Bundes-Forschungsanstalt für Viruskrankheiten der Tiere, Tübingen a. N.

Further Observations on the Behavior of the Cells in Murine LCM

By

Erich Traub and **Friedel Kesting**

With 3 Figures

An attempt has been made recently in this laboratory to study the immunological tolerance of mice infected congenitally with the virus of lymphocytic choriomeningitis (LCM) at the cellular level using lymph node and embryo cells from tolerant and non-tolerant mice. Lymph node cells (LNC) were chosen because the lymph nodes play an important part in the pathogenesis of murine LCM in particular and in immunity in general. Since the appearance of the first paper on the subject (22) additional observations have been made concerning the behavior of the cells in this disease. They will be reported in the present communication.

It may be concluded from recent work in other laboratories that the principal features of murine LCM as described years ago (14, 16, 17) are strikingly similar to those of murine (5) and avian lymphatic leukemia (3, 11, 12, 13). This is true especially for the natural modes of transmission of the respective agents and their long persistence in the tolerant host (tolerance has thus far been demonstrated in the avian disease only, but there can be little doubt that it occurs in the classical murine disease also). Moreover, there is evidence suggesting that LCM virus itself may cause lymphomatosis in mice under special conditions (18, 23). It is possible therefore that observations made on the virus-host relationship with the virus under study may be applicable in principle to lymphatic leukemia in different hosts and perhaps also to murine mammary carcinoma, in which immunological tolerance may be expected to play a role as well. These diseases are much more difficult to handle experimentally than murine LCM, which appears to be a useful model.

In the earlier experiments it was found that a difference exists between LNC from congenitally infected (tolerant) mice and those of non-tolerant

animals with regard to the way in which they support the multiplication of LCM virus *in vitro*. Whereas in tolerant cells infected in the animal of origin the viral growth curve approached a horizontal straight line, the curve obtained from non-tolerant cells infected *in vitro* resembled a sinus line at least during the first few weeks of cultivation. It appeared, however, that infected non-tolerant cells would acquire the character of tolerant cells upon prolonged cultivation.

The results of further growth curve studies are presented here. The paper includes observations concerning an indirect type of cell injury which occurs in non-tolerant mice infected experimentally and is probably caused by an immunological reaction.

Materials and Methods

The strain of virus used (W) and the mouse stocks from which the cell donors and test animals originated (III normal and III tolerant) were the same as in previous experiments (22). The leukemic mice came in part from a small colony of inbred AkR mice maintained at the Institute for special purposes.

Preparation of tissue cultures: In order to be able to compare the results of different tissue culture experiments in an investigation extending over a period of several years, the method of cultivating the cells (22) has not been changed in principle. There may be other media permitting more abundant growth of murine LNC. Such growth was not desirable, however, because we wanted to keep the cultures for long periods and, consequently, prevent the cells from detaching from the glass. This frequently happens in cultures showing luxurious growth, for instance, of mouse embryo cells and, occasionally, in cultures of LNC from very young mice.

In the earlier experiments, the cell suspensions obtained by fractionated trypsination were pooled before the cells were washed in PBS. Later, better results with regard to cell growth were obtained when the fractions were cultured separately. In the present experiments, the minced lymph nodes of mice without leukemia were trypsinized four times for 10 minutes each in a waterbath of 37° C and the fractions designated as I, II, III, and IV. For every fraction 15 ml. trypsin solution were used irrespective of the amount of tissue present. To hasten the dispersion of the cells, the tissue pieces were gently blown back and forth with wide-mouth Pasteur pipettes equipped with rubber bulbs. With soft lymph node or thymic tissue from leukemic mice, the first two periods were reduced to 5 minutes each and the fourth period omitted because there were often no viable cells left after the third trypsination.

Complement-fixation (c. f.) tests: These were carried out as described previously (20) using potent serum (heated at 56° C for 30 minutes) from hyperimmunized guinea pigs to demonstrate specific antigen in unheated 20 per cent lymph node or spleen extracts made with buffered saline. In tests for c. f. antibodies in spleen extracts from non-tolerant mice, these extracts were heated at 56° C for 30 minutes and then tested against unheated spleen antigen from tolerant mice (titer 1:32 in the presence of guinea pig hyperimmune serum 1:10). The c. f. titers given in Table 1 are expressed as the highest dilutions of 20 per cent extracts giving 4 + reactions with guinea pig hyperimmune serum diluted 1:10.

Infectivity tests and titrations: These were done by intracerebral (i. c.) mouse inoculation as before (22). The titers recorded in Table 1 represent the negative $\log_{10}$ of the number of SD_{50} (SD = symptom-producing dose) in 0,03 gms. of tissue.

Experimental

Growth capacity of LNC from normal, tolerant and leukemic mice

The cultivation of LNC from normal mice is a hazardous enterprise. Because of the small size of the organ, the submaxillary, axillary, subscapular, inguinal and mesenteric lymph nodes were harvested from groups of at least 6 mice of the same age.

In 20 tests made with lymph node tissue from normal mice 3 weeks to 7 months old, in which trypsination fractions I—IV were cultured separately, there was good cell growth in 12 cases, moderate growth in 1 case, poor growth in 5 cases and no growth whatever in 2 tests. Since fractions III and IV often gave the best results, pools of III and IV were subsequently used when normal LNC were needed for other experiments.

