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Observations on "Late onset disease" and Tumor Incidence in Different Strains of Laboratory Mice infected Congenitally with LCM Virus

I. Experiments with Random-bred NMRI Mice

By

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With 3 figures and 8 tables

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"Late onset disease", first described in 1964 (5) in mice infected neonatally with the virus of lymphocytic choriomeningitis (LCM), is now believed to be due to antigen-antibody complexes, which are formed in the blood and trapped in the glomeruli of the kidneys (9, 10, 11). In our previous experiments (14, 16) mice infected congenitally with LCM virus showed an increased incidence of lymphatic tumors pointing to a correlation of some sort with the persistent LCM infection.

The main subject of the present paper is the pathogenesis of glomerulonephritis, which is the dominating syndrome in "late onset disease", often ending with obliteration of the renal filter (6). The incidence of leucosis in congenitally infected and normal animals will be recorded and the relative merits of direct and indirect complement fixation (CF) as methods for the direct demonstration of antibodies in sera from persistently infected mice will be discussed. Sideline observations on the effect of 2 strains of LCM virus upon the reproductive capacity of congenitally infected mice will be reported.

Material and Methods

Mice: Ancestors of the NMRI mice were imported by the writer from the Naval Medical Research Institute, Bethesda, Md., U.S.A., in 1954. The previous history of this breed is not clear. The animals are relatively free of common pathogens and have a high fertility rate. Breeding material for the present experiments was obtained from Ivanovas & Co., Kisslegg, Allgäu. All mice were fed commercial pellets and given water in drinking bottles.

Virus strains: Strain "W", isolated from a naturally infected wild mouse in 1958 (16), was used predominantly. It is nearly non-pathogenic for

newborn baby mice inoculated intracerebrally (ic) and otherwise behaves typically in all respects. In the literature it is sometimes referred to as "Traub strain", but it is not identical with the strain isolated in 1934 from Princeton mice (14). The latter virus was lost during World War II.

Fewer experiments were carried out with the neurotropic strain "WCC" modified in the U.S.A. by some 370 ic mouse passages and highly pathogenic when injected ic into newborn mice (18).

Both strains are indistinguishable serologically.

Production of congenitally infected mice: Newborn baby mice were inoculated ic with either virus strain. Mice so infected would harbor the virus for many months and females would regularly transmit it to their embryos.

Hyperimmunization of congenitally infected mice with organ extracts: Female weanlings congenitally infected with strain "W" were grouped as indicated in Table 6. Those in groups 1, 2 and 3 were given 14 intraperitoneal (ip) injections (0.2 ml. each at 3 to 4-day intervals) of 10 per cent kidney extracts from mice infected congenitally with strain "W" and sacrificed at the age of 14, 21 and 28 days, respectively. The controls in groups 1 a, 2 a and 3 a received the same number of inoculations with kidney extracts from 14, 21 or 28 day-old normal mice.

The animals in groups 4 and 5 were injected 14 times ip with 0.2 ml. kidney extract from infected mice killed on days 21 or 28 and received in addition 4 ip inoculations (0.5 ml. each) with 10 per cent spleen extract from 28 day-old mice carrying strain "W". The controls in groups 4 a and 5 a were inoculated in the same way with the corresponding normal extracts.

All mice were exsanguinated 10 days after the last injection. Their sera were pooled groupwise and tested by direct and indirect CF.

Tests for complement-fixing (CF) antigen in organs of congenitally infected mice: 10 per cent suspensions (w/v) made by grinding with sand and an isotonic salt solution (8 gms. NaCL; 0.3 gms. KCl; 0.24 gms. $CaCl_2 \cdot 2\ H_2O$; 0.2 gms. $MgCl_2 \cdot H_2O$; 2 gms. $NaHCO_3$; 1.8 gms. glucose; 0.01 gms. phenol red water soluble; 1000 ml. dist. water) were frozen and thawed twice before clearing them by centrifugation. The supernatants were considered to represent 1 : 10 dilutions of the infected tissues.

The diluent for all reagents in CF tests was phosphate-buffered (m/60) saline with Ca″ and Mg″ and small tubes served as containers throughout.

In antigen titrations in which the fixation and lysis periods were 30 min. each (at 37 °C), these extracts were tested in serial two-fold dilutions against an optimal, usually 1 : 40, dilution of LCM-specific serum from hyperimmunized guinea pigs using a small excess (1.1 to 1.2 units) of complement (C'). The percentage of hemolysis was estimated visually after sedimentation of the unlysed cells.

