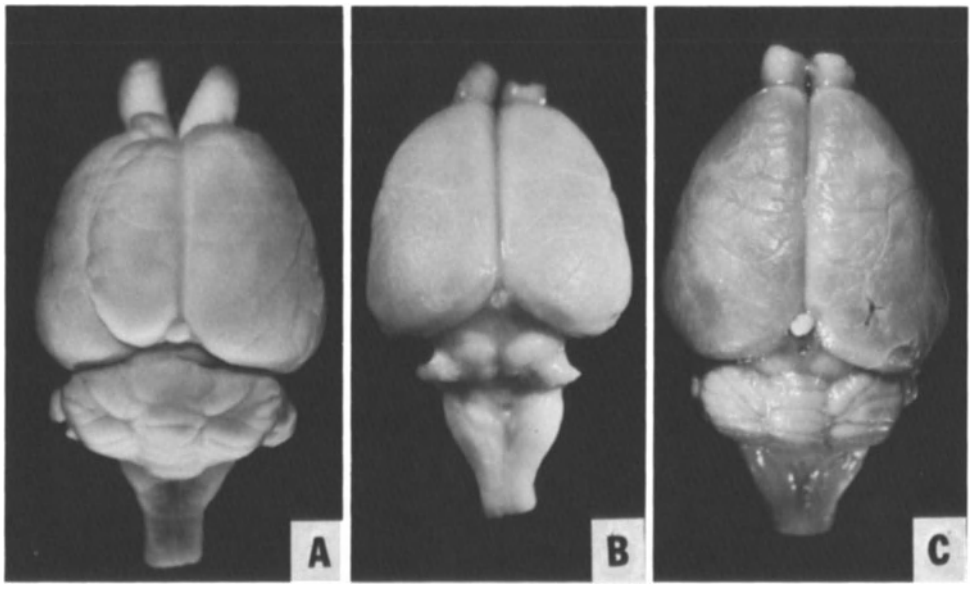


Fig. 2. Dorsal views of brains of rats injected intracerebrally at 4 days of age. (A) Control animal inoculated with diluent and sacrificed 21 days later. Magnification x2.5. (B) Experimental animal inoculated with LCM virus and sacrificed 21 days later. Magnification x2.5. (C) Experimental animal inoculated with LCM virus, treated with anti-lymphoid serum for 2 weeks, and sacrificed 52 days following infection. Magnification x2.5.

Virus could be found in all regions of the brain 9 days after inoculation. In addition to the cerebellum, other regions with intense fluorescence were the dentate gyrus of the hippocampus and the granule cells of the olfactory bulb. Occasionally, foci of necrotic cells could be seen in the olfactory bulb. The number of fluorescing cells began to decrease about 2 weeks after infection, and by 2 months viral antigen could be seen only in the hippocampal dentate gyrus and, minimally, in the lenticulostriate area, midbrain, and remaining cerebellar granule and Purkinje cells.

Brain virus titers reached a maximal level of 10^7 adult mouse LD50/0.03 g of brain 5 days after infection. These levels then declined until virus was undetectable by 4 to 5 months. The decrease in titer preceded the development of CF antibody, which was first observed 20 days after the intracerebral inoculation. Antibody titers rapidly attained levels of 200 and reached levels of about 1,000 from 50 to 110 days after infection. Titration of plasma revealed only a transient and intermittent viremia of less than 10 adult mouse LD50 during the first 2 weeks of infection.

Similar patterns of virus and antibody levels were found in animals inoculated intracerebrally at 1 and 7 days of age. Animals infected at 14 days of age evidenced only moderate levels of brain virus, about 10^3 LD50, becoming undetectable 1 month later. Brains from animals

infected as adults had a similar transient virus growth during the first week following injection of LCM virus.

Immunopathological basis of the cerebellar lesion. The immunopathological basis of LCM disease in mice has been well established. In order to determine whether this profound cerebellar lesion in rats also was mediated by an immunological mechanism or whether it was due to a cytocidal effect of LCM virus on the granule cell population, suckling rats were treated with ALS prior to and for 2 to 3 weeks after intracerebral inoculation of LCM virus at 4 days of age. Control animals received virus and treatment with either NRS or physiological saline or received no intraperitoneal injections. The efficacy of the ALS was evaluated by the ability of a corresponding treatment to prevent death in adult mice given a potentially lethal intracerebral inoculation of 1,000 LD50 of LCM virus.

While control rats uniformly developed the severe cerebellar pathology described earlier, no lesions appeared in the animals on the immunosuppressive regimen (Fig. 2C), although the brains contained large amounts of virus as shown by immunofluorescent staining (Fig. 3A) and virus titrations (Table 1). Distribution of antigen was more widespread than in the control animals and 3 weeks after infection was still present in these areas of the brain which in the non-suppressed infected rats had cleared (Fig. 3B).

Table 1. Virus and antibody levels in rats following intracerebral inoculation with LCM virus at 4 days of age. Effect of immunosuppression with anti-lymphoid serum

	Days after inoculation								
	3	5	7	9	15	18	23	52	115
Brain virus									
NRS	6.2[a]	6.6	6.2	5.6	4.7	2.2	3.6	1.0	n.d.[c]
ALS[b]	6.8	6.4	6.6	6.8	5.6	5.4	5.4	3.8	n.d.
Blood virus									
NRS	tr[d]	tr	0	0	n.d.	0	0	0	n.d.
ALS	0	0	≥1.0	≥1.0	n.d.	≥1.5	≥1.5	≥1.5	n.d.
CF antibody									
NRS	0	0	0	0	n.d.	0	60	120	>520
ALS	0	0	0	0	n.d.	0	0	0	168

[a] Virus levels expressed as $\log_{10}$ adult mouse LD50/0.03 ml inoculum.

[b] Anti-lymphoid serum was administered over a 2-week period, beginning 1 day before infection.

[c] Not done.

[d] Trace.

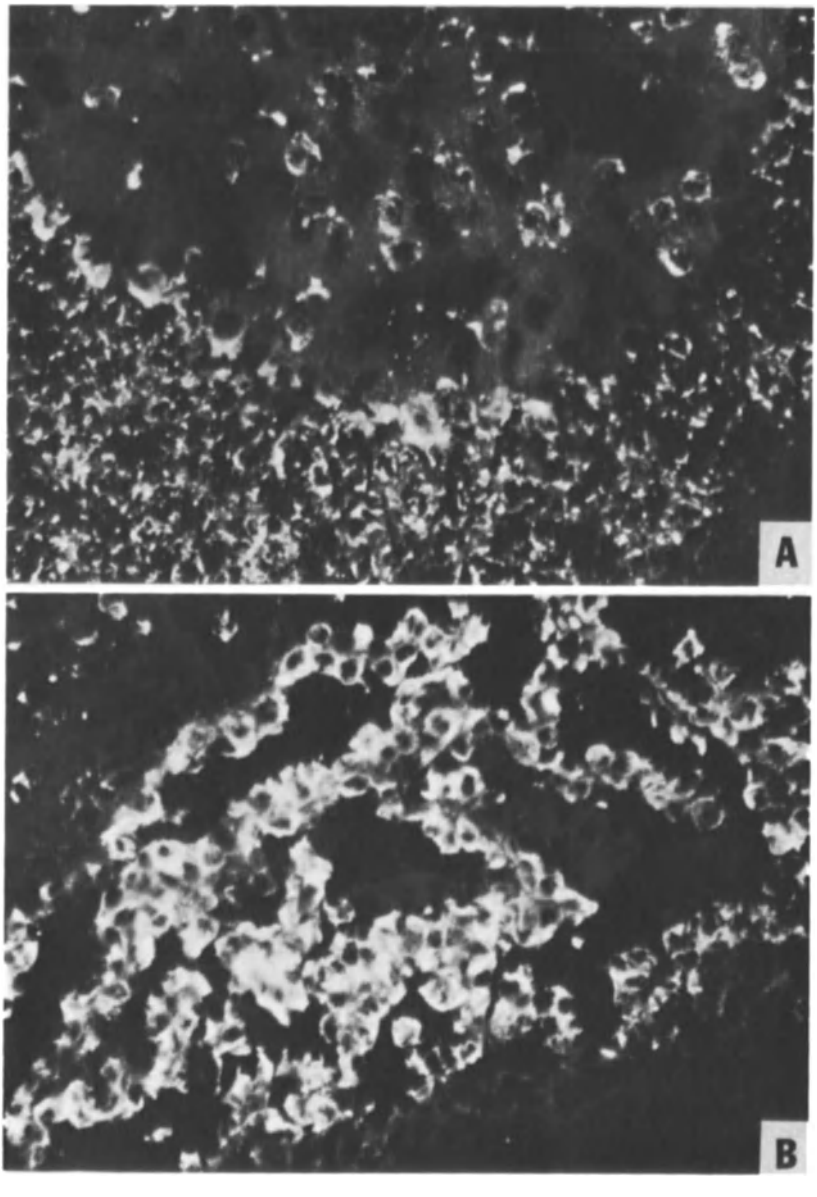


Fig. 3. Localization of infection following intracerebral inoculation of LCM virus into 4-day-old rats immunosuppressed with rabbit anti-rat lymphoid serum from 3 days of age until sacrifice 21 days after infection. (A) Folium of cerebellar cortex with immunofluorescent staining of internal granule cells, Purkinje cells, as well as cells in the molecular layer. Magnification x250. (B) Fluorescent antibody stain of LCM virus-infected cells in the choroid plexus and ependyma of the 4th ventricle. Magnification x250.

The long-term effects of a 2-week course of ALS administered on alternate days starting 1 day before intracerebral injection of LCM virus into 4-day-old rats is summarized in Table 1. Brain virus titers remained higher in the immunosuppressed animals than in the controls during the month following cessation of ALS treatment. In addition, a continuous viremia was detectable, unlike the minimal and transient viremia seen in the control animals. CF antibody was not found in the treated animals until 3 months after termination of the ALS administration. At no time were there signs of cerebellar pathology, either clinically or histologically, in the immunosuppressed animals.

Rats infected intracerebrally with LCM virus during the first 24 h following birth failed to develop cerebellar pathology, even though viral antigen could be seen in the cerebellar as well as in the cerebral parenchyma. If the absence of pathology in the infected cerebellar cells was due to the immaturity of the animal's immune response and, hence, the development of a state of relative tolerance as in the mouse, transplantation of immune spleen cells might induce the lesion developed by the older sucklings.

Therefore, newborn inbred Fischer rats were given either 1,000 LD50 of LCM virus or 1,000 LD50 of virus and 80 x 10^6 immune spleen cells, or an equal volume of diluent intracerebrally, and 80 x 10^6 immune spleen cells intraperitoneally during the first 24 postnatal hours. Cells alone produced no untoward effects. Rats receiving virus alone did not have any cerebellar pathology but, in contrast to the outbred Wistar rats, about 75% of these animals died 2 to 3 weeks after the intracerebral infection from an as yet undetermined cause. Animals which received virus and immune cells all died within 3 weeks, but death was preceded by a severe runting, starting at about 9 days of age, as well as by the development of cerebellar pathology.

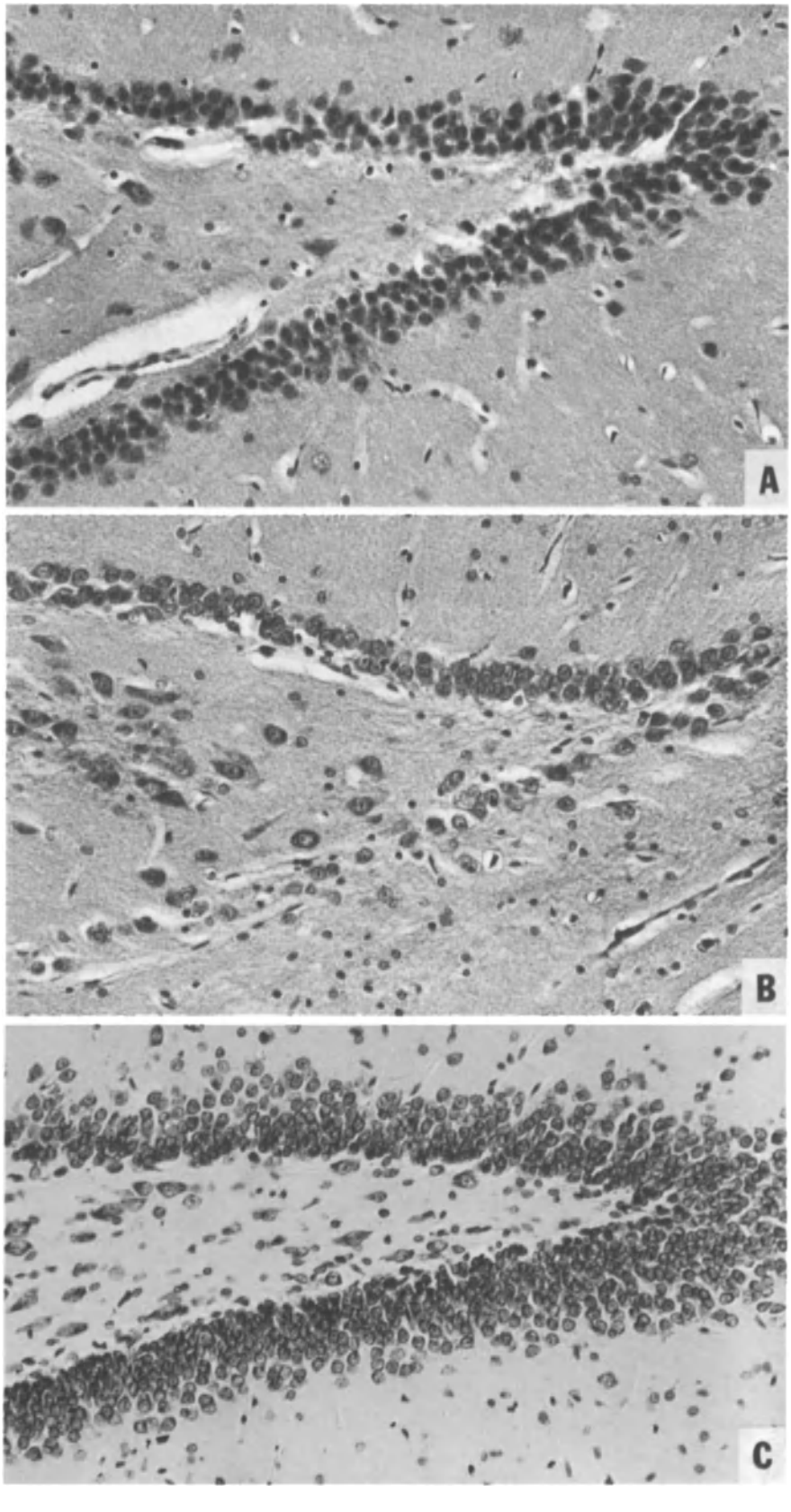
Thus, the immunopathological basis of the LCM virus-induced cerebellar pathology in neonatal rats has been demonstrated both by preventing it with immunosuppression of normally susceptible animals and by eliciting it by transfer of LCM virus-immune spleen cells into infected newborns who would not normally develop the lesion.

Late developing pathology. Rats infected intracerebrally with LCM virus at 4 days of age developed pathological changes within the cerebellum shortly after inoculation and, within 3 weeks, the evolution of the lesion was effectively complete. In some, but not all animals, foci of necrotic cells could be found within the olfactory bulb during this period. However, no alterations were evident within the hippocampus even though the dentate gyrus was heavily infected, as shown by fluorescent antibody staining.

On the other hand, pathological consequences of the hippocampal infection could be found long after the intracerebral inoculation. Three months after infection, a slight thinning out of cells was seen in the dentate gyrus (Fig. 4A). This continued until a marked loss of granule cells was evident 1 year after the neonatal inoculation (Fig. 4B) as compared with normal rat dentate gyrus (Fig. 4C).

A similar pattern could be seen in animals infected at 1 day of age, whilst those infected at 14 days of age were free of any observable changes. Investigations are still underway to determine whether this pathology also can be prevented by immunosuppression.

Ocular disease. While intracerebral infection of the newborn rat failed to produce cerebellar necrosis, it did result in a destructive


A
B
C

retinopathy (14) which was never seen in animals injected at 4 days of age or older. Pathological changes were first seen 10 days after infection as a progressive disorganization of the outer nuclear layer, followed by a loss of cells in the inner nuclear and ganglion cell layers, finally resulting in total disorganization and destruction of the retina. Although the outer nuclear layer evidenced the first morphological signs of LCM disease, virus initially was detected in the optic nerve and inner nuclear layer 4 days after inoculation, then in the pigment epithelium, and finally, at 2 weeks of age, in all layers of the retina.

Notwithstanding the widespread destruction of the retina, at no time was there a significant cellular infiltrate. The inflammatory cells consisted of lymphocytes and histiocytes, primarily found within the inner layers of the retina, and appeared in greatest numbers about 2 weeks after inoculation, diminishing thereafter despite the progressive retinal pathology.

Considering the immunopathological nature of the cerebellar disease, preliminary experiments were conducted in which LCM virus-infected newborn rats were given ALS for a period of 3 weeks. This treatment resulted in inhibition of the retinopathy, suggesting that it too is mediated by immunopathologic mechanisms.

Strains of LCM virus. In all of our experiments hitherto described, we have used the Armstrong E-350 strain of LCM virus. The ability of several other strains of the virus to produce cerebellar pathology after intracerebral inoculation into 4-day-old rats was tested, and the comparative results are shown in Table 2. Both the WE/UBC-A351 and

Table 2. Effect of LCM virus strain upon course of disease following intracerebral infection of 4-day-old rats

	Strain			
	E-350	WE/UBC-A351	CA 1371	Traub
Mortality	0	≥60%	≥70%	≥50%
Days to 50% of mortality	-	12	<15	12
Ataxia	+	+	+	-
Cerebellar pathology	Severe	Moderate	Mild	None
Choriomeningitis	Moderate	Mild	Mild	Trace
Protection by ALS	+	n.d.[a]	n.d.	+

[a] Not done.

◄ Fig. 4. Development of pathological changes in the dentate gyrus of the hippocampus following intracerebral inoculation of LCM virus into 4-day-old rats. (A) Slight thinning of cells, 3 months after infection. Hematoxylin and eosin. Magnification x200. (B) Severe fallout of cells, 1 year after infection. Hematoxylin and eosin. Magnification x200. (C) Normal adult rat dentate gyrus. Hematoxylin and eosin. Magnification x200.

the CA 1371 strains were able to induce cerebellar pathology and choriomeningitis, but to a lesser extent than did the E-350 strain. However, infections with the former 2 strains resulted in a high degree of mortality within the first 2 weeks after inoculation, a consequence never observed in the previous studies with the Armstrong virus. No signs of cerebellar pathology, either clinically or histologically, were observed after infection with the Traub strain, although there was a minimal degree of choriomeningitis. Immunosuppressive therapy with ALS successfully prevented deaths following infection with the Traub virus, but cessation of such treatment resulted in the same degree of mortality, delayed by the period of ALS administration. Thus, the LCM disease induced in neonatal rats by intracerebral inoculation of the Traub virus appears to be immunopathological in nature.

DISCUSSION

The study of LCM disease in the rat has been instructive in emphasizing the importance of the dynamics of the host-virus interrelationship. Intracerebral infection of the neonatal rat resulted in varying extents of CNS parenchymal pathology dependent upon host age and strain of virus. During the first 2 weeks of life, granule cells within the cerebellum, hippocampus, olfactory bulb, and retina are in a state of migration and proliferation (1,19), and it is especially within these cell systems that we have seen large amounts of fluorescent antigen. Thus, LCM virus appears to have a proclivity for infecting actively proliferating neuronal populations.

The pathological consequences of this infection, however, follow a different time course. Whereas animals infected as newborns have cerebellar granule cells containing LCM viral antigen, no lesion is produced unless they receive a transplant of spleen cells from immune syngeneic donors. Animals infected at 7 days of age have neither the massive lesion nor the heavy infection of the cerebellum which occurs following intracerebral inoculation of LCM virus at 4 days of age. Thus, in the rat the immunopathological lesion of the cerebellum is a dual function of the decrease in susceptible cells and the development of immunocompetence with increasing age.

This relationship between infected target cell and the host's state of immunological responsiveness has been shown also to apply in the mouse. Mice infected intracerebrally at 4 days of age with LCM virus develop cerebellar granule cell necrosis before dying of acute choriomeningitis between 8 and 14 days after inoculation (4). In addition, similar focal cerebellar lesions can be found in adult mice made virus carriers by intracerebral infection combined with cyclophosphamide treatment, followed by adoptive immunization with immune spleen cells 1 month later (7).

A number of questions remain unanswered about the pathological consequences of LCM virus infection in the rat. We cannot explain either why the cerebellum is so susceptible to the necrotic process, or what mechanisms are involved in this process. Nor do we yet know why it takes several months for changes to occur in the hippocampal dentate gyrus, why the heavily infected olfactory bulb so irregularly exhibits pathology, or why retinal lesions develop in the absence of cerebellar lesions in the rat infected as a newborn.

In contrast to the marked choriomeningitis is the surprisingly minimal inflammatory infiltrate seen within the lesions. This observation initially led us to the impression that the pathology was a consequence of

a direct cytolytic effect of the virus. However, immunosuppression with ALS prevents the lesion even in the presence of a continued widespread parenchymal infection. We have not yet fully investigated the role played by non-cellular immune mechanisms in the pathogenesis of the necrotic lesions. On the other hand, considering the late appearance of CF antibody in relation to the evolution of the cerebellar pathology, and the requirement of immune lymphoid cells for the transfer of adoptive immunity (16) or acute choriomeningitis (5,7) in the mouse, it would appear that LCM disease in the rat is primarily a function of cell-mediated immunological processes.

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In Vitro Measurement of the Time Course of Cellular Immunity to LCM Virus in Mice

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SUMMARY

The role of cellular immunity in LCM disease in mice has been well established. In order to investigate the cellular immune response in vitro, a quantitative method was developed based upon the 51Cr release following immunologically specific cytotoxic activity of sensitized lymphocytes upon LCM virus-infected and radioactively labeled L cells. The time course of the cellular immune response was determined for the acutely infected adult mouse as well as for the neonatally infected carrier mouse following transfer of lymphocytes from immune syngeneic donors. While lymphocytes from acutely infected mice specifically lysed LCM virus-infected L cells, no such activity was found when using similar cells from carrier mice. Furthermore, no evidence was obtained showing the presence of enhancing antibody in the virus carriers. Dose-response experiments indicated that a direct cell-to-cell interaction may be involved in the in vitro cell-mediated destruction of LCM virus-infected target cells.

INTRODUCTION

Substantial data indicating an independent cell-mediated immune response to LCM virus in mice have been presented by Volkert and Lundstedt in a review in 1971 (21). Since then, reports of experiments which further elucidate the problem have been published by several authors. By using the foot pad test in highly sensitive strains of mice, Tosolini and Mims have been able to demonstrate the time course in the development of a D-type hypersensitivity during acute infection (18). Furthermore, Oldstone and Dixon have shown that a cytotoxic substance is released when immune lymphocytes are stimulated by LCM virus antigen (15), and in 1971 Holtermann and Majde presented data (12) which confirm the cytotoxic effect of immune lymphocytes on infected target cells, first demonstrated by Lundstedt (13).

More recently, P.W. Wright and his colleagues have published a short communication which indicates that the cellular immunity to LCM virus can be suppressed by thymectomy, sublethal irradiation, and other immunosuppressive treatments (23). The degree of cellular immunity in these experiments was determined by the Hellström in vitro cytotoxicity test.

Because in vitro methods for the measurement of cell-mediated immunity to LCM virus have only recently been taken into use, many problems

connected with this immunity remain obscure. We know little about the time course, the magnitude, and the mechanism of the cellular type of immunity to the LCM virus.

In order to throw more light upon these problems, a highly reliable and reproducible in vitro method for measuring cell-mediated LCM virus immunity in mice was developed in our laboratory.

It is the purpose of this paper to present and discuss some of the results obtained by this method.

MATERIALS AND METHODS

Mice. Except for virus titrations, strictly inbred C3H mice, raised and bred in this laboratory, were used throughout the study. When the term "virus carrier" is used, it refers to mice infected at birth and carrying virus in high titers in blood and organs, but without antibody detectable by ordinary serological techniques.

Virus strain, virus titrations, the preparation of the cell suspensions, and the serological tests have been described in previous papers from this laboratory (3).

The lymphoid cell preparations employed in all experiments were mixtures of cells from the spleens and the lymph nodes in a proportion of about 4:1.

The cytotoxic assay. After preliminary attempts using a number of techniques, a method based on the in vitro cytotoxic effect of immune lymphocytes on infected target cells was developed. The technical data are presented in detail elsewhere (14). Briefly, the method is a combination of the technique described by Brunner for determination of transplantation immunity (4) and the Hellström technique (10).

The target cells were ^{51}Cr-labeled L cells which 18 h prior to the experiment had been infected with the LCM virus. With the technique used about 90% of the cells were infected. Suspensions of the target cells were added to Petri dishes, and shortly after they had settled and adhered to the bottom the syngeneic lymphocytes were added in an amount corresponding to a ratio between lymphocytes and target cells of 25:1.

Eighteen h later the degree of cell destruction was measured by determination of the amount of ^{51}Cr released into the tissue culture fluid. For this purpose a liquid scintillation counter (Beckman LS-250) was used. The results are expressed as a cytotoxic index, which reflects the ratio between the actual amount of ^{51}Cr released and the total amount of ^{51}Cr incorporated (4).

RESULTS AND DISCUSSION

The acute infection. Several experiments were carried out to determine the time course of the cellular immune reaction in the LCM virus infection of normal adult C3H mice, expressed as the immunologically specific cytotoxic activity of lymphoid cells from such animals. At different times before the cytotoxicity assay was made, groups of 6 mice were inoculated intraperitoneally with 10^3 LD50 LCM virus. On the day of the experiment suspensions of lymphocytes from each group of animals

were prepared and tested as described. Thus the various suspensions were assayed in one test.

Average levels of cellular immune activity from 4 of these experiments are shown in Figure 1. It may be seen that significant activity was already detectable 3 days after infection. It then increased rapidly to a maximum on day 9, when a cytotoxic index of 100 was reached. The activity then fell equally rapidly, reaching a cytotoxic index of about 40 on day 12. This steep decline was followed by a more gradual one from day 12 to day 27, while the cellular immunity during the last 3 weeks of the experiment remained at a low and relatively constant level.

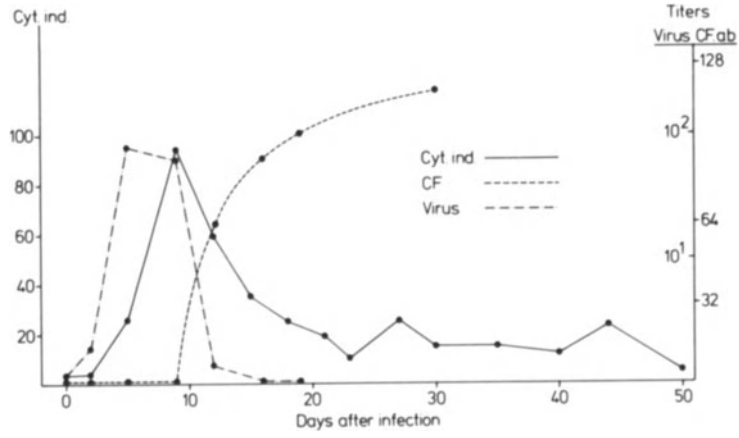

Fig. 1. Time course of immune response and viremia following acute LCM virus infection of adult C3H mice. Cellular immunity is expressed as cytotoxic index (Cyt. ind.) and humoral immunity is expressed in terms of CF antibody.

The time course for the cell-mediated immunity to LCM virus just described roughly follows that described by Tosolini and Mims who employed the foot pad method. Moreover, our data also resemble the results obtained by Brunner and others in transplantation and tumor immunity experiments (5,6,17); in these a 51Cr release method was employed.

The usual course of events with regard to blood virus titer and CF antibody in acutely infected normal adult mice was determined using 10 groups of 6 mice and employing standard titration methods. The resultant graphs are shown together with the time course of cellular immunity in Figure 1.

Already on day 2, low but significant virus titers can be detected in the blood, and from day 2 to 5 there is a steep rise in this parameter. With a delay of only 3 to 4 days this rise is followed by an equally fast increase in the cytotoxic activity.

When the blood virus titer falls drastically from day 9 to day 12, it is followed closely by the rapid decline in cellular immunity described above. From day 16 virus is no longer detectable in the blood, while the cytotoxic activity during this period gradually declines to a very low level.

CF antibody has reached demonstrable amounts on day 12. Cellular immunity at this time has dropped to less than half of its maximum, while virus has almost disappeared from the blood stream. Later in the experiment the CF antibody titer rises and remains quite constant at a high level, while the cellular immunity falls to very low values, and virus is totally absent from the blood.

The time course of these 3 parameters indicates that the development of the cellular and humoral responses is independent. Moreover, in our opinion the data obtained strongly support the supposition propounded earlier that it is the cellular immune reaction which eliminates the virus (21).

Adoptive immunization. When a sufficient number of spleen and lymph node cells from immunized mice are injected intravenously into syngeneic C3H virus carriers, the recipients develop CF antibody with titers 10 to 100 times higher than those developed when normal adult animals are infected (19). Simultaneously there is a marked drop in the blood virus titers. We therefore determined the cell-mediated immune response in this immunological situation, in order to investigate to what extent this immunity is responsible for elimination of the virus. The immune cells were obtained from female mice infected 1 or 2 months earlier by nursing their infected babies. Lymphoid cells, 100 x 10^6, prepared as described earlier, were injected intravenously into syngeneic virus carriers at various times before the cytotoxic test was carried out. Representative results are shown in Figure 2. It appears

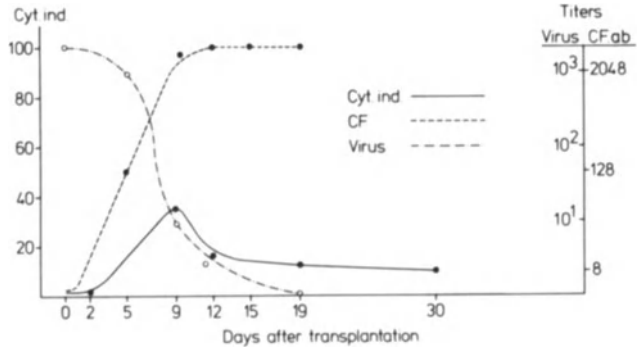

Fig. 2. Time course of immune response and viremia in C3H virus carriers adoptively immunized with 100 x 10^6 lymphoid cells from immune syngeneic animals. Cellular immunity is expressed as cytotoxic index (Cyt. ind.) (see text) and humoral immunity is expressed in terms of CF antibody.

that when 100 x 10^6 cells are transplanted, the time course of the cellular immunity is, except for the dramatic peak around the 9th day, very much the same as that observed in the acutely infected animals. Maximal cytotoxic indices are reached on day 9 but are usually less than 40.

When this cell dose is employed, there is no significant time difference between the development of antibody, the onset of cellular immunity, and the drop in the blood virus titer. It is therefore difficult to evaluate whether the decisive factor in the elimination of the

virus is one or the other of the two kinds of immunity - or both. It is striking, however, that the cellular immune response seems very weak when compared with the large amounts of rapidly produced antibody. When these data are considered in the light of the fact that adoptively immunized animals also produce neutralizing antibody (19), it seems obvious to assume that during adoptive immunization the humoral immunity may be an important factor in the elimination of the virus. As mentioned earlier, this is in contrast to the situation occurring during the acute infection. Here cell-mediated immunity seems to be both rapid and intense, whereas antibodies are produced slowly, reach relatively low titers, and show no neutralizing capacity. The mechanism of the elimination of the virus during the acute infection might therefore be different from that operating during adoptive immunization.

Cell-mediated immunity late in the infection. It appears from the data presented in Figure 1 and also from the experiments described by Tosolini and Mims (18) that cellular immunity is at a low level late in the infection. Since it is known that at this time the virus is still present in quite high titers in different organs, e.g. the kidneys (8,9), and since there is a great deal of experimental evidence which points to the cell-mediated immunity being the decisive factor in keeping the virus in check (20), the low level of the cellular immunity in this situation is rather surprising and difficult to explain.

In order to throw more light upon the cell-mediated immunity late in the infection of the normal adult mouse, the cytotoxic activity of lymphocytes from a group of immune mothers was compared with that of lymphocytes from normal mice. The mice in the 2 groups were tested individually. The immune mothers were from 2 to 5 months old and were immunized by nursing their infected babies at an age of approximately 2 months, and the controls were normal inbred female C3H mice. In 1 out of 2 experiments a significant difference between the groups was observed (Table 1).

Table 1. Cytotoxic effect upon infected L cells following incubation with lymphocytes from LCM virus-immune mice

Experiment	Mice[a]	Counts per minute ± S.D.	Cytotoxic index
1	Immune	5734 ± 144	2.9
	Normal	5574 ± 132	
2	Immune	6676 ± 182	7.5
	Normal	5900 ± 234	

[a] Each group consisted of 6 mice.

When these results are considered, the obvious difficulty other authors have had in demonstrating a difference in cytotoxic activity between lymphocytes from normal mice and those from mice late after LCM virus infection (immune mothers) is easily understood (13).

The question of tolerance in virus carrier mice. In the field of LCM virus immunology the problems connected with the existence and the degree of tolerance to the virus have been of great significance. For many years it seemed to be a well-established fact that a state of tolerance to the virus could be produced in mice. However, Oldstone and Dixon have recently presented data which may indicate that some cell-mediated immunity to the virus in apparently tolerant mice does exist (16). Moreover, Allison and others have expressed the opinion that enhancing antibody might be present in the virus carrier mice and that these obscure the presence of a cell-mediated immunity (personal communication). These problems were therefore investigated. In a series of experiments the cytotoxic effect of pooled lymphocytes from virus carriers was compared with that of lymphocytes from normal mice. Later, other groups of mice were tested individually and compared. In none of these experiments was it possible to demonstrate a significant difference between the activity of the lymphocytes from the 2 groups of animals. Our data thus provide no support for the suggestion of a cell-mediated immunity to LCM virus in carrier mice. If, nonetheless, such an immunity exists in these animals, it must be very weak.

In order to search for enhancing antibody, the following experiments were carried out: infected L cells were added to Petri dishes and allowed to adhere to the bottom. The medium was removed and the cells were rinsed with phosphate-buffered saline. Virus carrier serum or serum from normal mice, both diluted 6-fold, was then added, and incubation took place at 37°C in a CO_2 atmosphere on a rocking platform. The serum was inactivated at 56°C for 30 min before use. After the cells had been exposed to the serum in this manner, medium and lymphocytes from acutely infected mice which had received 10^3 LD50 intraperitoneally 9 days earlier, or from normal mice, were added. The reaction mixtures were then incubated for 18 h under the conditions already described. The results show that the degree of cell lysis and the amount of ^{51}Cr released was the same, regardless of whether the target cells

Table 2. Effect of virus carrier serum and normal serum upon the lymphocytic destruction of LCM virus-infected target cells

Serum	Pool[a]	Cytotoxic index	Mean ± S.D.
Virus carriers	1	54	51.6 ± 2.4
	2	45	
	3	52	
	4	47	
	5	60	
Normal mice	1	47	48.8 ± 1.9
	2	46	
	3	48	
	4	57	
	5	46	

[a] Each pool consisted of serum from 2 mice.

had been exposed to normal serum or to serum from virus carriers. The data from 1 of these experiments are shown in Table 2. Furthermore, the cytotoxic indices were in the range in which they are normally found when the target cells have not been exposed to mouse serum during the experiment. The presence of hypothetical enhancing antibody in the serum from virus carriers could therefore not be demonstrated by our technique.

As the two kinds of experiments just described revealed neither cell-mediated immunity nor enhancing antibody in virus carrier mice, they give indirect support to the old hypothesis that a state of tolerance to LCM virus has developed in these animals.

The question of soluble cytotoxic factors. Another in vitro method for measuring cellular hypersensitivity is the lymphocyte migration inhibition test. In this test it is well established that even as few as 2% sensitized lymphocytes mixed with 98% non-sensitized macrophages are sufficient to cause an inhibition of the migration of the whole population, and it has been demonstrated unequivocally that on contact with the antigen the lymphocytes produce a soluble substance which brings about an inhibition of the migration of the non-sensitized lymphoid cells (2).

It was also claimed by Granger that a factor resembling the migration inhibition factor and likewise released from immune lymphocytes following contact between the cells and the homologous antigen might be responsible for the cell-mediated cytotoxicity (7,22). Moreover, in a cytotoxic two-step system consisting of lymphocytes from LCM virus-immunized animals and virus-infected or non-infected target cells, Oldstone and Dixon (15) demonstrated the existence of a soluble cytotoxic factor. Here the immune lymphocytes were incubated with live or inactivated virus. In both cases a cytotoxic factor was produced, and while this production seemed to be immunologically specific, once released the factor was non-specific in that it destroyed both infected and non-infected cells. In contrast to these reports, other investigators have not been able to demonstrate that soluble factors play any role in the cytotoxic effect of immune lymphocytes, and it seems that most workers in this field believe that the destruction is the result of a direct contact between the lymphocyte and the target cell (1,11). In order to elucidate these problems, experiments were devised to analyze the dose effect of sensitized lymphocytes in our system.