Similar results were obtained with LNS from tolerant mice infected congenitally. In growth tests made with 21 groups of tolerant mice 3 weeks to 7 months of age, good cell growth was noted in 8 cases, moderate growth in 6 cases, poor growth in 3 cases and no growth in 4 experiments. LNC from very young tolerant mice 18 to 25 days old furnished the best results. Such cells appeared to grow better than those of normal mice of the same age. In general, fractions III and IV gave better results than fractions I and II.

As would be expected, the growth capacity of LNC from mice presenting the classical picture of lymphatic leukemia was considerably greater than that of normal LNC. The tests were usually made with tissue from one leukemic animal only. There was good cell growth in all 8 tests in which leukemic mice not infected with LCM virus were used. The result was better with fractions II and III than with fraction I. Similarly, of 11 tolerant mice of line 11 presenting the picture of more benign lymphomatosis, described and designated as type II in a previous communication (23), 9 animals furnished well-growing LNC. There was moderate growth in 1 and poor growth in the remaining case. In general, the cell density in the cultures grown from fractions II and III was at least twice as great as in those from fraction I.

In cultures prepared from thymic sarcomas of 5 mice not infected with LCM there was hardly any cell growth from fraction I. Fraction II gave moderate growth in 3 and poor growth in 2 cases. The best result was obtained with fraction III (good growth in 4 cases and moderate growth in one).

Special tests have shown that the poor cell growth often observed in cultures of fraction I and, less frequently, those of fraction II is independent of the number of cells originally present in the cultures. Microscopically, fraction I did not differ so much from the other fractions that this could readily explain the differences in cell growth, which were most marked with thymic sarcoma cells. Cells differing greatly in size were present in the trypsination fractions. It has been easy to differentiate small and large lymphocytes, but virtually impossible to classify the larger cells, from which cell growth appears to start.

*Microscopic evidence suggesting that lymphocytes contain a substance
inhibiting the growth of reticulocytes*

As a rule, cultures with good or poor growth potential could already
be recognized after incubation for 24 hours. In the former, the media
showed a distinct color change towards the acid side (yellow), in the latter
in the alkaline direction (red or purple). At this early stage, well-growing
cultures would show clusters of small lymphocytes attached to larger
cells with many small lymphocytes floating freely in the medium or
loosely attached to the glass. They appeared round and healthy with sharp
contours. In badly growing cultures, the small lymphocytes, intermingled
with cellular debris, were firmly attached to the glass. They appeared
smaller than those in well-growing cultures and most of them would show
signs of severe damage. There were clusters of small, damaged lymphocytes
around larger cells, but these also appeared to be inactive metabolically.

On the following days, more or less extensive outgrowth of cells oc-
curred in the cultures with normal metabolism. The small lymphocytes,
originally present in large numbers, would gradually disappear from the
picture except some attached to larger outgrowing cells and apparently
destined for phagocytosis by the latter. The impression has been gained
that the ingestion of lymphocytes or lymphocytic debris stops the growth
of reticulocytes at a certain point either forever or at least temporarily.
Cultures prepared from trypsination fraction I would frequently be very
active metabolically (yellow in color on the second day of cultivation,
the small lymphocytes appearing normal) and still not show any appreci-
able growth later, the bulk of these cells remaining in the resting state
for weeks. In metabolically inactive cultures, there was either no evidence
of cell growth for weeks or some isolated reticular cells appeared in the
course of time, often phagocytizing the lymphocytic debris in their
neighborhood. Such phagocytosis, however, obviously harmed many of
these cells. They would gradually retract their plasmatic processes and
change into large round cells without showing any evidence of further
growth in the future.

The observations made have raised the suspicion that lymphocytes
contain a substance inhibiting the growth of a cell type considered to be
reticulocytes. It is possibly identical with the substance extractable from
lymph nodes (and other organs) and capable of inhibiting or destroying
reticulocytes in tissue cultures (22).

*Remarks concerning a possible cytopathogenic effect of LCM virus in murine
LNC*

It is practically impossible to identify all cell types present in well-
growing cultures of LNC from normal or leukemic mice. After a few days
of cultivation involving 2 or 3 changes of media, when the bulk of the

small lymphocytes has disappeared, there are round cells of the larger variety and many cells with shoots in one or more directions. They apparently have started to grow but stop to do so at a certain point, possibly for lack of a growth-promoting substance or advent of an inhibiting factor. There is some evidence of organization, even of rudimentary capillary formation, but this is not an important feature. The only cell type which really grows are fibroblast-like cells described previously (22) and believed to be reticulocytes. Some of these cells may gradually change into round cells or shrink, losing most of their cytoplasm without any known reason. Obviously, the morphology of the descendants of the stem cells of the reticulo-endothelial system is just as complex and variable *in vitro* as *in vivo*.