Antigen titers are expressed as $-\log_{10}$ of the highest extract dilutions giving 50 per cent C′ fixation. These dilutions were determined graphically.

Direct CF tests with individual mouse sera: The sera heated at 58 °C for 30 min. prior to use. They were sometimes anticomplementary. C′ was therefore titrated in the presence of every serum (1 : 5) and, separately, of every antigen (LCM and normal). Since cold fixation for 21 hours at + 3 °C was practised in all direct CF tests with murine sera, the titration mixtures were refrigerated in the same way before addition of sensitized sheep erythrocytes and subsequent incubation at 37 °C for 30 min.

In the fixation test, mouse serum 1 : 5 was tested and, in some cases where this was possible, titrated against 1 : 2 and 1 : 16 dilutions of 10 per cent spleen extracts from congenitally infected and normal adult mice in presence

of 1.1 to 1.2 units of C′ including the usual controls in every test. The various reagents were used in 0.05 ml. doses, distributed with 0.1 ml. pipettes.

A reaction was regarded as positive when the estimated degree of hemolysis was 87 per cent or less and all controls were in order.

Indirect CF tests: The method outlined in a thesis (1) just completed in this laboratory was employed. However, it was found preferable to increase the amount of C′ to 1.5 units (1 unit representing the highest C′ dilution effecting 100 % hemolysis in the C′ tiration at 30° for 30 min. in presence of undiluted serum under test) when the serum quantity obtained from an individual mouse did not permit variation of the C′ dose in the test proper. When larger amounts of serum were available, they were tested with 2 or more C′ doses, for instance, 1.25, 1.5, 1.75 and 2 units (see Table 6).

A test with one C' dose only was limited to 3 tubes each of which contained 0.05 ml. undiluted mouse serum under test + 0.05 ml. LCM spleen antigen 1 : 8 (optimal dilution determined by previous titration against positive murine control serum). These mixtures were incubated in a waterbath at 37 °C for 60 min. Thereafter, 0.1 ml. guinea pig immune serum 1 : 40 (optimal dilution as shown by previous box titration) were added to tube 1, 0.1 ml. normal guinea pig serum 1: 40 to tube 2 and 0.2 ml. buffered saline to tube 3. 0.1 ml. C'dilution equivalent to 1.5 units were then pipetted into tubes 1 and 2. After incubation at 37° for 30 min. 0.2 ml. hemolytic system containing 2 units of rabbit hemolysin and 2 per cent washed sheep erythrocytes were added to each tube. Hereupon the mixtures were again incubated for 30 min. and the degree of hemolysis in each tube was estimated visually after sedimentation of the cells overnight in the refrigerator. A comparison of the supernatants and of the cell sediments in tubes 1 and 3 (the latter representing the color control facilitating the reading) allowed an estimate of the degree of antigen neutralization by the murine serum. Direct CF, which usually does not occur with sera from congenitally infected mice under the conditions of this test (1), would have been recognizable in tube 2. This tube, however, showed complete hemolysis in every case.

A positive control serum and one or more normal sera were included in every test. Since the latter showed a certain degree of non-specific variation independent of the age of the donors (see Table 6), a serum reacting like N2 in this table was chosen as negative control but not a serum like N8.

Reactions were arbitrarily judged as follows: positive, when the mean degrees of hemolysis between serum under test and normal serum differed by

Table 1

Pathogenicity of LCM strains W and WCC for mouse embryos*

Virus strain	Seasonal variation			Overall	
	Season	No. of litters	Average litter size	No. of litters	Average litter size
W	warm **	76	10.84	111	10.5
	cold +	35	9.77		
WCC	warm	20	10.55	50	9.4
	cold	30	8.63		
Normal controls	warm	39	11.28	125	11.3
	cold	86	11.30		

* first litters and live embryos counted only
** April through September
\+ October through March

Table 2
Size of successive litters from females carrying virus strains W or WCC

Virus strain	Mother mice		Average litter size					
	No.	No. of successive litters	Litter No. 1	2	3	4	5	6
W	42	2	10.9	11.0				
	21 *	3	11.0	10.3	10.9			
	7	4	10.3	10.4	10.9	10.4		
	5	5	10.0	10.4	10.8	10.8	12.0	
	2 **	6	11.5	13.0	11.0	12.5	12.5	10.5
WCC	26	2	10.8	9.4				
	12	3	11.2	9.2	9.3			
	5	4	10.8	10.6	10.4	6.4		
	4	5	10.2	9.7	9.5	5.6	5.8	
	2 **	6	10.5	6.0	10.0	7.5	5.5	4.5

* of the aforementioned 42 mothers, etc.
** one female of either group produced in 9 successive pregnancies 93 (W) and 54 (WCC) live baby mice, respectively

12.5 per cent or more; suspicious, when the difference was below this figure, but still clearly recognizable; and negative, when there was no distinct difference.