Lymphocyte suspensions from C3H mice inoculated intraperitoneally 9 days earlier with 10^3 LD50 of LCM virus, and from normal mice of the same sex and age, were prepared as described. Mixtures of the 2 suspensions containing increasing numbers of immune lymphocytes and decreasing numbers of normal lymphoid cells were produced. These mixtures were assayed for cytotoxic activity against LCM virus-infected L cells.

The results from a representative experiment from this series are shown in Figure 3. The cytotoxic indices are plotted against the percentages of immune lymphocytes in the reaction mixture, and it is seen that there is a direct linear relationship between the percentage of immune cells and the degree of cell destruction. The graph is a "one hit kinetic" curve, and thus reveals that only one type of immune cell is necessary to bring about the destruction of a target cell. Similar results have been obtained in other systems (6,11,17). Furthermore, it can be seen that using a ratio of 1:25, maximum cell destruction is already reached at 60% immune cells, which means that 15 immune lymphocytes per L cell are necessary to ensure that all target cells are destroyed.

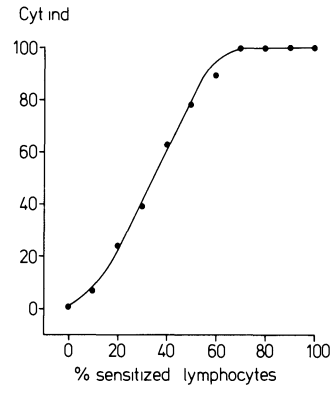

Fig. 3. Dose effect of sensitized lymphocytes upon LCM virus-infected L cells. The cytotoxic indices were determined in different reaction mixtures containing the same total number of lymphocytes but increasing percentages of sensitized cells.

Thus it may be concluded that if a cytotoxic factor is produced, it must be of very low potency or stability, or it must be produced by very few of the sensitized cells. On the basis of the results reported here, the results of other workers in the same field, and of our present knowledge of the migration inhibition factor, we feel that the most likely mechanism of in vitro lymphocyte cytotoxicity is a direct cell-to-cell interaction.

ACKNOWLEDGMENT

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LCM Disease of the Adult Rat: Morphological Alterations of the Brain

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SUMMARY

LCM virus, strain WE, multiplies readily in the brain of young adult Sprague-Dawley rats with maximum titers of close to 10^8 ID50/g of tissue, which are reached by day 5. Five to 6 days after the intracerebral inoculation of 10^6 mouse ID50 clinical signs develop which closely resemble the ones seen in mature mice infected via the brain. The morphological alterations, too, are similar in both species, although in the rat they are more widespread, involving parts of the brain which remain essentially normal in the mouse. In the rat, inflammatory reactions are found in the meninges, predominantly in the basal regions, as well as in circumventricular organs, such as plexus chorioidei, subfornical organ, area postrema, organum vasculosum laminae terminalis, and median eminence. In severe cases, the ventricular ependyma too is largely destroyed, and beneath the ependyma distentions of the intercellular spaces and structural changes of myelinated axons occur. Here, perivascular round cell infiltrates dominate the picture, whereas in other areas, such as the hypothalamus, the thalamus, and the cerebellar nuclei, a diffuse distribution of inflammatory elements is accompanied by a moderate glial reaction.

The observed predilection of pathological alterations in circumventricular organs suggests that peculiarities of this structurally and functionally unique system may play a relevant role in the pathogenesis of experimental LCM of the rat.

INTRODUCTION

LCM virus has a wide host range and numerous species support its multiplication. However, replication of the agent is not always followed by disease, and the spectrum of host responses to the virus includes severe pathological alterations with marked clinical signs as well as inapparent infections. For a variety of different species the patterns of virus multiplication and the pathological consequences are well established (11). In the case of the adult rat the evidence is contradictory. Several authors have described apparent disease and/or pathology (1,4,5,15,26), while others have failed to observe a clinical response (2,12). In an attempt to resolve these discrepancies, we have taken up this problem anew and have investigated the ability of LCM virus to multiply and cause clinical and morphological

alterations in the rat. Observations concerning the central nervous system are recorded here.

MATERIALS AND METHODS

Animals. Male specific pathogen-free Sprague-Dawley rats, designated FW 49/Bib. and NIH/Han., were used when 5 to 6 weeks old.

Virus. A few preliminary experiments were done with strain E-350 of Armstrong, but in general the WE strain of Rivers was employed. It was passaged and propagated in L cells, and for quantitation the ID50 was determined in mice as described previously (10). Both virus strains were tested and shown to be free of the lactic dehydrogenase agent. Growth of virus in the brain was determined after the intracerebral inoculation of 10^3 ID50 contained in 0.1 ml. At intervals the brains of 2 rats were homogenized separately using as diluent balanced salt solution with 1% inactivated calf serum and antibiotics followed by treatment with an ultrasonic drill for 45 s. After centrifugation, the supernatant was immediately assayed for infectivity in weaned mice.

Light microscopy. Rats were perfused under deep hexobarbital anesthesia via the aorta, first for 3 min with Haemaccel (Behringwerke, Marburg, Germany) or balanced salt solution and subsequently for 10 min with 4% formaldehyde in pH 7.2 phosphate-buffered saline. The brains of some of the moribund animals were fixed in situ by immersion in 10% formaldehyde. They were embedded in Paraplast, and 10 µm frontal sections, sometimes in series, were cut at different levels so as to include the organum vasculosum laminae terminalis, the subfornical organ, the subcommissural organ, the 4th ventricle at the level of the nucleus vestibularis and nucleus cochlearis and the area postrema. The sections were stained with cresyl-violet and hematoxylin-eosin.

Electron microscopy. Rats maintained by artificial respiration were anesthetized with hexobarbital and, after having reduced blood coagulation by intracardial administration of heparin, their brains were fixed via the aorta by a 2-step perfusion procedure, employing dilute and concentrated mixtures of formaldehyde and glutaraldehyde (19). The initial perfusion pressure of 80 mm Hg was diminished to 40 mm Hg after 10 min and the brain was left within the skull in the concentrated aldehyde solution for at least 4 h. Thereafter, small tissue blocks were prepared from the organum vasculosum laminae terminalis, the roof of the 3rd ventricle (choroid plexus), the ventricular wall of the posterior horn of a lateral ventricle, and the medulla oblongata (area postrema). These were rinsed in 0.2 M Na-cacodylate, postfixed in 2% osmic acid buffered with 0.2 M Na-cacodylate, dehydrated and embedded in Epon. Ultrathin sections were prepared with diamond knives in an ultramicrotome OmU_2 (Reichert, Vienna, Austria), subsequently stained with lead citrate and uranyl acetate, and examined with an EM 96 electron microscope (Zeiss, Oberkochen, Germany). Semithin (1 µm) sections were stained with toluidine blue or were silver-impregnated.

RESULTS

Signs of the disease. The intracerebral inoculation of 10^6 ID50 of strain E-350 did not lead to overt disease in Sprague-Dawley rats. In contrast, the same dose of the WE strain virus causes clinical signs which very much resemble the ones seen in adult mice. After an interval of 5 to 6 days a syndrome develops which consists of loss of

weight, conjunctivitis and huddling in a hunched-back posture. When the animal is suspended by the tail, a characteristic fine tremor is noticed which through coarse fasciculations and myoclonic jerks may develop into generalized seizures with rotating movements around the longitudinal axis. A few animals die 6 or 7 days after infection.

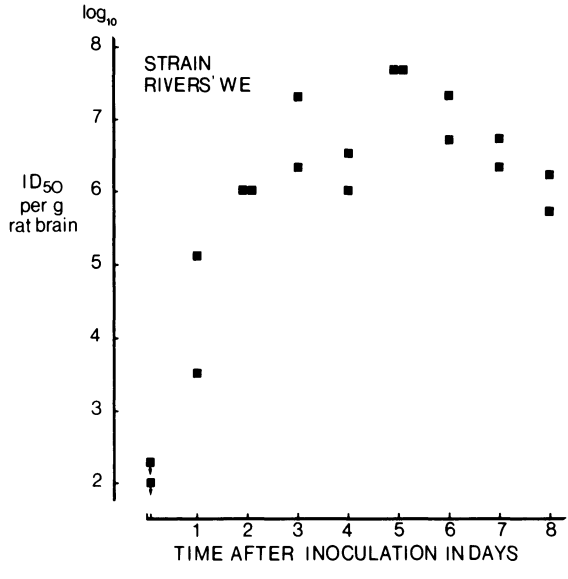


Fig. 1. Multiplication of LCM virus, strain WE, in the rat brain after intracerebral infection.

Virus multiplication. Growth of the virus in the brain of the rats following the intracerebral inoculation of 1,000 ID50 is depicted in Figure 1. No infectivity can be detected at 2 h, but 24 h after infection progeny virus is readily demonstrable and a peak of infectivity is reached after 5 days. This growth curve is almost identical with the one determined for the brain of the mouse (10), although titers are slightly lower.

Morphological alterations. Around the 5th day after the intracerebral inoculation of 10^6 ID50 of LCM WE virus, morphological alterations make their appearance in the brain. Characteristically, they are localized in the leptomeninges, the walls of the ventricles and certain circumventricular organs (3), namely organum vasculosum laminae terminalis, subfornical organ, area postrema, median eminence, and plexus chorioidei, which have in common a characteristic localization around the 3rd and 4th ventricle and a special differentiation of the glial, ependymal, neuronal and mesenchymal tissue elements, resulting in functional peculiarities.

The leptomeninx (Fig. 2) is infiltrated by lymphocytic cells predominantly in the basal regions, which is frequently accompanied by glial proliferation in the molecular layer. The lymphocytic infiltrates may extend into adjacent brain structures, such as the nucleus supraopticus, the corpus mammillare or, in the region of the medulla, the tractus cortico-spinalis. In the plexus chorioidei the lymphocytic in-

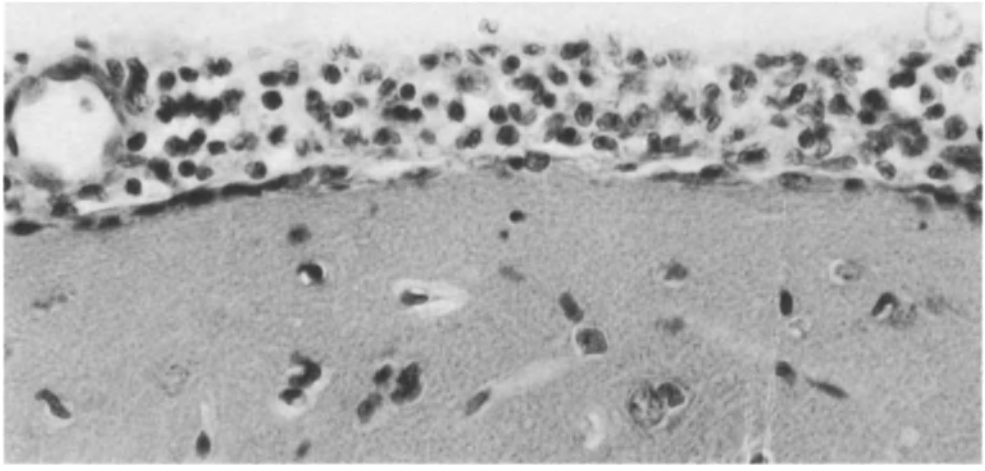
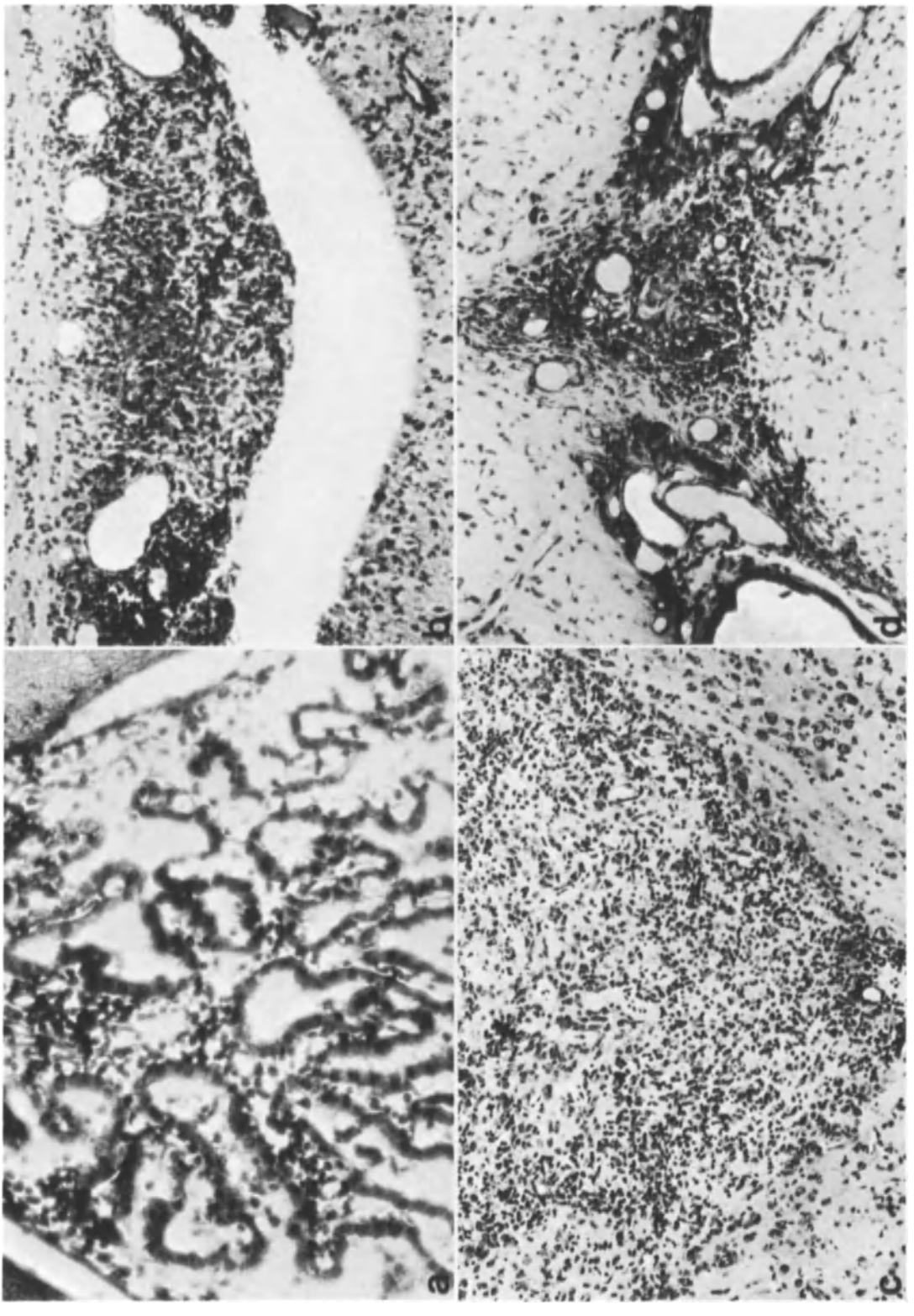


Fig. 2. Rat cerebral cortex 6 days after intracerebral inoculation of LCM virus, showing severe meningitis. Hematoxylin-eosin. Magnification x460.

Fig. 3. Circumventricular organs of rat brain 6 days after intracerebral inoculation of LCM virus, showing characteristic mononuclear cell infiltration. a = plexus chorioideus of the 3rd ventricle (recessus pinealis). Hematoxylin-eosin. Magnification x250. b = organum subfornicale. Cresyl-violet. Magnification x150. c = area postrema. Hematoxylin-eosin. Magnification x110. d = organum vasculosum laminae terminalis. Cresyl-violet. Magnification x150.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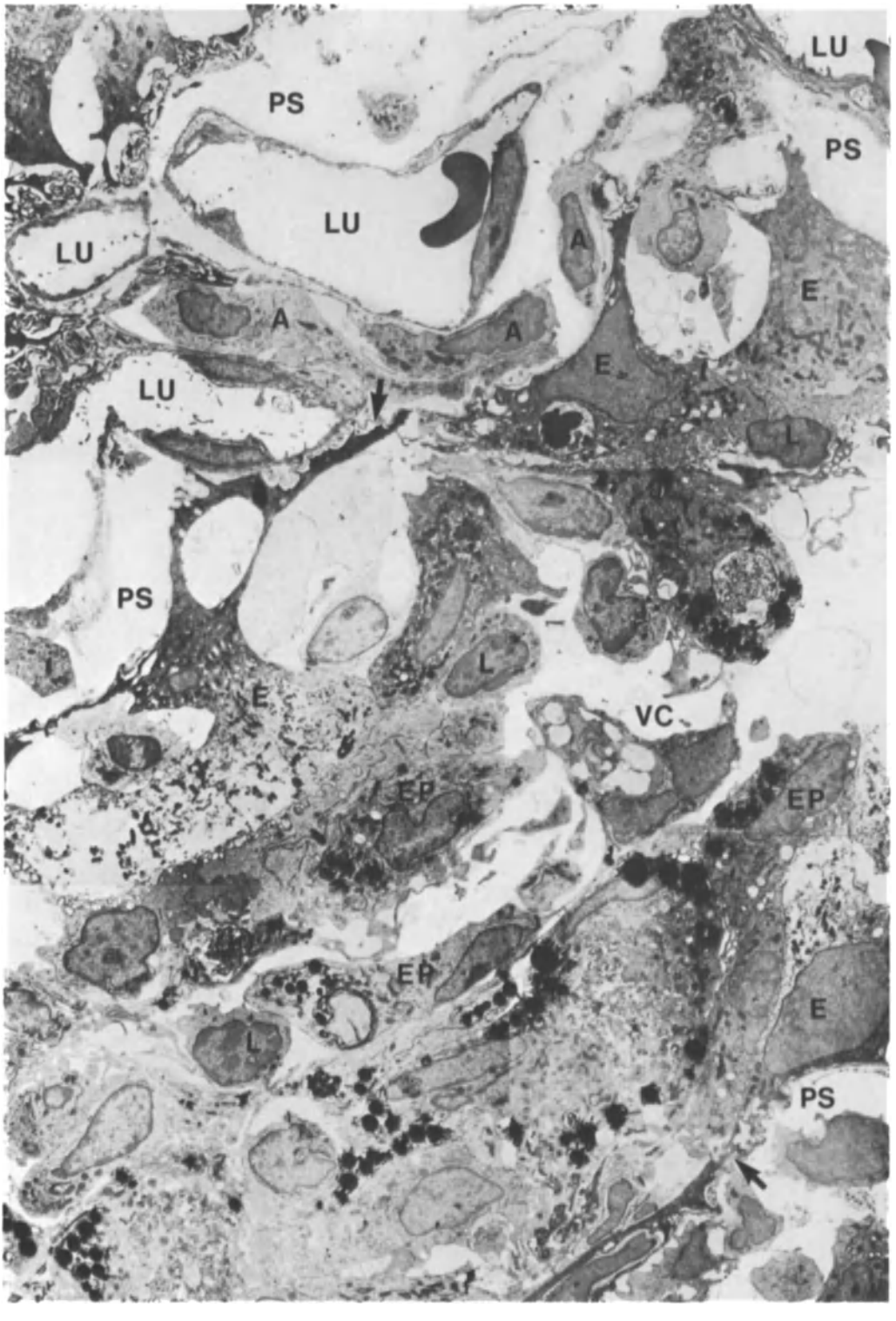
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filtrates originate in the stroma of the villi (Fig. 3a). Furthermore, lymphocytes accumulate together with detached ependymal cells and a few macrophages on the epithelia of the plexus, forming a more or less dense cell coating (Fig. 4). With the help of the electron microscope we see, in addition to rather inconspicuous plexus epithelia, epithelial cells whose microvilli and apical cytoplasms are edematously swollen (Fig. 4 and 5c). This swelling may involve the whole cell, but more often the basal parts of the cytoplasm show an increased electron density. The proliferated endoplasmic reticulum is then largely covered by ribosomes and the free ribosomes in the cytoplasm have increased in number. Even with severe edematous swelling, the epithelial cells of the plexus remain attached at their apical membrane junctions (Fig. 5c). Sometimes the basement membrane of the epithelial cells is detached from the basal epithelial cell membrane (Fig. 5d). The inner perivascular basement membrane may likewise have separated from the endothelium (Fig. 5d). Typical lymphocytic cells are located in the perivascular space, in intercellular gaps between the epithelial cells of the plexus (Fig. 5a) and, together with detached ependymal cells, may be found on the brush border in the ventricular cavity (Fig. 5b and 5c). The detached ependymal cells contain many lipid droplets and phagosomes which may also be observed in adventitial cells and occasionally in the epithelial cells of the plexus.

Inflammatory alterations are also found in other circumventricular organs, i.e., the subfornical organ (Fig. 3b), the median eminence, the area postrema (Fig. 3c) and the organum vasculosum laminae terminalis (Fig. 3d). In the subfornical organ, the lymphocytic infiltrates originate in the marginal venules, and this is also the case in the area postrema. In the organum vasculosum, too, dense lymphocytic infiltrates are found. Here the electron microscope reveals that the perivascular spaces contain lymphocytic and monocytic cells in addition to stationary reticular connective tissue elements. The outer basement membrane of the perivascular space is in some places separated from the membrana limitans gliae perivascularis. Despite extremely enlarged intercellular spaces (Fig. 6)*, the junctional complexes of the ependymal cells, which are here specifically differentiated, are preserved. As in the case of the epithelial cells of the plexus, the endoplasmic reticulum is proliferated. Both the glial and the adventitial tissue elements in the organum vasculosum contain numerous lipid droplets and phagosomes.

In cases where the alterations are more pronounced, the process may spread from the affected circumventricular organs to the adjacent structures, for instance, from the tela chorioidea to the ventricular wall or from the basis of the lamina terminalis to the dorsal chiasma. Electron microscopic examination reveals enlargement of the intercellular spaces.

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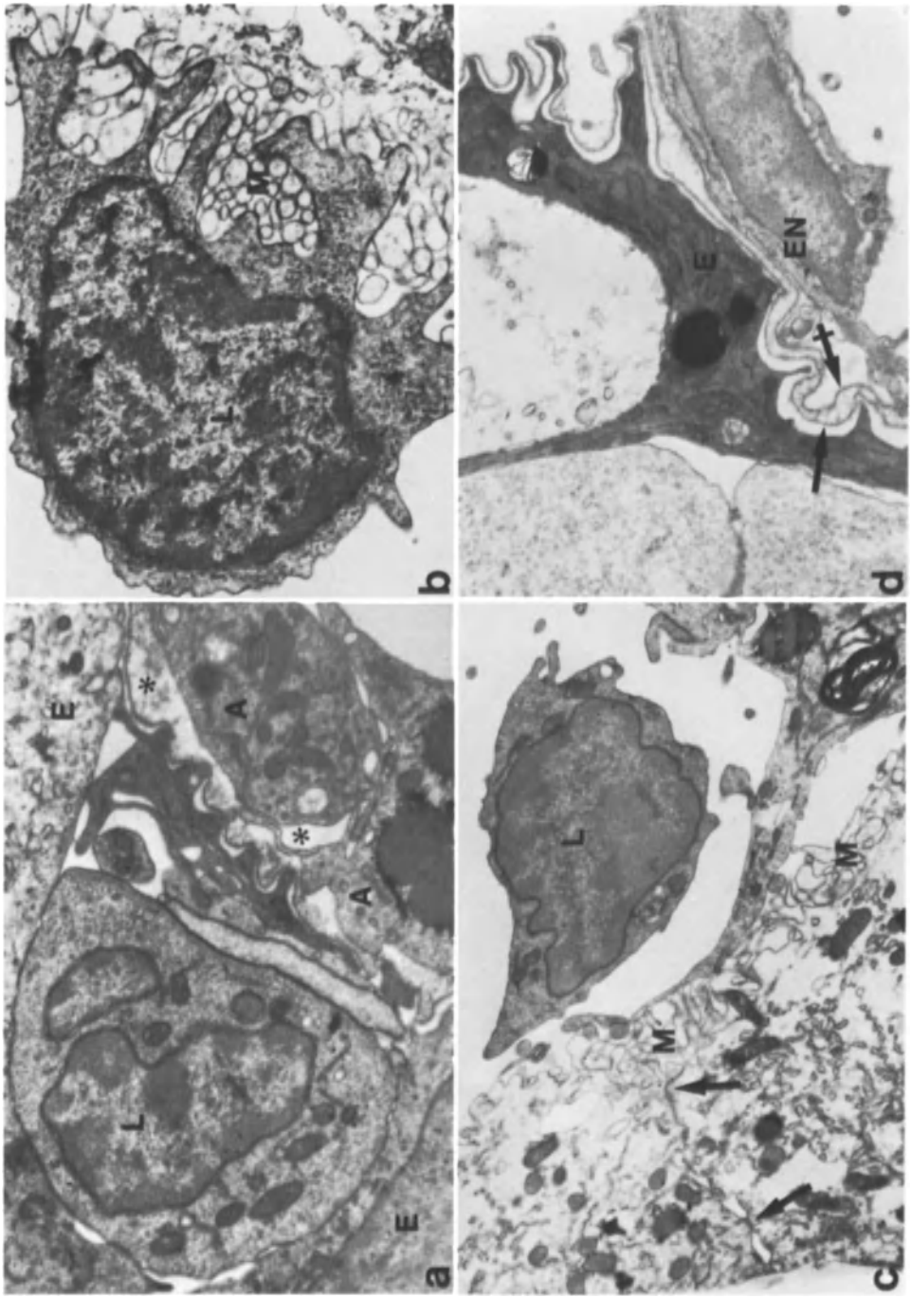
*Fig. 6 see p. 226.

◄ Fig. 4. Photomontage of electron micrographs of portion of 2 plexus villi. Lumina of capillaries of plexus (LU) surrounded by perivascular spaces (PS) with adventitial cells (A). Arrows: outer basement membrane of the perivascular spaces. On the epithelial cells (E) in the ventricular cavity (VC) a pathological cell coating is visible. L = lymphocytes; EP = detached ependymal cells. Magnification x2,500.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In cases with advanced lesions pathological alterations extend from the recessus olfactorii to the central canal of the spinal cord. Otherwise, the posterior horns of the lateral ventricles, the site of the foramina interventricularia, the 4th ventricle, and especially the recessus infundibularis of the 3rd ventricle are mainly affected. The ependymal cells disconnect, round off, and separate from subjacent tissues (Fig. 7). In the subependyma diffuse and perivascular lymphocytic

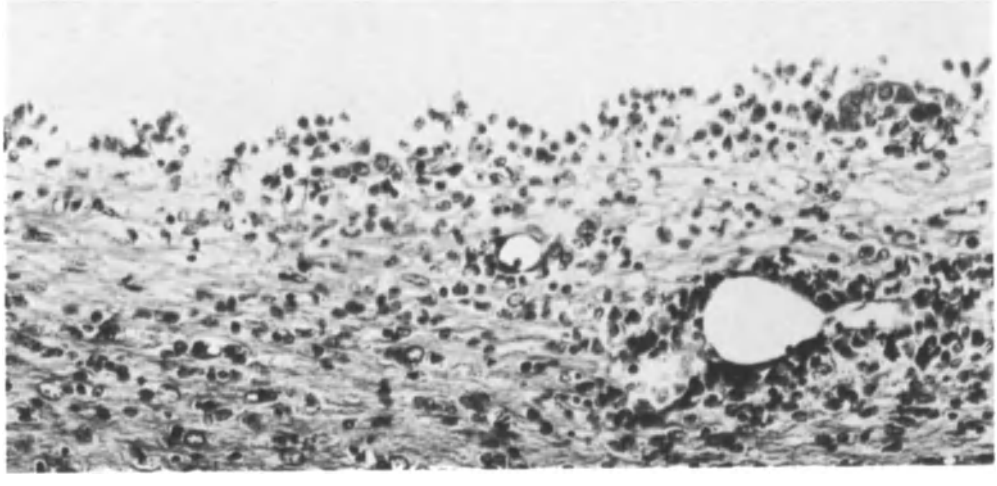

Fig. 7. Ventricular wall of posterior horn of a lateral ventricle, showing disconnection and destruction of the ependymal cell layer. In the subependyma diffuse as well as perivascular lymphocytic infiltrates and proliferation of glial cells can be seen. Hematoxylin-eosin. Magnification x280.

infiltrates occur as well as proliferation of glial cells (Fig. 7). The subependymal fiber layers separate so that subependymal cells may pass into the cerebrospinal fluid. With the electron microscope (Fig. 8) enlargement of the intercellular spaces is observed, which is as a rule confined to the subependyma. Subependymal myelinated axons may show different stages of degeneration; the axolemma increasingly shrinks away from the myelin sheath.

◄ Fig. 5a. A lymphocyte (L) is located between 2 plexus epithelial cells (E) which have migrated from the perivascular space (x). A = adventitial cells. Magnification x10,100. 5b. Lymphocyte (L) attached to microvilli (M) of a plexus epithelial cell. The microvilli are edematously swollen. Magnification x14,000. 5c. Microvilli (M) and apical parts of 2 epithelial cells which are edematously swollen. Arrow: epithelial cell junctional complexes. L = lymphocyte in the ventricular cavity. Magnification x6,800. 5d. Detachment of the outer perivascular basement membrane (arrow) from the basal membrane of an epithelial cell (E) and the inner basement membrane (crossed arrow) from an endothelial cell (EN). Magnification x7,900.

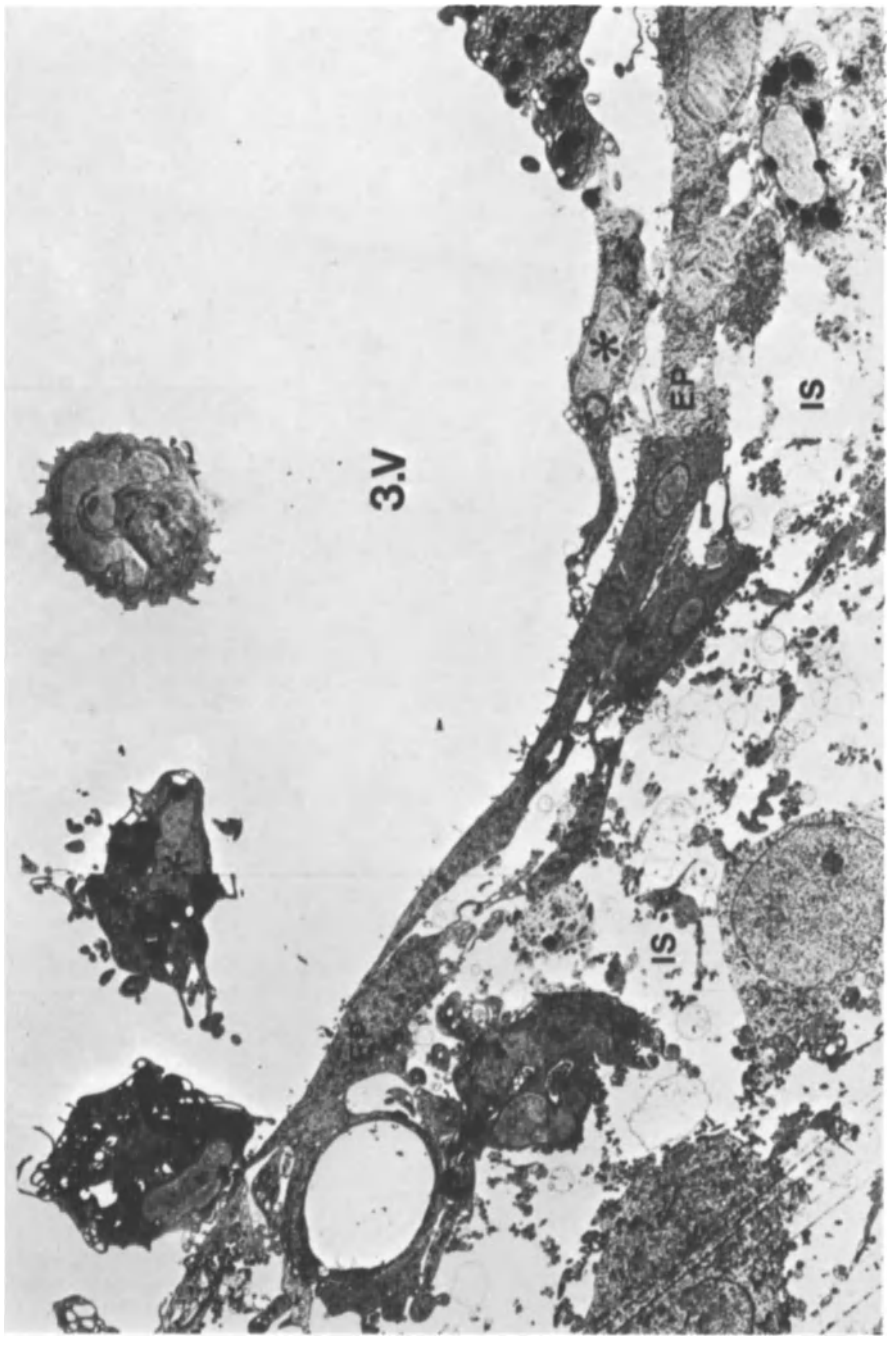

Fig. 6. Extremely dilated intercellular spaces (IS) of the organum vasculosum. The special ependymal cells (EP) have retained their connections. 3.V = optical recess of 3rd ventricle with exfoliated cells (x) which contain numerous lipid droplets. Photomontage of electron micrographs. Magnification x2,300.

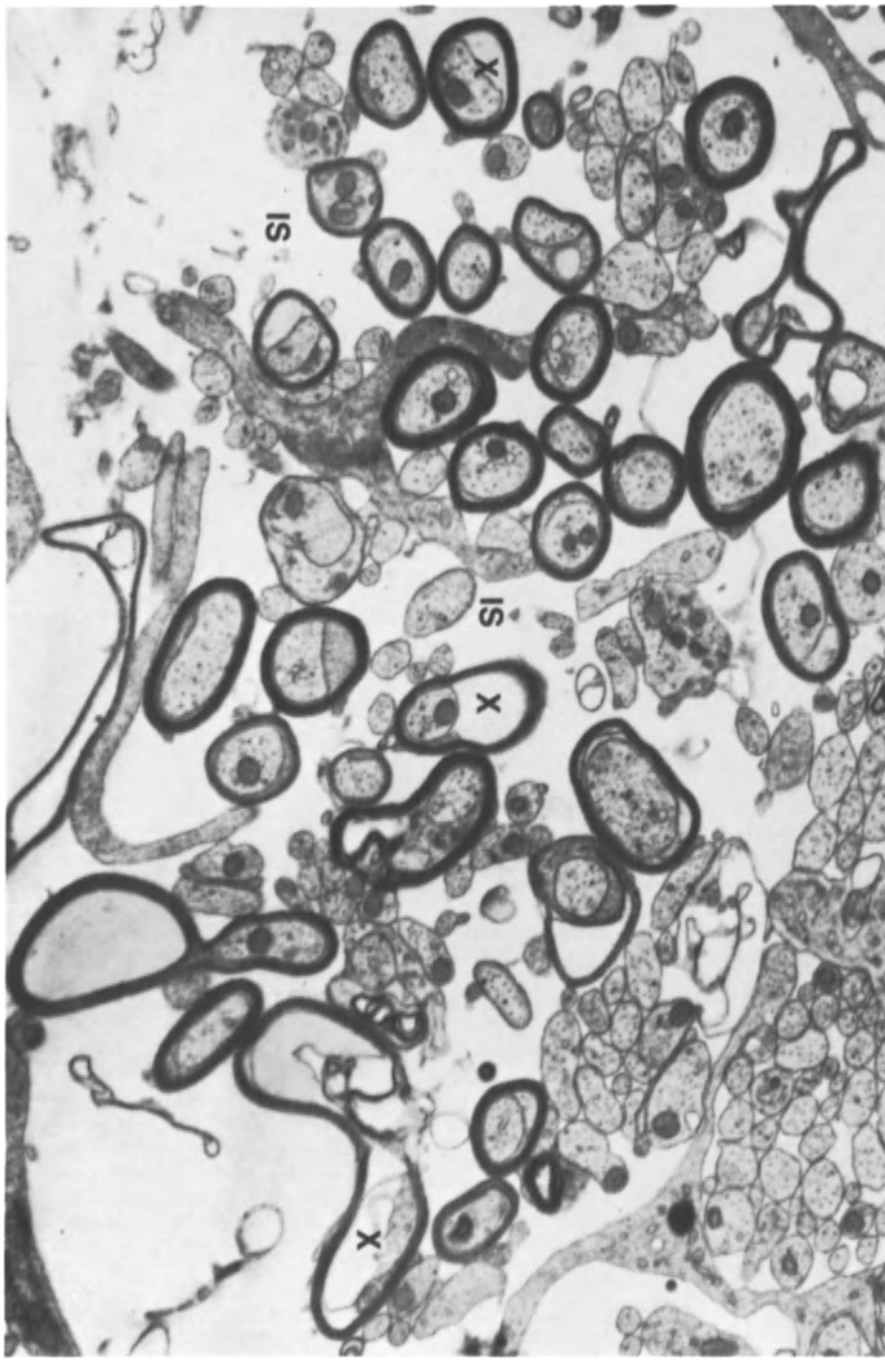

Fig. 8. Ventricular wall of posterior horn of a lateral ventricle, showing different stages of degeneration of subependymal myelinated axons (x). Interstitial spaces (IS) are dilated. Magnification x17,900.