Under such conditions, it is very difficult to identify a slight cytopathogenic effect of a virus (as reference viruses those of EEE and vesicular stomatitis, type "Indiana", were used, which have a moderate and very marked cytopathic effect, respectively, in murine LNC). Such effects reported for LCM virus by others (2, 1) were observed with other cells and with other strains of virus which may have been modified by animal passage. It has been known for a long time that this procedure may quickly change the pathogenic properties of this agent (15). Cell damage by the natural strain W has not been observed in outgrown cells, normal or leukemic. However, it has long been questionable whether or not the virus may cause slight changes in cells infected immediately after trypsination. The bulk of the evidence is against it. The experiments recorded in Fig. 3 clearly indicate that the virus *per se* is harmless for the cells.

Multiplication of LCM virus in normal LNC infected immediately after trypsination or after the outgrowth of cells

Different viral growth curves were obtained from normal and leukemic LNC when infected with culture filtrate either immediately or on the 7th to 14th day of cultivation. A representative experiment is presented graphically in Fig. 1.

The normal LNC used originated from 8 week-old females (stock III). Test tube cultures were prepared from the four trypsination fractions as described in section Materials and Methods. Four cultures of every fraction were infected at once and four on the 14th day of cultivation, the infecting medium consisting of 50 per cent Berkefeld N filtrate of infectious culture fluid from tolerant cells (stored at −20° C) and 50 per cent standard medium as used throughout this work (22).

In the following weeks, the cultures infected immediately showed good cell growth in fractions I, II and III, and moderate growth in IV. Those infected on the 14th day showed moderate growth in I and II and good growth in III and IV.

For comparison, a similar series of cultures was prepared from tolerant LNC infected *in vivo*. There was good cell growth in all fractions, but shrinkage

of reticulocytes (see below) was noted in the second half of the cultivation period.

Periodic titrations of culture fluids were made as indicated by the dots in the curves.
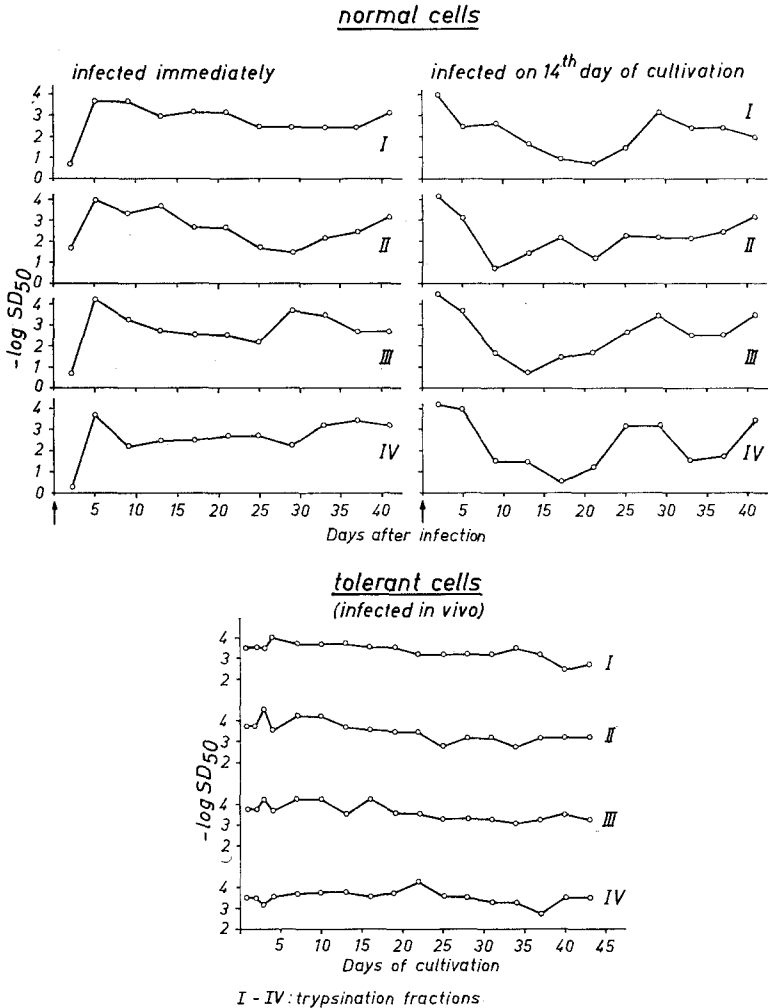

Fig. 1. Multiplication of LCM virus in normal lymph node cells infected immediately after trypsination or on 14th day of cultivation: for comparison cultures of lymph node cells from tolerant mice.

The viral growth curves shown in Fig. 1 may be briefly characterized as follows:

Normal cells infected immediately: slow start of virus multiplication and slow remission after first peak, least distinct with fraction I; evidence of a second peak.

Normal cells infected on 14th day: rapid start and rapid decline after first peak; remission much more marked than in former case; distinct second peak.

Tolerant cells: continuous multiplication of virus at about the same level from the very start despite the fact that the cultures (like those of normal cells infected immediately) showed first evidence of cell growth only on second day of cultivation; no significant remissions.

In view of the marked heat-lability of LCM virus, which may lose its infectivity completely during incubation for 24 hours (9), there can be little doubt that virus multiplication started rapidly in the tolerant cells. Apart from the slow start of viral growth in normal cells infected immediately after trypsination, their reaction to the virus appears to be intermediate between those of outgrown normal cells and tolerant cells.