Experimental Design and Results

Pathogenicity of LCM strains "W" and "WCC" for congenitally infected breeding mice and embryos

Observations concerning this still controversial problem extended over a period of 2 years. Congenital carriers and normal mice were kept in an animal room where the temperature was kept all over the year as close to 25—26 °C as possible. Infected and normal mice were fed and otherwise treated alike. Litters born by normal mothers were susceptible to neonatal ic infection with "WCC", while congenitally infected litters proved to be resistant or

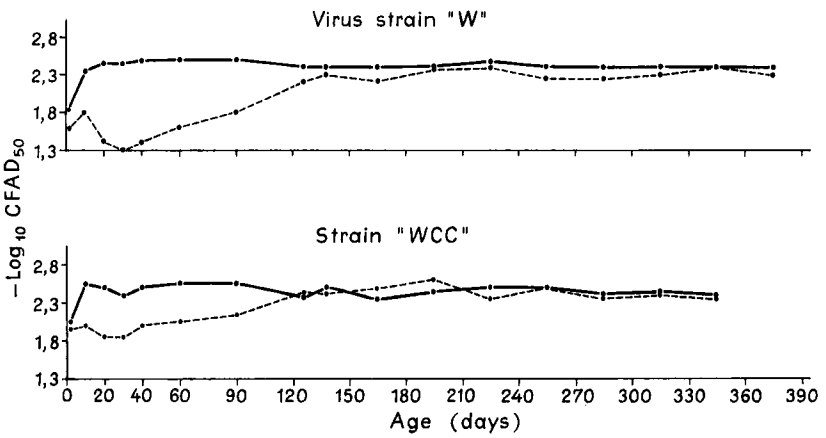

Fig. 1. CF antigen titers of spleen (—) and lidneys (- - - -) at different age levels in mice infected congenitally

were shown by demonstration of CF antigen in individual baby mice at birth to consist exclusively of infected animals.

It can be seen in Table 1, in which only first litters and baby mice born alive are recorded, that there was practically no seasonal variation in the size of litters from normal mothers, whereas those from infected mothers ("W" and "WCC" taken together) averaged 10.8 baby mice in the warmer season and 9.24 mice in the colder months. Average litter sizes calculated for the entire year are 11.3 baby mice in normal litters, 10.5 in litters infected with "W" and 9.4 in litters infected with "WCC".

Higher pathogenicity of "WCC" compared with "W" is also evident from Table 2 in which successive litters of females congenitally infected with either strain are registered. The first litters of these mothers were included in the above calculations. Whereas the average size of litters from females infected with "W" did not change appreciably in 6 successive pregnancies, it dropped considerably with mothers carrying strain "WCC".

Antigen content of spleen and kidneys at different age levels

Extending an earlier observation (18) on the antigen content of the kidneys in congenitally infected mice, which was higher in older animals, the CF antigen titer of spleen and kidneys was studied more systematically in mice from 11 litters, 7 infected congenitally with strain "W" and 4 with "WCC". In every case, litter mates were killed at intervals (two of each litter on day 2 due to the small size of the organs, and one at each of the higher age levels) as indicated in Fig. 1 for titration of the antigen contents of spleen and kidneys. The last animal of each litter was sacrificed on day 126. Definite kidney lesions were not seen in these mice. The dots in the curves up to day 126 indicate arithmetical means of individual titers compiled from 7 and 4 individual litter curves, respectively. At higher age levels, mice of similar age from other litters were substituted for those of the 11 litters just mentioned.

The composite curves in Fig. 1 show that spleen titers rose sharply between days 2 and 10 and soon reached a plateau, whereas kidney titers dropped to a low on or about day 30. This was much more marked with strain "W" than with "WCC". The low varied somewhat in duration with different litters. Thereafter, the kidney titers rose steadily to reach a plateau at an age of about $4^1/_2$ months and at a level similar to that of the spleens.