In animals which exhibit generalized seizures or which have died, the inflammatory processes involve also brain structures which are close to the ventricular system. In particular the vicinity of the recessus infundibularis with the hypothalamus (nucleus periventricularis and nucleus arcuatus) is affected together with the nucleus triangularis septi as well as those portions of the nuclei vestibularis, cochlearis and dentatus which are located close to the ventricular wall. Also the corpus callosum and the commissura fornicis are involved. Besides marked perivascular and diffuse lymphocytic infiltrates, there is a moderate glial reaction.

In animals which have survived a severe disease, 4 weeks after infection the ventricular wall shows in some places a fresh granular ependymitis, characterized by an unusually severe glial cell proliferation of the subependyma. The ventricular system of these animals appears to be dilated and the plexus epithelia have bizarre shapes containing numerous vacuoles.

DISCUSSION

Knowledge concerning the ability of the LCM virus to multiply in the rat is limited. Our data leave no doubt that the WE strain of LCM virus multiplies readily in the brain of the adult Sprague-Dawley rat. Volkert and Hannover Larsen (24) employed the Traub strain of LCM virus, and their observations indicate that very young rats can be infected, while animals older than 2 weeks are resistant. The same conclusion may be drawn from the study of Monjan et al. (15,16,17) who worked with the E-350 strain. In rats infected intracerebrally when 1 or 7 days old, the virus multiplied to high concentrations, reaching titers of $10^{8.5}$ mouse LD50/g of brain tissue. In contrast, multiplication took place to a limited extent in older animals and after maturity was reached only transient virus growth for 1 week was observed.

Thus, LCM virus strains seem to differ in their ability to multiply in rat tissues, and this may explain why previous investigators arrived at different conclusions concerning pathological lesions following inoculation of LCM virus.

The pathomorphological alterations in young adult Sprague-Dawley rats infected experimentally with the WE strain of LCM virus are marked by accumulations of inflammatory lymphoid cells distributed in a characteristic pattern, an altered ultrastructure of plexus cells and ependymal cells as well as of myelinated axons in the subependymal regions and enlargement of the intercellular spaces of the subependymal structures. Damage to the neuronal cells themselves is conspicuously absent. The alterations are reminiscent of the ones seen in the central nervous system of the mouse suffering from acute experimentally induced LCM disease (2,13,22), although there are differences. For instance, in the mouse inflammatory infiltrates are restricted to choroid plexus and meninges, while in the rat the walls of the ventricles and deeper structures also participate in the pathological process. Especially striking is the frequent inflammatory involvement of the hypothalamic nuclei.

It is likely that the intraventricular pressure is increased in the LCM virus-infected rat. This is indicated by the enlarged interstitial space between glial cells and between myelinated axons in the subependyma. Similar signs of distention were described by Ogata et al. (18) and by Weller and his colleagues (25) who induced acute hydrocephalus in young cats and dogs by injecting kaolin or silicone into the cisterna magna.

The cells of ependyma and plexus epithelia, which differ morphologically and functionally from other tissue elements of the central nervous system, appear to be particularly susceptible to a variety of other viruses. Johnson et al. (7,8,9) described the development of an acute inflammation of the ependyma with partial destruction of its ventricular lining, subependymal lymphocytic infiltrations and internal hydrocephalus in young hamsters inoculated intracerebrally with mumps, influenza A, and parainfluenza II viruses. A similar combination of pathologic lesions was seen by Margolis and Kilham (14) in ferrets, rats, and mice consequent to the intracerebral inoculation of reovirus I. These alterations closely resemble the ones described here, although we are not yet certain whether internal hydrocephalus is a regular feature in the LCM disease of the rat.

Predilection of the circumventricular organs for pathological reactions in LCM of the rat suggests that structural peculiarities, i.e., perivascular spaces with reticular connective tissue and unique properties of the blood-brain barrier, may play a role. This assumption is supported by the fact that the subcommissural organ, which in the rat does not share these special properties, remains unchanged, as seen by light microscopy. Considering the important role of the circumventricular organs for the production and resorption of the cerebrospinal fluid, the regulation of the osmotic balance between blood and cerebrospinal fluid as well as their participation in neuro-secretory functions (20,21,22), damage to these organs may be of major relevance for the development of the cerebral symptomatology in LCM disease of these rodents.

ACKNOWLEDGMENTS

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Epidemiologic Aspects of Lymphocytic Choriomeningitis in Man

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SUMMARY

Lymphocytic choriomeningitis is an anthropozoonosis with the house mouse as main host, and normally this rodent is the only source of human infections. Therefore, human LCM diseases usually occur in areas where mice carry the virus. The close association between LCM virus-infected mice and human cases of LCM could be established serologically. In the north and northwest of Germany, i.e., in areas where LCM virus carrier mice are frequently found, 9.1% of the rural population had neutralizing antibody. In contrast, only 1.2% of the people residing in southern Germany, which is essentially free of LCM virus-carrying mice, had antibody. On the basis of these figures, it can be estimated that in the Federal Republic of Germany approximately 1,000 persons are newly infected each year.

The epidemiological features of LCM vary when animals other than M. musculus are involved. Thus, LCM transmitted from Syrian hamsters has occurred during recent years in districts where infected mice are not found. Altogether 47 human cases could be traced to these pets. We have seen several family outbreaks though no epidemics. Clinically inapparent infections were diagnosed only twice.

INTRODUCTION

Since the LCM virus was first isolated from man by Rivers and Scott (14), its significance for human pathology has been under discussion. The most relevant discoveries in the past were the peculiar behavior of this agent in the mouse as described by Traub (17) and the close association which was found by Armstrong and Sweet (4) to exist between virus-excreting carrier mice and human LCM. Since then it has become increasingly clear that the house mouse is an important reservoir for the virus being the main source for human infections. As a rule, the infectious chain ends with man.

It was not so easy to obtain a clear picture of the disease. The early assumption that aseptic meningitis as described by Wallgren was caused solely by the LCM virus was soon disproved. Indeed, LCM virus infections in man are quite rare, and it is revealing that few statistics exist which contain proved cases. The most extensive survey is the one conducted in the U.S.A. by Rasmussen (13), Adair et al. (3), and Meyer et al. (12) who studied during 18 years 1,568 virus

diseases of the central nervous system which occurred in military personnel and their relatives. Only 7 cases per year were found to have been caused by the LCM virus. A comparable low incidence was reported by the World Health Organization for recent years. Only vague assumptions can be made concerning the number of undiagnosed cases.

The significance of M. musculus as the source of human infections is underlined by studies performed in Germany. These results also allow conclusions as to the frequency of LCM in man.

RESULTS AND DISCUSSION

Mus musculus as a source of LCM virus. Very early we realized that human LCM virus infections are rare. Thus, specific tests conducted with diagnostic materials from several large hospitals revealed an incidence of seldom more than 1 case per year (15). In a selected group of people antibody was found in less than 1%. Although these low figures contrasted markedly with results obtained previously by Wooley et al. (18), our studies were not extended because of the great technical efforts which a thorough re-evaluation would have entailed. Rather, it appeared preferable to study M. musculus: if this animal, as previous observations indicated, was the main source for human infections, then knowledge on the distribution of virus-carrying mice should give a clue as to the distribution of the virus among populations.

Out of 1,795 house mice trapped in Germany, 65 carried LCM virus (1). In 44 of 376 trapping areas infected mice were found. Interestingly, the areas where infected animals were trapped are quite unevenly distributed. Whereas in South Germany only a few places were found in which infected mice lived, many positive areas were detected in Northwest and North Germany. In accordance with other authors, few mice other than M. musculus were found to harbor the virus, even in areas which were inhabited by virus-carrying house mice (10).

In a later serological survey we established the importance of the zoonosis for man (8). The proportion of people with LCM virus-specific antibodies was significantly higher in rural areas where infected mice were known to exist as compared with zoonosis-free districts. Thus, it is not surprising that those human LCM virus infections which had come to our attention between 1947 and 1968 stemmed exclusively from areas inhabited by infected house mice.

From these results it may be concluded that the infected house mouse is the principal source for LCM virus infecting man. Even in zoonosis areas the proportion of people with serological proof of a recent infection is 9.1%, and one may ask why this figure is so low. Presumably, man is the last link of the infectious chain, and spread of the virus from mouse to man is rare. In one infested village, for instance, neutralizing antibody was found in only 6% of the inhabitants. The 5 positive persons had resided there an average of 23 years (7). Thus, human LCM occurs exclusively in areas of zoonosis, and even there usually less than 1/10 of the population contracts the infection. Our results confirm and extend the work of Armstrong and Sweet (4) who concluded from their findings that "gray mice constitute a reservoir of choriomeningitis infection from which human cases may be contracted".

From the frequency with which antibodies are found in the rural population of the German Federal Republic, the overall frequency of LCM virus infections may be calculated. All persons investigated lived in rural areas, and approximately 6 million people live under comparable conditions. If only these are exposed to infected mice and assuming a minimal overall infection rate of 1.2%, as in the areas free of zoonosis, one can calculate that in West Germany at least 72,000 persons must have had an infection. Thus, with an average life expectancy of 70 years, each year ca 1,000 new LCM virus infections occur. If this estimation is correct, most infections are not diagnosed; alternatively, a high proportion of cases remain inapparent.

Mesocricetus auratus as a source of infection. In March and April, 1970, we saw 2 cases of LCM in one section of the city of Cologne. Both patients denied having had contact with gray mice, but one of them had bought a Syrian hamster 3 weeks previously and the other had worked for 2 weeks in the animal shop in which this hamster had been bought.

In collaboration with many colleagues we have discovered a total of 47 LCM cases, all of which were presumably transmitted from Syrian hamsters (2). According to Hannover Larsen and Volkert (9), neonatally infected hamsters do not become virus carriers. Smadel and Wall (16) detected virus not longer than 8 weeks after infection of the adult animal. Both these findings are borne out by our observations. All the human infections we have seen were transmitted from young hamsters bought recently. Since contacts between people and hamsters usually are very close, many persons are exposed to the risk of infection. We have seen 6 families where more than one member fell ill at about the same time; in one household no fewer than 4 people were infected. Since Syrian hamsters are kept predominantly in urban districts, it is not surprising that these infections occurred outside the known areas of house mouse zoonoses.

The widespread occurrence of this disease and the sudden increase of the number of cases within a short period of time may be explained by infestations of hamster breeding colonies into which the virus presumably was introduced by gray mice.

In laboratory personnel who, unknowingly, worked with LCM virus-infected hamsters, Baum et al. (6) saw 10 LCM cases, all with "influenza-like" illnesses, which is unusual. In contrast, the symptomatology in our patients did not deviate from the one known from previous experience. Only 2 infections remained inapparent. If the history given by these patients was correct, then they represent the first LCM virus infections on record which did not lead to clinically recognizable illnesses.

In accordance with the reports of Lewis et al. (11), Baum et al. (6), and D. Armstrong et al. (5), our findings demonstrate that the epidemiological pattern of LCM virus infections change if new hosts are involved. In the case of spread of virus from the hamster, epidemic dimensions are not reached, but several persons in close contact may be infected at the same time. Since this animal is kept as a pet in many households, infections occur independent of LCM virus-infected house mice.

Serological investigations of families revealed that most human infections with the LCM virus become clinically apparent. This leads to the question why are most infections with this virus not diagnosed?

The answer is manifold. The disease is rare and few physicians are familiar with its symptomatology which furthermore is quite uncharacteristic. The areas in which infected mice exist are largely unknown. The virological diagnosis is time-consuming and technically difficult, and because of the rarity of the disease few laboratories are willing to make the necessary arrangements.

In conclusion, it may be accepted as a general rule that M. musculus is the principal host for the virus and that man acquires the disease from this animal. Though LCM is an uncommon disease, being clinically apparent in most cases, in areas where zoonoses exist transmission to man does occur more frequently. Only exceptionally may other animals, e.g., Syrian hamsters, become hosts and may then transmit the virus to man. In such instances the distribution within the human population depends on the pattern of infection in the animal host and the type of its contact with humans.

Inasmuch as our knowledge of the hamster zoonosis is still incomplete and our diagnostic means limited, an evaluation of the health risk which infected hamsters may pose is difficult at present.

LITERATURE

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Other Arenaviruses

Hemorrhagic Fever Group

Chairman: K. M. Johnson

Biology of Tacaribe-Complex Viruses

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SUMMARY

Tacaribe viruses have basic properties in common with LCM virus. Eight distinct serotypes, all found in the Western Hemisphere, have been collected as an antigenic complex because of extensive sharing of complement-fixing antigens, although there is a demonstrable immunological relationship, particularly as detected by immunofluorescent antibody, between members of the complex and the Old World arenaviruses LCM and Lassa.

Seven of the Tacaribe viruses are strongly rodent-associated, and accumulating evidence suggests that clinically silent, chronic infection of one or two rodent species with both horizontal and vertical intraspecific virus transmission represents the fundamental mechanism for natural maintenance of these agents. Experimental study of Machupo virus in its natural host, the field mouse Calomys callosus, disclosed many features in common with the behavior of LCM virus in Mus musculus. Salient differences also were documented. These included: 1) absence of acute disease in infected adult Calomys, 2) rapid development and long-term persistence of microcytic hypochromic anemia in animals infected shortly after birth, 3) major reduction in reproductive potential following neonatal infection, 4) definite but minimal evidence of formation of circulating and renal-bound virus-antibody complexes, and 5) two distinct response patterns among animals infected after the 8th day of life. About half of the Calomys had all the markers of newborn infection; the remainder developed neutralizing antibody and cleared virus from the blood.

Both Machupo and Junin viruses cause acute hemorrhagic fever in man, a disease characterized by fever, leukopenia, gastrointestinal hemorrhage, shock, and neurological signs. Mortality varies from 10 to 50%. Clinical and experimental evidence suggesting that virus infection in non-natural hosts is immunosuppressive is reviewed, and progress toward development of vaccines suitable for use in man is reported.

INTRODUCTION

Between 1956 and 1958, workers at the Trinidad Regional Virus Laboratory recovered 11 strains of a mouse-pathogenic virus from tissues of fructivorous bats belonging to the genus Artibeus. Although rabies virus was suspected, this diagnosis was soon ruled out. Nor could

these isolates be serologically linked with any of the then recognized arboviruses. The new agent was named Tacaribe virus (11) and was consigned to the freezers of the Rockefeller Foundation laboratories to await its future. It did not wait long. The etiologic agent of a newly described human disease called Argentine hemorrhagic fever was isolated in 1958 and designated as Junin virus (38,44). Junin and Tacaribe viruses were found to share CF antigens (32). When Machupo virus, causative agent of Bolivian hemorrhagic fever, was uncovered in 1963, it proved to be related to both Junin and Tacaribe viruses (17). We are not sure about the proper status of 2 relatives, but it has long been axiomatic that 3 is a crowd - or a complex. Tacaribe virus, being the figurative first-born, has the honor of naming this complex which has now grown to 8 members.

We will not review in detail how the Tacaribe complex led eventually to the concept of arenavirus which stimulated this symposium. The definitive steps have been traced by preceding speakers. What may not be appreciated is why. In 1963 it was thought that Tacaribe and Junin viruses were arboviruses. Mites were the hypothesized biological vectors of the Junin virus from reservoir rodent to man (39). Our study of the Bolivian disease and Machupo virus originally was oriented along these lines. But by 1964 we had changed our minds. We could incriminate no arthropod vector in virus transmission. The virus was found to induce chronic infection in the rodent Calomys callosus, and some of its fundamental properties suggested to us that it might be related to LCM virus. We formulated our doubts regarding the role of arthropods and our speculations regarding the identity of Machupo virus at a symposium held at the annual meeting of the American Society of Tropical Medicine and Hygiene late in 1964 (20). Acceptance of these heresies was at best mixed and very tentative.

During the next 4 years we tried to obtain virological proof. On two occasions we exchanged reagents with Dr. Wallace Rowe, looking for a CF link between Machupo and LCM viruses. There was none. With Dr. Gernot Bergold in Caracas, we repeatedly tried to concentrate virus and find negatively stained virions with his electron microscope. We failed, mainly because the techniques employed apparently broke the virus at least as rapidly as centrifugal force concentrated it - a worthwhile clue since the record indicates a similar problem with LCM virus. Finally, the puzzle was solved with the very first bits of virus-infected lymphoid tissue from rodents that we sent Dr. Murphy (35). But Dr. Bergold did find the first particles. On one or two attempts he got a few pictures of Tacaribe virus showing spheroids of about 85 nm with the spikes (2), just as subsequently revealed by Murphy et al. (35).

VIRUS PROPERTIES AND CLASSIFICATION

As arenaviruses the Tacaribe-complex viruses contain ribonucleic acid, are inactivated by lipid solvents, and share a common morphology marked by the incorporation of electron-dense granules which at least resemble host cell ribosomes. Beyond these properties, members of the complex are joined by the sharing of readily detectable CF antigens and are separated by nearly complete absence of antigenic overlap with respect to antigens which determine virus neutralization (56). At present 8 viruses, all from the Western Hemisphere, fulfill these criteria (5,11, 17,38,42,53,56,58). Their names and geographic origins are shown in Figure 1. A candidate 9th serotype, Portillo virus (31), has not been shown to be a distinct agent.

Fig. 1. Partial map of Western Hemisphere showing geographic localities in which viruses of the Tacaribe complex have been recognized.

Progress in understanding the behavior of a virus at any level depends in the first place upon the relative availability of practical systems for recognition, replication, and assay of the agent and of the specific host-mediated responses to it. In terms of many such systems, some of the Tacaribe-complex viruses appear to offer possibilities superior to those available for study of LCM virus, although data presented during the first 2 days of this symposium show how far this imbalance has been redressed. All members of the complex are pathogenic for suckling hamsters and produce large amounts of infectious virus and CF antigen in the brains of these animals. Adult hamsters and mice, in contrast, are not killed by any of the viruses and may be employed for preparation of complex- and type-specific antisera suitable for use in CF, neutralization, and immunofluorescent tests.

All of the Tacaribe viruses also replicate in mammalian cell cultures. Some of them induce cytopathic effects in certain cells and all have been shown to produce plaques under agar, most notably in the Vero (green monkey) continuous cell line (15,45,50,56). Although we found suckling hamsters more sensitive than cell cultures for detection of Machupo virus in human field materials (19), the plaque assay has proven to be a valuable standard technique for a variety of purposes with

this and other viruses of the complex. The quantity of virus produced in Vero and other cell cultures, however, generally has been less (about 10^5 to 10^6 infectious units/ml) than desired by the investigator wishing to pursue detailed work on the biochemistry and molecular biology of the complex. The solution of this problem and some of the consequences in the case of Pichinde virus will be presented by Dr. Rawls.

Many properties of Tacaribe viruses are, not surprisingly, similar to those of LCM virus. The agents may be lyophilized without difficulty and can be stored for long periods at -60°C or colder if protein-containing stabilizers are added. Thermal inactivation kinetics depend on specific conditions. For example, we found that all Machupo virus contained in a 10% suspension of hamster brain was killed in 30 min at 56°C, whereas Calomys callosus serum containing 10^5 PFU/ml still had a small amount of infectivity after incubation under these conditions. Brain suspensions incubated at 24°C contained readily detectable virus 48 h later, while virus present in rodent urine disappeared within 12 h at this temperature (27,54). Although other arenaviruses probably share this attribute, investigation would be worthwhile since subgroups have been elucidated among picornaviruses and reoviruses based on acid resistance and lability.

The buoyant density of Parana virus in cesium chloride gradients was between 1.18 and 1.20 g/ml (56). A value of 1.18 was found for Pichinde virus in sucrose (33). We wish to add to the record previously unpublished observations on other Tacaribe viruses, summarized in Table 1. Virus source was hamster brain, and gradients were centrifuged for 16 h in a SW39 rotor. Many of these data were obtained by Dr. Manoel Bruno-Lobo of Rio de Janeiro, working in our laboratory. He also found that the density of Junin virus was altered to 1.20 in cesium chloride. It is now clear that arenaviruses have a buoyant density of about 1.18 g/ml and that they produce a non-infectious CF-reactive antigen of 1.09 to 1.11 g/ml.

Table 1. Buoyant density of some Tacaribe viruses

Virus	Density (g/ml) of peak activity	
	Infectivity	CF antigen
Machupo	1.17[a]	1.10
Junin	1.17	1.09
Tacaribe	1.17	1.11
Amapari	1.18	1.09

[a] Density determined in 10 to 50% (w/v) sucrose gradients.

HUMAN DISEASE POTENTIAL

Only two of the Tacaribe viruses are clearly infectious for man. Junin and Machupo viruses cause a severe febrile illness marked by hemorrhagic diathesis. The diseases are called Argentine and Bolivian hemorrhagic

fever (AHF) (BHF), respectively, and they are so similar that joint description is indicated. The first symptoms are insidious progressive fever and myalgia which begin 7 to 14 days after infection. Headache, hyperesthesia of the skin, and conjunctival hyperemia are common. Fever is high and unremittent and is accompanied by a generalized leukopenia (30,34,46,51). In many patients no further progression occurs and convalescence is uneventful except for transient loss of hair and the appearance of Beau's lines in the nails (non-specific consequences of the sustained high fever). Hemorrhagic phenomena begin about the 5th day of illness and frequently are heralded by the appearance of fine scattered petechiae in the palate and on the upper chest and axillae. Bleeding then is noted from the gingivae, the stomach and intestines, occasionally the nose and vagina. Blood loss is not great, and renal and hepatic function are not impaired; the ominous findings are a rising hematocrit and the appearance of an intention tremor of the tongue. Hemoconcentration leads initially to "warm" shock, with a narrowing of arterial pressure without change in pulse rate or the skin changes of classical shock. Failure to recognize this condition and correct it by judicious use of intravenous fluids and blood volume expanders is tragic, for when patients slide into full-blown clinical shock they nearly always die. In this stage, attempts to correct blood volume lead to refractory pulmonary edema and rapid death; vasopressor drugs prolong life for not more than 48 h.

The tremor of the tongue may progress to frank intention tremors of the extremities and to such dysfunction of the muscles of the throat that patients cannot speak coherently or swallow. When generalized convulsions occur the prognosis is grave. Depending on the proximity and quality of medical attention available, AHF and BHF cause death in 10 to 50% of infections. In one series of about 700 cases treated in San Joaquin, Bolivia, the figure was 18%. Maintenance of fluid balance, preferably without use of intravenous solutions, was the single most important factor in prevention of the severe form of the disease. There was a definite direct correlation between day of illness when patients reached the hospital and subsequent mortality rate.

Patients surviving the disease recover completely and develop CF, immunofluorescent and neutralizing antibodies, the last type persisting for years (52,55). Virus has not been recovered during convalescence, and the progressive decline in CF antibody also suggests that chronic infection does not occur in man.

Virus can be isolated from the blood and throat washings during acute illness. Junin virus is more regularly obtained from these sources than Machupo virus (4). Only about 1 in 5 samples yielded the latter agent in patients studied through 12 days of illness (19). Recovery of virus from urine is infrequent. At autopsy, spleen and lymph nodes are most commonly positive for Machupo virus. Despite the conspicuous neurological symptoms and signs, and the fact that circulating antibodies have not appeared when patients die, virus is not found in brain or cerebrospinal fluid.

Histologically there are few virus-specific lesions. There is little or no destruction of parenchymal cells of any vital organ. Small focal hemorrhages are seen in many tissues, there are scattered inclusion bodies reminiscent of Councilman bodies in liver cells (the pigment observed was periodic acid-Schiff, but not iron, positive), and there are the expected changes associated with terminal shock, as well as frequent evidence of secondary bacterial bronchopneumonia (6,7,12,13).

We still know far too little about disease pathogenesis. Infection is always primary in the sense that no immunological evidence exists for prior infection with any other Tacaribe-complex virus. Furthermore, clinically inapparent infection is very rare. A single attack confers long-lasting immunity to the homologous virus, since no confirmed natural reinfections have been found, and immune persons have worked with Machupo virus for 9 years in our laboratory without incident.

It has been postulated that disseminated intravascular coagulation plays a role in the pathogenesis of the disease and data compatible with this phenomenon have been obtained in a few patients with AHF (1, 49). What is striking, however, is the fact that no markers have been found during the first 4 or 5 days of illness which can be used to predict the eventual severity of disease.

No laboratory animal has been found that reproduces the clinical features of the Bolivian disease when inoculated with Machupo virus. Guinea-pigs, however, appear to provide a reasonable model in the case of the XJ strain of Junin virus. Virus multiplies in the lymphoid tissues of these animals, there is viremia and leukopenia, and fever appears at about 7 days. Most guinea-pigs die after another week during which they lose weight. There are widespread subcutaneous and focal intestinal hemorrhages (3). Junin virus also has been shown by Argentine workers to suppress both primary and secondary humoral immune responses to sheep erythrocytes in guinea-pigs (40), and to inhibit "Jones Mote" hypersensitivity and the dermal component of the Arthus reaction (37, 37a). The humoral antibody effect was not due to suppression or destruction of initiator macrophages since in vitro phagocytosis and in vivo clearance functions were normal in infected animals. It was thus proposed that virus action was at the level of antibody-producing lymphocytes (36). Mortality in guinea-pigs was reduced by 50 to 60% when specific antiserum was administered either 24 h before, or 5 days after Junin virus inoculation (60).

Plasma from convalescent patients has been used as an adjunct in therapy of both AHF and BHF, and physicians attending patients are convinced that it is of value if given during the first 5 days of disease. No controlled trials have been done. Nevertheless, we believe that passively administered anti-viral antibodies may be clinically useful if the following speculative and admittedly simplified view of pathogenesis is correct: The virus gains entry by the respiratory or alimentary routes and is sequestered in lymphoid tissues. After several cycles of multiplication, virus is disseminated throughout the reticuloendothelial system where it 1) arrests the normal temporal evolution of humoral (and possibly cellular) immune response, and 2) directly causes, or activates host mechanisms which produce, widespread vascular damage leading to functional hypovolemic shock. There is no direct viral damage to vital parenchymal tissues. Virus is thus largely exposed in the circulatory system where antibody can effectively neutralize it. Our evidence for immunosuppression in man is admittedly fragmentary. But it is unusual that a systemic virus infection requires 5 to 6 weeks to produce detectable circulating antibodies as do both AHF and BHF. Further, these diseases, unlike LCM in man, are rarely marked by a biphasic pattern of fever and clinical symptoms, one of the suggestive hallmarks of immune-mediated pathology. Finally, we have tested the ability of all 8 Tacaribe-complex viruses to multiply in serially cultured human lymphoblasts. Only low-passage isolates of Machupo and Junin viruses have this capacity and both viruses grew to exceptional titers in such cells.

ECOLOGY

In ecological terms, Tacaribe virus is atypical of the agents in the antigenic complex. It has been recovered only from bats, and only during a two-year period in the mid-1950's. Since then it apparently has disappeared from the island of Trinidad. All the other members of the Tacaribe complex are rodent viruses. Furthermore, all the rodent hosts are members of the family Cricetidae, the largest and most rapidly evolving group of rodents in the New World. Table 2 lists the hosts of the respective agents. One or two species are usually incriminated. It is of interest that the 2 human pathogens have rodent hosts of the same genus and noteworthy that 1 animal, Calomys callosus, is host to 2 viruses. Also, it is almost certainly true that the present list of Tacaribe viruses reflects the activity of virologists rather than natural reality. There must be many more serotypes awaiting discovery.

Table 2. Principal natural vertebrate hosts of Tacaribe viruses

Virus	Vertebrate	Habitat
Tacaribe	Artibeus lituratus	Tropical forest
	Artibeus jamaicensis	Tropical forest
Junin	Calomys laucha	Hedgerows, farm fields
	Calomys musculinus	Hedgerows, farm fields
	Akodon azarae	Hedgerows, early secondary forest
Machupo	Calomys callosus	Grassland-forest edge, farm fields, houses
Amapari	Oryzomys goeldi	Tropical wet forest
	Neacomys guianae	Tropical wet forest
Tamiami	Sigmodon hispidus	Grassland, early secondary forest
Pichinde	Oryzomys albigularis	Tropical cloud forest
Parana	Oryzomys buccinatus	Tropical dry forest
Latino	Calomys callosus	Grassland-forest edge, farm fields, houses

Many of the agents have been shown to produce chronic infection in one or more of their natural hosts (18,43,48,53). In most cases, such infection is characterized by persistent viremia and absence of detectable circulating antibodies, a type of infection similar to that long since described for LCM virus in Mus. But this is by no means always true. Viremia was transient in Sigmodon rodents experimentally infected with Tamiami virus, although long-term presence of virus in kidneys and urine was documented (16). Newborn Calomys rodents inoculated with Latino virus also cleared virus from the blood after many weeks and developed antibodies but had virus in their tissues (57).

Chronic infection of rodents with associated viruria represents the basic mechanism for transmission of Junin and Machupo viruses to man. Arthropods have not been implicated. Argentine hemorrhagic fever is a disease of the humid pampa where maize is the principal crop, adult males engaged in the maize harvest are the principal victims, and hundreds to a few thousands of cases are reported each year between March and June (8). Both Calomys laucha and Calomys musculinus are present in the fields and along the hedgerows. In Cordoba Province, the latter species predominates and is more often infected naturally (48).

Although agriculture is much more primitive in eastern Bolivia, Calomys callosus occupies much the same ecological position as its Argentine relative. In addition, this field mouse has a definite propensity to migrate into human habitations whenever such territory is not occupied by the common Old World rodents Mus and Rattus (29). We found about 50% of Calomys infected during the 1963/64 hemorrhagic fever epidemic in San Joaquin and dramatic proof of the role of these mice in disease transmission was afforded when no new cases occurred after an intensive trapping campaign. That study is depicted in Figure 2.

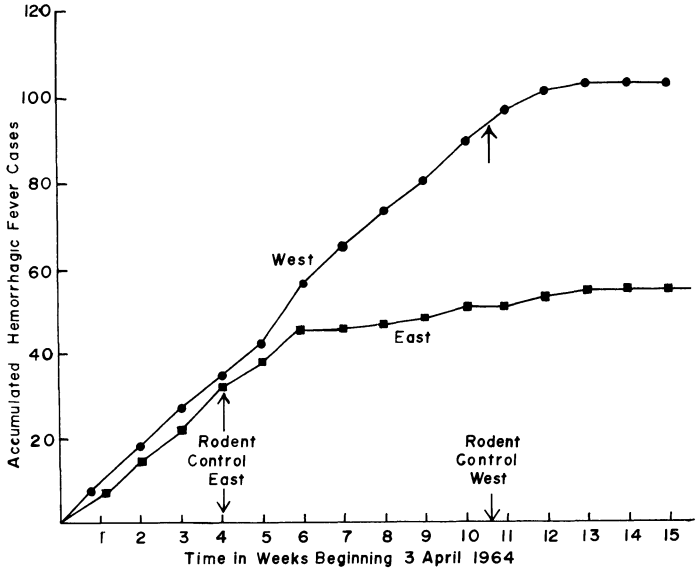

Fig. 2. The effect of intensive rodent trapping on incidence of hemorrhagic fever in man, San Joaquin, Bolivia. Arrows indicate dates when 15 traps were placed in each house in respective sectors. An average of 11 Calomys callosus rodents was captured per house and most of them were taken during the first 5 days of trapping. Effect on disease curve shows that human incubation period was about 14 days.

Direct proof is lacking, but virus transmission to man is probably by contamination of food, water, or air with infectious rodent urine, or by inoculation of skin abrasions with virus as has been postulated in the case of Argentine maize harvesters. Human-to-human transmission also can occur. This has happened twice with Machupo virus wherein

wives in close contact with their sick husbands subsequently contracted hemorrhagic fever (10,23). In 1971, an even more dramatic outbreak occurred in Cochabamba, Bolivia, a city located at an altitude of nearly 9,000 feet, far from the known habitat of Calomys callosus. An index patient from the endemic region was treated there and 5 contact infections ensued among family members and medical personnel (41). Acute liver damage was seen in at least 3 of these cases, and there was only 1 survivor. The virus was identified antigenically as a strain of Machupo virus.

EXPERIMENTAL BIOLOGY OF MACHUPO VIRUS IN CALOMYS CALLOSUS

In 1963, before we knew anything about chronic infection, we brought 6 pairs of Calomys callosus to Panama and initiated a breeding colony. Fortunately, none of these animals were infected, and we have maintained the colony through more than 20 outbred generations as a source of animals to study the behavior of Machupo virus. All work here discussed was done with the Carvallo strain which had been passed only twice in suckling hamster brains.

No amount of virus administered by whatever route to rodents of any age was capable of inducing acute illness or death in Calomys callosus. Animals could be infected parenterally and by nasal and oral administration of virus.

Infection of Newborn Animals

Virus multiplication and distribution. Suckling animals always developed chronic infection marked by continuous viremia and viruria. Virus could be easily recovered from oral swabs (24). Such animals failed to produce neutralizing antibody in detectable amounts but were capable of making antibodies to other viruses; thus we designated them as tolerantly infected (25). Sequential assays disclosed that virus multiplied first in the lymph node proximal to inoculation site, was found in other nodes, thymus, and spleen by the 3rd day and within 3 more days could be found in every organ (59). Viremia became detectable about day 4 and urinary shedding was established between 7 and 10 days. Tissue virus titers were almost uniformly higher than those of blood. We could find no evidence that virus was bound to, or present in, erythrocytes. Immunofluorescent studies using a direct anti-Machupo conjugate prepared from immune mouse ascitic fluid revealed brilliant cytoplasmic staining of lymphoid elements in the cortical areas of nodes and thymus, in the germinal centers of the spleen, and of cells of the myeloid series in the bone marrow. Megakaryoblasts also were stained. Connective tissue, vascular endothelium of many organs, and the endothelial lining of the atria of the heart were positive. Fixed phagocytic cells of liver and lung contained antigen. Parenchymal cells, however, were largely spared. Exceptions were neurons in all areas of the brain and secretory cells of the salivary glands. Occasional small foci of hepatic cell fluorescence were found in some animals during the 2nd to the 4th weeks after infection, but these disappeared as animals aged. After the 1st month of life, virus titers in all organs began to decrease and appeared to stabilize at 6 months at levels about 100-fold less than peak titers which had been as high as 10^7 to 10^8 PFU/g. Intensity of fluorescent staining also declined, except in salivary glands and reproductive organs. Little or no antigen was ever observed in renal glomeruli, but collecting tubules were often strongly positive in older animals.

Pathophysiological effects. Virus-infected Calomys failed to gain weight as rapidly as their normal cohorts. Most of the deficit was incurred during the 1st month of life and by 4 months of age respective mean weights for 20 males were 55 and 62 g, for 20 females 40 and 52 g (59). Otherwise, infected animals appeared grossly healthy. Females developed estrus cycles of 6 to 7 days and male testicular development was histologically normal. Virus was present, however, in the reproductive organs of both sexes and could be recovered in modest amounts from seminal fluid. Fluorescent antigen was widely distributed in ovaries including germinal epithelium and thecal cells of follicles and even the eggs. Antigen was seen in seminiferous tubules only in an occasional animal older than 9 months, but intertubular connective tissue and Leydig cells were brightly stained.

The animals, however, were not normal. They all developed enlarged spleens; statistically significantly within 2 weeks after infection and very grossly by 21 days. Histologically, such spleens showed a marked increase in erythropoietic elements and had persistent "primitive" malpighian corpuscles with very few small lymphocytes. As the animals aged, there was a marked accumulation of iron-containing pigment in phagocytic cells of spleen, liver, and nodes (62).

These changes suggested anemia, probably hemolytic. As shown in Figure 3, this was readily confirmed. There was a sharp fall in hemoglobin of infected rodents at 3 weeks which correlated temporally with

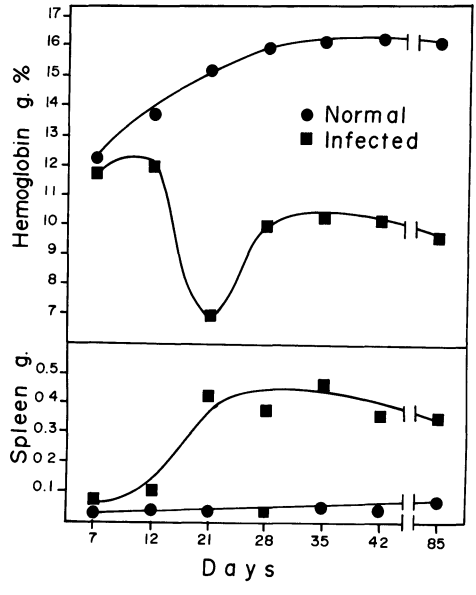

Fig. 3. Evolution of anemia and splenomegaly in Calomys callosus rodents infected with Machupo virus less than 4 days after birth.

rapid enlargement of the spleen. At this time, erythrocyte numbers were approximately half those of controls. Hemoglobin rebounded somewhat and stabilized at about 10 g% and red cells returned nearly to normal numbers. The anemia was thus microcytic and hypochromic. There

also was a mild chronic reticulocytosis. Osmotic fragility studies disclosed that cell lysis began at the same salt concentration as in normal cells but that much lower ionic strength was needed to lyse all cells as compared to the controls (22).