Viral growth in different kinds of cells

An attempt has been made to construct composite curves from a number of individual viral growth curves obtained from the same kinds of cells in different experiments. It was feared beforehand that such a procedure might not lead to characteristic curves because the rhythms of the individual curves might be too different. As Fig. 1 shows, peaks and remissions do not occur at exactly the same time in comparable curves, but there is a sufficient degree of uniformity permitting the construction of composite curves which will at least show the general trend of viral multiplication in the respective system.

As previously reported (22) and since confirmed by additional tests, LNC from leukemic mice showed the clearest remissions and a considerable uniformity in the rhythm of viral reproduction during the first few weeks of cultivation. In the course of time, they appeared to acquire the character of tolerant cells. Their behavior towards LCM virus was different from that of embryo cells. This is worthy of note with regard to the embryo cell theory of cancer genesis. Like normal LNC, leukemic cells infected immediately after trypsination showed a lag period and a less marked remission after the first peak. No evidence has been obtained suggesting that a leukemia agent was interfering with the virus under study.

The difference between normal LNC infected either immediately or after the outgrowth of cells is shown by curves 1 and 2. It seems possible that growing cells get a stronger shock by the infection than cells whose growth has been stopped by trypsination. They react to it accordingly. In contrast to leukemic LNC, the behavior of normal LNC from different groups of mice was not uniform. Among the 14 curves combined in curve 2, there were two cases resembling curve 1. In spite of such variations, the general rhythm of reproduction of LCM virus is similar to that in leukemic cells.

Virus-free LNC from non-tolerant immune mice (curves 3 and 4), although susceptible to *in vitro* infection, were found to differ from normal LNC in two respects: (a) in cells brought in contact with virus immediately
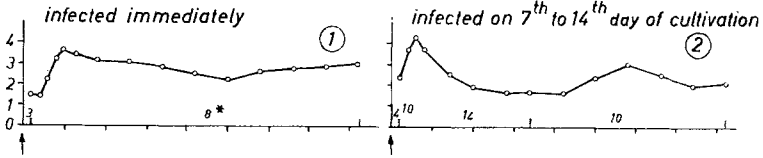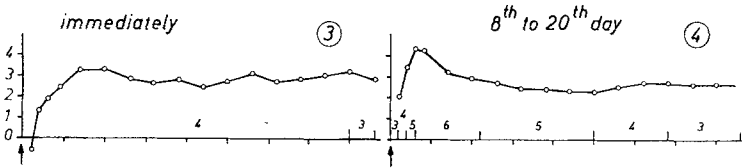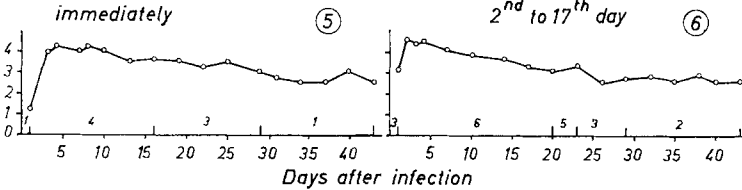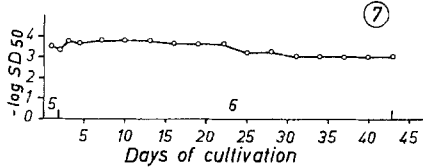

Fig. 2. Growth curves constructed from mean titers of LCM virus in different kinds of cells.

after trypsination, infection was delayed and the multiplication of the virus inhibited in its initial phases; (b) the viral growth curve in cells infected later failed to show a distinct second peak after the first remission. The behavior of cells from immune animals and its possible causes will be dealt with in more detail in another paper.

Due to their abundant growth and the rapid detachment of the cell sheet from the glass, normal embryo cells (curves 5 and 6) were difficult to handle over long periods of time. Multiplication of LCM virus started and declined somewhat more rapidly in grown cells than in those infected after trypsination. In contrast to normal LNC, there was no significant second rise after the slow remission.

In trypsinized LNC from tolerant mice (curve 7) viral multiplication continued without interruption apparently at the same level as *in vivo*. A significant lag period was missing in embryo cells from tolerant mice also. There were no definite remissions. The slight decrease of virus production in the course of time can be readily explained by aging of the cultures.

Indirect cell damage in mice infected experimentally with LCM virus

Since several attempts to cultivate cells from the lymph nodes of mice sacrificed during the acute stage of the disease were unsuccessful in the past (22), it was suspected at that time that the failure of the cells to grow was due to a temporary harmful effect of LCM virus upon the cells. It was difficult to reconcile this concept with the missing pathogenicity of strain W in cultures of LNC from normal mice. The results of a more systematic investigation of the problem are presented here.

Five to 6 week-old mice were injected subcutaneously (s. c.) in the right flank with infectious serum from congenitally infected animals. In mice kept for observation, the inoculation was followed by swelling of the regional lymph nodes by the 4th day and of the other lymph nodes and the spleen 1 — 3 days later. The majority of the mice showed no overt signs of disease but developed more or less extensive s. c. edema in the injected area by the 7th or 8th day. A small percentage of the animals succumbed to serous pleuritis and/or peritonitis. In some such cases the lymph nodes and the spleen were unusually small.