In another experiment with 5 litters infected congenitally with strain "W", CF antigen in spleen and kidneys was titrated at an age of 2, 4, 7, 10, 14, 21, 35 and 42 days confirming the rapid rise and levelling out of the spleen titer as well as the marked decline of the kidney titer at 2 to 4 or 5 weeks in every case. The latter phenomenon was again much less marked with 3 litters infected with "WCC".

In contrast to the spleen, there was no parallelism between early kidney titers in baby mice infected congenitally with strain "W". During the first 10 days of life the antigen content of the kidneys increased in baby mice from 7 out of 12 litters reaching a minor peak between days 7 and 10 and declining thereafter. This peak was missing in the curves for 5 other litters.

Antigen titers of spleens and kidneys were determined in baby mice from 5 more litters sacrificed at birth. Due to their small size, these organs were pooled and tested litterwise. Medium titers of 2.10 (1 : 106) for the spleen and of 2.00 (1 : 126) for the kidneys were obtained. The latter values are much lower than those calculated for adult mice with glomerulonephritis (see Fig. 2).

Organ titers of newborn baby mice, in turn, were higher than antigen titers of fully developed total embryos weighing more than 1 gm. They averaged 1.74 (1 : 55) and showed considerable uniformity in embryos of 7 highly pregnant females tested individually.

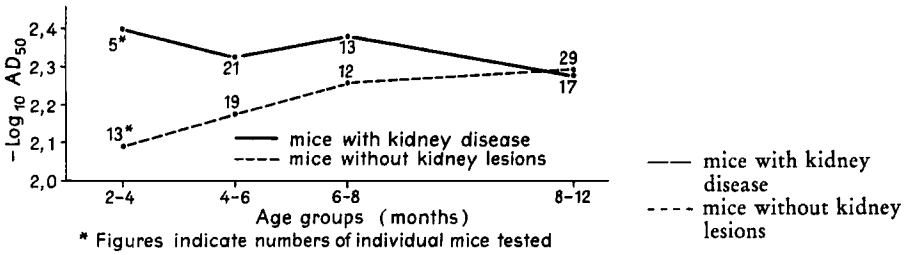

Fig. 2. Mean antigen titers of kidneys in mice with and without macroscopic kidneys lesions

Antigen titers of kidneys in congenital carriers of strain "W" with or without kidney lesions

Since routine histological examinations were not possible, nephritis was diagnosed macroscopically using as main criteria color changes of the kidneys (yellowish brown, yellow, gray or even grayish white) and an increase in volume and weight. Serous exudation (pleural, peritoneal and/or subcutaneous) in acute cases provided additional circumstantial evidence for the presence of the disease. The serum would often show increased opalescence and a yellow-greenish color with a tendency to form precipitates after freezing and thawing.

CF antigen was titrated in individual mice of different age groups with and without macroscopic kidney lesions and arithmetical means were calculated for the respective age spans. Results are shown in Fig. 2. In the age group of 2 to 4 months only 5 mice with kidney disease were encountered. The antigen content of their kidneys was uniformly high, on the average twice as high as that of 13 animals without obvious kidney disease. A distinct, though smaller difference was still noticeable at 4 to 6 and 6 to 8 months, but at 8 to 12 months the difference was nil. The slope of the curve for the mice with normal appearing kidneys (broken line) resembles that of the corresponding curve in Fig. 1.

In 7 mice with kidney disease the CF antigen titer of the enlarged suprarenal lymph nodes was compared with that of a pool of the other lymph nodes. No antigen at all could be detected in 5 per cent extracts of the suprarenal nodes in 4 animals, whereas such extracts of the other lymph nodes had a medium titer of 2.375 (1 : 237). In the remaining 3 mice the mean titer of the suprarenal nodes was 2.19 (1 : 155) and that of the other nodes 2.369 (1 : 234).

Attempts to correlate glomerulonephritis with the presence of CF antibodies in the blood

Data on the age distribution of this disease were obtained from a batch of 82 female mice observed for one year, 66 infected congenitally with strain "W" and 16 with "WCC". A control group of 49 normal females was kept under observation for the same period. The animals were exsanguinated either when seriously ill or at the end of the observation period if they remained outwardly healthy. This was the case with 27 individuals in the infected

group and with 41 mice of the normal batch. However, in some of these mice reaching the age of 1 year pathological changes were found at autopsy.