Another adverse consequence of Machupo virus infection in young Calomys was the fact that reproductive potential was subsequently impaired. In 2 separate trials, we found that male fertility was only about half of normal cohorts. Furthermore, the average number of offspring born to normal females mated with infected males was only about 60% of normal. Infected females exposed to normal males produced only 5% of the number of offspring born when both parents were uninfected. Preliminary evidence indicates that developing embryos die and are resorbed by infected females, although we have not yet determined precisely when this happens nor whether this is the sole mechanism for the reduced fertility (26).

Infection of Adult Animals

As previously noted, we did not observe acute disease, such as that classically induced by LCM virus in Mus musculus, when weaned Calomys were inoculated with Machupo virus. All animals had viremia for at least 1 month. By 60 days, however, many had cleared virus from the blood and had detectable neutralizing antibody (21). By 90 days, 2 major patterns had emerged. About half the rodents had antibody and no viremia, although many were chronically infected for many more weeks or months as evidenced by persistent shedding of small amounts of virus in urine and oral secretions; the others, however, exhibited persistent viremia and absence of virus-neutralizing antibody. These animals also developed persistent anemia. We found, further, that this dichotomy of response was sharply established on the 9th day of life; all animals inoculated prior to this age developed chronic viremia, and equal fractions of rodents beyond this age pursued the viremic and non-viremic courses. There were no sex differences. We also had a very few animals with chronic viremia and persistent low-titered neutralizing antibody, and some with neither detectable virus nor antibody in the blood. The latter rodents were infected, however, since we found infectious virus and/or specific viral fluorescent antigen in their tissues. This spectrum of response was somewhat reminiscent of that observed by Hannover Larsen in mice 2 to 9 days old when infected with LCM virus (14).

We suspect that these different responses to infection are at least partially determined by host genetics - of which we presently know next to nothing. In a difficult series of breeding studies (the offspring must be weaned and back crosses done prior to testing the parents for phenotype because of the infertility of chronically viremic females) we were able to alter the 1 to 1 ratio of viremic to non-viremic responders, but the data showed that control was not based at a single gene locus. In addition, we have discovered that virus dose clearly influences response. About 60 to 70% of animals develop antibodies if undiluted hamster brain virus is administered (about 10^6 PFU, plus an unknown but probably large amount of non-infectious viral antigen) whereas more than 75% exhibit persistent viremia when the same inoculation is diluted to 10^2 PFU. We hope to find out why.

Is chronic viremic infection truly tolerant? Probably not. We have demonstrated that virus is precipitated from the blood of such animals by anti-Calomys globulin antibody. Several months after infection of both newborns and adults, circulating fluorescent anti-viral antibody

became detectable at low levels in a proportion of rodents. Many also exhibited specific fluorescence of renal glomeruli when tissue sections were reacted with conjugated rabbit anti-Calomys globulin antiserum. Histological changes in glomeruli, however, were absent or minimal in 7 animals infected for 13 months, and abnormal proteinuria was not found in any of them. We have not yet begun to elucidate the role of cellular immune mechanisms. But the striking difference in response manifested by individual adult rodents offers a unique opportunity for future investigation of these and other problems. In our opinion, it also provides another argument for retention of a term such as "tolerant" or "persistent tolerant" to describe the chronic viremic type of response induced in appropriate hosts by certain arenaviruses. Some of our Calomys just do not tolerate Machupo virus forever in their blood; others do.

PROSPECTS FOR CONTROL OF ARGENTINE AND BOLIVIAN HEMORRHAGIC FEVER

In our view, the restricted natural host range and highly evolved state of parasitism in specific indigenous rodents strongly suggests that Junin and Machupo viruses have lived in South America for millennia. We suspect that recent human alteration of natural ecologic systems has resulted in greatly increased rodent populations and what are known as "new" diseases. We cannot foresee that agricultural practices in Argentina or Bolivia are likely to change in ways that might be expected to reduce such rodent populations. In the case of Machupo virus, however, there is reason to suspect that the auto-sterilizing tolerant type of infection may be exerting a degree of natural biological control. We have laboratory data showing that a female rodent horizontally infected by contact with an infected male will be fertile but viremic when she has her litter 3 weeks later. The offspring are not infected at birth, but all are converted to tolerant "zero growth" disciples of Paul Ehrlich, the virus being provided by maternal milk. Virus maintained within a given breeding group of rodents could spread rapidly through an entire population when animal density increased to the point where frequent contact between members of independent breeding populations occurred. Hemorrhagic fever in humans followed by a sharp decline in rodent numbers are the likely consequences.

Evidence from the field is compatible with such a concept. We found that prevalence of infection among Calomys was 5 times higher in Bolivian localities where human disease had occurred within the previous year than in places where no disease had been reported for 3 or more years. In Argentina, hemorrhagic fever cases and Calomys populations fluctuate in roughly coincident cycles of about 4 years. Possible ways to control hemorrhagic fever in Bolivia by biological manipulation of rodents and distinct Tacaribe viruses will be discussed by Dr. Webb. Simple killing of Calomys does offer some protection, particularly in Bolivia where the mice so freely invade the simple houses of the rural villages, but few people consider that this approach represents a reasonable long-term solution.

The classical answer, of course, is immunization of humans. An experimental, live, attenuated Junin virus vaccine has reached the point of cautious human trial in Argentina. The virus used was the XJ strain that had been passaged an unknown but large number of times in suckling mice, then plaque-purified in a continuous line of rabbit kidney cells. This virus, unlike its parent, did not produce hemorrhage or death in guinea-pigs. Animals infected with high-passage virus developed neutralizing antibody and resisted challenge with the field strain (9). Seven human volunteers were vaccinated and none experienced clinical

illness while all subsequently formed specific neutralizing antibody (47,61). Seventy-one persons then were vaccinated in a preliminary field trial with similar results (40a). Patterns of virus excretion, in vitro markers to distinguish vaccine and wild virus, and tests for reversion to virulence remain to be worked out. Argentine scientists also are attempting to find alternate host systems for vaccine production, since the pilot material was grown in suckling mice which may have harbored latent murine viruses.

We have made a start with Machupo virus. A human strain was reisolated and serially passaged 100 times in brains of suckling specific pathogen-free mice. At the 80th passage, this agent showed numerous differences from its parent. It would not replicate in human lymphoblastic cells, it formed smaller plaques in Vero cells, it failed to kill guinea-pigs, and was partially attenuated for marmosets. These animals experienced no viremia. Many survived and were clinically immune to challenge with low-passage virus. The 80th passage agent also was highly pathogenic for suckling Calomys rodents. Furthermore, survivors did not develop splenomegaly or anemia. We are sure that this strain does not contain another arenavirus, and antibody production tests in adult mice and Calomys failed to disclose evidence for presence of other murine viruses detectable by this technique. We next propose to try to inactivate this strain and assess its immunogenicity in animals. If successful, we can begin clinical testing. An inactivated vaccine is proposed because we are concerned that virus attenuation for laboratory animals may not have that same significance for man, because the problem of reversion to virulence is clearly less, and because we do not feel confident that Machupo virus infection has no long-term adverse consequences.

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Biophysical and Biochemical Studies of Pichinde Virus

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SUMMARY

Pichinde virus, a member of the arenavirus group, was found to contain single-stranded RNA. The RNA consisted of 5 discernible components corresponding to 31S, 28S, 22S, 18S and 4 to 6S. The 28S and 18S RNA's appeared to be of host cell origin and the 4 to 6S RNA was probably from the same source. The 31S and 22S RNA's appeared to be components which coded for viral products. The virus was found to contain 4 structural polypeptides. Two were glycopeptides and 2 were polypeptides. One polypeptide (V_I) and 1 glycopeptide (V_{II}) remained associated with the viral RNA after treating the virus with nonionic detergent, while the other glycopeptide (V_{III}) was removed with such treatment. V_{IV} was a minor peptide and its fate following treatment of the virus with nonionic detergent could not be determined.

INTRODUCTION

Immunological tolerance has been considered the mechanism responsible for the chronic virus infections associated with the arenaviruses. Neutralizing antibody has not been demonstrated in persistently infected animals. However, it has recently been shown that virus antigens and antibodies to the antigens occur in mice chronically infected with LCM virus (19,20). This latter observation does not necessarily negate the hypothesis of tolerance since the animal may be tolerant to antigens of the virion which are associated with neutralization but not tolerant to all virus-induced antigens. Thus, knowledge of the structure of the arenaviruses may be important in resolving the mechanisms of persistence and of the pathogenesis of the diseases caused by the virus.

Pichinde virus produces a persistent infection in its natural host, Oryzomys albigularis (33). The virus replicates well in tissue culture, can be accurately assayed, and does not appear to be pathogenic for man (17,33). Biochemical studies of Pichinde virus were therefore undertaken. The results indicate that the virus contains segmented single-stranded RNA and 4 structural polypeptides (4,23).

MATERIALS AND METHODS

Virus and virus assay. Pichinde virus strain AN 3739 (33) was kindly provided by Dr. Carlos Sanmartin (Cali, Colombia). The virus was originally isolated from the serum of an Oryzomys albigularis. It was sub-

sequently passaged 12 times in newborn hamster brain. In our laboratory the virus was passaged an additional 2 to 4 times in Vero cells or BHK21 cells. Virus stocks were prepared in BHK21 cells and Vero cells were used for virus assay. The virus was assayed by the plaque counting method previously described (17).

Isotopically-labeled Pichinde virus was prepared in BHK21 cells. Monolayers of cells in 16 oz prescription bottles were washed with Eagle's medium and covered with 2 ml of virus stock which was diluted to a concentration of about 1 PFU/cell (17). After 60 min at 37°C, the unadsorbed virus was removed by washing the monolayers once with Eagle's medium. The monolayers were covered with 10 to 20 ml of maintenance medium and incubated at 37°C. Radiolabeled virus was prepared by supplementing the culture medium with the various radiolabeled precursors. Culture fluids were collected 48 or 72 h after infection.

Concentration and purification of virus. The extracellular fluids from the infected cultures were clarified of cell debris by centrifugation at 1,500 rev/min for 10 min in an IEC Model PR-6 centrifuge. The virus was concentrated by polyethylene glycol (PEG) precipitation (16). One hundred ml of the clarified extracellular fluid was mixed with 2.2 g NaCl and 6 g PEG 6,000 (Union Carbide Corporation). After mixing by magnetic stirring at room temperature, it was stored overnight at 4°C. The precipitate was pelleted by centrifugation at 10,000 rev/min for 45 min in a Spinco model L 3-40 centrifuge with rotor 30. The pellet was resuspended in 3 ml of 0.01 M tris buffer, pH 7.4, containing 0.001 M EDTA and 0.1 M NaCl (TNE) and sonicated in a Raytheon Sonic Oscillator Model DF 101 for 30 s at 50 kc. The material was layered over a discontinuous 20 and 50% (w/w) sucrose gradient prepared in TNE and then centrifuged at 25,000 rev/min for 2 h using rotor SW 50. The visible lower band was collected by side puncture, diluted with TNE and placed on a continuous 20 to 50% sucrose gradient which was centrifuged for 2 h at 35,000 rev/min using an SW 50 rotor. Fractions of 20 drops were collected by piercing the bottom of the centrifuge tube. The radioactivity of each fraction was determined in a Beckman LS-250 scintillation counter, the refractive index was measured in an Abbe-3L refractometer, and the virus infectivity was determined by the plaque counting method.

Isolation of RNA from virus and cells. The fractions of the linear sucrose gradient which contained the virus (density = 1.14 to 1.18 g/cm^3) were pooled and the nuclease inhibitors 2-mercaptoethanol and diethyl oxydiformate were added to 0.1% and 0.2% in the final volume, respectively. Sodium lauryl sarcosinate (SLS) was added to 1% in the final volume and the mixture was diluted 4-fold in TNE. After gently mixing for 10 min at 4°C, an equal volume of cold phenol, m-cresol solution (4) was added and mixing was continued for 10 more min. After separation of the phases by centrifugation (12,000 x G for 10 min) the phenol phase was reextracted once with 1/4 volume of TNE. The aqueous phase was removed, pooled with the aqueous phase from the 1st extraction, and deproteinized again. After centrifugation as above, the aqueous phase was carefully collected, made 2% with respect to potassium acetate and placed in ice. The nucleic acid was precipitated for at least 2 h with 2 volumes of ice cold 95% ethanol at -20°C. The precipitate was then washed twice in cold 70% ethanol and centrifuged at 18,800 x G for 20 min. After the final wash the precipitate was dried in the cold and then suspended in 1 ml TNE.

RNA was extracted from confluent monolayers of HeLa cells which were grown in 16 oz bottles and labeled for 24 h with 14C-uridine. The cells were collected, suspended in 0.01 M acetate buffer, pH 5.1, and

the RNA was extracted by the hot phenol procedure described by Scherrer and Darnell (25). RNA was extracted from uninfected BHK21 cells using a method which was modified from that previously described by Biswal et al. (3).

Velocity sedimentation of viral and cell RNA. An 0.2 ml sample of RNA was layered on top of 5.0 ml of a 5 to 20% (w/w) linear sucrose density gradient. A little mineral oil was layered above the sample to insure uniform distribution of the sample on the sucrose. Centrifugation was carried out for 3.25 h at 43,000 rev/min in a Spinco SW 50 rotor at 4°C. After centrifugation, fractions (0.15 ml) were collected by bottom puncture of the tube. To determine the radioactivity, 200 µg of bovine serum albumin was added as carrier to each fraction and the RNA was precipitated in an equal volume of cold 10% trichloroacetic acid (TCA) at 4°C for 15 min. The radioactivity of the precipitate was determined by Millipore filtration. 14C-labeled HeLa cell RNA was used as reference marker and the sedimentation coefficients of viral RNA were calculated by the method of Spirin (29).

Determination of base composition. The base composition of RNA species from the virion or from BHK21 cells was determined by a slightly modified method previously described by Biswal et al. (3). Briefly, the 32P-labeled viral or cellular RNA species were mixed with 500 µg of yeast "carrier" RNA and precipitated with 5% TCA at 4°C for at least 15 min. The precipitate was centrifuged at 12,000 x G for 10 min, washed in cold distilled water, dried, and digested in 0.05 ml of 0.5 M KOH for 16 h at 37°C. The hydrolysates were spotted on Whatman 3 M paper for electrophoresis and the nucleotides were electrophoresed for 3 h at 1,500 volts in 0.05 M ammonium formate, pH 3.5. The ultraviolet light-absorbing spots on the paper were cut out and the radioactivity of each was determined.

Disc polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was used to characterize the proteins and the nucleic acid of Pichinde virus. Detailed descriptions of the methods used may be found in previous publications (2,4,5,7,23,28,32).

Ultraviolet and γ-irradiation of Pichinde virus. Pichinde virus was diluted 10-fold in tris-buffered saline, pH 7.4, supplemented with 1% fetal bovine serum and 1.0 ml aliquots were delivered to 60 mm tissue culture dishes. The virus samples were UV-irradiated at a dose of 3.5 ergs/mm^2/s, and after appropriate exposure times, each sample was diluted 10-fold in Eagle's medium and residual infectivity assayed by the plaque counting method. Sindbis and rubella viruses were irradiated under identical conditions.

To measure the sensitivity to γ-radiation, triplicate samples of lyophilized virus were irradiated for various time intervals. The source of radiation was the uranium-235 reactor located at the Nuclear Science Center, Texas A&M University, College Station, Texas. The reactor was operated at power levels of either 200 kw or 1,000 kw designed to deliver 4 x 10^5 rads/h or 2 x 10^6 rads/h at a distance of 40 cm. After irradiation the ampules were held at 4°C until assayed for residual infectivity. Lyophilized Sindbis virus and rubella virus were irradiated under identical conditions.

RESULTS

Polypeptides of Pichinde virus. Pichinde virus was grown 24 to 72 h in the presence of radiolabeled arginine or leucine. The virus was

purified and the polypeptides were examined by electrophoresis in 15% polyacrylamide gels which were cut in 2 mm slices. Three peaks were discernible. The 2 slower migrating peaks were also labeled with glucosamine (Fig. 1). The radioactivity of virus labeled with both glu-

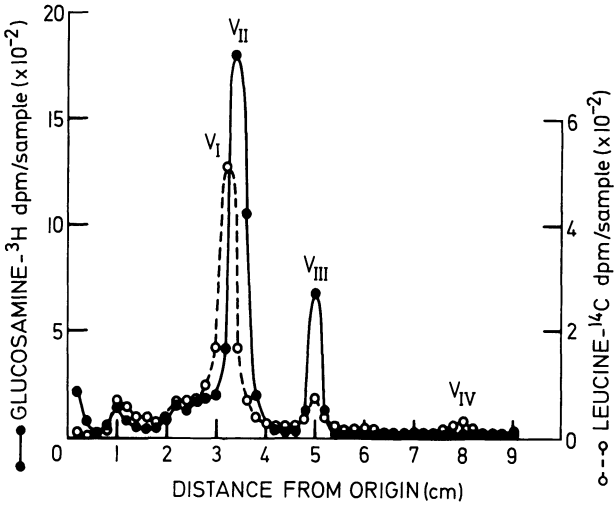

Fig. 1. Polyacrylamide gel electropherogram of polypeptide and glycopeptide from Pichinde virus. Pichinde virus was labeled with 14C-leucine (1 μCi/ml) and 3H-glucosamine (5 μCi/ml) from 24 to 72 h after infection, purified and prepared for gel electrophoresis. The sample was electrophoresed on 15% concentration of polyacrylamide gels and sliced in 2 mm portions.

cosamine and leucine was not coincident in the major peak. When the gels were sliced at 0.5 mm, the slowest migrating peak was found to be composed of 2 peaks (Fig. 2). Thus, Pichinde virus was found to have

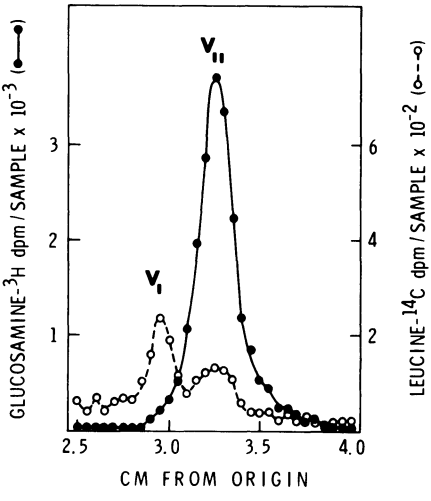

Fig. 2. Polyacrylamide gel electropherogram of the polypeptides of Pichinde virus. The portions of the gel which migrated in the region of 2.5 to 4 cm from origin (Fig. 1) were sliced at 0.5 mm instead of 2 mm.

4 polypeptides; 2 of these were glycopeptides. After treatment of the virus with the nonionic detergent NP40, the 2 components of the slowest migrating peak remained associated with viral nucleic acid (23). The polypeptide V_I and glycopeptide V_{II} appear to be functioning as nucleoproteins, while V_{III} appears to be an envelope glycopeptide. The function of the minor V_{IV} polypeptide is not known.

Nucleic acid of Pichinde virus. The nucleic acid of Pichinde virus was found to have the characteristics of single-stranded RNA. The virus labeled with radiolabeled uridine but not thymidine (unpublished observations). Extracted RNA from purified virus was rendered acid soluble with pancreatic ribonuclease but not deoxyribonuclease and was sensitive to treatment with 0.5 M KOH. The buoyant density of the viral RNA in cesium sulfate was found to be between 1.669 to 1.685 g/cm^3, densities characteristic of single-stranded RNA.

By sedimentation in 5 to 20% sucrose density gradients, the nucleic acids of the virus were separated into 4 components. The major component, corresponding to 28S, was consistently observed as were 22S and 4 to 6S components. A minor 18S component was observed which sometimes appeared as a shoulder on the 22S peak. When the viral RNA was examined by electrophoresis in 2.4% polyacrylamide gels, 31S, 28S, 22S and 18S components were observed. The low molecular weight component (4 to 6S) migrated off of the gel under the conditions of electrophoresis. Thus, the nucleic acid of the virus appears to be composed of 5 components, 31S, 28S, 22S, 18S, and 4 to 6S RNA.

Replication of Pichinde virus in the presence of actinomycin D. Low concentrations of actinomycin D added to the culture medium during replication of Pichinde virus consistently resulted in the production of increased amounts of infectious virus. Representative experiments are shown in Table 1. The incorporation of 0.05 µg/ml of actinomycin D increased infectious virus by 0.5 $\log_{10}$ or more in all experiments conducted. The increased yield of virus appeared to be due to an increased production of infectious particles, while the total particle yield as determined by electron microscopy was not significantly affected. The particle to infectivity ratio ranged from 77 to 185 for viruses grown in the absence of actinomycin D and from 5 to 16 for viruses grown in the presence of actinomycin D.

Table 1. The effect of low concentrations of actinomycin D on the replication of Pichinde virus in BHK21 cells

Actinomycin D (µg/ml)	Experiment 1		Experiment 2	
	Yield[a] (PFU/ml)	% of control	Yield (PFU/ml)	% of control
0.00	2.0×10^7	100	7.3×10^6	100
0.05	1.5×10^8	750	8.3×10^7	1137
0.10	1.9×10^7	95	2.7×10^6	36

[a] Monolayers of BHK21 cells infected with Pichinde virus at a multiplicity of infection 0.6 PFU/cell. Following adsorption, medium containing actinomycin D was added to the cultures. The extracellular virus yield was determined after 48 h of incubation.

The basic patterns of polypeptides obtained by gel electrophoresis were not altered by the addition of 0.05 µg/ml of actinomycin D in the culture medium. However, the relative incorporation of glucosamine when compared to leucine was less in the virus grown in the presence of the drug than in the virus grown in the absence of the drug.

In BHK21 cells, 0.05 µg/ml of actinomycin D was found to inhibit the synthesis of host cell ribosomal 28S and 18S RNA. This is shown in Figure 3 and confirms the observations of others (24). Pichinde virus RNA grown in the presence of 0.05 µg/ml of actinomycin D was found to be

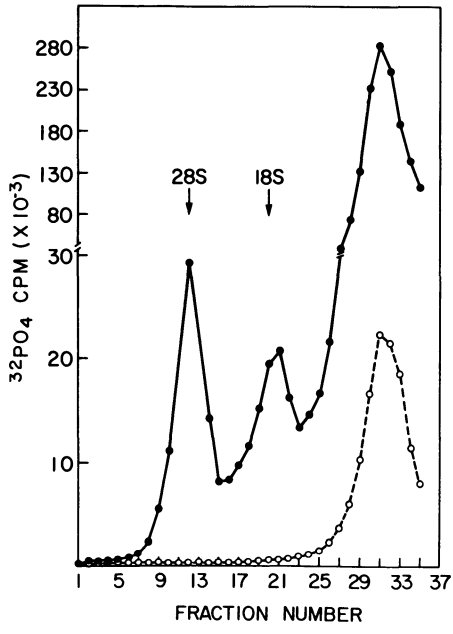

Fig. 3. Sucrose velocity centrifugation of BHK21 cellular RNA isolated from cells treated or not treated with actinomycin D. Confluent monolayers were placed in medium supplemented with 32P-orthophosphate (5 µCi/ml) or in medium supplemented with 32P and actinomycin D (0.05 µg/ml). The cellular RNA was extracted and 0.2 ml samples of RNA from actinomycin D-treated (O--O) or from untreated cells (●——●) were layered on top of 5.0 ml 5 to 20% linear sucrose gradients and centrifuged as described in Materials and Methods. Fractions were collected by bottom puncture and the TCA-precipitable radioactivity of each fraction was measured. 14C-uridine-labeled HeLa cell ribosomal RNA was centrifuged in a separate tube and used as marker.

devoid of newly synthesized 28S and 18S RNA's. Figure 4 demonstrates the sedimentation pattern of the virus RNA in sucrose velocity centrifugation and shows the absence of 28S RNA and the absence of the shoulder of the 22S RNA peak when the virus was grown in the presence of actinomycin D.

Analysis of the RNA by polyacrylamide gel electrophoresis produced similar results (Fig. 5). The 31S RNA was better resolved by electrophoresis and this component was not altered by replicating the virus in

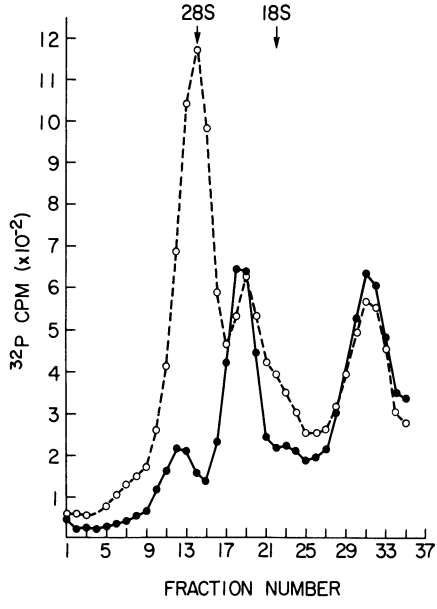

Fig. 4. Velocity sedimentation of 32P-labeled viral RNA synthesized in the presence of actinomycin D. The 32P-labeled RNA was extracted from Pichinde virions grown in the presence (●——●) or absence (O---O) of actinomycin D (see Materials and Methods) and centrifuged as described in Fig. 3. Fractions were collected by bottom puncture of the tube and the TCA-precipitable radioactivity of each fraction was measured.

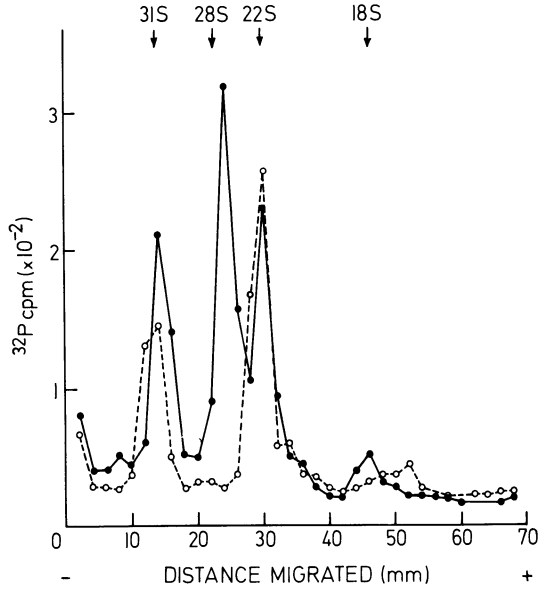

Fig. 5. Polyacrylamide gel electrophoresis of Pichinde virus RNA synthesized in the presence of actinomycin D. 32P-labeled RNA was extracted from virions grown in the presence (O---O) or absence (●——●) of actinomycin D and electrophoresed. 14C-uridine-labeled HeLa cell ribosomal RNA was used as reference marker.

the presence of the actinomycin D. The 28S component was not detected in the virus grown in the presence of the actinomycin D, while the 22S component was not affected by the drug.

Host cell ribosomal RNA in Pichinde virus. The above data suggest that host cell ribosomal RNA was incorporated into the virus. Cellular ribosomal and transfer RNA's have been found to contain methylated nucleotides (10,36), while such nucleotides have not been found in animal viruses. When Pichinde virus was grown in medium containing 3H-methyl methionine, the RNA extracted from the purified virus was found to contain radiolabel only in the 28S, 18S and 4 to 6S components, indicating that the nucleotides of those RNA species had been methylated. Virus grown similarly but in the presence of actinomycin D contained radiolabel only in the 4 to 6S component.

Table 2. Nucleotide composition of Pichinde viral RNA and BHK cell RNA

Mononucleotides	Viral RNA			Cell RNA			Viral RNA (+ actinomycin D)	
	28S	22S	4S	28S	18S	4S	31S	22S
CMP	28.5[a]	25.8	26.9	29.0	27.4	26.6	22.4	21.8
AMP	19.7	23.0	21.3	18.7	21.6	20.3	27.5	25.5
GMP	31.8	27.6	31.0	34.7	30.4	31.4	19.3	24.7
UMP	19.9	23.6	20.9	17.5	20.6	21.7	30.8	27.8
GMP + CMP	60.3	53.4	57.9	63.7	57.8	58.0	41.7	46.5

[a] Expressed as % mononucleotides.

The nucleotide compositions of the different RNA species of virus and BHK21 cell origin are shown in Table 2. The base composition of the 28S RNA from viruses replicated in the absence of actinomycin D was similar but not identical to host cell RNA. Both RNA's had a high guanosine and cytosine content. The base composition of the virion 22S RNA was distinct from either host cell 28S or 18S RNA's. There was a decrease in the guanosine and cytosine content of the viral 31S and 22S RNA's when the virus was grown in the presence of actinomycin D. Thus, the base composition of the virus RNA components are compatible with the 28S, 18S, and 4 to 6S RNA's being of host cell origin.

Finally, it was found that ribosomal RNA synthesized prior to infection was incorporated into the virus progeny. BHK21 cells were cultured in the presence of 3H-uridine and prior to infection the labeled uridine was removed. The cells were then infected with Pichinde virus and, after adsorption, medium containing 0.05 μg/ml of actinomycin D and 14C-uridine was added. The virus grown under these conditions was purified and the extracted RNA was analyzed. The results are shown in Figure 6.

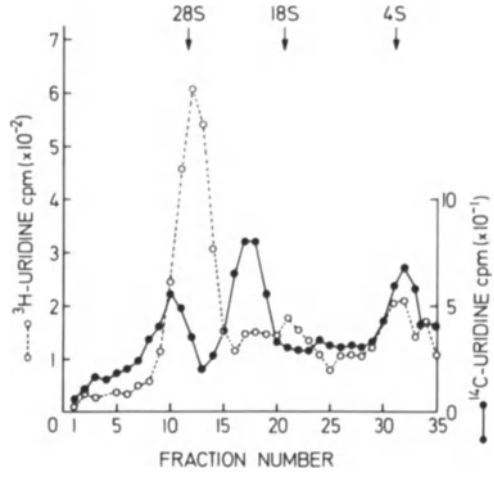

Fig. 6. Sucrose velocity centrifugation of Pichinde virus RNA labeled with 3H- and 14C-uridine. BHK21 cells were prelabeled for 48 h with 3H-uridine (2 µCi/ml) followed by 18 h incubation in medium containing excess unlabeled uridine (25 µg/ml). The cells were then infected and incubated for 48 h at 37°C in culture medium containing 14C-uridine (0.2 µCi/ml) and actinomycin D (0.05 µg/ml). Virus was concentrated and purified from the culture fluid and the viral RNA was extracted as described in Materials and Methods. The RNA was analyzed by centrifugation (see Fig. 3).

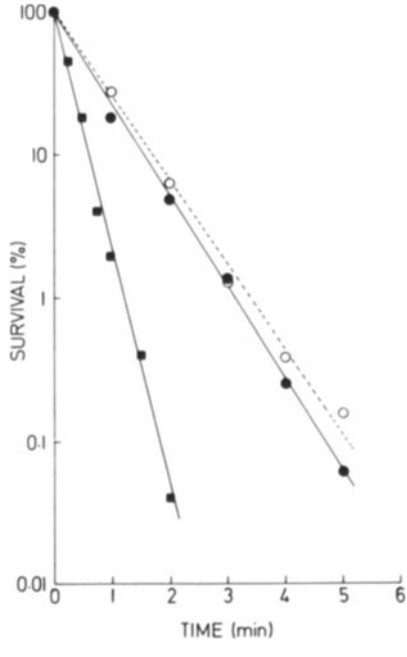

Fig. 7A. Inactivation of Pichinde virus by ultraviolet (UV) irradiation. One ml of a 1:10 dilution of Pichinde virus in tris-buffered saline was irradiated at different doses as described in Materials and Methods. Surviving virus was assayed by the plaque counting method. Sindbis and rubella viruses were irradiated under identical conditions. Pichinde virus (■——■); Sindbis virus (●——●); rubella virus (O---O).

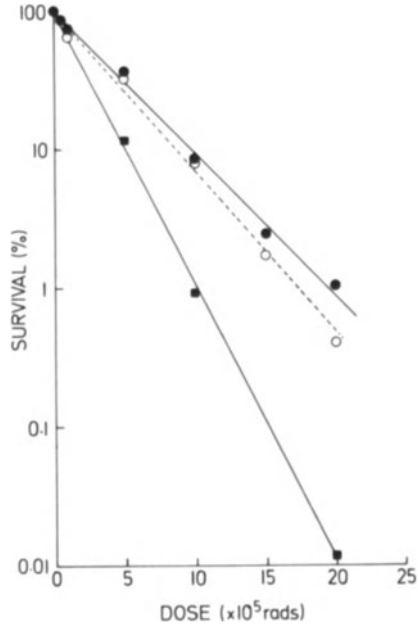

Fig. 7B. Inactivation of Pichinde virus by γ-irradiation. Triplicate samples of lyophilized Pichinde virus were irradiated at different doses as described in the section on Materials and Methods. The samples were held at 4°C until the residual infectivity of each sample was determined. Each point represents the average surviving infectivity in the 3 samples. Lyophilized triplicate samples of Sindbis and rubella viruses were irradiated under identical conditions. Pichinde virus (■——■); Sindbis virus (●——●); rubella virus (O---O).

The 3H-uridine incorporated prior to infection was found primarily in the 28S, 18S and 4 to 6S RNA's, while the 14C-uridine incorporated after infection was found in the 31S, 22S and 4 to 6S RNA's.

Inactivation of Pichinde virus by irradiation. The sensitivity of viruses to ionizing irradiation, with some exceptions, is related to the size of the functioning virus genome (1,9,11,13,15). The sensitivity of Pichinde virus to inactivation by ultraviolet irradiation and to γ-irradiation was compared to the inactivation of Sindbis virus and rubella virus. The results are shown in Figure 7. The kinetics of inactivation by ultraviolet irradiation (Fig. 7A) is that observed from a single hit. In addition, Pichinde virus was found to be more sensitive to ultraviolet light than Sindbis virus and rubella virus. Under the conditions of exposure to γ-irradiation, an initial loss of titer was observed which was not dose related. Following this initial loss of infectivity, the inactivation was linearly related to dose and the sensitivity of Pichinde virus to γ-irradiation was greater than the sensitivity of Sindbis and rubella virus to this form of irradiation (Fig.7B).

DISCUSSION

Morphologically, the arenaviruses are unique. The virus particles contain dense granules instead of a discernible core; the granules are similar in size to ribosomes (6,18). The results of the study of the nucleic acid of Pichinde virus suggest that the granules may indeed be ribosomes of host cell origin. Pedersen reported that the RNA of LCM virus could be separated into 4 components corresponding to 31S, 28S, 22S and 18S (21,22). The same components were found in Pichinde virus and in addition a 4 to 6S RNA was observed. Like Pedersen, we observed a suppression of incorporation of newly synthesized 28S and 18S RNA into the virion when the virus was replicated in the presence of low concentrations of actinomycin D. On the basis of these studies, it would appear that the RNA of the arenaviruses consists of single-stranded RNA which contains components derived from the host cell; the host cell contribution has the characteristic of ribosomal RNA.

Two components of the RNA, 31S and 22S, appear to represent the viral genetic material. These components have a base composition uniquely different from host cell ribosomal RNA; guanosine plus cytosine make up 64 and 58% of 28S and 18S ribosomal RNA from BHK21 cells, respectively, but only 42 and 46% of the viral 31S and 22S RNA, respectively. The 31S and 22S RNA's are not methylated, while the ribosomal RNA's are methylated. The 31S and 22S components correspond to molecular weights of 2.1×10^6 and 1.1×10^6 daltons, respectively. If one of each component were incorporated into an infectious virus particle, the RNA responsible for viral coded products would be about 3.2×10^6 daltons.

Of the polypeptides apparently coded for by the virus, 4 are incorporated into the virion. Two polypeptides (V_I and V_{II}) have molecular weights of about 72,000 daltons; one is a polypeptide and the other is a glycopeptide. These 2 polypeptides remain associated with the nucleic acid of the virus after treatment with a nonionic detergent and one or both of these components may function as a nucleoprotein. A second glycopeptide (V_{III}) with a molecular weight of about 34,000 daltons is associated with the virus envelope. A fast migrating polypeptide (V_{IV}) with a molecular weight of about 12,000 daltons is present; however, a putative function for this small polypeptide could not be assigned.

The estimated genome size of 3.2×10^6 daltons for the viral coded products of Pichinde virus does not appear unreasonable when compared with other small lipid containing RNA viruses. Three structural polypeptides have been found in members of the group A and group B arboviruses (12,26,31,34,37) which have genomes of about 3×10^6 daltons (8,30). A similar number of structural polypeptides have been found in rubella virus in which the genome has a molecular weight of about 3.2×10^6 daltons (27,35). Thus, a similar molecular weight RNA of Pichinde virus may code for 4 polypeptides. It is, therefore, of interest that the inactivation of Pichinde virus by ionizing irradiation is more rapid than the inactivation of Sindbis virus and rubella virus. If the inactivation is mediated through the interaction of ionizing irradiation with nucleotides as proposed (9), the functional nucleic acid of Pichinde virus is about 6×10^6 daltons. Since ribosomal function is sensitive to ionizing irradiation (14), these observations suggest that the host cell-derived RNA of the virion is required in virus replication. It may be further speculated that the ribosomal RNA derived from the host cells is in the form of functioning ribosomes. The RNA coding for viral products may thus exist in the virion as "messenger" RNA, from which proteins are translated early in infection.