From groups of 6 mice each the lymph nodes were removed for tissue culture tests various periods of time after inoculation as indicated in Fig. 3, and the mean weight of the spleen was determined in every case. The spleens were deep-frozen for c. f. tests, in which 20 per cent extracts in buffered saline were examined first for c. f. antigen and then, after heating at 56° C for 30 minutes and clearing by centrifugation, for c. f. antibodies.

Test-tube cultures were prepared from the lymph nodes using fractionated trypsination as described above. The cultures were examined microscopically every two days during a cultivation period of 16 days. The media were changed on the second day of cultivation and every 3 — 4 days thereafter. Samples of the culture fluids were tested for infectivity in mice.

Fig. 3 gives the results of two experiments. The shaded areas in the four rectangles of each column indicate the estimated cell density reached by the cultures prepared from trypsination fractions I — IV. The growth of fibroblast-like cells believed to be reticulocytes is indicated by plus or minus signs on the right of each rectangle ($\pm$ meaning that only one or a few isolated reticulocytes, failing to show further growth, were seen in the cultures). The mean weights of the spleens of the cell donors and the results of i. c. tests for presence of LCM virus in the cultures are recorded on top of the columns.

As can be seen in Fig. 3, there was a period in both experiments in which the cells grew very poorly or not at all (7—8 days after s. c. infection). At that time the trypsinized cells showed a marked tendency to clump when washed with PBS. Before and after this period, there was normal cell growth despite the fact that the respective cultures were infected with LCM virus. In experiment 1, the growth capacity of the cells was again relatively low on the 21st and 28th day, but this result could not be reproduced in experiment 2.

In the first experiment, infectious cultures were no longer obtained after the 10th day, while in experiment 2 virus was present in all cultures except those prepared 1 hour after inoculation.

The average spleen weight (like that of the lymph nodes, which was not measured) increased in the course of the disease, reaching more than twice its normal value by the 10th day, and then gradually returned to normal or almost normal.

In the second experiment, two growth tests were made on the 8th day using sick mice in one and healthy-appearing animals in the other test. At autopsy, the sick mice showed marked exudation (s. c., pleural and peritoneal) and subnormal size of spleen and lymph nodes, while the healthy-appearing animals presented only moderate s. c. edema, but enlarged spleens and lymph nodes. There was rather poor cell growth in both cases.

While all tests for c. f. antibodies in the spleen gave negative results, such antibodies were present in the serum on the 21st and 28th day (titer uniformly 1:5). Earlier samples were not tested. Small amounts of c. f. antigen were demonstrable in the spleen on the 6th and 8th day only (1+ to 3+ reactions with undiluted 20 per cent extract). The great difference in antigen content of such spleens and those of tolerant mice (20) is again evident.

It may be of significance (see Discussion) that, in contrast to spleen extracts from tolerant mice, those originating from s. c. infected animals killed during the period of decreased growth capacity of the LNC had a slight but definite anticomplementary effect.

Other experiments indicated that cell damage lasts longer in young mice than in older ones, specially in animals recovering from severe illness. The effect was not demonstrable in kidney cells.

When trypsination was omitted and the cells dispersed mechanically by mincing the tissue with scissors and blowing the fragments back and forth with a wide-mouth Pasteur pipette, LNC harvested on the 7th or 8th day after s. c. infection maintained their metabolic activity. The result with regard to cell growth was inconclusive, however, because normal LNC also failed to grow well under such conditions. This result has led to the tentative conclusion that trypsination enhances or completes an injurious effect which the cells are exposed to *in vivo*.
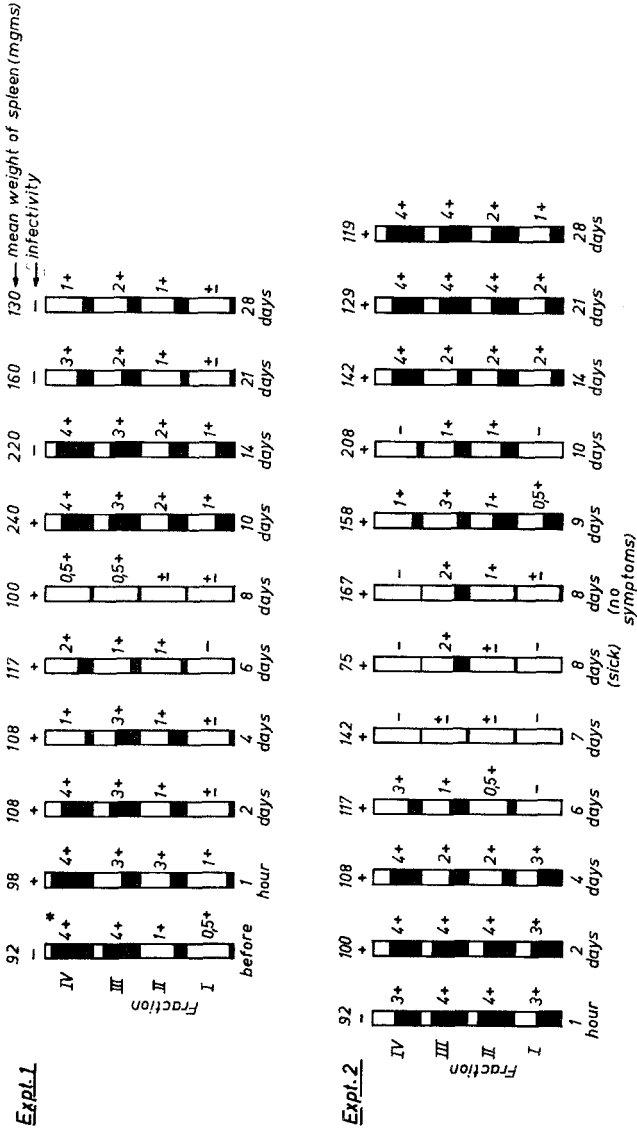
Fig. 3. Growth capacity of murine lymph node cells different periods of time after subcutaneous inoculation of LCM virus.