Intra vitam and post mortem examinations revealed the following pathological conditions in infected mice (No. of cases given in brackets): nephritis with exudation (16); ditto. without exudation (27); emaciation (23); ataxia (3); hyperesthesia and extreme nervousness (11); leucosis (12 definite and 5 suspected cases including 1 thymic sarcoma); moderate enlargement of spleen and /or peripheral lymph nodes (14); degeneration of the liver (6); impaction of the stomach (2); impaction of the cecum (1). Some mice were afflicted with more than one of these conditions. Nephritis and leucosis, for instance, were seen in 6 animals.

In the normal group were observed: nephritis without exudation (2); leucosis (7, including 1 thymic sarcoma); probable early stages of leucosis (4); impaction of the stomach (1).

Even though histological confirmation is still lacking, daily comparison of the behavior of infected and normal mice left no doubt about a more or less marked (in general slight) involvement of the central nervous system in "late onset disease". It was more pronounced in animals carrying strain "WCC" than in those infected with strain "W". Numerous tests showed that "WCC" -infected mice on the average harbor about 3 times more CF antigen in their brains than carriers of strain "W". The mean brain titers were 1.342 (1 : 22) and 1.851 (1 : 71) calculated from 36 and 21 individual titrations, respectively.

Leucosis will be dealt with in a later section of this paper.

The age distribution of nephritis (38 cases (58 %) among 66 mice infected with strain "W" and 5 (31 %) among 16 females infected with "WCC") is shown in Fig. 3. There were no cases in the age group of 0 to 3 months. At 3—9 months more than one half of the afflicted animals showed the dangerous acute form of the disease characterized by marked exudation. In the age group of 9—12 months there was only one such case. The disease became more chronic with increasing age. Apparently, some of these older mice could survive with severe kidney lesions for several weeks at least.

The relative frequency of the acute and chronic forms of nephritis was similar in mice from other experiments. Of 21 congenitally infected animals taken ill at an age between 68 and 214 days 12 showed marked exudation.

Mean weights of both kidneys were 650 mgms. in 43 mice with glomerulonephritis and 449 mgms. in 39 infected animals without macroscopic kidney changes. In 11 four months-old normal females the kidneys averaged 425 mgms. and in 19 normal females killed at the age of 1 year 421 mgms.

In the normal control group of 49 mice 2 cases of nephritis were noted at an age of 99 and 258 days, respectively.

All sera from 82 individuals of the infected batch were tested for CF antibodies using cold fixation. However, only 7 of 43 mice with kidney lesions and 7 of 39 mice with normal appearing kidneys gave weakly positive reactions. From them a clear picture of the age distribution of positive cases could not be obtained. It was therefore necessary to consider the results of a larger number of CF tests carried out in other experiments with sera from congenitally infected mice of different age including those of the 82 infected mice just mentioned (see lower curves in Fig. 3). All serum donors were shown to harbor specific CF antigen in their spleens and kidneys.

Of a total of 365 individual sera, including 63 samples from mice with nephritis, 44 (12 %) gave positive reactions in dilutions of 1 : 5. In general, the reactions were weak in contrast to the strong ones obtained with

control sera from immunized adult mice. Reactions with no hemolysis at a serum dilution of 1 : 5 were very rare. In the great majority of the cases the CFD_{50} could not be calculated by conventional methods. More details of the tests are given in Table 3 which also shows that some sera positive with LCM antigen reacted slightly with normal spleen extract as well. With the exception of one serum in the age span of 91—180 days (see Table 5, mouse 2), non-specific reactions were generally weaker than specific ones. Since the tests were well controlled (the serum controls without antigen and the antigen controls without serum were completely lysed in every case), these slight reactions probably have some meaning. In Fig. 3 the age distribution of nephritis can be compared with that of positive reactions in CF tests.

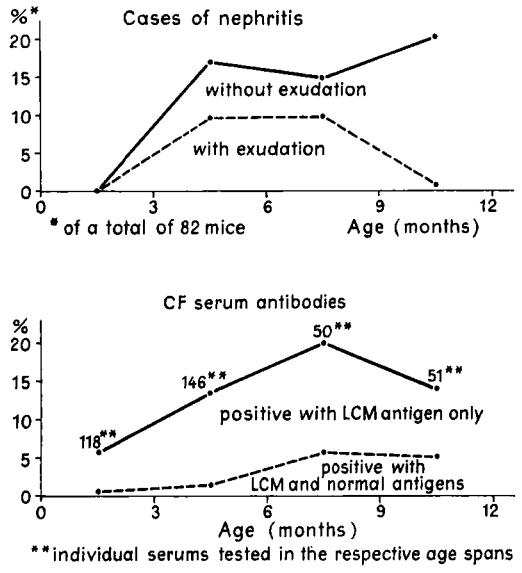

Fig. 3. Nephritis in correlation with serum antibodies

As can be seen in Table 4, the frequency of specific reactors among mice showing nephritis at autopsy and in those without macroscopic kidney lesions did not differ much. However, a difference is apparent in the percentage and the intensity of non-specific reactions with normal spleen antigen.