There are a number of questions raised by the finding of host cell RNA in the virion. For example, does the ribosomal RNA incorporated into the virion become immunogenic? If so, do antibodies to the host cell RNA play a role in the pathogenesis of the chronic infections associated with the arenaviruses? Hopefully, these and other questions will be answered as investigation of this fascinating group of viruses continues.

ACKNOWLEDGMENTS

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Ultrastructural Studies of Arenaviruses

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SUMMARY

The morphology and morphogenesis of all arenaviruses are remarkably similar: 1) virus particles are round or pleomorphic and vary widely in size (60 to 350 nm diameter), 2) they have a membranous envelope and surface projections, and 3) they have characteristic ribosome-like granules in varying numbers within an unstructured interior. Viral morphogenesis occurs via budding upon plasma membranes. Despite the distinctiveness of these virion characteristics, ultrastructural study of in vivo arenavirus infections proved difficult. In salivary gland cells of Calomys callosus, Machupo virus clearly matured upon plasma membranes and yielded salivary secretions containing large numbers of particles. However, in other tissues, such as lymph nodes, thymus, spleen, and central nervous system, where widespread infection of particular host species was anticipated from immunofluorescence, only modest numbers of virus particles were found. Inclusion bodies, easily identified in infected cells in culture, were impossible to discern in vivo. Some cytopathology was spatially associated with virus particles in lymphoid tissues. Ultrastructural study of in vivo arenavirus infections has proven excessively difficult without an immuno-electron microscopic contribution.

INTRODUCTION

Similarities in virion morphology and morphogenesis were the initial bases for uniting LCM virus and the members of the Tacaribe serogroup into the Arenavirus genus (3,8,9,10). Electron microscopic observation remains the key in defining this genus because the broadest serologic approach, involving indirect immunofluorescence for detection of a "group-specific antigen", is technically difficult (11) and characterization of other virion physical properties is just beginning to emerge (13; W.E. Rawls, this symposium). It is the purpose of the present report to expand morphologic-morphogenetic comparisons of cell culture-propagated arenaviruses and to relate these observations to correlative ultrastructural events in in vivo infection. In the latter case, Machupo virus infection of its natural host, Calomys callosus, was used as the primary model, although other virus-host combinations were studied also. The ultimate purpose of such study is, of course,

to further understanding of virus-host interactions in infections which range from simple subclinical episodes to fatal hemorrhagic fevers.

MATERIALS AND METHODS

Identification of most of the viruses used has been tabulated previously (9). Lassa virus (strain L.P.) was obtained from the Yale Arbovirus Research Unit, New Haven (2). Stock Lassa virus was a 2nd passage suckling mouse brain suspension originating from a human serum specimen collected in Nigeria; it was not titrated for this study. Methods for culture of Vero green monkey kidney cells, infection, and harvest of cells, as well as standard electron microscopic techniques employed on specimens derived from these infected cell cultures, have been described (9).

Newborn Calomys callosus rodents were infected with Machupo virus via intraperitoneal inoculation. For the purposes of this study, specimens of lymph nodes, thymus, spleen, and salivary gland were collected 9, 10, 14, 24, and 30 days after inoculation. Similarly, tissues from a) newborn C. callosus infected with Latino virus, b) newborn Syrian hamsters infected with Junin virus, and c) Swiss ICR mice infected with Tacaribe virus, were prepared for electron microscopy. Techniques for electron microscopic preparation of animal tissues have been described (8).

RESULTS

Cell culture infections. Previous studies from these laboratories indicated remarkable similarities in virion morphology and morphogenesis among 9 arenaviruses grown in parallel in Vero cells (9). Virus particles were round, oval, or pleomorphic in shape and ranged in diameter from 50 to more than 300 nm (mean diameters were 110 to 130 nm). Particles consisted of a dense membranous envelope with closely spaced projections and lucent interior containing varying numbers of dense ribosome-like granules. To further this direct comparative study, Lassa virus-infected Vero cells were examined by thin-section electron microscopy: cells exhibited an increased density at discrete sites in plasma membranes, an apparent covering of these sites with projections, and a concomitant distention of these sites to yield budding virus particles (Fig. 1). The characteristic presence of multiple dense, ribosome-like granules within budding and free virus particles was identical to other arenaviruses and consistent with all observations on Lassa virus reported by Speir et al. (12).

Negative contrast electron microscopy of 7 arenaviruses revealed round or pleomorphic particles with a very wide range of diameters (60 to 350 nm; most particles between 90 to 140 nm). Typical particles had closely-spaced surface projections affixed to a membranous envelope without apparent symmetry. Particle variances of 3 viruses, not previously shown, are illustrated in Figure 2. Continuing attempts to resolve internal constituents by varying negative staining techniques failed; conditions which ruptured viral envelopes did not yield ribosome-like granules. Attempts to offset the hazard of conventional negative contrast microscopy of Machupo and Junin virus specimens by technique modifications also failed; negative contrast microscopy has not been attempted with Lassa virus.

Distinctive intracytoplasmic inclusions increasing in number, size and density throughout the course of Vero cell infection are characteristic

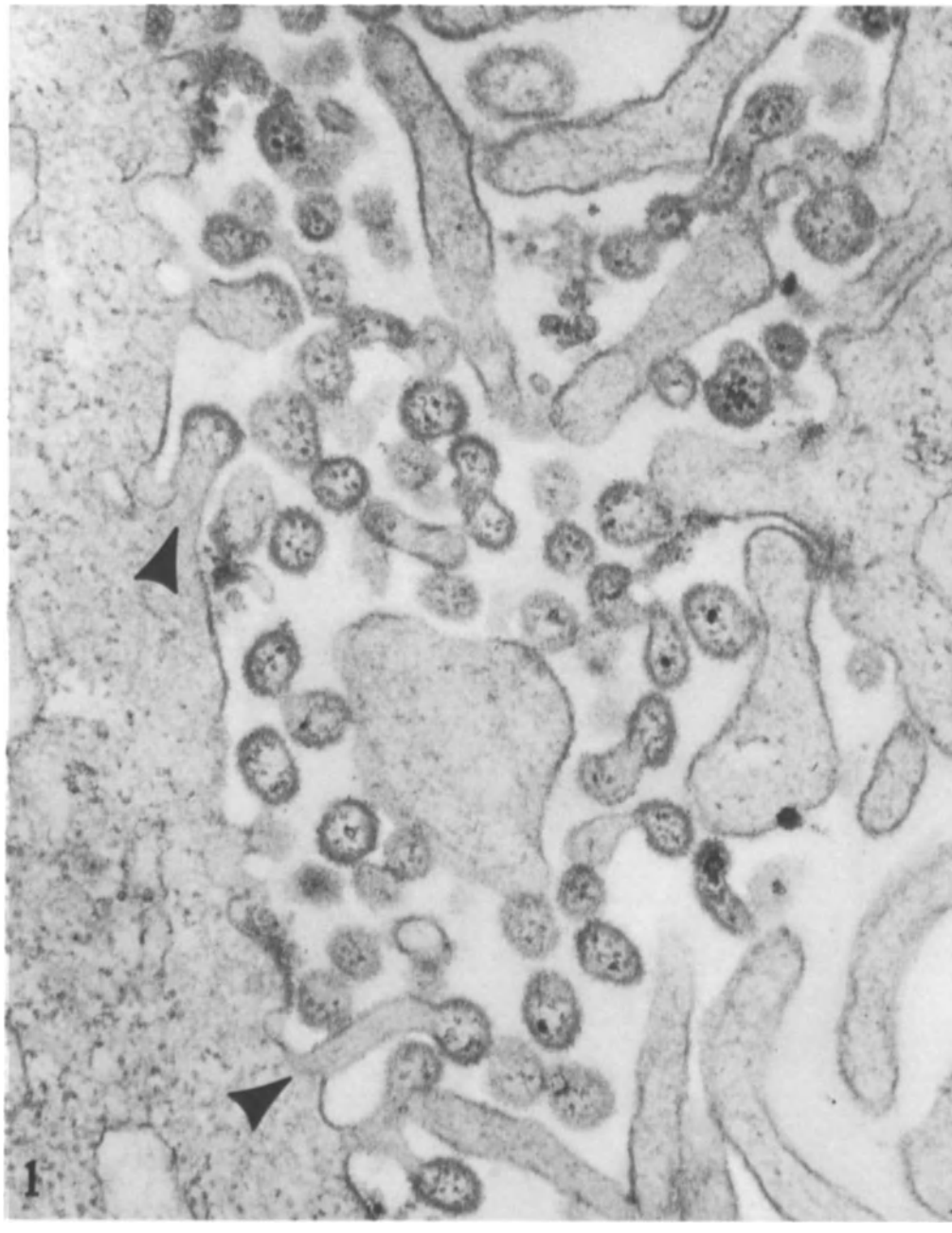
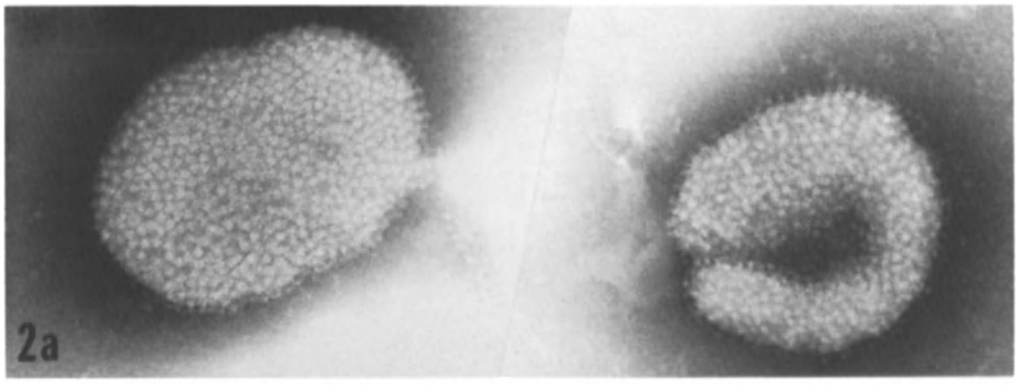


Fig. 1. Lassa virus budding from the plasma membrane of Vero cells and accumulating within intercellular space. Arrowheads point to mature or nascent virus particles in all figures. Magnification x75,000.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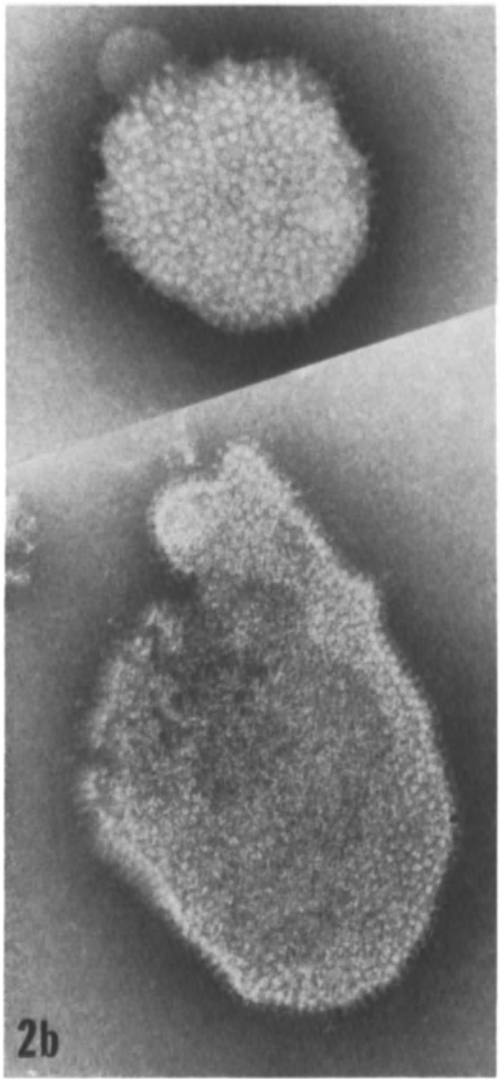
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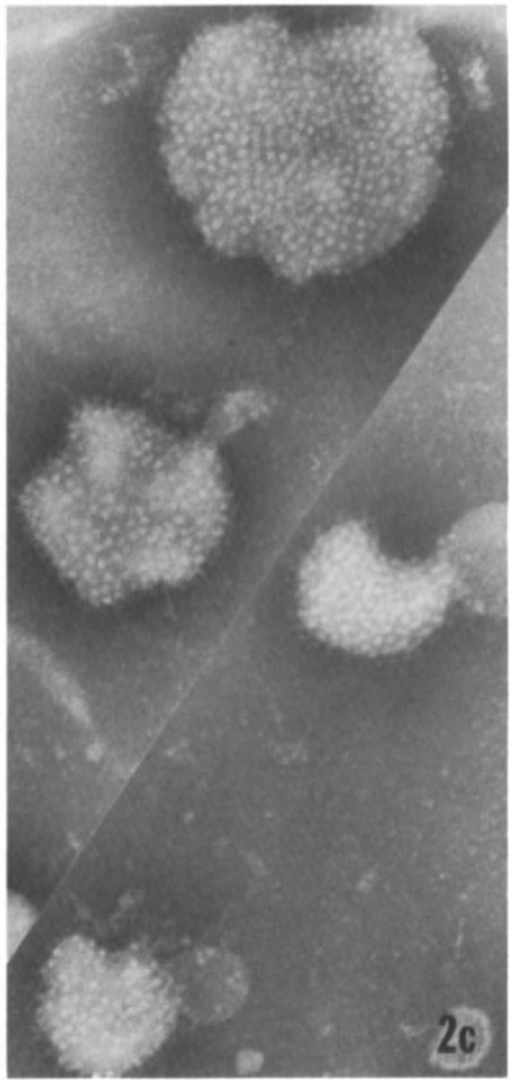
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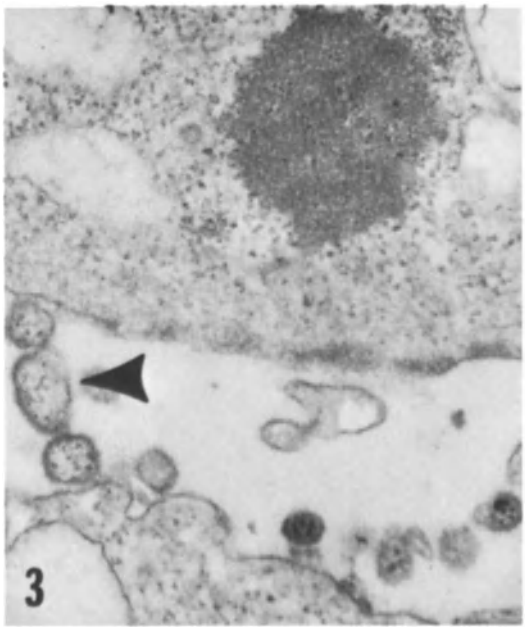
of all arenaviruses (9). The primary constituent of these inclusions are ribosome-like granules like those found within virions. Recently, further attention was directed toward the moderately dense, smooth matrix in which granules are occluded. The ratio of smooth matrix to granules in inclusions varied more widely than originally described; although inclusion density often increased late in infection due to granule compaction, some inclusions consisting primarily of the smooth matrix material occurred at all stages of infection (Fig. 3 versus 4). Attempts to correlate virus particles (infectivity assay or particle



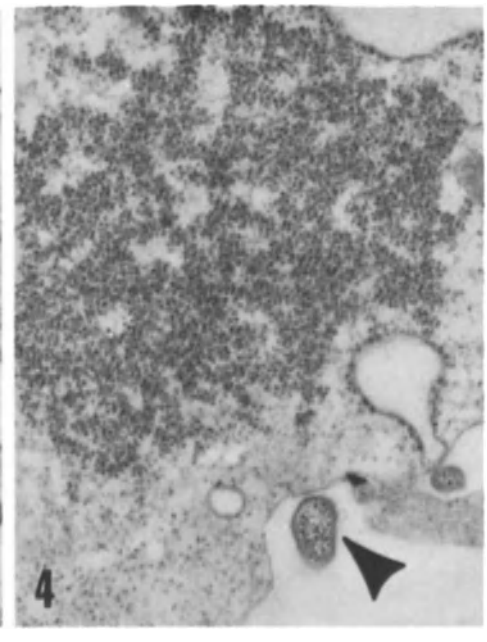


Fig. 3. Lassa virus-infected Vero cell illustrating an inclusion primarily consisting of smooth matrix material. Magnification x54,000.

Fig. 4. Junin virus-infected Vero cell illustrating a rather diffuse inclusion primarily made up of ribosome-like granules. Magnification x31,000.

count in thin-section specimens) with the number and/or size of inclusions failed to indicate any simple predictability of viral yield from low magnification evaluation of inclusions. Whereas previous studies of infected Vero cells showed that inclusions were very common, some recent cultures yielding comparable amounts of infectious virus (about 10^6 PFU/ml) contained few or no detectable inclusions. These two described characteristics of viral inclusions, that is their range in structure and their range in number, were predictive of difficulties in studying inclusions in vivo.

◄ Fig. 2. Composite illustrating variation in size, shape, surface projection spacing, and degree of negative contrast medium penetration. a, Latino virus; b, Tamiami virus; c, Amapari virus. All magnifications x192,000.

Machupo virus in C. callosus tissues. Organs from newborn rodents infected with Machupo virus were originally selected for electron microscopic study on the basis of anticipated parallels with hamsters infected with Junin virus. The latter were studied extensively by Bruno-Lobo et al. (1) using sequential organ titration and immunofluorescence. Subsequently, similar comprehensive studies of C. callosus infected with Machupo virus were undertaken (K.M. Johnson, to be published). Lymphoid-reticuloendothelial tissues were examined a) because rates and ultimate titers of viral synthesis indicated their role as primary targets and b) because they represented sites of mediation of immunopathologic disease and persistent viral carriage. In lymph nodes, thymus, and spleen, typical arenavirus particles were found in specimens from the 9th, 10th, and 14th day, but not later. These particles were widely dispersed in cortical areas of nodes and thymus and randomly in spleen; they usually occurred individually in extracellular spaces. Because of the occurrence of many simple membrane bound particles and villi cut in cross-section, a most conservative approach to virus particle identification was necessary. Particle accumulation was minimal, probably due to the high rate of fluid movement in extracellular spaces in all of these organs (a phenomenon of particular disadvantage in electron microscopy). In those few sites where there were larger numbers of virus particles, they were always associated with the plasma membrane of one cell type (Fig. 5). These were large lymphoblastoid cells characterized by a moderately dense cytoplasm containing large numbers of mono- and poly-ribosomes, moderate numbers of mitochondria and Golgi membranes, but no endoplasmic reticulum. These cells were clearly dis-

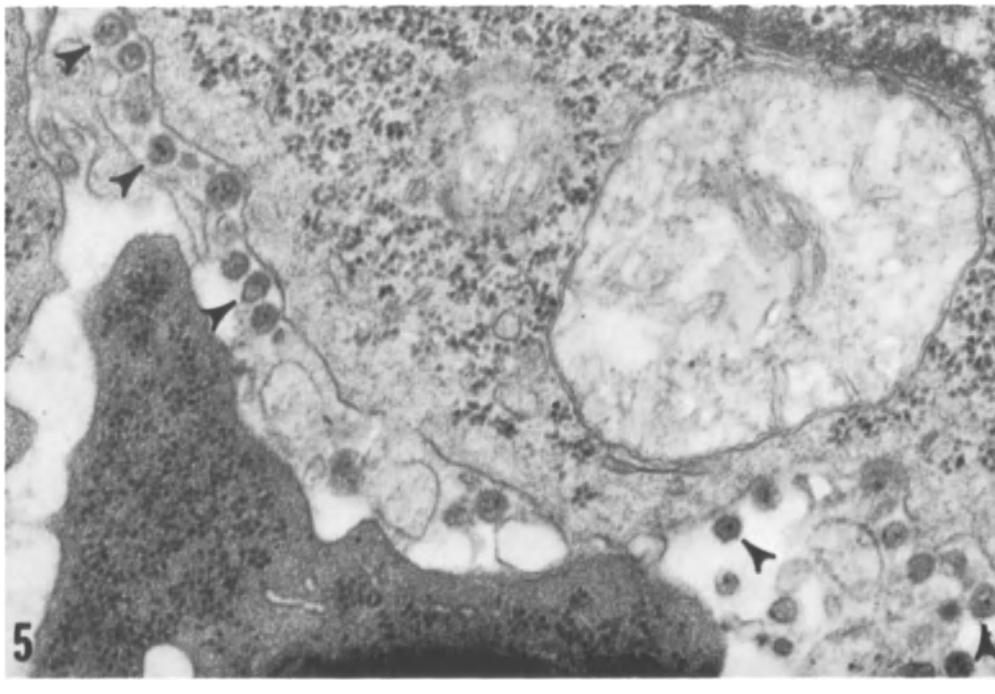


Fig. 5. Machupo virus in a lymph node of a Calomys callosus at 14 days. Particles are associated with a large lymphoblastoid cell which appears normal. Magnification x43,000.

tinguishable from small lymphocytes, macrophages, etc., but closely resembled the cultured human lymphoblastoid cells previously shown to support Machupo virus growth to high titer (8). The normal presence of these large numbers of ribosomes, especially in monodispersed array, made inclusion body identification impossible (Fig. 5). Supportive elements, including connective tissue (fibroblasts), and vasculature (endothelium and perithelium) were never associated with virus particle localization.

Expected high cell turnover rates in lymphoid tissues, especially thymus (evidenced ultrastructurally by asynchronous cytonecrosis), made viral cytopathology difficult to discern; nevertheless, changes which were spatially associated with virus particle accumulation are of interest. Most virus particles were located at margins of otherwise normal cells. In fewer instances particles were near cells undergoing various stages of disarray of cytoplasmic architecture or amid the debris left from complete cell disruption (Fig. 6). There was no macrophage activity associated with this damage. Lymphoid cytopathology has been described in LCM virus-infected mice (4; reviewed in 5,7).

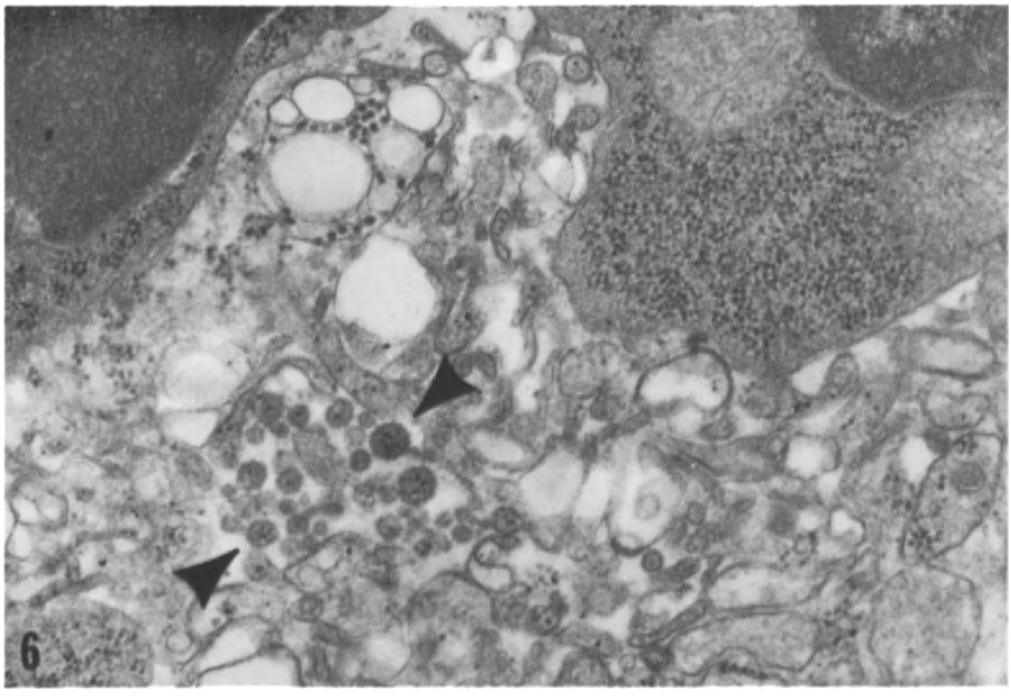


Fig. 6. Machupo virus in C. callosus thymic cortex at 10 days. Virus particles are contained in the debris of a disintegrated cell; 2 lymphoblasts remain intact. Magnification x38,000.

Salivary glands of infected C. callosus were examined because large amounts of infectious Machupo virus were previously recovered on oral swabs (6). Submandibular salivary glands of animals contained large numbers of virus particles at 14, 24, and 30 days. Plasma membranes

of mucogenic cells at the level of intercellular canaliculi and apical borders of acinar lumina were sites of viral budding (Fig. 7). This budding was still seen in 30-day specimens. Salivary gland anatomy favors the accumulation of virus particles in interstices of the se-

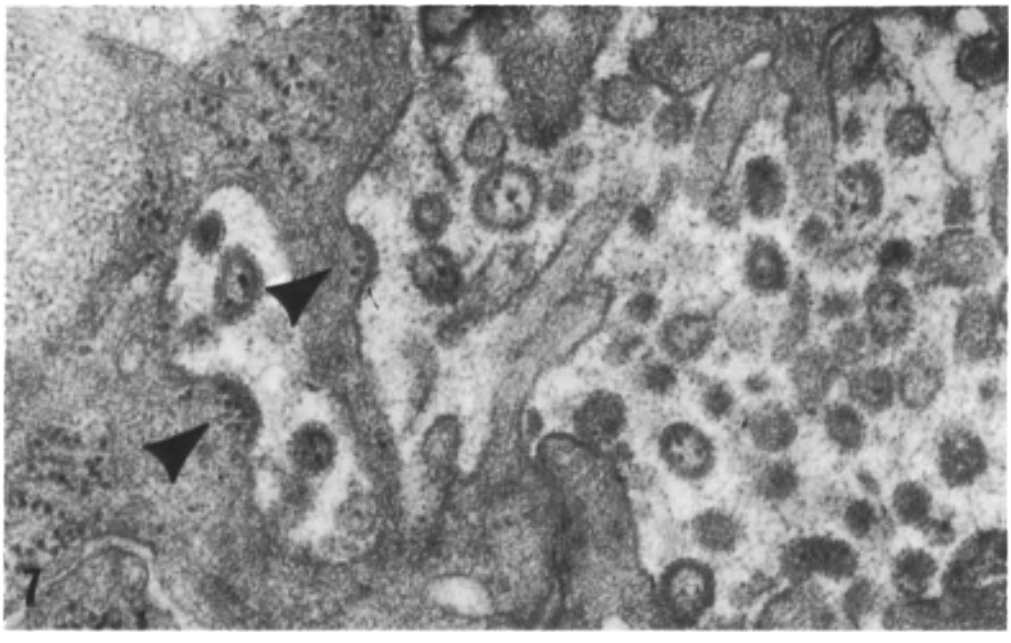


Fig. 7. Machupo virus in C. callosus submandibular salivary gland. Virus particles are budding into the lumen of an intercellular canaliculus of a mucogenic acinus. Magnification x58,000.

cretion product space. Particles were found in all such spaces, especially distended acinar lumina proximal to union with ducts (Fig. 8). In some instances these and smaller spaces were packed with virus and some cell debris (Fig. 9). No salivary gland cytopathology could be associated with infection.

Other arenavirus infections. Similar observations of lymphoid and salivary gland tissue infection were made in C. callosus infected with Latino virus (a natural host-virus combination) (Fig. 10). However, in hamsters infected with Junin virus no particles were found, and in mice infected with Tacaribe virus only a few particles were found in salivary glands (Fig. 11). The neurotropism of this virus, easily demonstrated by immunofluorescence, was not resolved at an ultrastructural level.

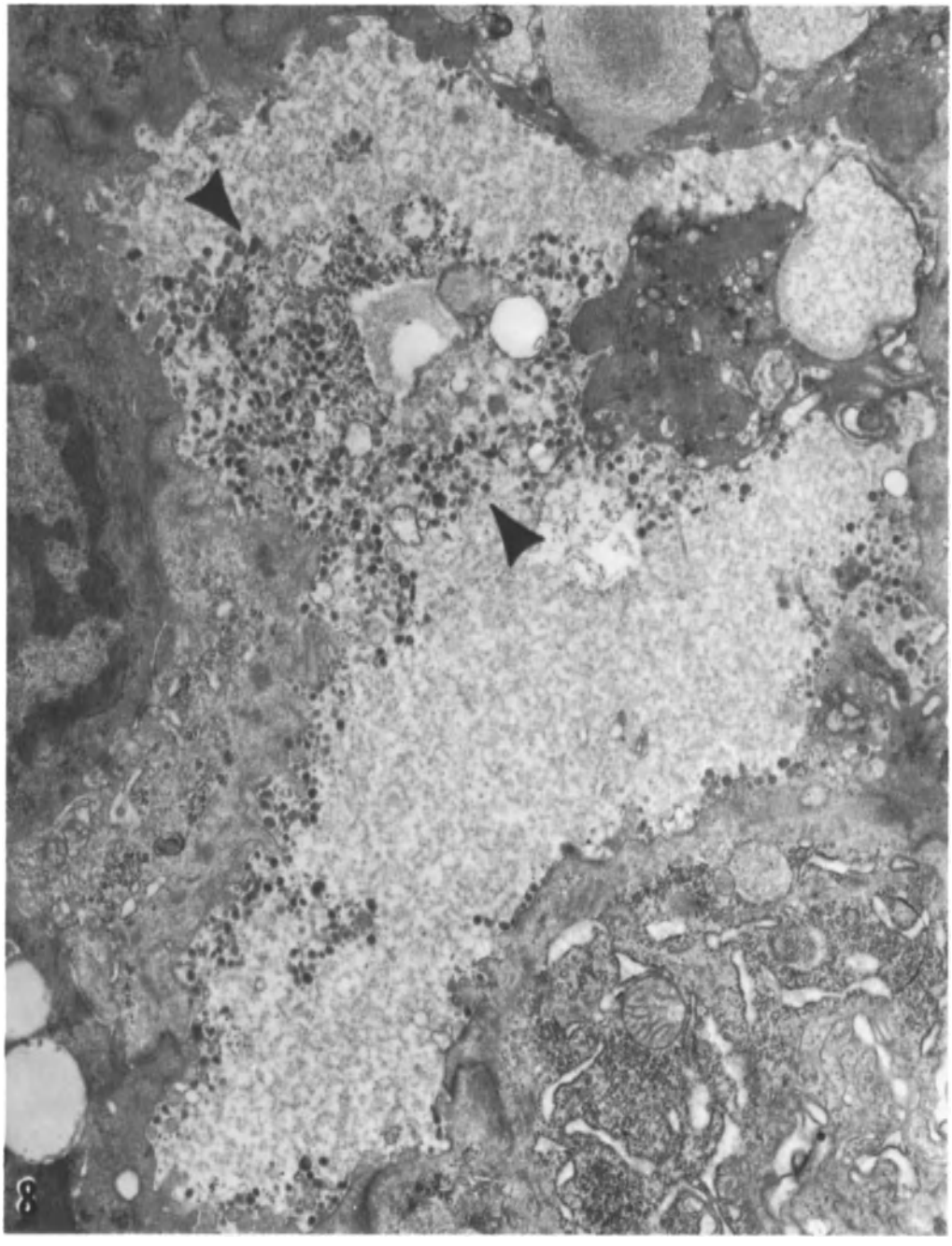


Fig. 8. Machupo virus in salivary gland acinar lumen at 24 days. Large numbers of virus particles accumulated in interstices normally containing salivary secretions. Magnification x15,000.

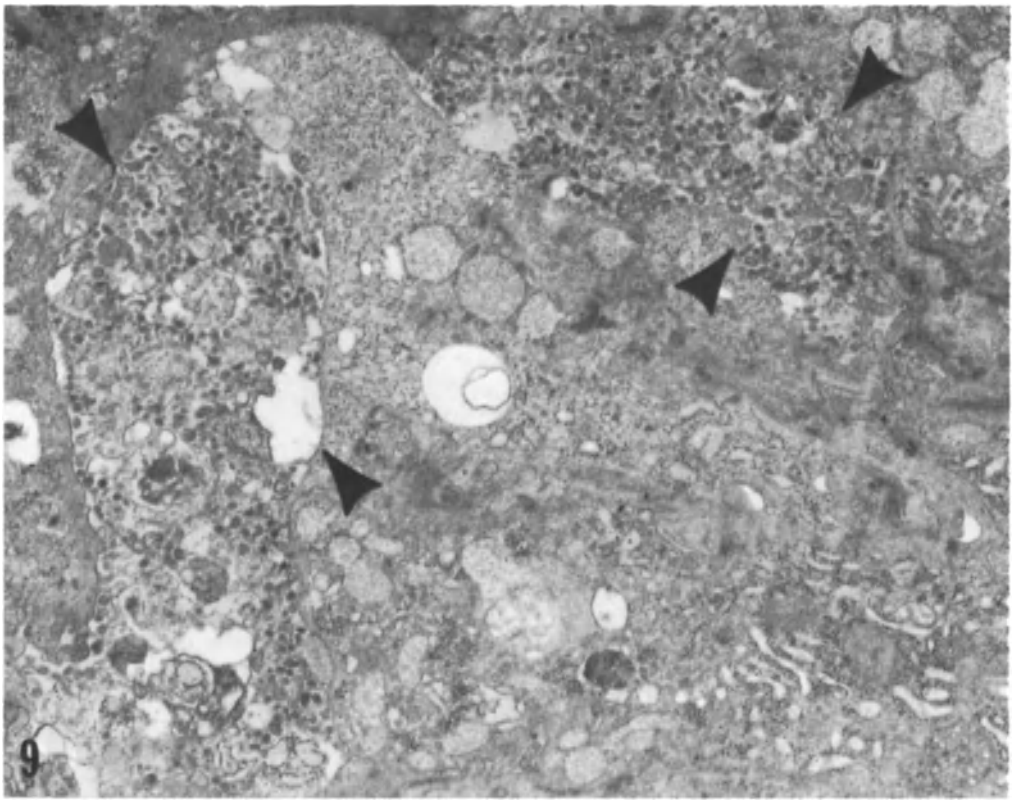
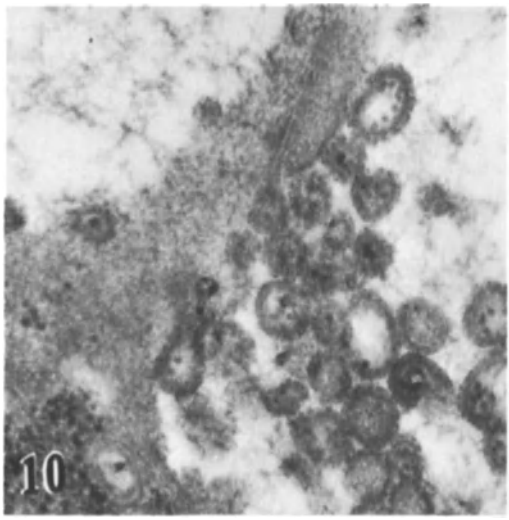


Fig. 9. Machupo virus particles filling salivary gland lumina at 30 days. No cytopathology was associated with acinar infections yielding this amount of virus. Magnification x15,000.

DISCUSSION

The morphology and mode and site of morphogenesis of all arenaviruses in cultured cells appear identical and so distinctive as to allow presumptive identification of a new isolate as a member of the genus. The minor virion variations described appear to reflect differences in growth conditions or stages of infection. It must be anticipated by analogy to other virus groups that the arenaviruses will constitute a most homogeneous genus when defined by biochemical-biophysical comparison of multiple virion properties (RNA, polypeptides, replication mechanisms, etc.). As with other virus groups then, a major enigma arises concerning similar agents and varying in vivo tropism, diseases, and relationships to host immunologic-immunopathologic mechanisms. In this regard, in vivo ultrastructural study of acute and persistent arenavirus infections should be a valuable adjunct to other methods of experimental pathogenesis investigation - organ titration, light microscopic histology, immunofluorescence, etc. But, so far it has not;



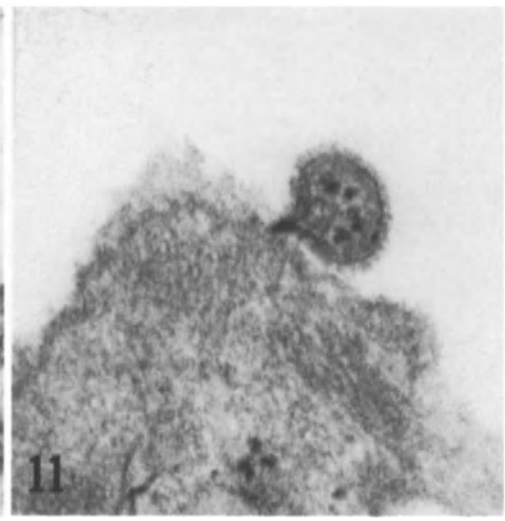


Fig. 10. Latino virus in C. callosus salivary gland acinar lumen. Typical arenavirus particles are distinguishable from transected microvilli. Magnification x58,000.