Attempts to affect malignant cells by infecting leukemic mice s. c. with LCM virus have thus far been more or less unsuccessful. The number of tests is still very limited. LNC from a leukemic mouse sacrificed 8 days after s. c. infection showed a practically normal growth potential. However, there was evidence of decreased growth capacity of these cells in leukemic mice killed 13 or 15 days after s. c. infection. The results show that a single shock is not sufficient to destroy or inhibit the bulk of the malignant cells. Theoretically, better results may be expected from repeated shocks. However, these are not possible with the same virus. The experiments are being continued along different lines.

Multiplication of virus in cultures prepared from injured cells

It was known from previous experiments that considerable multiplication of LCM virus may occur in cultures containing non-growing ("resting") cells only, as this is not infrequently the case in cultures prepared from trypsination fraction I. This observation has been confirmed by repeated infectivity tests of the following cultures (set up and tested in duplicate) in experiment 1, Fig. 3: (a) 6 days, fraction I; (b) 8 days, fraction I and (c) 8 days, fraction II.

While the two cultures of (a) never showed any growing reticulocytes, a few isolated cells of this type were detected in cultures (b) and (c) between the 10th and 14th days of cultivation. They had passed into the "resting" state by the 16th day and never showed any tendency to multiply thereafter. The number of "resting cells" present in all three culture pairs was small from the beginning and decreased progressively upon prolonged cultivation. In spite of this fact, all cultures produced virus for at least 32 (a) or 30 days (b and c), when the tests were discontinued. Judging from the incubation periods in the mice injected i. c. with the culture fluids, the amounts of virus regularly manufactured by the "resting cells" were considerable.

Lymphoid hyperplasia in tolerant mice and growth capacity of tolerant cells in vitro

Except in a special genetic line (No. 11) of tolerant mice in which lymphomatosis is very frequent (23), congenital LCM infection as a rule leads to moderate enlargement of the lymph nodes and of the spleen. In the latter, the Malpighian bodies show an increase in volume. This is most distinct in old animals. The hyperplasia of lymphatic tissue, not yet detectable in very young mice (see Table 1), possibly indicates that a defensive reaction of some sort is proceeding in tolerant animals.

The results of numerous growth tests with LNC from tolerant mice of different age have led to the conclusion that these cells undergo a change in the course of time. While their growth capacity was normal or

definitely enhanced at the age of about 3 weeks, reticulocytes from older tolerant mice showed a greater tendency to shrink than comparable normal cells.

Table 1 gives information on the average weight of the spleen, which roughly parallels that of the lymph nodes, in tolerant and normal mice of

Table 1. Average spleen weight, infectivity and complement-fixation titers of spleen and lymph nodes, and growth capacity of lymph node cells in tolerant and normal mice of different age

Age of mice	Tolerant mice						Normal mice	
	Weight of spleen (mgms.)	Infectivity titer ($-\log_{10}$ SD_{50})		Complement fixation titer		Growth of LNC	Weight of spleen (mgms.)	Growth of LNC
		spleen	lymph nodes	spleen	lymph nodes			
19 days	57	6.5	5.5	1:16	1:8	good	61	good
25 days	78			1:16		good	59	good
28 days	90	6.8	5.8	1:32	1:8	good	69	good
32 days	114			1:16		none	72	good
7 weeks	133			1:16		good	92	good
3 months	150			1:32		moderate	97	none
4 months	167			1:16		moderate	110	good
4½ months	183	7.5	6.8	1:16	1:16	very poor	100	very poor
5 months	192			1:32		poor	117	very poor
6 months	150			1:32		none	100	very poor
7 months	179			1:32		good	117	good
7½ months	200	6.8	6.8	1:32	1:32	moderate		
10 months	192			1:32		moderate		
10½ months	200	7.0	6.5	1:32	1:16	good		
12 months	216	7.0	6.8	1:32	1:16	very poor		
13 months	183			1:32		good		
14 months	250	6.8	6.8	1:16	1:16	—*		
18 months	200			1:32		very poor		
20 months	350	6.8	5.8	1:16	1:8	—*		

* no cultures made.

different age (normal mice older than 7 months were not available), the infectivity and c. f. titers of the spleen and lymph nodes as far as determined, and the growth capacity of the cells. The data were compiled from different experiments carried out for different purposes. A relative increase of the average spleen weight after the third week of life in tolerant mice and the persistance of infectious virus and c. f. antigen to high titer despite the hypothetical defensive reaction are evident. The table also shows the irregular results of growth tests with LNC from tolerant and

normal animals older than 4 weeks. They do not confirm the impression gained in earlier, less extensive, experiments (22) that chronic LCM infection generally stimulates cell growth. This appears to be the case only with cells from very young mice, which have not yet undergone the change just mentioned.