Fig. 11. Tacaribe virus particle budding from a mucus cell into an acinar lumen in a mouse salivary gland. Typical arenavirus morphology is discernible. Magnification x114,000.

there is the impression, for example, in examining infected lymphoid tissues that findings represent the "tip of an iceberg". In several attempts in our laboratory to study arenavirus central nervous system infections at the ultrastructural level (with parallel immunofluorescent support) we have failed to find more than the occasional virus particle; these studies in mice have included a) LCM virus meningitis (M.S. Hirsch, unpublished), b) Tacaribe virus encephalitis (E.C. Borden, unpublished), c) Lassa virus encephalitis (S.G. Whitfield, unpublished), d) Tamiami virus encephalitis (W.C. Winn, Jr., unpublished). In fact, the only organ where electron microscopic findings have matched other approaches has been the salivary gland.

There are several possible reasons for disparity between results on lymphoid and central nervous system tissues anticipated from other approaches, and those actually found by thin-section electron microscopy. If infections are focal, necessarily small samples might not contain involved sites. Immunofluorescence often is focal in central nervous system tissue, but it is not in lymphoid tissue. If fluid flow through intercellular space is high, virus particles would be carried away from sites of synthesis on plasma membranes and thereby obviate the best marker of infection, the accumulation of virus particles. The large extracellular spaces of normal lymphoid tissue favor this possibility, but normal central nervous system cells are closely joined and should favor particle accumulation. (This has been shown repeatedly with other viruses.)

Finally, disparity primarily between immunofluorescent and electron microscopic observations might reflect variation in the propensity of a given cell type or tissue to accumulate viral antigen (immunofluorescence) relative to its capacity to yield infectious virus particles (electron microscopy). The repeated failure to resolve viral inclusions (massed viral antigen) in vivo indicates a critical need to apply immuno-electron microscopic techniques. Certainly, the accumulation in cell culture of arenavirus antigens without synthesis of infectious virus is a documented phenomenon; if the contrary, the production of virus in the absence of detectable antigen buildup, is also characteristic of some cells, and if this occurs in vivo, then some aspects of viral pathogenesis will need re-evaluation (5,7). In this regard, the described lymphoid cytopathology, which would not have been evident by light microscopy, will need to be evaluated further.

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Tamiami Virus-Induced Immunopathological Disease of the Central Nervous System

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SUMMARY

Tamiami virus, a member of the arenavirus group, produces an acute central nervous system disorder in suckling mice characterized by cerebellar ataxia and less frequently by paralysis, convulsions, and death. Histopathologic changes in acutely ill mice include vasculitis, meningitis, and impaired migration of the external granule cell layer of the cerebellum and, in survivors, asymptomatic cerebellar heterotopia. All animals carry virus in their brains for at least 2 months following infection, as detected by immunofluorescence and virus titration.

Neonatal thymectomy prevents the development of acute central nervous system disease both clinically and pathologically in Tamiami virus-infected suckling mice despite the high titers of virus present in their brains. This strongly suggests that acute central nervous system disease produced by Tamiami virus is immune-mediated.

The presence of prolonged virus infection and immune-mediated central nervous system disease induced by Tamiami virus further serves to identify the arenaviruses as a taxonomic group with similar biologic properties.

INTRODUCTION

Tamiami virus, a member of the Tacaribe group of arenaviruses, derived its name following its original isolation from cotton rats in the Everglades area of South Florida crossed by the Tamiami Highway (11). Tamiami virus has been identified as an arenavirus by its physicochemical (22), morphologic (18), and antigenic (23) properties.

Initial investigations of Tamiami virus in rodents revealed a pathogenicity for suckling mice with histologic evidence of meningo-encephalitis and cerebellar pathology characterized "by scattered foci of necrosis, most prominent in the granule cell layer" (4). Moreover, in the suckling mouse, experimental infection with Tamiami virus resulted in extraneural infection for as long as 3 months in some animals (29).

In light of these preliminary findings, studies were undertaken to determine: 1) the correlation of histologic changes with the clinical course of Tamiami virus-induced disease; 2) the nature of the cerebellar lesion and a comparison with cerebellar lesions induced by other arenaviruses; 3) the ability of Tamiami virus to produce the virus carrier state in mice; 4) the influence of immunosuppression by neonatal thymectomy on the outcome of virus infection. An overview of these studies is presented here.

MATERIALS AND METHODS

Animals. Suckling white mice of the ICR strain were obtained from Flow Laboratories, Dublin, Virginia.

Virus. Tamiami virus, strain W-10777, was obtained from Dr. C.H. Calisher, Arbovirology Unit, Center for Disease Control, Atlanta, Georgia. Stock virus was prepared as follows: brains were harvested 4 to 6 days after intracerebral inoculation of suckling mice. A clarified 10% homogenate in phosphate-buffered saline (PBS), pH 7.2, supplemented with 0.75% bovine plasma albumin, was filtered through a Millipore membrane (450 nm) and stored at -80°C. For titration studies, brains were removed from perfused mice, homogenized, and prepared as 10% suspensions in PBS. Decimal dilutions of brain homogenates were titrated intracerebrally in ICR suckling mice. Titers were calculated by the Kärber method (12) and expressed as the AD50/0.02 ml or 0.02 g. The appearance of severe cerebellar ataxia was used as the end-point (ataxic dose = AD). This pool had a suckling mouse intracerebral AD50 titer of $10^{4.4}$/0.02 ml and was used at a 10^{-2} dilution to give a standard inoculum of about 175 AD50/0.02 ml. The titer of this same inoculum by plaque assay (see below) was 165 PFU/0.02 ml.

Virus titration in tissue culture. Tamiami virus was assayed by inoculating confluent monolayers of Vero cells grown in 50 mm Falcon plastic Petri dishes with serum and brain homogenates from Tamiami virus-infected animals. The medium was removed from the Petri dishes and 0.2 ml of inoculum in decimal dilutions (2 dishes/inoculum) was added to the cell monolayer. After adsorption at 37°C for 1 h, infected monolayers were overlaid with 4 ml of a mixture containing 2 ml of 1% agarose in water and 2 ml of nutrient medium composed of Eagle's basal medium (BME) at twice the normal concentration supplemented with 0.28 g/100 ml sodium bicarbonate and 4% fetal bovine serum; to each ml 200 units of penicillin G and 100 µg of streptomycin were added. After 4 days incubation at 37°C in 5% CO_2 in air, the cultures were refed with 2 ml of a mixture of equal parts nutrient medium and agarose. After an additional 4 days, each culture received 2 ml of medium-agarose mixture containing neutral red at a final concentration of 1:15,000. Plaques were counted 48 h later.

Light microscopy. Mice were perfused with isotonic saline followed by 1% acetic acid in 10% formalin. Paraffin sections of brain tissue were cut at 7 µm and stained with hematoxylin and eosin.

Immunofluorescent staining. Brains from saline-perfused mice were cut in 8 µm parasagittal sections in a cryostat, fixed in acetone for 5 min, and stored at -20°C. The direct method of staining was used, following conventional techniques (9). Adult rabbits were hyperimmunized (at 0, 2, and 3 weeks) with large intraperitoneal and intramuscular doses of Tamiami virus mixed with equal volumes of complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan), and bled 1 and 2 weeks following the final immunization. Serum was pooled and an ammonium sulfate-

precipitated globulin fraction was conjugated with fluorescein isothiocyanate (Baltimore Biologic Laboratories). Prior to staining, 2 parts of conjugate, 1 part of pH 9.4 phosphate-buffered saline (PBS), and 1 part Evans blue counterstain were mixed. The counterstain was prepared as 0.5% dye solution in PBS, pH 9.4.

The specificity of the reagent was ascertained by: 1) its failure to stain uninfected brains; 2) an approximate correlation of numbers of stainable cells with infectious virus; 3) the identical fluorescence of Tamiami virus-infected brain with a different Tamiami virus conjugate prepared from hyperimmune mouse ascitic fluid by Dr. Washington Winn, CDC, Atlanta; 4) the ability of Tamiami virus hyperimmune sera prepared in rabbits to block fluorescence of Tamiami virus-infected mouse brain by the Tamiami virus conjugate prepared in mice; 5) the failure of normal mouse sera to block the fluorescence of Tamiami virus-infected mouse brain by the Tamiami virus conjugate prepared in mice. Sections were overlaid with conjugate, incubated, washed, dried, and mounted as previously described (7).

A Leitz Orthoplan fluorescence microscope with a mercury HBO200 bulb and a Schott BG12 exciter filter with a K530 barrier filter were used.

Neonatal thymectomy. Within 24 h of birth, mice were thymectomized, following the technique of Roosa et al. (20). Animals were chilled on ice and anesthetized with ether. They were then secured on a contoured sponge rubber pad and the thorax was swabbed with alcohol. An incision was made in the skin over the thymic area, and a part of the sternum was removed. The thymus was lifted out with a glass pipette connected to low suction. The wound was then sprayed with a fine powder of antibiotics (Powdalator-es, Abbott Laboratories) and closed with a single mattress suture. Control animals were sham-thymectomized by the same procedure with the exception that the thymus was left intact. Following surgery the animals were warmed and then returned to their mother.

Tamiami virus was inoculated intracerebrally into thymectomized animals the following day (24 to 48 h after birth) corresponding to the time of infection of the non-thymectomized animals. Clinical, histologic, and virologic data for the two groups of mice were correlated at similar times.

RESULTS

Clinical disease in suckling mice. Neonatal ICR mice inoculated intracerebrally between 24 to 48 h after birth with 175 AD50 Tamiami virus develop neurological signs characterized primarily by cerebellar ataxia and less often by convulsions, hind leg paralysis, and death. CNS disease is heralded by slower growth of infected animals which is usually apparent by 7 days post-infection (p.i.). Eight to 11 days p.i., mice are tremulous, irritable, and often exhibit hunched posture and ruffled fur. From days 12 to 21 p.i., the animals display a staggering wide-based gait, truncal instability, and have difficulty righting themselves from a position on their backs. Ataxia in many mice is severe, and they are unable to walk without falling. Others are less affected, but a slight push results in the animal falling to the opposite side. All infected animals are ataxic for approximately 10 days, and in those mice which survive the ataxia disappears by the end of the 3rd week.

Of the mice infected with Tamiami virus intracerebrally, 40 to 50% not only become ataxic, but develop paralysis, have convulsions (tonic sei-

zures can often be elicited by spinning the animal by the tail) and usually die 13 to 25 days p.i.

Those mice which survive the Tamiami virus-induced acute neurological disease have no permanent CNS sequelae but are smaller than non-infected control animals for several months.

Neuropathological observations. Pathologic changes in the brain consist of a diffuse intense vasculitis, moderate meningitis, particularly involving the meninges between the cerebellar folia (Fig. 1A), and an extensive cerebellar lesion characterized by impaired migration of the external granule cell layer resulting in cerebellar heterotopia (Fig. 1B). The inflammatory infiltrate is composed of mononuclear cells of varying size with noticeable absence of a polymorphonuclear leukocyte response. Other lesions seen more irregularly throughout the brain are a mild choroiditis, and rare foci of necrosis and softening surrounding areas of intense vasculitis. The latter feature occurs primarily in the brain stem and pyramidal cell layer of the hippocampus.

The appearance of acute CNS histopathologic changes parallels the clinical course and is most marked between 2 to 3 weeks p.i. and begins to be resolved after 21 days p.i. The permanent cerebellar heterotopia is characterized by the persistence of numerous granule and rare inflammatory cells in the molecular layer of the cerebellum (Fig. 1B). In occasional cerebellar neurons, unusual dark inclusion-like masses are seen in the cytoplasm.

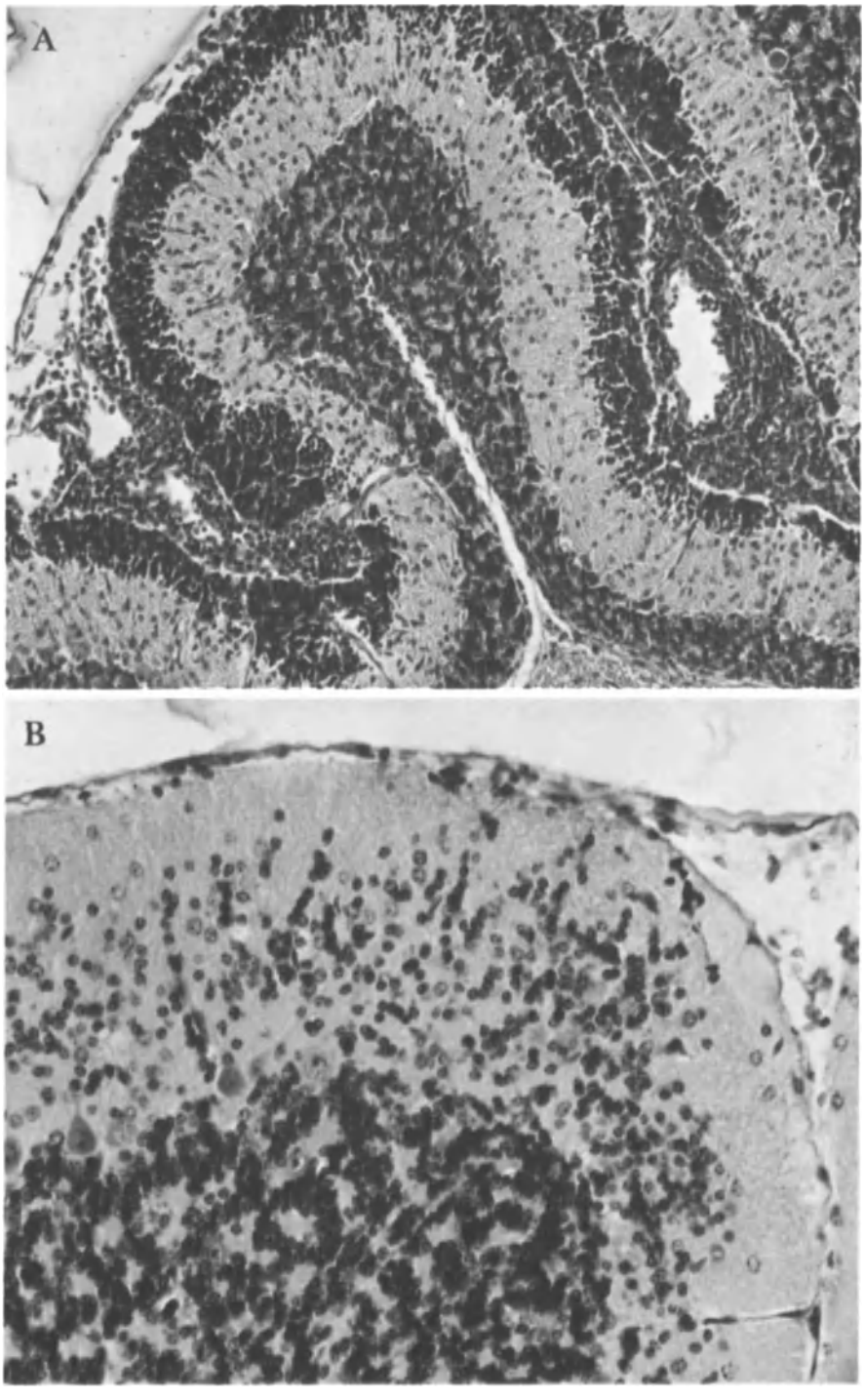
Virological studies. Following intracerebral inoculation of 175 AD50 of Tamiami virus in suckling mice, virus was detectable in the brain by the 2nd day and rose rapidly thereafter to peak titers by day 5 (greater than 10^7 PFU/ml). Plaque assay titrations correlated well with the presence of virus as detected by immunofluorescence. Brain titers remained at this peak for at least 30 days, and virus was detectable for at least 66 days p.i. (more than 10^2 PFU/g). An example of Tamiami virus plaquing in Vero cells is seen in Figure 2.

Viremia only developed transiently and inconsistently up to 9 days p.i. and titers were generally low (between 10^1 and 10^2 PFU/ml).

Immunofluorescence. Viral antigen detected by direct immunofluorescence was most intense in the granule cell layer of the cerebellum (Fig. 3) and hippocampus; however, virtually all other areas of the cerebrum, subcortical nuclei, cerebellum and brain stem were infected to varying degrees. The number of fluorescing cells in the meninges, choroid plexus, and ependyma was not as extensive as in parenchymal structures. Vascular endothelium, however, fluoresced throughout all CNS structures. Viral antigen was detected by immunofluorescence for as long as 66 days after infection.

Fig. 1A. Cerebellum and overlying meninges from a mouse inoculated intracerebrally with Tamiami virus and perfused at time of neurological illness 18 days later. Hematoxylin and eosin. Magnification x150. ►

Fig. 1B. Cerebellum from a mouse inoculated intracerebrally with Tamiami virus and perfused after resolution of neurological disease 50 days later. Hematoxylin and eosin. Magnification x300. ►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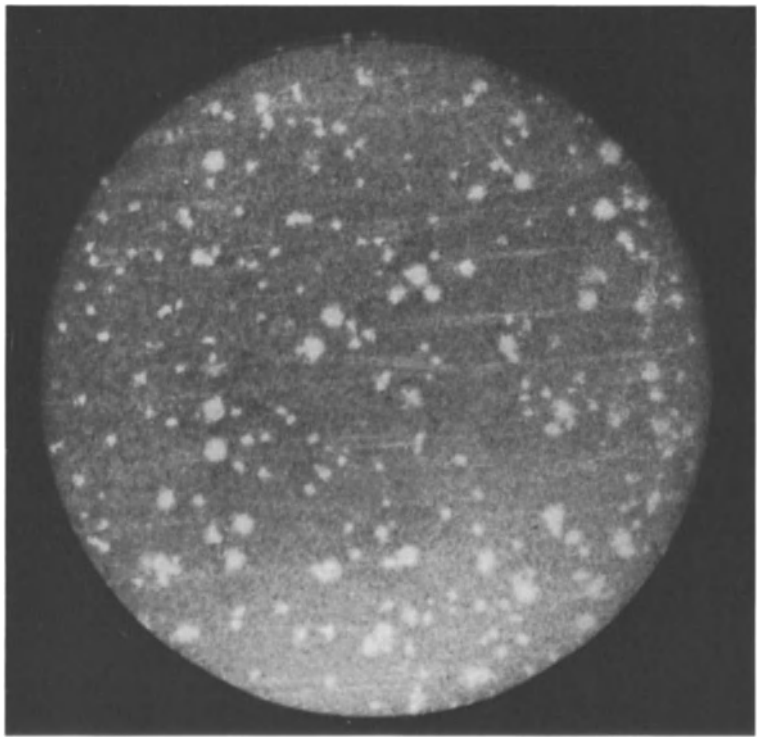

Fig. 2. Plaque formation in Vero cells following infection with Tamiami virus. The variety of plaque size is characteristic.

Effect of neonatal thymectomy. Neonatal thymectomy prevented ataxia and other neurological signs from developing in more than 90% of animals infected intracerebrally with Tamiami virus (Fig. 4). The sparing effect of neonatal thymectomy on clinical disease was corroborated histologically by the absence of intense inflammation and cerebellar heterotopia. Only a few mice developed ataxia (less than 10%), which was reflected histopathologically by minimal cerebellar heterotopia and mild vasculitis.

At similar intervals all asymptomatic neonatally thymectomized mice infected intracerebrally with Tamiami virus had titers of virus in brain equivalent to those in mice receiving virus only. In addition, immunofluorescent monitoring for viral antigen revealed dissemination in the CNS earlier after infection in immunosuppressed mice. Both virus plaquing and immunofluorescent studies also indicated that Tamiami virus titers in the brain persisted longer in neonatally thymectomized mice than in non-thymectomized mice.

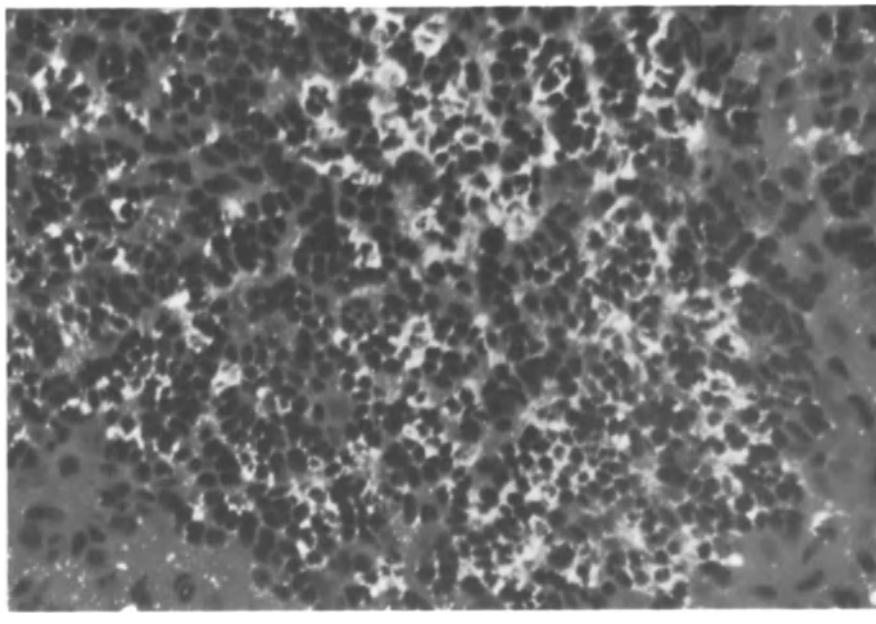

Fig. 3. Cerebellar folium of a mouse demonstrating infection of granule cells after intracerebral inoculation of Tamiami virus; perfusion at time of neurological disease 15 days later. Anti-Tamiami virus immunofluorescent stain. Magnification x250.

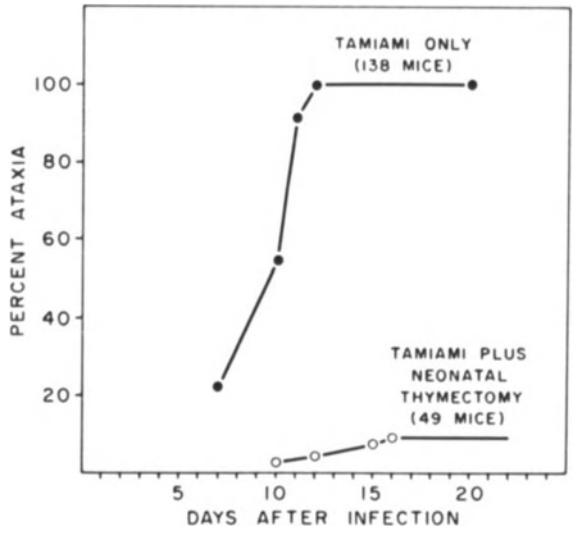

Fig. 4. Ataxia following intracerebral inoculation of Tamiami virus, $10^{4.4}$ AD50/0.02 ml, in 1 to 2-day-old ICR mice, and the modification of ataxia by neonatal thymectomy within 24 h of birth.

DISCUSSION

CNS disease. Following inoculation of Tamiami virus into suckling mice, the neurologic signs which occur (convulsions and cerebellar ataxia) are similar to those described in suckling rodents after other arenavirus infections (15,16,24). The susceptibility of suckling mice to CNS disease and the increasing resistance with age following intracerebral inoculation with Tamiami virus is similar to clinico-pathologic data reported for Junin (15) and Tacaribe (2) viruses. The intense meningitis and vasculitis together with the moderate choroiditis produced by Tamiami virus resemble the changes described by Borden (2) in suckling mice inoculated with Tacaribe virus. Only the prototype of the arenavirus group, the LCM virus, produces acute CNS disease more readily in the adult than in the newborn mouse (26).

Tamiami virus infects the external granule cells, the most actively proliferating layer in the post-natal cerebellum and the largest contributor to cerebellum mass and cell population (13). In normal infant rats between day 5 and 17, cerebellar DNA increases by 8.5 times as compared to increases of 2.5 times in the cerebrum (28). Thus it is not surprising that the cerebellum, particularly the granule cell layer, bears the brunt of virus infection more severely than does the cerebrum. Other viruses which have an affinity for the rapidly dividing cerebellar granule cells (rat virus and feline panleukopenia virus) produce cerebellar hypoplasia after neonatal infection (14).

Cerebellar necrosis is produced by LCM virus in suckling rats (16) and, with less regularity, in suckling mice (5). Tacaribe virus also causes cerebellar necrosis in newborn mice (2). The main difference in pathology following Tamiami virus infection is cerebellar heterotopia as opposed to cerebellar necrosis. It can be speculated that both heterotopia and necrosis are a continuum of lesions which depend in part upon immunologic responsiveness. Because the lymphoid system of the newborn mouse is not fully mature (1), the development of immunopathological cerebellar heterotopia may have been propitiously produced by inoculating the partially immunocompetent 1 to 2-day-old mouse. It could be anticipated that cerebellar necrosis might result from adoptive transfer of Tamiami virus-immune lymphocytes to neonatally inoculated Tamiami virus carriers. These studies are in progress.

Although both the affinity of Tamiami virus for the actively dividing cerebellar granule cells and the host immune response are important in producing cerebellar disease, the relative influence of each mechanism is still unclear.

Effect of immunosuppression. Immunosuppression by neonatal thymectomy spared mice from acute disease, indicating that Tamiami virus-induced disease of the CNS is immune-mediated, and depends in part on the integrity of the thymus-dependent immune system. It is becoming increasingly clear that thymus-dependent lymphoid tissue plays a major role in acute disease after arenavirus infection (6). Rowe (21) originally demonstrated that neonatal thymectomy protected weanling mice from acute CNS disease if infected with LCM virus. Weissenbacher (27) found that neonatal thymectomy spared mice from acute CNS disease induced by Junin virus. More evidence that cerebellar disease is immunopathologic comes from the studies of both Borden (2) and Monjan (17) who demonstrated that the cerebellar lesion is reduced by anti-lymphocytic serum following arenavirus infection. Additional evidence is the observation of necrosis in adult mice following adoptive immunization of cyclophosphamide-induced LCM virus carriers with LCM virus-immune spleen cells (8).

Virus persistence. The persistence of detectable virus for months after experimental arenavirus infection is well documented. The presence of Tamiami virus for at least 1 to 2 months after neonatal infection is comparable to that reported for Lassa fever virus (3), Pichinde virus (24), and LCM virus (25). Even the clinical features of "wasting disease" (small size, ruffled fur, and blepharitis) described by Hotchin in neonatally induced LCM virus carrier mice (10) are present in some Tamiami virus-infected mice a few months after resolution of acute CNS disease. LCM "late disease" is thought to result from virus-antibody complexes leading to renal glomerular disease (19). Preliminary examinations of kidneys from Tamiami virus-infected carriers by immunofluorescence with anti-mouse immunoglobulin were negative for the accumulation of antibody, but direct immunofluorescence as described for CNS studies revealed the presence of viral antigen in some kidney arterioles.

ACKNOWLEDGMENTS

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The Pathogenesis of Tamiami Virus Meningoencephalitis in Newborn Mice

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SUMMARY

Tamiami virus produced a lethal meningoencephalitis when inoculated peripherally into 1- to 5-day-old mice, while older and younger animals were spared. Comparison of lethally and non-lethally infected animals suggested that host factors, especially the extent of inflammatory response, determined the outcome of infection. Intracellular localization of antigen-antibody complexes coincided with recovery from disease and elimination of virus and viral antigen, suggesting a beneficial, rather than deleterious role for antibody in this disease.

INTRODUCTION

To date only two arenaviruses have been isolated in North America - LCM virus, the prototype of the group, and Tamiami virus, a member of the Tacaribe subgroup. Tamiami virus was first isolated by Calisher et al. (4) along the Tamiami Trail in the Florida Everglades, where its natural host is the cotton rat, Sigmodon hispidus. Initial field and laboratory investigations (4,9) suggested that the virus was maintained in nature by rodent-to-rodent contact and that a chronic infection might be present. Repeated isolations were made from body fluids of cotton rats. When newborn mice were exposed to excreta from these cotton rats, infection without clinical illness resulted. The present studies were undertaken to investigate further the host-virus relationship.

MATERIALS AND METHODS

Virus strain. The virus was a 7th suckling mouse brain passage of the prototype strain, W-10777. A 20% suspension of infected mouse brain was clarified by centrifugation and stored at -60°C. The stock virus had an infectivity titer of $10^{7.5}$ LD50/ml in 2- to 3-day-old mice.

Mouse inoculations. Swiss ICR mice were used throughout the experiments. Mice from each age group were pooled, inoculated with 10^4 LD50 of virus suspension, and redistributed to the mothers. For age-mor-

tality comparisons, groups of 50 mice ranging from newborn (less than 18 h of age) to 8 weeks old were inoculated and deaths occurring after day 5 recorded. In the sequential pathogenesis study, 2-day-old mice and mice less than 18 h old were inoculated similarly (referred to as 2-day and 0-day groups); 3 to 5 mice were sacrificed at frequent intervals for virus and antibody determinations and morphological studies.

Virus titration. Virus titrations of tissues were performed by a fluorescent focus assay. Confluent monolayers of Vero cells were grown in slide chambers under a 5% CO_2 atmosphere. Ten-fold dilutions of clarified tissue homogenates were adsorbed for 1 h at 37°C before adding media. After 3 days infected cells and groups of cells were identified by immunofluorescence and recorded as fluorescent foci/g of tissue.

Antibody determinations. CF antibody was measured in a micro-titer system by the LBCF method (5). Neutralizing antibody was assayed in tissue culture by reduction of fluorescent foci; virus and 2-fold dilutions of serum were incubated for 1 h at 37°C, then inoculated into slide chambers. Antibody end-points were taken as the reciprocal of the highest dilutions which caused an 80% reduction of 25 to 50 fluorescent foci.

Light microscopy. Tissues were fixed in neutral-buffered formalin, embedded in paraffin, and cut at 6 μm. Sections were stained with hematoxylin-eosin.

Fluorescent microscopy. Hyperimmune mouse ascitic fluid to Tamiami virus (4) was fractionated on DEAE-Sephadex and the globulin fraction conjugated with fluorescein isothiocyanate. Unbound fluorescein was removed by chromatography on G-25 Sephadex. (Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.)

Other reagents were purchased commercially. Fluorescein-conjugated anti-mouse immunoglobulin was obtained from Hyland Laboratories, Los Angeles, Calif.; fluorescein-conjugated IgG fraction of rabbit anti-mouse B_1C globulin and anti-mouse albumin were obtained from Cappel Laboratories, Downington, Pa.; fluorescein-conjugated equine anti-guinea-pig serum (complement) was obtained from Progressive Laboratories, Baltimore, Md.

Tissues were embedded in Tissue Tek O.C.T. compound and frozen on dry ice or by immersion in liquid nitrogen. Sections were cut at 4 μm in a cryostat at -16 to -20°C, air-dried, fixed in cold acetone, and stained with fluorescent antiserum for 30 min at room temperature.

Specificity controls, where indicated, consisted of blocking of fluorescence by a) prior staining with unconjugated antiserum, and b) preincubation of antigen with conjugate at 37°C for 60 min. Tissues of age-matched mice inoculated with normal mouse brain were stained in parallel.

Fixation of exogenous complement was detected by an indirect technique, using a pool of commercial guinea-pig complement followed by fluorescein-conjugated equine anti-guinea-pig serum. The technique was similar to that of Goldwasser and Shepard (6) and Burkholder (3). Phosphate-buffered saline or heat-inactivated guinea-pig complement (56°C for 60 min) was used as a first layer for a specificity control.

To demonstrate antibody elution, unfixed frozen sections were incubated with citrate buffer, pH 3.2, or phosphate-buffered saline for 60 min at room temperature or 18 h at 4°C. Sections were then fixed in cold acetone and stained for immunoglobulin.

RESULTS

Peripheral inoculation of 10^4 LD50 of virus produced a complex set of age-related mortality curves (Fig. 1). As expected, adult animals did not become ill, while mortality approached 100% in 2- to 4-day-old

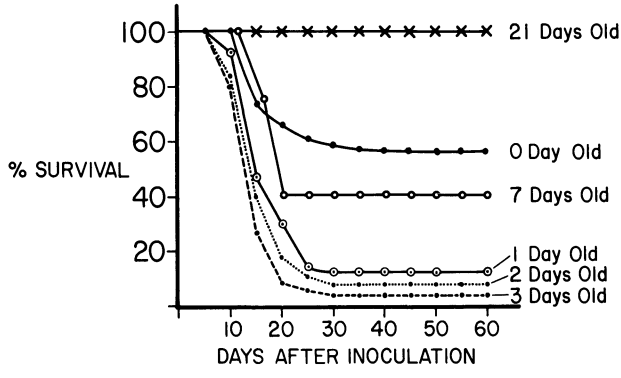


Fig. 1. Relationship of age at inoculation to survival in Tamiami virus infection. Mice from less than 18 h old (0 day) to 3 weeks old were inoculated by the intraperitoneal route with 10^4 LD50 of the prototype strain.

mice. The most striking finding was the increased survival of mice inoculated in the first day of life. Mortality ranged from 40 to 50% whether animals were inoculated under 6 or under 18 h of age. Illness and deaths began 10 to 14 days after infection and most of the deaths occurred within the next 10 days. Affected mice were sometimes hyperexcitable and exhibited circling movements. Cerebellar signs, especially difficulty in walking and righting themselves, were prominent and some mice developed limb pareses before progression to a prostrate state. Many surviving mice appeared ill during the acute infection, then recovered except that they were smaller than control animals for several months following. Control mice, inoculated with normal mouse brain, uniformly survived, though after 3 months there were a few deaths.

Pathogenesis in 0-day and 2-day-old mice. To investigate the status of surviving mice and possible mechanisms for their recovery, the course of illness in 2-day-old mice with high mortality and 0-day-old mice with low mortality was compared.

After intraperitoneal inoculation, a transient low level viremia appeared in 2-day-old mice and for a more prolonged period in 0-day-old mice, but it ended by day 17. In both groups visceral infection preceded viral invasion of the brain, though virus was detected earlier in both kidney (Fig. 2) and spleen of the 0-day group. Virus had disappeared from the spleen of both groups by day 17 but was present in kidneys of the 0-day group for a month after infection (Fig. 2).

Virus was first detected in the brain of both groups on day 9. Titers rose rapidly at first, then began to decline from a peak at day 13 in the 2-day group. In contrast, the 0-day group produced increasing amounts of virus until day 17, after which titers leveled off for several weeks and then declined (Figs. 2 and 3).

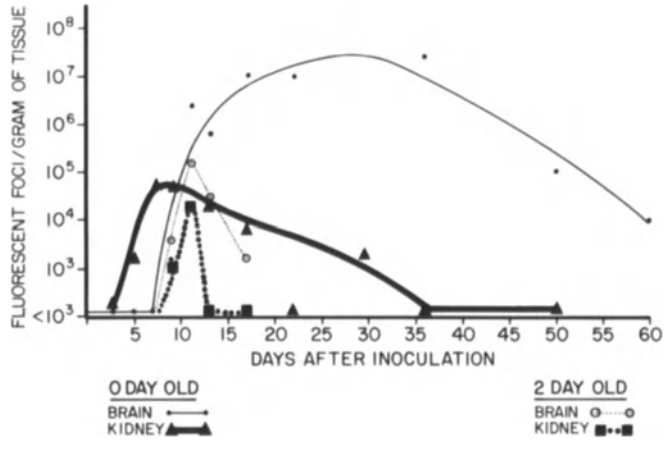


Fig. 2. Comparison of brain and kidney virus titers in 0-day and 2-day mice inoculated at day 0 with 10^4 LD50 of Tamiami virus.

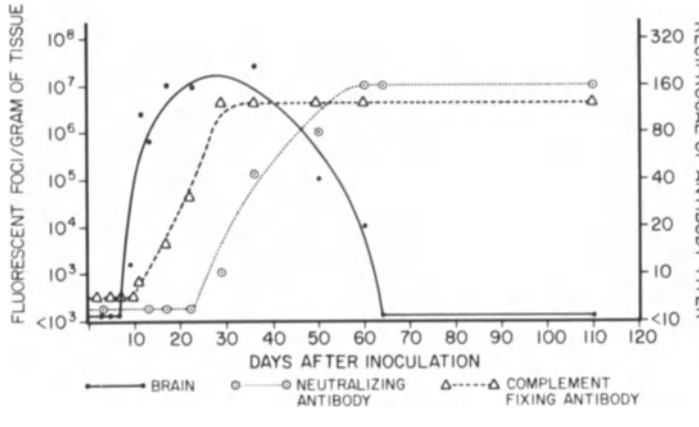


Fig. 3. Serum antibody and brain virus titers in mice inoculated when less than 18 h old with 10^4 LD50 of Tamiami virus.

Localization of viral antigen by immunofluorescence followed a pattern similar to infectious virus during the acute illness. Results in the 2 age groups were similar and will be described together. By day 5 small amounts of antigen were seen in viscera, primarily in the cap-

sules of abdominal organs. By day 10 multiple small foci of fluorescence were present throughout the brain and spinal cord. Over the next few days accumulation of antigen proceeded rapidly to involve virtually all areas of the central nervous system. The fluorescence occurred as discrete cytoplasmic aggregates, often limited to neurons, though in heavily involved areas cellular staining was more homogeneous and all cells appeared infected (Fig. 4). Neuronal processes could be traced

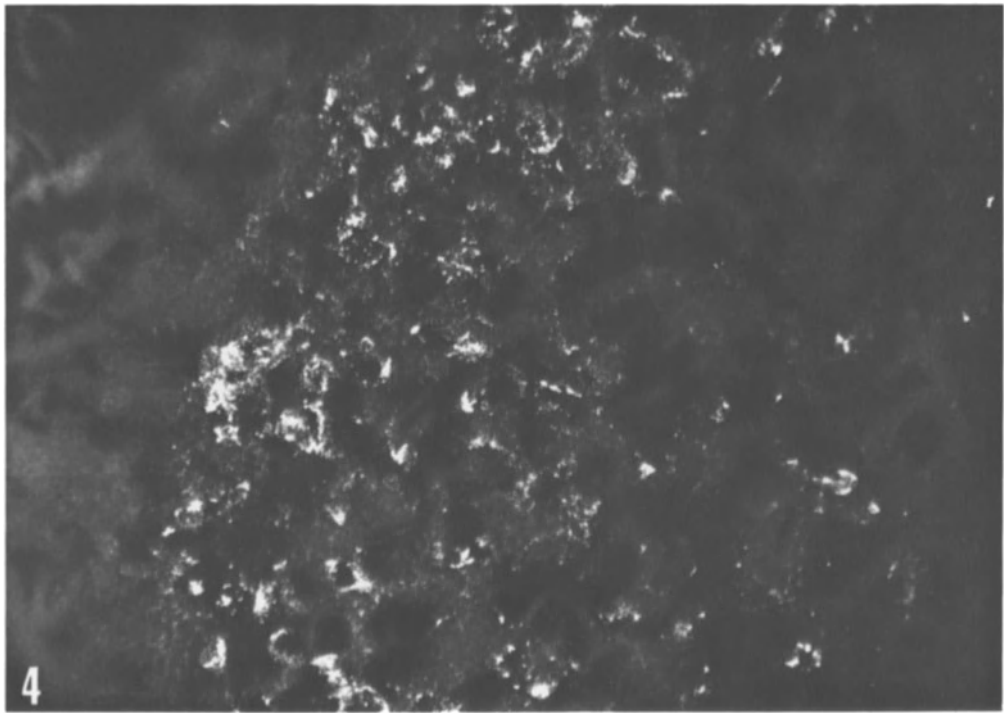


Fig. 4. Cerebral cortex from a 0-day mouse 2 weeks after inoculation. Accumulation of viral antigen is primarily in neurons, but some fluorescent cells are probably glial. Antigen in cell processes gives such areas a "peppery" background. Fluorescein-conjugated anti-Tamiami virus globulin. Magnification x250.

by pinpoint aggregates of fluorescent antigen in their cytoplasm. In all instances there was complete absence of antigen in meninges, choroid plexus, and ependyma. The only difference between the 2 groups was an increased amount of antigen in the hippocampus (Fig. 5) and cerebellar granule cells of the 0-day group. Antigen was frequently focal in distribution but involved inner and outer granular layers as well as granule cells migrating through the molecular layer.

Histologic evidence of acute meningoencephalitis first appeared in both groups on day 11. The initial reaction consisted of a mixture of mononuclear cells, neutrophils, and eosinophils. Inflammatory cells appeared focally in the meninges, especially over the cerebellum, and infiltrated through the walls of small vessels into the surrounding

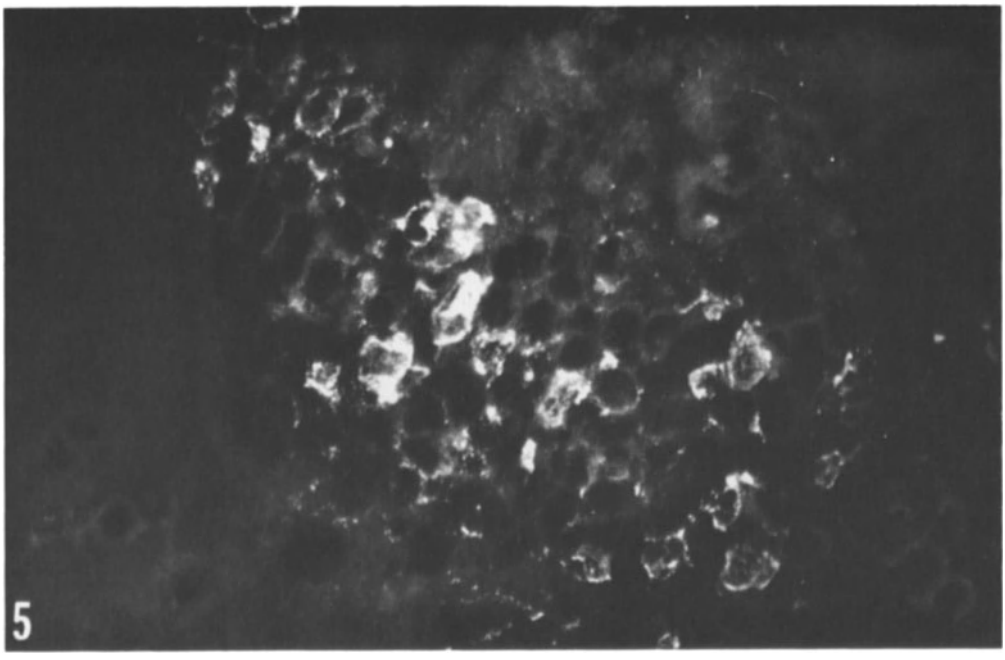


Fig. 5. Pyramidal neurons of the hippocampus from a 0-day mouse 17 days after inoculation. Some neurons show heavier and more uniform staining. Adjacent areas of the hippocampus were negative. Fluorescein-conjugated anti-Tamiami virus globulin. Magnification x400.

brain substance. Necrosis and fibrinoid degeneration of vessel walls were not seen. The inflammatory response in the 2-day group was more intense and included more polymorphonuclear leukocytes, especially in the midbrain, brainstem, and spinal cord. In a few mice from the 2-day group frank parenchymal necrosis occurred with accumulation of large numbers of acute inflammatory cells, including many eosinophils (Fig. 6).

In the 0-day group histopathologic changes were concentrated in the cerebellum and hippocampus where focal necroses involved the granular and molecular layers and the pyramidal neurons, respectively. Extensive cell debris and some acute inflammatory cells were seen in the necrotic foci at the height of illness, but all evidence of acute inflammation disappeared very quickly. In many of the animals arrest of granule cell migration from outer to inner layers was also prominent.

For several weeks following the period of acute mortality, a progressive increase in the cerebellar and hippocampal necrosis dominated the histologic picture in the surviving 0-day mice. In contrast, the inflammatory response became increasingly less prominent, leaving hypocellular lesions containing stringy amorphous debris (Fig. 7). The perivascular and meningeal infiltrate became predominantly mononuclear (Fig. 8) and glial nodules were found. By 6 months all signs of inflammation had disappeared from most animals, but the focal acellular lesions remained.

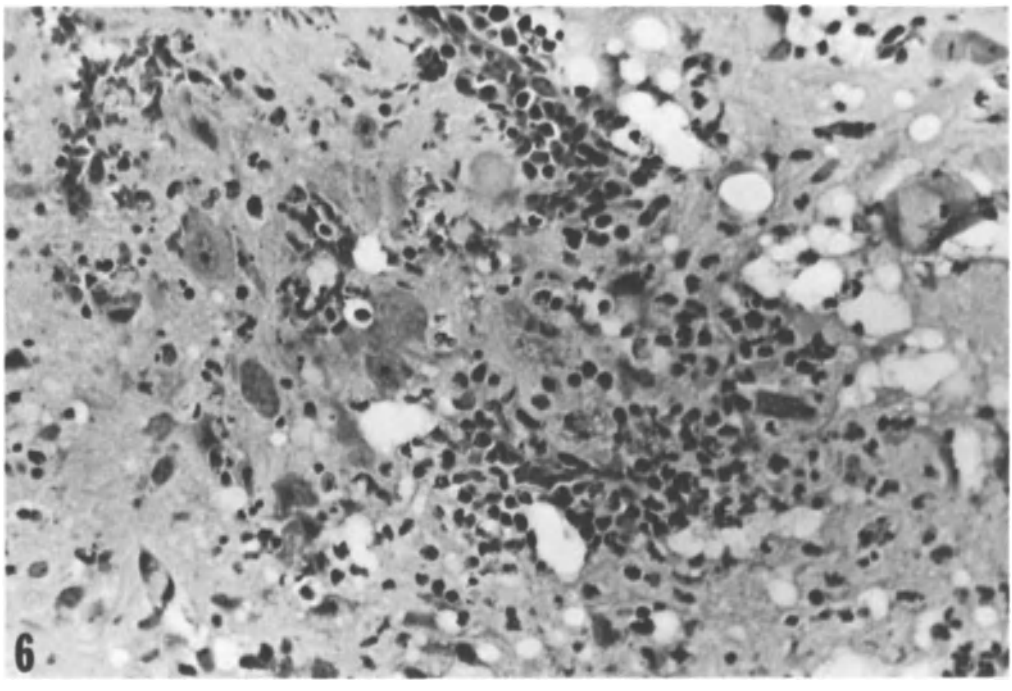


Fig. 6. Extensive tissue destruction in the midbrain of a 2-day mouse 17 days after inoculation. The inflammatory response consists of mononuclear cells and polymorphonuclear leukocytes, including many eosinophils. Hematoxylin-eosin. Magnification x250.

During the 2nd month of infection, titers of infectious virus declined until, by day 65, no virus was detectable. Additional attempts to isolate virus at 4 and 5 months were also unsuccessful (Fig. 3). Viral antigen, on the other hand, was still present in varying amounts 6 months after infection. Though there was considerable variation among animals, the trend was toward decreasing quantity with time till some animals were nearly negative as early as 4 months after inoculation. In addition to quantity, staining intensity tended to decrease. The areas of most conspicuous involvement in the older animals were cerebellum, cerebral cortex, and brainstem nuclei.

Concomitant with the disappearance of infectious virus and the decreasing quantities of fluorescent viral antigen was the appearance of both CF and neutralizing antibodies (Fig. 3). CF antibody was detected first on day 13 and was just beginning to rise when the animals in the 2-day group died. Serum neutralizing antibody was delayed in appearance but rose to plateau levels, along with CF antibody, over the 2nd month of infection.

At the time when CF antibody was rising and neutralizing antibody first appearing, immunofluorescence demonstrated the presence of mouse immunoglobulin in infected brains. Diffuse staining in meninges and around blood vessels was seen to some extent in uninfected animals and was discounted. In the brains of infected mice, however, immunoglobulin was

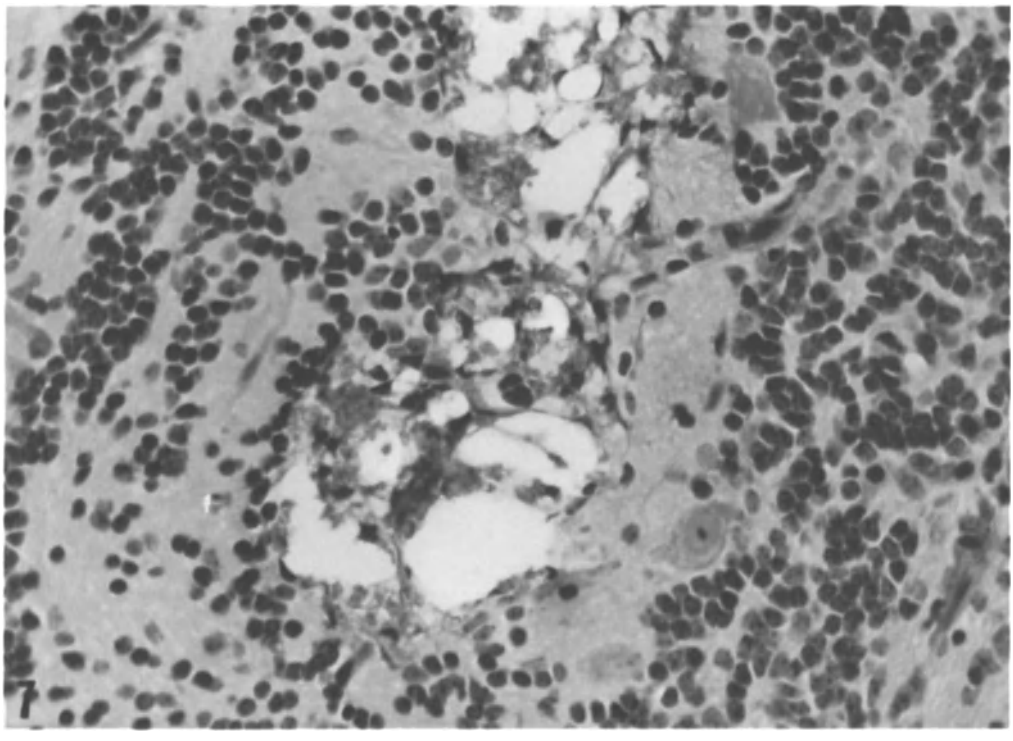


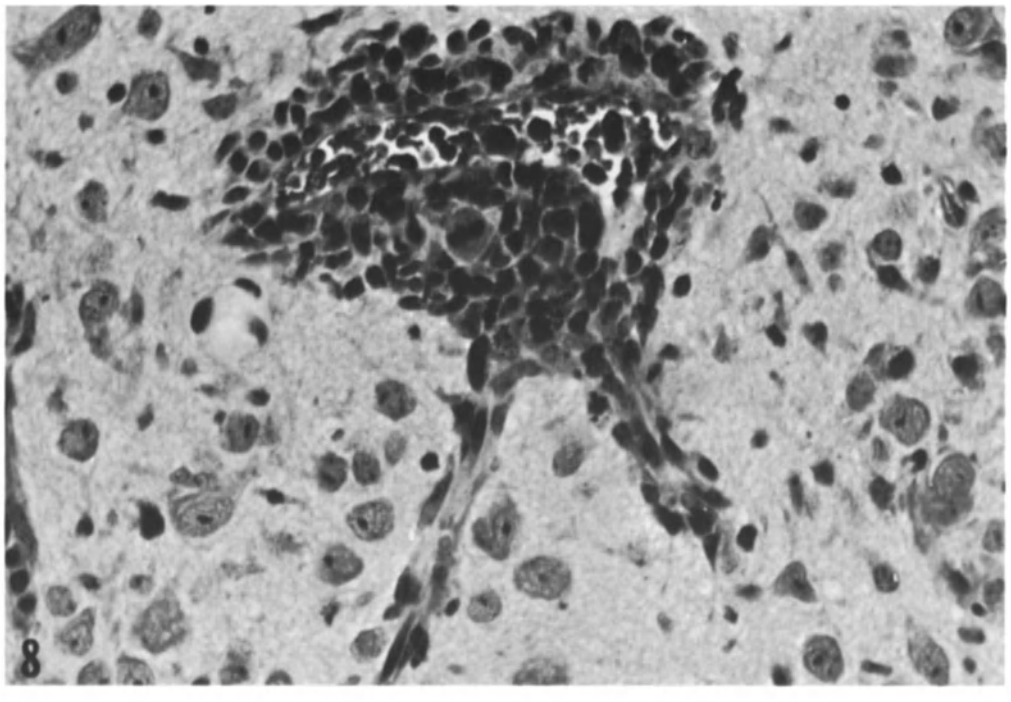
Fig. 7. A destructive lesion containing amorphous debris and a few reactive glial cells in the cerebellum of a 0-day mouse 36 days after inoculation. Here necrosis has occurred in the granular cells arrested in their migration through the molecular layer. Hematoxylin-eosin. Magnification x400.

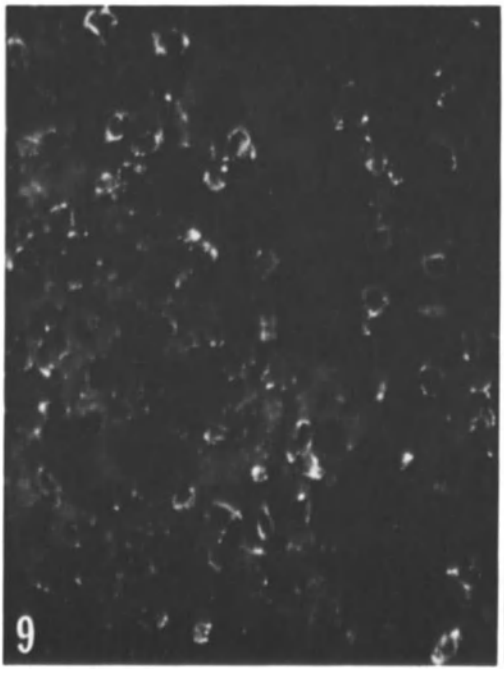
Fig. 8. A perivascular cuff, predominantly of mononuclear cells (with 1 cell in mitosis), in the brain of a 0-day mouse 17 days after inoculation. A few inflammatory cells extend into the surrounding brain. After this time infiltrates became increasingly mononuclear in character. Hematoxylin-eosin. Magnification x400.

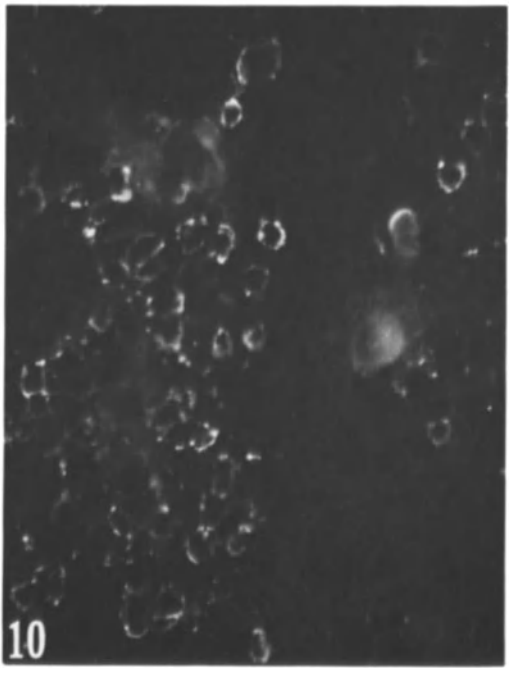
Fig. 9. Immunoglobulin-containing cells in the cerebellar granular layer of a 0-day mouse 2 months after inoculation. Fluorescence is cytoplasmic and generally discrete. Fluorescein-conjugated anti-mouse immunoglobulin. Magnification x400.

Fig. 10. Fixation of guinea-pig complement in the cerebellar granular layer of a section adjacent to that in Figure 9. Guinea-pig complement followed by fluorescein-conjugated anti-guinea-pig serum. Magnification x400.

strikingly located in the cytoplasm of neurons. Staining had either a finely granular pattern or more diffuse cytoplasmic fluorescence.







Immunoglobulin-containing cells occurred in sites where viral antigen had been located, including cortex and granular layer of cerebellum (Fig. 9). Although amount and location of immunoglobulin varied, in general it increased to a maximum about 2 months after infection, then followed the same declining course as viral antigen. All specificity controls confirmed the identity of the reaction as mouse immunoglobulin. Cellular staining did not occur with fluorescein-conjugated anti-mouse albumin. Fixation of exogenous complement occurred with the same distribution as antigen and immunoglobulin staining (Fig. 10). Fluorescence was again finely granular, sometimes diffuse, and cell processes could be traced by punctate staining of cytoplasm. Uninfected brain sections were negative; phosphate-buffered saline or inactivated complement abolished staining when substituted as the first layer. Fluorescein-conjugated anti-mouse B_1C globulin also produced a similar pattern of staining, primarily in the cerebellar granular layer, though not as extensive as with the guinea-pig complement method. Uninfected sections were negative, but satisfactory specificity controls for this reaction were not available.

After incubation of these brain sections with citrate buffer for either 60 min at room temperature or 18 h at 4°C, globulin staining was almost completely eliminated, while adjacent sections similarly treated with phosphate-buffered saline were unchanged.

DISCUSSION

The central observation in these investigations is the high survival rate of mice inoculated within 18 h of birth. It is clear that inability of the virus to replicate within cells is not the basis for host survival under these conditions. Serial virus titration demonstrated that virus multiplication proceeded to even higher levels in the surviving group than in the older, fatally infected animals. Fluorescent staining of viral antigen documented the high proportion of infected cells in the brains of survivors.

The most apparent differences between the 2 groups were the extent and predominant localization of histopathological damage. The concentration of injury in relatively non-vital areas of the 0-day group (cerebellum and hippocampus) contrasted with more severe destruction in the brainstem and spinal cord of the 2-day mice. Differential susceptibility of the cerebellum to injury with age at inoculation was shown by Monjan et al. (13) in LCM virus infection of neonatal rats. Focal cerebellar necrosis was reported by Borden et al. (1) in Tacaribe virus infection, though the effect of age on development of the lesions was not stated.

Although the amount of viral antigen in the cerebellum was greater in 0-day than 2-day mice, an equivalent amount of fluorescence was present in more functionally critical areas, such as brainstem and midbrain. The more acute and extensive inflammatory response in these areas of the 2-day group suggested the importance of host reaction in the development of disease. The key role of host response and immunological status has been demonstrated in animals infected with several members of the arenavirus group. In LCM (7,11), Machupo (10), Junin (17), and Tacaribe virus (1) infections, persistent viral carriage with absent or decreased host response has been found by inoculation of the natural host or by immunosuppressive maneuvers.

The character of the histological lesions is not sufficient basis for separating cellular and humoral immune mechanisms in the pathogenesis

of acute Tamiami virus encephalitis (15). A possible role of host response to antigen-antibody complexes was suggested by the prominence of neutrophils and eosinophils in many of the acute lesions. Clinical illness and histological damage, however, began at a time when serum antibody was not yet detectable and immunoglobulin staining of brain was not significantly different from controls. Theoretically the acute inflammatory response could result from target cell damage and release of chemotactic factors (2,16) by any mechanism, including viral cytopathic or cellular immune mechanisms. If humoral immunopathologic mechanisms are important in the acute disease, then undetected differences in rate, titer, specificity, or localized synthesis of antibody between the groups would have to be hypothesized.

The most interesting questions raised in this study of murine Tamiami virus infection concern the mechanism of virus suppression and antigen elimination at the cellular level. Unlike the typical pattern in mice neonatally infected with LCM virus (8), Tamiami virus-infected mice developed CF and neutralizing antibodies and infectious virus was eliminated by 2 months of age. The correlation of antibody appearance with virus decline and the presence of immunoglobulin in infected cells strongly suggests a role for humoral immunity in the recovery phase of illness. Lack of staining with fluorescein-conjugated anti-mouse albumin indicated that the intracytoplasmic globulin did not result from non-specific leakage into damaged cells. Furthermore, the identification of the intraneuronal globulin as specific antibody was strengthened by the finding of both endogenous and exogenous complement fixation and elution of immunoglobulin from coincident sites of acid-treated sections.

Intracellular antigen-antibody complexes have been noted in at least 2 human diseases. Ter Meulen et al. (12) reported the occurrence of antigen-antibody complexes in neurons and glia of human brains infected with the measles-subacute sclerosing panencephalitis (SSPE) agent. More recently, Nowoslawski et al. (14) have demonstrated complexes of Australia antigen and immunoglobulin in both hepatic and extra-hepatic tissues from human cases of hepatitis and liver cirrhosis. In both of these instances continuing tissue damage was apparent. In Tamiami virus infection, on the other hand, the appearance of intracellular immunoglobulin coincided with the period of clinical recovery. There was some evidence of continued host reaction, such as perivascular and meningeal inflammatory cells and glial nodules, in the surviving 0-day animals. Similarly, the site of most striking complex formation, the cerebellar granular layer, was also the site of most extensive tissue destruction. The absence of appreciable damage, however, in areas of brain, such as cerebral cortex, where immunoglobulin-viral antigen complexes were found, also weighs against a deleterious role for the complexes.

Although antibody may be important in suppression of infectious virus, the disappearance of both viral antigen and neuronal immunoglobulin over the succeeding months remains unexplained. Whatever the mechanism of antigen clearance in the late stages of infection, there was little evidence of the extensive cellular reaction that should be necessary for the task of removing the infected cells. A slow, random removal, however, or a simple outfall of neurons without host response might be difficult to appreciate histologically.

ACKNOWLEDGMENTS

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Behavior of Machupo and Latino Viruses in Calomys callosus from Two Geographic Areas of Bolivia

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SUMMARY

Latino virus, a new member of the Tacaribe complex, was isolated from Calomys callosus, the rodent host of Machupo virus. Laboratory colonies of Calomys designated SJ (Machupo virus) and JL (Latino virus) were established with animals from their respective virus-specific areas of Bolivia. Newborn SJ animals gave a split response to infection with Latino virus; some had persistent viremia while others developed complement-fixing antibody and cleared virus from their blood. When these animals were super-infected with Machupo virus as adults, they evolved a similar pattern of split response unrelated to prior type of Latino virus infection.

Adult JL Calomys inoculated with Latino virus had immunofluorescent antibody, but no viremia or anemia 60 days later. Machupo virus-infected animals from this colony cleared virus from their blood more slowly and a few still were viremic and anemic 5 months after infection. Infection of suckling animals with Machupo virus resulted in uniform viremia among SJ rodents, whereas about half of the JL Calomys eventually were non-viremic. Latino virus was cleared from the blood by all JL Calomys after 4 months, but by only half of the SJ animals. Anemia was Machupo virus-specific rather than host-determined. Responses to both viruses in F_1 hybrid rodents inoculated as newborns were strongly related to origin of the male parent. The data indicate that factors specific to both virus and host influence the outcome of infection by related arenaviruses in a single rodent species. They also suggest that control of human disease caused by Machupo virus cannot be achieved by introduction of Latino virus or its geographic race of Calomys callosus.

INTRODUCTION

All but one of the Tacaribe-complex viruses are highly adapted rodent parasites, although 3 of the agents (Junin, Amapari, and Pichinde) have been isolated from more than one rodent genus (8,10,11). Machupo virus, causative agent of Bolivian hemorrhagic fever, is transmitted directly to man by contamination of the environment with virus contained in excreta of the rodent Calomys callosus (4). The mechanism apparently depends on the common occurrence of chronic viremic infection of the rodent accompanied by prolonged viruria. Several years ago,

we obtained wild Calomys laucha rodents from Venezuela and were able to establish a breeding colony. In Argentina this species is a major reservoir host for Junin virus, an etiologic agent of hemorrhagic fever. Our purpose was to compare the biology of Machupo virus in this relative of its natural host and to delineate the behavior of the Junin virus in its taxonomically homologous rodent. To our surprise, Junin virus did not induce chronic infection in the Venezuelan rodent when given before 3 days of age. No acute illness was observed and circulating antibody was formed following peripheral inoculation of both newborn and adult animals. Since the virus employed was a mouse-passaged strain not pathogenic for guinea-pigs, as are low-passage field isolates, we were not sure whether this pattern was attributable to unknown virus or host factors.

In 1965, we recovered 18 strains of a virus sharing CF antigens with members of the Tacaribe complex by inoculation of spleen and kidney suspensions into suckling hamsters. All isolates were from Calomys callosus recovered from an area in Bolivia and Brazil about 200 miles from the region known to be endemic for Machupo virus (Fig. 1). Great difficulty was encountered in establishing the identity of the new agent because 1) virus plaques were difficult to obtain and could be elicited at very low efficiency only by prior serial passage in Vero

Fig. 1. Map showing known geographic distribution of Machupo (shaded area) and Latino (stippled circles) viruses in rodent Calomys callosus.

cells, and 2) because specific neutralizing antibody was difficult to produce. After 6 inoculations of virus and adjuvant, hamsters developed homologous neutralizing antibody with titers of only 16, whereas CF antibody reached levels of 8,000 or greater. This agent was finally shown not to induce neutralizing antibody which would interact with any other member of the Tacaribe complex, and a paper naming it Latino virus will appear shortly (13).

Latino virus apparently is non-pathogenic for man. Hemorrhagic fever has not been recognized in its geographic region; no CF antibody was found in human sera obtained from the villages where infected rodents were captured; and persons, both immune and non-immune to Machupo virus, have worked with it in our laboratory for 7 years without clinical incident or development of CF antibody.

This is the first instance in which 2 distinct Tacaribe viruses have been associated with a single rodent. With our Venezuelan Calomys laucha experience in mind, we established separate colonies of Calomys callosus from each virus-specific geographic region. Colonies homologous for Machupo and Latino viruses were designated San Joaquin (SJ) and Juan Latino (JL), respectively. The work here reported was designed to assess the practical possibility of achieving biological control of Bolivian hemorrhagic fever by selective manipulation of the viruses and rodents, to elucidate the basic parameters of Latino virus infection, and to examine the reciprocal specificity of "immune tolerance" between virus and rodent.

MATERIALS AND METHODS

Rodents. The SJ Calomys callosus colony was started from 6 breeding pairs brought to the laboratory of the Middle America Research Unit from San Joaquin in 1963 (7) and now is in the 21st generation. The JL colony was initiated from 2 breeding pairs obtained in 1970 and is in the 7th generation. After F_1 litters were established, the parent animals were examined exhaustively for arenaviruses and found to be negative. Samples of sera from each colony were collected at periodic intervals for antibody determinations to 14 murine viruses; none was found. Animals from both colonies have been classified as Calomys callosus by Dr. Ronald Pine of the Smithsonian Institution. Chromosome patterns of male animals from each colony, made by Dr. Robert Baker of Texas Tech University, were identical. Females from the SJ colony were bred with JL males and vice versa, and the offspring rebred with success.

Viruses. The Carvallo strain of Machupo virus obtained from human spleen and strain 10924 of Latino virus recovered from the kidney of a Calomys callosus rodent were used in all experiments. The viruses were employed at the 2nd and 5th hamster brain passages, respectively. Stock pools were diluted so that approximately 1,000 suckling hamster LD50 units were contained in inocula of 0.03 or 0.1 ml which were administered by intraperitoneal route to suckling and adult rodents. Assays for Machupo virus in blood of infected animals were carried out by a plaque technique in Vero cell monolayers (3). The presence of Latino virus was detected by intracerebral inoculation into suckling hamsters. Quantitative studies were not done for the latter agent. Representative isolates from each experiment were reidentified by passage in suckling hamsters and assay of brain suspension in a standard CF test using hyperimmune anti-viral mouse ascitic fluids or hamster sera.

Measurement of anti-viral antibodies. Neutralizing antibody to Machupo virus was determined by a plaque test as described previously (12). Reduction of plaque numbers by 80% or greater was taken as evidence for antibody. Latino virus CF antibody was measured by overnight incubation at 4°C of serially diluted serum with 2 exact units of complement and 4 units of hamster brain antigen. Infected Vero cells served as source of antigen for assay of immunofluorescent antibody to each virus (9). Monolayer cultures were infected and 3 (Machupo virus) or 11 (Latino virus) days later cells were dispersed with EDTA, suspended in Eagle's medium containing 10% fetal bovine serum and transferred in 8 discrete "spots" to glass slides. These were incubated at 37°C in a humidified incubator having 4% CO_2 atmosphere in order that the cells would adhere tightly to the glass. The slides then were fixed and stored in acetone at -60°C.

An antiserum to Calomys γ-globulin was prepared in rabbits. The globulin was obtained by Pevicon block electrophoresis, emulsified with complete Freund's adjuvant and repeatedly injected into muscle of rabbits. Antiserum was harvested when immunodiffusion tests showed equilibrium in mixtures containing 2 volumes of antiserum to 1 volume of Calomys globulin. The antiglobulin did not contain antibody to serum albumin detectable by immunoelectrophoresis. Rabbit antiserum was fractionated with A-50 Sephadex and conjugated with crystalline fluorescein isothiocyanate according to Dedmon et al. (2) at an F/P ratio of 1:20. Sephadex G-25 and mouse tissue powder were used in the indirect immunofluorescence technique for detection of antibody in Calomys callosus sera to both Machupo and Latino viruses.

RESULTS

Sequential infection of SJ Calomys with Latino and Machupo viruses. Thirty-eight animals, 1 to 4 days of age, were inoculated with Latino virus. Four months later, 9 of these were viremic and had no detectable CF antibody, but 19 had developed CF antibody (many to titers of at least 256) and had no discernible virus in the blood. Three weeks later, 5 viremic and 5 non-viremic rodents were given Machupo virus. After a further 60 days, only 4 of the 9 survivors had neutralizing antibody for Machupo virus, the expected result when this agent is administered to previously uninfected adult SJ Calomys. Machupo virus-immune individuals were found in both types of Latino virus responders, showing that infection with the latter agent had no readily apparent effect on outcome of Machupo virus infection.

Infection of adult JL Calomys. As reported elsewhere (5), Machupo virus in SJ Calomys induces either chronic viremia without neutralizing antibody or an "immuno-competent" infection wherein virus is eventually cleared from the blood and antibody becomes detectable. The first type of infection is accompanied by continuous urinary excretion of large amounts of virus which leads finally to infection in man. Non-viremic rodents, even if chronically infected, shed little or no virus in urine. Thus it seemed likely that chronic viremia was a good indicator of rodents having virus vector potential. Sixteen JL animals, between 4 and 11 months of age, were given Latino virus and 14 age-matched animals received the Machupo agent. Rodents were bled at 30, 60, 83, 120, and 133 days, then sacrificed and spleen weights were determined. Virus, antibody, and hemoglobin patterns for these animals are shown in Table 1. No animal had circulating Latino virus at 60 days, nearly all had strongly reacting immunofluorescent antibody, and there was never any anemia; mean spleen weight of this group was normal (0.077 g). Machupo virus, in contrast, persisted for many weeks; even at the termination

Table 1. Some parameters of response in adult JL Calomys callosus following infection with Machupo and Latino viruses

Days	Machupo virus				Latino virus			
	Hgb g/%	Viremia	Antibody[a] N	IF	Hgb g/%	Viremia	Antibody N	IF
30	12.7				15.7			
60		5/14[b]	5/14	14/14		0/16	n.t.[c]	15/16
83	15.0				15.6			
120		2/12	9/12			0/14		
133	15.2	2/11	9/11	10/11	17.2		n.t.	13/13

[a] N: neutralizing antibody; IF: immunofluorescing antibody.

[b] Number positive over number tested.

[c] Not tested.

of the experiment, 2 animals were viremic and without detectable neutralizing antibody. Initially, all these rodents displayed mild anemia. At the time of sacrifice, the 2 viremic animals had hemoglobin values averaging 12.5% and spleens that weighed 0.400 g.

Reciprocal infection of suckling SJ and JL Calomys with Machupo and Latino viruses. The fact that most adult JL animals were able eventually to suppress Machupo viremia suggested that these rodents might either 1) not transmit the virus from generation to generation, or 2) continuously transmit virus, but with much less urinary excretion of it into the environment. Virus behavior in suckling animals was thus important to establish. To do this, groups of approximately 70 animals from each colony were inoculated with the respective viruses during the first 3 days of life. Between 10 and 15 individuals taken from different litters of each group were sacrificed for study at the intervals shown in Figure 2. Viremia, as expected, was uniformly pre-

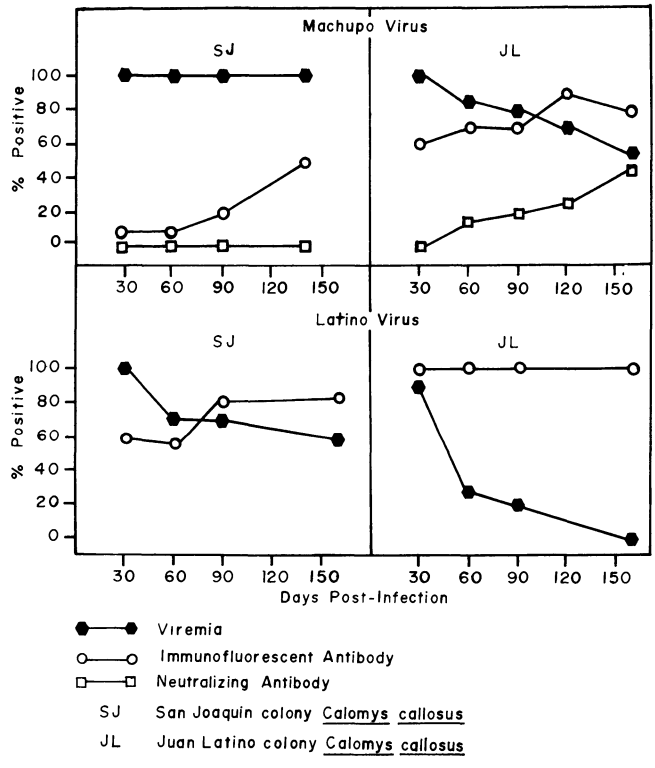

Fig. 2. Response of newborn SJ and JL Calomys to infection with Machupo and Latino viruses.

sent in the homologous Machupo-SJ combination. A few animals finally showed barely detectable immunofluorescent antibody. Some of the Machupo-JL animals eventually suppressed viremia, and immunofluorescent antibody in this group was more common and more strongly reactive than in the Machupo-SJ group. Latino virus also was cleared more quickly from the blood by JL than by SJ rodents. Immunofluorescent antibody was produced by nearly all JL animals and by many of the SJ Calomys. In general, the intensity of staining was greater than that seen with Machupo virus infection.