Discussion

It appears opportune to give here a clear definition of "tolerance" as a property of cells with respect to the virus under study. The term signifies that the cells tolerate the virus and make no recognizable effort to impede its multiplication at the same high level for a considerable period of time. Non-tolerant cells, on the other hand, will hinder the initially rapid growth of the virus using a mechanism whose nature is not yet clear. It has not been possible to demonstrate either interferon or c. f. antibodies in such cultures. The antiviral efforts of the cells show a definite periodicity, which is most marked in leukemic cells.

Cultures of LNC from tolerant mice seem to represent pure populations of tolerant cells, whereas those from non-tolerant animals may a priori contain some cells having the character of tolerant cells. These may gain dominance in the course of time (22).

Viral growth curves from normal cells differ depending on the state of development in which the cells are infected in vitro. LNC in grown cultures show a more marked reaction to infection than young cells just about the grow out. The latter thus form a bridge to embryonic cells, which in general react only slightly or not at all. There is good evidence suggesting that LCM virus is transmitted from generation to generation by way of the female genital cells (19). They and their descendants in many cell generations throughout life are fully tolerant. Normal embryo cells cultured and infected in vitro in the third week of pregnancy have reached a higher degree of physiological maturity than their more primitive ancestors and consequently show a slight reaction to in vitro infection. The highest degree of maturity is shown by outgrown cells from adult mice, and their antagonistic response to infection is therefore most pronounced. These conclusions have been drawn from numerous viral growth curves in cells from embryos in different stages of development and in LNC from mice of different age.

A property differentiating tolerant from non-tolerant mice is the ability to form antibodies, which is lacking in tolerant animals. It is not known if there is a correlation between the reactivity of the cells and the capacity to produce specific antibodies.

Although the unmodified strain of LCM virus used in our studies does not have a visible cytopathogenic effect in cultures of murine lymph node or embryo cells, a period of decreased growth capacity of trypsinized

LNC was noted in non-tolerant mice during the acute stage of the experimental disease (Fig. 3). Doubts about the significance of this observation were at first raised by the fact that LNC from normal mice will sometimes not grow well under the same experimental conditions. These doubts, however, were dispersed by the frequency with which the phenomenon occurs. It is not an "all or none" effect and its duration is short except in young mice which make a slow recovery from severe illness.

The possibility that the phenomenon is caused directly by LCM virus can be ruled out because cultures made before and after the inhibition period showed good cell growth in spite of the fact that they were infected. The period of decreased growth capacity coincides roughly with the exudative phase of the disease and not with the period of lymphoid hyperplasia, which begins earlier and lasts longer. When the phenomenon becomes manifest, the infectivity titer of the lymph nodes, which generally has its peak on the fourth day, is already decreasing and the animal has theoretically had time to form specific antibodies. It is not known whether the phenomenon is confined to lymph node and related cells. Efforts to demonstrate it in kidney cells were unsuccessful. It is possible that these cells do not contain specific antigen.

Hotchin's theory (6), which attributes the symptoms and lesions in murine LCM to an immunological reaction, is in line with certain observations made by other investigators in studies on homograft reactions [see reviewing article by *Gorer*, (4)]. It can also explain the effect of X-irradiation on the experimental disease in mice as reported by *Rowe* (10) and confirmed by *Hotchin* and *Weigand* (7). Confirmatory evidence has come from experiments with suckling mice descending from immune and normal mothers and inoculated i. c. with suitable amounts of LCM virus (21). Mortality was significantly higher in the progeny of the immune females. However, it is hard to believe that a reaction between the virus itself and specific antibody is responsible for the phenomenon under discussion because there was good growth of LNC from mice treated with mouse or guinea pig hyperimmune serum and injected with different amounts of LCM virus thereafter (unpublished experiments). It is more likely that an immunological reaction takes place with an antigen arising in the body in the course of the disease and being recognized as foreign by the immunological apparatus. An example of this sort is the precipitin reaction in yellow fever described by *Hughes* (8).

The anticomplementary effect of spleen extracts from mice showing the phenomenon points to another simultaneous reaction binding complement. The spontaneous clumping of trypsinized and washed LNC from such animals may also be an indicator of an antigen-antibody reaction. Such clumping, however, was often much more intense than in other cell-antibody systems. It appears that mucoid substances may be involved.

For reasons outlined above, it is probable that cells primarily injured by the hypothetical immunological reaction *in vivo* are killed or damaged further by trypsination *in vitro*, while uninjured cells survive such treatment. An inhibiting substance released by decaying lymphocytes *in vitro* probably increases the primary damage suffered by the reticulocytes *in vivo*.