Anemia and splenomegaly were virus- rather than host-associated. Latino virus failed completely to induce these changes, but chronic viremic infection by Machupo virus did so in both groups (Table 2).

Table 2. Mean spleen weights and hemoglobin concentrations in SJ and JL Calomys infected as newborns with Machupo and Latino viruses

	Days	Machupo virus			Latino virus	
		SJ	JL		SJ	JL
			Viremic	Antibody		
Spleen weights g	30	0.403	0.346	--	0.089	0.076
	60	0.201	0.114	0.060	0.066	0.070
	90	0.243	0.192	0.106	0.104	0.073
Hemoglobin g/%	30	9.8	10.5	--	14.6	14.7
	140-160	10.6	10.6	13.3	14.7	15.1

Infection in suckling SJ-JL hybrid Calomys. As part of our assessment of the species identity of the SJ and JL colonies, cross-breeding experiments were carried out which proved reciprocally successful. When Machupo virus was administered to suckling hybrid rodents, it was found that the response pattern was strongly influenced by the male parent. Thus, by 120 days after infection, 5 of 10 offspring of JL males mated

with SJ females had neutralizing antibody and no viremia, anemia, or splenomegaly. Eight of these rodents had immunofluorescent antibody. In contrast, no offspring of the SJ male-JL female cross ever developed neutralizing antibody, and immunofluorescent antibody was not detected until 120 days. All were viremic, anemic, and had large spleens. Latino virus patterns also were male-directed. Response of JL male-SJ female offspring resembled that of pure JL rodents in that viremia was cleared in every instance by 120 days, whereas all 3 of the SJ male-JL female hybrids available for testing were viremic at that time. Immunofluorescent antibody was regularly produced in both groups and there was no anemia.

DISCUSSION

These data permit small prospect that human disease caused by Machupo virus can be effectively controlled by introduction of either Latino virus or its homologous race of Calomys callosus into the endemic region of Bolivia. Latino virus infection established early in life caused no discernible change in response of SJ rodents to Machupo virus and the chronic viremia exhibited by many of the JL animals, particularly those infected in the first days of life, strongly suggests that Machupo virus would continue to survive if the JL race were imported into the San Joaquin area. The fact that hybrid response to Machupo virus was apparently sex-linked only reinforces this conclusion.

Although we did not put the matter to direct test, results of these studies also raise the possibility that endemic Latino virus infection may afford no protection to the Calomys callosus of the southern Bolivian plains should Machupo virus be introduced into that region. This point may soon become something more than a virologist's hobby since road and rail links between Santa Cruz and Beni Provinces of the country are now under construction and the increased flow of human commerce that must ensue will surely increase the risk of such an occurrence. Should the virulent virus gain access to Santa Cruz, Bolivian hemorrhagic fever could reliably be predicted to appear in hundreds to thousands, rather than the current tens, of cases annually because this province is the active growing point of contemporary Bolivian agriculture.

This study did, however, provide some new information concerning the behavior of Tacaribe viruses in natural rodent hosts. In the first place, we obtained strong evidence that the capacity to cause anemia and splenomegaly in Calomys is virus-specific. Machupo virus elicited this response in animals from both colonies, Latino in neither, despite the fact that the latter virus did produce chronic viremic infection in many SJ animals. Thus anemia and splenomegaly could not be attributed to the presence in Calomys of a latent microbial agent which was activated during infection by any given arenavirus. Such a possibility based on an unknown but specific attribute of Machupo virus is, of course, not eliminated. It also might be argued that the erythropoietic lesion caused by Machupo virus is due to the presence of an unrecognized pathogen derived either from the human from which the virus strain employed was originally recovered or from the suckling hamsters in which it was twice passaged. This is unlikely since we have been able to produce anemia in young Calomys with strains of virus derived from man and maintained only in monkey cell cultures, and from unpassaged infected wild Calomys tissues (6).

If the colonized JL Calomys are representative of natural populations

(and caution is necessary because they were initiated from very few animals), then we must conclude that chronic infection with persistent viremia does not in fact characterize the natural ecology of all arenaviruses. A pattern similar to that of Latino virus in Calomys callosus also has been found for Tamiami virus in Sigmodon hispidus (1).

The split response displayed by suckling JL animals infected with Machupo virus provides another potential comparative model, supplementing that of adult SJ rodents inoculated with this agent. The detection of immunofluorescent antibody in viremic animals of both colonies infected with each virus suggests that strict immunological tolerance is probably not the rule in any of these host-parasite pairings. Moreover, the stronger reactions obtained with Latino virus might tempt the speculation that infection with this agent may be accompanied by greater antibody-mediated immunopathology than that with Machupo virus. But we do not yet have any definite evidence that either virus produces such disease; and the observed difference in immunofluorescent antibody may be due to greater accumulation of antigen in Latino virus-infected cultured cells used in the tests rather than to qualitative or quantitative differences in antibody production.

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Lassa Fever Virus

Chairman: J. Casals

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INTRODUCTION

Lassa fever is a virus disease of man characterized by generalized organ involvement resulting in manifestations of myositis, myocarditis, pneumonitis, encephalopathy, and signs of kidney involvement and hemorrhagic diathesis.

HISTORY

The disease was first described as seen in early 1969 in a missionary nurse stationed in a locality in northeastern Nigeria, Lassa; she was evacuated to a hospital in Jos, Benue-Plateau State, Nigeria, where she died 30 h after admission. Two other missionary nurses at that hospital, who took care of the first, developed the disease in rapid succession; one died 14 days after onset, the other was moved to the U.S.A., where after a severe course requiring 2 months hospitalization she finally recovered.

In June of 1969, an investigator contracted the infection while working in the laboratory with the newly isolated virus; after an initial severe period lasting 5 or 6 days followed gradual improvement, and he was discharged after 30 days hospitalization, fully recovered. Later in the year, a technician in the same laboratory contracted the disease and died; he was at no time involved in research with the virus and had no known contact with it.

In January, 1970, a restricted but severe outbreak occurred in Jos, seemingly hospital-centered; 28 persons were affected with 13 deaths, including among the latter an attending physician. The index case, hospitalized in the last days of December, 1969, acquired the disease in another town; the source of her infection was not determined.

The last episode, at this writing, took place at Zorzor, Liberia, in March, 1972. The outbreak involved 10 persons of whom 4 died and was strikingly similar to that of 1970 in the fact that all the patients were connected with a hospital and only the index case had acquired the disease elsewhere.

THE VIRUS

Owing to the risk involved and to restrictions imposed on work with the virus, its properties have not as yet been investigated in depth;

strains of laboratory-propagated virus are now available only at the Center for Disease Control, Atlanta, Georgia.

Morphology

Electron microscopy of African green monkey kidney cells in cultures (Vero) infected with the virus reveals the presence of pleomorphic particles of variable size. In thin sections of packed sedimented cells are seen extracellular particles of diverse sizes and shapes; among the smaller are round particles surrounded by an electron-dense limiting membrane, containing a variable number, up to 8 or 10, electron-dense granules. On the surface of the particles are seen projections or spikes (20). Intracellular particles are observed in what appear to be either vesicles or extracellular invaginations; the presence of particles right under the limiting membrane, which appears thickened and as though pushed out, suggests that the virion may reach its extracellular stage by budding. While no size was stated for the particles, it appears as though the smaller ones may have been of the order between 70 and 80 nm, which estimate agrees with that of filtration studies (4).

Biochemical Properties

Replication of the virus in Vero cell cultures is not affected by the addition of bromodeoxyuridine (BUdR) to the maintenance medium; it appears, therefore, that the virion's nucleic acid is RNA.

Treatment of a tissue culture fluid virus suspension with an initial titer of $10^{7.5}$ TCD50/ml with 0.5% sodium deoxycholate results in complete inactivation of the cytopathogenic activity of the virus. In this respect Lassa virus behaves in a manner similar to that previously observed with Junin virus (15). From this observation it was concluded that Lassa virus contains structural lipids in an envelope; morphological studies seemed to bear out the conclusion.

Betapropiolactone (BP) at a final concentration of 0.1% completely inactivates the virus in the fluid phase of infected Vero cell cultures; a pool of fluid with an initial titer of $10^{6.5}$ TCD50/ml loses its cytopathogenic capacity when held with that concentration of the chemical at 4°C during 24 h. On the other hand, treatment with concentrations of 0.2 and 0.3% of BP fails to affect the CF activity of the fluid.

The infectivity of the virus propagated in newborn mouse brain tissue is also inactivated by 0.15% BP after 2 days at 4°C. In a single attempt made in our laboratory, no infective virus was recovered from an antigen prepared by the sucrose-acetone method from brain tissue of newborn mice harvested on the 7th post-infection day, following exposure to the chemical under those conditions. From experience with similar antigens it seems unlikely that acetone as used would inactivate completely the infectivity of Lassa virus; it is, therefore, assumed that the loss of virulence was due to the action of BP.

Biologic Properties

Natural host range

The virus has been isolated in nature thus far only from man; it has been found in serum, pleural, pericardial, and peritoneal fluids, throat washings and urine. It appears that no other human materials have been tested. Tissues from a number of small mammals - mostly rodents - have been tested for virus, but no isolations have been reported.

Experimental host range

The choice host-system for direct isolation of the virus from clinical specimens, also for experimental studies including sero-epidemiological surveys, is cultures of African green monkey kidney cells, specifically the Vero cell line, either in fluid medium or under an agar overlay. The virus has failed to infect continuous cell lines derived from either Aedes aegypti or A. albopictus (4).

For reasons of safety only limited attempts have been made to determine the susceptibility of laboratory animals to the virus; in fact, only mice of the CD(R)1 Yaru colony, ICR/HaM CDC colony and Webster-Swiss have been used.

Infectivity in Vero cell cultures. Virus isolations from patients have been successfully made in 2 laboratories, Yale Arbovirus Research Unit and Center for Disease Control. The number of patients supplying specimens is 18 and successful isolations have been reported in 17 (4,2, 10). The total number of different specimens tested is 39, of which 29 sera yielded virus in 25 instances; 3 pleural, 1 pericardial and 1 peritoneal fluids all contained virus; 2 throat washings and 3 samples of urine likewise all yielded virus.

Cytopathic changes appear as early as 4 days after inoculation of infected clinical specimens on 1st passage; when attempted, 2nd and subsequent passages are readily accomplished and end-points in titrations are usually reached 7 days after inoculation. The virus in the fluid phase of infected cultures generally has a titer of $10^{7.5}$ TCD50/ml. On monolayers under agar the virus produces distinct plaques about 2 mm in diameter, which develop from the 5th to the 8th day; assays by plaque formation give titers similar to those obtained in fluid cultures by cytopathic changes.

Cytopathic changes, similar in all strains examined, begin as single necrotic cells throughout the monolayer along with minute holes caused by falling out of such cells; also as groups of 10 to 20 cells rounded and granulated localized within focal areas, as well as large empty spaces left by the detachment of such cells. Later, from the 5th to the 8th day, rounding and granulation of about 50% of cells occur with detachment which in a few tubes proceeds to complete destruction of the cell layer. Acid production goes along with cell destruction.

Infectivity for mice. Only limited observations are available. Intracerebral inoculation of the virus into 1-day-old mice generally results in inapparent infection (4,10). In one series of reported tests, inoculation of virus-containing serum from a patient into 20 mice resulted in no apparent ill effects in 19, which survived and were discarded 5 months later; one mouse was found dead on the 9th post-inoculation day. A pool of urine collected on the 83rd day contained Lassa virus with a titer of $10^{3.5}$ TCD50/ml; at that time, a pool of sera from the same mice

had CF antibody with a titer of 32 to 64. A passage of the brain tissue from the mouse found dead on the 9th day by intracerebral inoculation to 1-day-old mice again resulted in no illness during a 30-day observation period, at which time a pool of their sera had CF antibody with a titer of 64 to 128 as well as plaque reduction neutralizing antibody. These results seem to indicate that the virus was propagated in mice for 2 passages. Multiplication of the virus in newborn mice occurs in all the organs tested, with the highest titers in brain, lung, and muscle (10).

Young adult mice, 25 to 30 days old, respond differently to intracerebral inoculation of the virus. In tests in which infected tissue culture fluid was inoculated simultaneously to 1-day-old and to young adult mice, the former developed no illness during a period of observation of 60 days but had virus in the urine on the 46th day. The adult mice remained well for 5 days, and on the 6th day 11 of 15 inoculated mice were found dead; the remaining ones when held by the tail communicated to the hand a characteristic, fine tremor and when spun by the tail went into tonic convulsions, with rigidly extended hind legs and temporary arrest of respiration (4). These signs and the time of their appearance closely resemble those seen in adult mice intracerebrally inoculated with LCM virus and were the first indication that Lassa virus might be related to LCM virus. There is some evidence that the susceptibility of young adult mice to the intracerebral inoculation of the virus may vary between colonies (10).

Cytopathology

Light microscope examination of infected Vero cell cultures stained according to Giemsa clearly shows basophilic pleomorphic aggregates localized in the cytoplasm of single cells or in cells grouped together in foci; subsequently, complete cellular destruction occurs. These cytoplasmic inclusions are similar to those seen in this laboratory (15) in cell cultures infected with Junin virus, which fact led to suspect a possible relationship with the Tacaribe group agents.

Microscopic examination of tissues from weanling mice intracerebrally inoculated with the virus shows alterations only in the brain, consisting of a perivascular and meningeal infiltration by mononuclear cells and diffuse increase in glial cells. Tissues from newborn mice - brain, lung, muscle, kidney, liver, spleen, heart, and thymus - all of which contained virus, show no abnormality under light microscopy, nor is the virion seen by electron microscopy (10).

Antigens

CF antigen. Inoculation of Vero cell cultures with the virus results in its multiplication, measured by the infectivity titer, and also in development of CF antigen in the fluid and accumulated debris. Following inoculation of approximately 200 TCD50 of virus, the infectivity titer of the combined fluid and cell phases increases from undemonstrable virus shortly after inoculation to a height of $10^{8.2}$ to $10^{8.5}$ TCD50/ml on the 4th and 5th post-inoculation days; between the 6th and 9th (when last tested) days the titer is lower, $10^{7.5}$ to $10^{6.2}$ TCD50/ml. In the same materials, CF antigen is first detected on the 3rd post-inoculation day with a titer of 8; the titer rises to 16 on the 4th day and remains unchanged through the 8th day; on the 9th and last day of observation the titer was 8.

Fluid and cellular debris from infected Vero cell cultures can be used as source material for the preparation of a non-infectious CF antigen;

the fluid is first inactivated with BP and, in order to increase its titer, concentrated by dehydration. This type of antigen was employed in the earlier studies of identification of the virus (4).

One- and 2-day-old mice intracerebrally inoculated with 1st and 2nd mouse or tissue culture passages of the virus remain seemingly well; brain tissue from these mice sacrificed on the 7th post-inoculation day contain high titers of CF antigen demonstrable by the standard sucrose-acetone method. In a single attempt in our laboratory, an antigen inactivated with 0.15% BP had a titer of 128; no virus was recovered from this antigen on repeated inoculations of Vero cell cultures, including 2 serial blind passages. Similar brain tissue antigens have subsequently been prepared in another laboratory (10).

The manner of development of CF antigen in the brain tissue of newborn mice inoculated with Lassa virus, closely duplicates observations with LCM virus (9). With the latter, too, newborn mice intracerebrally inoculated remain well but when sacrificed on the 7th post-inoculation day their brain tissue yields, following sucrose-acetone treatment, CF antigens with titers of 128 to 512. It is worth stressing, furthermore, that other members of the arenavirus group, Junin (16), Pichinde, and Tacaribe, also yield their higher titers of CF antigen in the brain tissue of newborn mice when these are sacrificed on the 6th or 7th post-inoculation day, at which time hardly any of the inoculated animals show signs of illness, nor will, if allowed to live, show them for the next 2 to 8 days.

Antigenic characterization. Neutralizing and CF antibodies develop in man during convalescence; mice inoculated with the virus at birth and young adult mice, repeatedly inoculated by the intraperitoneal route, also develop such antibodies. Using sera from these 2 species and CF antigen prepared with infected newborn mouse brain tissue, inactivated with BP, Lassa virus has been compared with agents from nearly all groups of viruses of vertebrates in a Universal System of Virus Classification (23). In particular, Lassa virus has been serologically compared, directly or through cross-reacting links, with practically all known arboviruses and arenaviruses.

Antigen and/or antisera for Lassa virus have failed to react by CF or HAI tests - the latter as applicable - or both, with sera or antigens derived from more than 117 different arboviruses. Since many of the monotypic immune sera or ascitic fluids were prepared with viruses that belong in small or minor antigenic groups, in addition to the ungrouped ones, it can be reasonably stated that the serological comparison covers a larger number of agents than the figure given above. In addition, grouping polyvalent sera or ascitic fluids for groups A, B, C, California, Bunyamwera, Simbu, phlebotomus fever and vesicular stomatitis viruses have failed to react with Lassa virus antigen. Nor have cross reactions been detected with ectromelia, reovirus type 3, herpes, rabies, Newcastle disease, Marburg, simian hemorrhagic fever, mouse encephalomyocarditis, mouse polioencephalitis and mouse hepatitis viruses.

Additional serological tests have been done with the serum from a patient known to have Lassa CF antibody against antigens for influenza A, parainfluenza 1, 2, 3 viruses, adenovirus, Mycoplasma pneumoniae, psittacosis-lymphogranuloma venereum group agents, Q fever, rickettsialpox, typhus, Rocky Mountain spotted fever rickettsiae as well as proteus OX19 and proteus OX2. No antibodies were found in the patient's serum or, if present, they had the same, low titers in two paired samples, the first one of which had no antibodies against Lassa virus (19).

The similarity between Lassa and LCM viruses on inoculation to newborn and adult mice promoted intensive efforts to see whether an antigenic relationship existed between the 2 viruses and between them and members of the antigenic group Tacaribe; the study was done mainly by CF test and to a lesser, but significant, degree by fluorescent antibody technique. It is now established that there is a low-titered, but on the whole reproducible, reciprocal cross reactivity between Lassa and LCM viruses by CF test, provided that highly potent hyperimmune mouse sera or ascitic fluids are used; on occasion, cross reactions are also observed between Lassa sera and Tacaribe group antigens. On the other hand, sera from convalescent persons with titers between 32 and 128 against Lassa antigen, have failed consistently to react positively with LCM antigen. Results of a number of CF tests are combined and given in Table 1.

By means of the fluorescent antibody technique, two laboratories (7,17) independently have shown cross reaction between the serum from a Lassa fever convalescent and LCM virus; the observation has been easily confirmed in this laboratory (3).

To the extent to which they have been investigated, and only by CF test, all strains of Lassa fever virus have been found alike.

Taxonomy

On the basis of morphology, morphogenesis, RNA content, and susceptibility to sodium deoxycholate indicating the presence of essential lipid constituents in the envelope, Lassa virus is included in a group designated arenaviruses (18). In addition to the morphological and biochemical similarities with all the viruses of this group, Lassa virus shares common antigens with LCM virus and, to a lesser extent, with other agents of the group.

THE DISEASE

Clinical Features

The incubation period is generally estimated to be between 7 and 16 to 18 days; in 1 case, it has been definitely established to have been 6 or 7 days. The onset is insidious; sore throat is often the first symptom, followed by generalized muscle aches which when localized in the thigh can be extremely painful on locomotion; very soon also appear headache, asthenia, lassitude, malaise, abdominal pain, loss of appetite, vomiting and diarrhea. Fever develops very soon and can later reach 40.0 to 41.7^{o}C; it is accompanied by somnolence, indifference and blurred vision. On physical examination the patient appears very ill, toxic, listless, but with little to account for the obvious marked severity of the illness. The temperature is at this time in the 38.9 to 40.0^{o}C range; a few petechiae may be seen on the chest or abdominal walls, but the infection, although it has a definite bleeding diathesis, at no time seems to have been so severely hemorrhagic as those caused by other arenaviruses. Marked pharyngitis is apparent, with white patches in the pharynx, soft palate and tonsillar pillars; ulcerations of the buccal and pharyngeal mucosae can occur; the tongue is dry, coated. Voluntary movements and to a lesser extent passive ones can be painful. At this time the blood pressure is adequately normal and the pulse has a somewhat increased rate but is, generally, regular and full. Diarrhea, dysphagia, and slight dysuria are common.

Table 1. Arenavirus group: complement fixation tests with mouse hyperimmune sera or ascitic fluids

Antigen	Serum											
	Lassa			LCM								
	1	2	3	1	2	3	AMA	JUN	PICH	TAC	TAC group	Controls[b]
Lassa	256[a]	256	256	4	2		0	0	0	0	0	0
LCM	16	16	4	256	256	256	4	0	4	0	2	0
Amapari	4	16	2			4	256				128	0
Junin	4	2				8		256			128	0
Pichinde	0	0							256		16	
Tacaribe	4	4	0			8				256	256	
Tamiami	0	2									32	0
Controls[b]	0	0	0	0	0	0	0	0	0	0	0	
Homologous												128-512

[a] Reciprocal of serum titers; 0, no fixation at dilution 1:2 or 1:4.

[b] Control antigens and sera prepared with viruses outside this group. (Six different sera, 3 different antigens tested.)

When the disease is progressive, the patient becomes increasingly toxic, indifferent, almost lethargic; there appears a suffusion or flush of the skin of face and thorax, the face is puffed, the neck seems swollen, the vision is blurred and the tongue is very dry, heavily coated in the middle, red and cracked on the margins. There is also an increased dysuria and moderate oliguria. Additional petechiae appear, sometimes slightly larger subcutaneous hemorrhages on the arms, abdominal wall or legs; pleural effusions may occur, causing acute costo-vertebral pains. In fatal cases there is a sudden circulatory collapse; the blood pressure which up to now was normal falls to as low as 60 to 80 systolic. Death is generally attributed to cardiac failure; in 1 case the immediate cause of death was acute laryngeal edema (2,8,13,22). Patients who recover do so gradually after long convalescence. Recovery seems to be complete with the exception that there may have been some impairment of hearing with partial deafness in some survivors.

There is definite evidence that infection with the virus also occurs in a mild, perhaps even inapparent form (see Epidemiology), particularly in children; in the course of a well investigated epidemic in 1970, 4 persons were found to have high titer CF antibody shortly after they had minor complaints that required no hospitalization.

Too little is as yet known about the overall pattern of infection of man by the virus in nature to appraise the risk of severe and fatal disease following exposure. Clinically diagnosed, hospitalized cases, with laboratory confirmation either by isolation of virus or development of antibodies, have a serious prognosis; to this date, 39 such cases have been recognized of which 20 have died. The disease has been particularly severe when occurring in hospital or laboratory Caucasian staff, with 5 deaths of 7 hospitalized persons.

Following the disease man develops neutralizing and CF antibodies. The antibodies are relatively late in appearing, being first noted between the 20th and 25th day from onset. The titer of CF antibody reaches a level between 32 and 128 in persons who have survived; in one survivor, the CF titer was 16 after 3 years from onset, in another also 16 when last tested 18 months later.

Clinical Laboratory

The blood initially reveals moderate leukopenia, 2,500 to 4,000 leukocytes/mm^3, with relative lymphopenia, 10 to 12%, moderate shift to the left, monocytes elevated to 10 to 13%, and 5% eosinophils; in the few reported observations platelets were found near normal in one case, markedly reduced, 60,000/mm^3, in another. Later, the leukocyte count returns to normal or is elevated, between 11,000 and 37,000. Erythrocyte sedimentation rate has been within normal limits early, from 2 to 8 mm/h, increased later to 40 to 80 mm/h. Hemoglobin, hematocrit and erythrocyte count have been within normal limits; results of blood-clotting studies, including prothrombin time, venous clotting time, and thrombin time are either within normal limits or moderately elevated, but detailed studies are reported in only few instances. The reported results of blood chemistry studies, also in few patients, show essentially normal values for serum calcium, phosphorus, and cholesterol and normal protein electrophoresis pattern; blood urea nitrogen is either normal or slightly elevated with maximum values of 32 to 44 mg/100 ml. Serum enzymes are considerably increased: lactic dehydrogenase, 715 to 1,025 µg/ml; glutamic-oxaloacetic transaminase (SGOT), 425 to 1,132 µg/ml; and creatinine phosphokinase, 648 units.

The urine shows protein, from 2 to 4 plus, a few red and white blood cells and granular casts. Electrocardiograms have been abnormal due to axis and intra-atrial block and lowered T-waves (8,13,22).

Diagnosis

A specific diagnosis is made only on the basis of isolation and identification of the virus or of development of antibodies between early and late samples of serum. Virus has been easily isolated from the blood between the 3rd and 14th day from onset and from urine and throat washings. Since this virus has no known close antigenic relatives, development of either CF or neutralizing antibodies between 2 paired serum samples is considered diagnostic; it should be remembered, however, that antibodies do not appear until the middle or end of the 3rd week after onset.

A presumptive diagnosis of Lassa fever can be made clinically on the basis of a severe, toxic and progressive illness with compatible signs and symptoms as described in a patient residing in certain geographic areas; the disease can, however, be confused with various other infections that bring about a toxic febrile condition such as typhoid fever, typhus, certain types of malaria, and viral pneumonitis with viremia.

Treatment

As this is presently considered an extremely dangerous virus, causing a severe disease, particularly among medical and nursing staffs, it is imperative that on suspicion of Lassa fever the patient be placed under rigorous isolation and that attending personnel use mask and gown as well as strict precautions for self-protection. Urine, feces, sputum, and nasal discharges from the patient should be treated with strong disinfectants.

The patient should be given supportive measures, particularly intravenous saline infusions, in order to combat dehydration and intravenous feeding; care must be taken to prevent obstruction of the respiratory passages by exudates. Convalescent plasma has been given to 2 patients. In one case (13) transfusion on the 9th day of illness resulted in a seemingly favorable response, with the severe signs subsiding within 2 or 3 days and eventual recovery; in another patient, convalescent plasma given on the 13th day of illness did not prevent death 2 days later (22).

Pathology

Gross examination at autopsy has revealed from moderate to very marked edema and congestion of the lungs with fluid in the pleural cavity; edema and congestion of the larynx has been noted in one case and considered the immediate cause of death. The heart appears normal but pericardial exudate has been observed. The liver is normal or slightly enlarged in size, pale on the surface and on the cut section; petechiae are seen on the gastric mucosa which has also been described as hemorrhagic. Large amounts of dark blood have been found in one case in small and large intestine as well as moderate amounts of peritoneal fluid. Kidneys have shown congestion, are hemorrhagic at the cut surface, and in one instance the cut surface was gelatinous. Brain edema has been reported. Petechial hemorrhages are seen on the skin of neck, face, shoulders, and back.

On microscopic examination severe involvement has been detected in lungs, liver, intestinal mucosa, spleen, and lymph nodes. In the lungs is noticeable acute interstitial pneumonitis with edema and congestion. The liver shows severe acute fatty degeneration in the parenchyma with most lobules presenting necrotic cells surrounded by polymorphonuclear leukocytes, located centrally, mid-zonally, and periportally. Striking, edematous changes have been observed in the submucosa of the intestine with disruption of normal architecture due to the presence of much fluid, with few cells. Congestion of the spleen with greatly atrophied malpighian bodies is observed; there is widespread involvement of the lymph nodes with atypical hyperplasia or with follicles strikingly depleted of lymphocytes with, in their place, prominent reticulum with many histiocytes (2,6,8).

EPIDEMIOLOGY

Reservoir and Portal of Entry

The naturally acquired disease has been reported in Nigeria and Liberia; on the grounds of circulating antibodies the infection has retrospectively been reported in Guinea. The reservoir is unknown as is the manner of transmission to man; once a human case has appeared, secondary cases arise, very likely by person to person transmission and, judging from the timing, even tertiary infections. It is not definitely established how the transmission from person to person takes place; the fact that the virus is present in throat washings in the acute stage of the disease, probably even during the prodromal stage, offers good grounds for suspecting upper respiratory or digestive portals of entry, particularly since one of the early symptoms is sore throat with definite pharyngitis and tonsillitis.

Contamination of food and drinks and household objects must also be considered as a possible source, for the virus has been found in the urine of a patient 32 days after onset, far longer than in the throat.

Laboratory Infections

Accidental infections have not settled the question of route of entry. In one case the patient developed the disease while working with infected mice; the presence of the virus in the urine of the animals with consequent contamination of the cage bedding and inhalation of dust points to the upper respiratory route since the patient definitely had a sore throat. However, this patient might also have been infected through the cutaneous route as it was later established that, at the time when the infection occurred, he had several small cracks in the periungual skin-hangnails (13). In the second laboratory-acquired infection, no known contact was established between the patient and the virus, except that virus suspensions in plastic tubes were held in an electrical deep freezer which was used for other purposes as well; the patient did at no time work with the virus (2).

Two other patients, both of whom died, related a history of possible transcutaneous infection. A nurse taking care of a patient with the disease used a gauze dressing wrapped around her finger to clear the secretions from the patient's mouth; she realized at the time that there was a small cut in her finger. The other patient was a physician who cut herself while performing an autopsy in a victim of Lassa fever. These 2 patients, however, had also been in close contact with hospitalized persons suffering from the disease (8,22).

Outbreaks

The 3 described outbreaks of Lassa fever have been hospital-associated. The index case, having acquired the disease elsewhere in an unknown manner, is brought to a hospital; within 10 to 20 days appear a crop of cases involving other patients, hospital staff and bedside visitors. In one of the outbreaks was also observed, still later, another cluster of infections, tertiary cases, affecting relatives of the previous patients; these tertiary cases were milder.

In the initial episode, 1969, the index case was a nurse in a mission hospital in Lassa, northeastern Nigeria; she was evacuated to a larger hospital in Jos, Nigeria, where 2 other nurses who took care of her were taken ill on the 7th and 10th to 12th day, respectively, after her admission. No additional cases were reported at Lassa, Jos or at the hospital in New York where the surviving nurse of the 2 contacts was evacuated (8).

In the 2nd and thus far largest outbreak were involved 28 patients, 24 of whom were hospitalized. The index case was admitted to a hospital at Jos on December 30, 1969, with a febrile, progressive illness which became severe during the next few days; she, however, recovered and was discharged on January 12, 1970. The illness was diagnosed as Lassa fever nearly 1 year later, on the basis of a positive test for antibodies and the clinical history. Between January 11 and February 7, 1970, 23 cases were clinically diagnosed and hospitalized at the same Jos hospital, except 3 that were hospitalized in a nearby town, Vom. These cases appeared in 2 groups, by date of onset: 18 occurred between January 11 and 25 and 5 had onsets between February 2 and 7; the latter consisted of a physician and 4 family contacts of earlier cases. A thorough investigation showed that nearly all 18 cases with onsets in mid-January shared only one common experience in the 10 to 20 days prior to their illness and that was an association with the hospital in which the index case had gained admission. The association was definitely established in 15 patients; the other 3 could not be located after dismissal and there was no way of determining whether or not they had had association with the hospital. The 15 patients with hospital association included 4 persons in the nursing staff; the rest were either patients in the hospital for other reasons or relatives and friends who had visited patients in the hospital. The late cases with onsets in February, in addition to the physician, were 3 in one family and 1 in another; in both families there had been earlier, fatal cases; possibly the late cases represent extra-hospital transmissions (5,21).

The last 4 cases in the outbreak were not hospitalized, as they had had only minor complaints at the time when relatives were in the hospital; the infection was diagnosed in them through the chance finding of CF antibody in their blood (5).

The 3rd and, at this time, last outbreak occurred in Zorzor, Liberia, and involved 10 patients. The index case was a maternity patient, 5 months pregnant, who was admitted to the hospital with high fever and severe vomiting; she aborted twins on the day after admission, recovered and was discharged from the hospital 17 days later. The clinical picture as well as subsequent events led an attending physician to suspect Lassa fever; the etiology was established later by the development of CF antibody in the index case. Between March 19 and 25, 9 additional persons connected with the hospital were taken ill and clinically diagnosed Lassa fever; in this number are included 2 other patients in the same ward, both of whom died on the 8th and 15th day of illness, respectively, and 7 of the nursing staff, 6 Liberians and

an American. One of the Liberian aids and the American missionary nurse died. The last patients were discharged from the hospital on April 10 (1,14).

Serological Surveys

In order to determine the distribution of human infection by the virus, either clinical but heretofore undiagnosed or, if it exists, inapparent infection, are required extensive sero-epidemiological surveys preferably by plaque reduction neutralization tests. Similar surveys with sera from lower animals may point to the natural reservoir.

A small survey (4) first showed the feasibility of detecting evidence of past infection with this virus; the results of a larger survey (10) showing the existence of a fair number of past infections are given here in some detail. In the survey were included sera from 712 missionaries who had resided in Africa between 1965 and 1969, representing about 20 different countries with the largest groups being from Nigeria, Ethiopia, Liberia, Zaire and Kenya; plaque reduction tests revealed 5 positives, 1 from a person stationed in Nigeria, the other 4 from a mission at Telekoro, Guinea; 4 of these 5 persons had experienced while residing in Africa a severe illness with symptoms and signs compatible with Lassa fever.

A second group of sera consisted of 458 specimens collected in 1965 to 1966 by a WHO survey team in the area of the Benue river around Makundi, Nigeria, mostly from adults; 10 had antibodies.

The last set, 281 sera, represented several areas of Nigeria and different years. Among 54 sera from itinerant cattle herders collected in 1965, 3 were positive; no sera were positive of 27 obtained from adults at Bornu Ranch, northeastern Nigeria, in 1966. Among sera taken in 1969 in 3 localities on the Jos plateau, 3 of 52 children and 5 of 56 adults were positive. Finally, sera obtained in 1970 in Jos revealed 2 positives of 35 children and 10 positives of 57 adults.

Ecology

The 1969 and 1970 episodes occurred in December-January, which in Jos coincides with the middle of the dry season; the outbreak at Zorzor, in March-April, was also in the dry part of the year. It is said that at that time, when the harvests have been gathered and stored near human habitations, small rodents are seen in appreciable numbers in the fields and near or in houses, often being chased by children, either as a source of food or as a pastime. Certain facts, discussed later, led to consider the possibility that rodents may in some way be involved in the natural cycle of the virus. In attempts to isolate virus from wild caught small mammals, 39 specimens were trapped in and near houses where Lassa fever patients lived (5); among the animals were: Mus musculus, M. menutoides, Mastomys natalensis and Crocidura sp. Tissues from all these animals failed to yield virus, and urine collected from some was also negative. Sera from another 78 small mammals, comprising 48 rodents and 2 hedgehogs, collected in northern Nigeria in 1970, and those from 28 bats caught near Ibadan in 1966, were negative for neutralizing antibody (5).

Human Transmission

There seems to be good evidence to the effect that, once the index case has appeared, further human cases can be explained by person to person transmission, by means as yet undetermined. The problem of how is the disease transmitted from the natural reservoir to man is still completely unsolved.

The morphological similarity and serological relationships between Lassa, LCM and Tacaribe group viruses led early to entertain the opinion that Lassa virus may have a rodent reservoir. Particularly weighty in this respect is the similarity of behavior of Lassa and LCM viruses when intracerebrally inoculated into newborn and adult mice and the fact that Lassa virus persists in the urine of mice, inoculated when 1 day old, for as long as 80 days; evidently a model in which an animal infected at birth develops at all appearances normally and sheds virus in its urine for extended periods, perhaps a lifetime; and when the animal is a domestic or peridomestic pest, with the potential to soil and contaminate food, dust, and household objects, then the model seems to fit a possible natural situation. The opinion is strengthened by similar situations prevailing with the diseases caused by other arenaviruses, particularly Machupo and probably Junin viruses.

It is of interest to speculate about certain similarities between Lassa fever as seen in Jos in 1970 and the disease designated savanna typhus observed in 1940 to 1942 in the areas of the Ubangui-Chari rivers and in Upper Volta (11,12). The disease was described as a toxic hemorrhagic fever of man, causing high mortality and prevailing during the dry season, at which time large numbers of rodents were seen on the harvested fields; the indigenous populations trapped the rodents as food source. At the time, the disease was considered rickettsial in etiology, though the evidence was weak.

PREVENTION

In the absence of knowledge of the natural reservoir and of the mode of transmission to man it is not possible to recommend effective preventive measures; however, on the grounds that there is some theoretical reason for suspecting that rodents may be involved in the cycle of the virus, and that at any rate no harmful effects would ensue, it seems that measures aimed at controlling rodent population and diminishing contact between man and rodent should be advised. Hospitalized patients should be kept under strict isolation and the practices described under Treatment should be rigorously followed, including thorough personal hygiene of patient and attendants.

Medical and nursing personnel in hospitals in African countries where Lassa fever has been recognized or is suspected should be particularly alert to possible reappearances of the disease. Owing to the high mortality in clinically diagnosed and laboratory confirmed cases, it would appear that development of a vaccine should be promoted for use by personnel at high risk in emergency situations.

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