The occurrence of "resting cells" in the lymphatic organs and the morphological diversity of cells descending from the reticulo-endothelial system leave the possibility open that similar events as described for tissue cultures may take place in the animal. One may speculate further that a mechanism like that prevailing in LCM-infected mice may also be operative in cases in which LNC from apparently normal mice fail to grow and the lymphocytes are badly damaged by trypsination. Theoretically, this may be due to immunological reactions involving other antigens with which the body has to deal. Our observations indicate that, while the age factor appears to have an influence on the growth capacity of murine LNC, it is not always decisive. Good cell growth was repeatedly noted in cultures of LNC from old mice and *vice versa*.

The observation that continuous multiplication of LCM virus over a period of several weeks occurs in cultures consisting only of "resting cells" may also have a parallel *in vivo*, namely, in mice recovering from severe illness. One may expect a considerable number of injured cells in the lymphatic system of such animals. In such cases, the virus usually persists much longer than in mice recovering rapidly (16). Other experiments have shown that it is practically impossible *in vitro* to inactivate virus multiplying in "resting cells" by adding guinea pig hyperimmune serum of relatively high potency to the cultures. The inactivation of the virus in such cells *in vivo* by autologous antibodies may therefore be equally difficult.

The changes which LNC of tolerant mice undergo in the course of time are obviously different from those just discussed. They probably have another cause, which is still subject to investigation.

Summary

Extending earlier observations on the multiplication of LCM virus in cells from non-tolerant mice, viral growth curves are presented depicting the production of LCM virus over periods of 6 weeks in lymph node cells (LNC) from normal and immune (non-tolerant) mice, normal mouse embryo cells, and LNC from congenitally infected (tolerant) animals. The curves obtained from non-tolerant cells differ more or less depending on the stage of growth at which the cells were infected *in vitro*.

LNC from mice infected by s. c. inoculation with LCM virus showed a reduced growth capacity during a limited period coinciding roughly

with the exudative phase of the disease with a maximum on the 7th or 8th day after inoculation. This phenomenon was not observed with kidney cells.

It is not caused directly by the virus, which has no definite cytopathogenic effect in murine LNC, but appears to be due to an immunological reaction. The antigen involved is probably not the virus itself, but a complex formed in the course of the disease and recognized as foreign by the immunological apparatus.

Cells affected by this reaction *in vivo*, including the small lymphocytes, are killed or damaged when subjected to trypsination. Some reticulocytes may survive, but their growth is obviously inhibited. Such cells were designated as resting cells. Cultures containing "resting cells" and lymphocytic debris only were found to support the multiplication of LCM virus for at least one month.

There is evidence suggesting that lymphocytes contain a substance capable of inhibiting the growth of reticulocytes *in vitro*.

The possibility is discussed that the poor growth capacity sometimes shown by LNC from adult normal mice may be connected with immunological reactions involving other unknown antigens.

Reticulocytes from adult tolerant mice have a tendency to shrink during prolonged cultivation. The cause of this phenomenon is not known.

References

1. *Ackermann, R.:* Arch. ges. Virusforsch. **10**, 183 (1961).
2. *Benson, L. M.,* and *J. E. Hotchin:* Proc. Soc. Exp. Biol. a. Med. **103**, 623 (1960).
3. *Burmester, B. R.:* Ann. N. Y. Acad. Sci. **68**, 487 (1957).
4. *Gorer, P. A.:* Cellular Aspects of Immunity. Ciba Foundation Symp.; p. 330. London: J. and A. Churchill (1960).
5. *Gross, L.:* Oncogenic Viruses. Oxford, London, New York, Paris, Pergamon Press (1961).
6. *Hotchin, J. E.:* Symposium on Latency and Masking in Viral and Rickettsial Infections, Minneapolis, Burgess Publ. Co., p. 59 (1958).
7. *Hotchin, J. E.,* and *H. Weigand:* J. Immunol. **87**, 675 (1961).
8. *Hughes, T. P.:* J. Immunol. **25**, 275 (1933).
9. *Lehmann-Grube, F., R. Ackermann, K. A. Jochheim, G. Liedtke,* and *W. Scheid:* Arch. ges. Virusforsch. **9**, 64 (1959).
10. *Rowe, W. P.:* Proc. Soc. Exp. Biol. a. Med. **92**, 194 (1956).
11. *Rubin, H.:* Nature **195**, 342 (1962).
12. *Rubin, H., A. Cornelius,* and *L. Fanshier:* Proc. Nat. Acad. (U. S.) **47**, 1058 (1961).
13. *Rubin, H., L. Fanshier, A. Cornelius,* and *W. F. Hughes:* Virology **17**, 143 (1962).
14. *Traub, E.:* J. exper. Med. **64**, 183 (1936).
15. *Traub, E.:* J. exper. Med. **66**, 317 (1937).
16. *Traub, E.:* J. exper. Med. **68**, 229 (1938).
17. *Traub, E.:* J. exper. Med. **69**, 801 (1939).

18. *Traub, E.:* Zbl. Bakt. I Orig. **147,** 16 (1941).
19. *Traub, E.:* Zbl. Bakt. I Orig. **177,** 453 (1960).
20. *Traub, E.:* Arch. ges. Virusforsch. **10,** 303 (1960).
21. *Traub, E.:* Arch. ges. Virusforsch. **10,** 289 (1960).
22. *Traub, E.:* Arch. ges. Virusforsch. **11,** 473 (1961).
23. *Traub, E.:* Arch. ges. Virusforsch. **11,** 667 (1962).