Antigen titers of spleens and kidneys in mice with and without demonstrable serum antibodies

As numerous titrations have shown, the presence of CF antibodies in the serum has no significant effect on the antigen titers of spleens and

Table 3

CF tests with individual sera from 365 congenitally infected mice of different age groups

Age span (days)	No. of serums	Positive with LCM spleen antigen				Positive with normal spleen extract			
		No.	%	medium degree of hemolysis	trace reactions*	No.	%	medium degree of hemolysis	trace reactions*
18 - 90	118	7**	5.9	71	12	1+	0.8	87	0
91 - 180	146	20	13.7	54	16	2	1.4	43	3
181 - 270	50	10	20	74	9	3	6	83	7
271 - 395	51	7	13.7	71	7	3	5.9	79	5

* not counted as positive
** no positive reactions before day 35
\+ donor 80 days old

Table 4

CF antibodies in sera of congenitally infected mice, 2 to 12 months old, with or without macroscopic kidney lesions

Kidney lesions	No. of mice	Positive in CF test				Mean degree of hemolysis (%)		Trace reactions*			
		LCM antigen		normal antigen				LCM antigen		normal antigen	
		No.	%	No.	%	LCM antigen	normal antigen	No.	%	No.	%
with	62	10	16.1	6	9.7	74	88	7	11.3	6	9.7
without	230	34	14.8	3	1.3	63	98	33	14.3	10	4.3

* not counted as positive

kidneys. With one exception, the titers of these organs were on the average about equally high in congenitally infected mice of similar age with and without antibodies. Spleen titers averaged 2.393 (1 : 247) and kidney titers 2.301 (1 : 200) in mice with CF antibodies. The corresponding values for animals without antibodies were 2.396 (1 : 249) and 2.316 (1 : 207). This is in line with the results of extensive studies by VOLKERT and coworkers (for references see (3)).

The exceptional animal just mentioned (mouse 1 in Table 5), is remarkable inasmuch as it represents the first case of autosterilization observed by us in many years of experience with LCM. It was congenitally infected with strain "W" and immune to ic infection with "WCC" at birth. When 2 months old, it was mated with a congenitally infected male. Ten of 12 baby mice of the first litter were immune to neonatal ic infection with "WCC" and CF antigen was demonstrated in each of the remaining 2 animals at birth. The situation changed with the second litter consisting of 15 baby mice of which 10 had no cerebral immunity to "WCC" and 5 gave a negative result in a test for CF antigen. The mother was exsanguinated during her third pregnancy at the age of 157 days. Infectious virus could not be detected in the organs (spleen, kidneys, liver, ovaries and uterus) or in embryos and placentas by ic mouse inoculation. Tests for CF antigen were likewise negative. The serum contained specific CF antibodies with a titer of 1 : 14.

The serum of mouse 2 in Table 5, congenitally infected with "WCC" and sacrificed at the age of 127 days, reacted equally well with LCM and normal spleen extracts with titers of 1 : 27 and 1 : 23, respectively. However, there was no autosterilization in this case. Spleen and kidneys both gave positive CF tests in high dilutions.

Table 5

Serum antibody titers and antigen titers of spleens and kidneys in two congenitally infected females positive in direct CF tests

Mice		CFD_{50} of serum		AD_{50}	
No.	Age (days)	LCM spleen antigen	normal spleen antigen	spleen	kidneys
1*	157	1 : 14	1 : 5	1 : 10	1 : 10
2**	127	1 : 27	1 : 23	1 : 275	1 : 442

* lost the virus
** remained infected

Erratum: Titers calculated for mouse 1 should read 1 : 14, > 1 : 5, > 1 : 10 and > 1 : 10, respectively

Attempt to stop the progressive increase of the renal antigen titer by prolonged treatment with kidney extracts obtained from 2 to 4 week-old mice

The observation that the antigen titer of the kidneys always drops markedly during the second to fourth weeks of life in mice infected congenitally with strain "W" suggested a test of the possibility that the kidneys might at that age contain a factor capable of inhibiting the continuous accumulation of viral antigen in the kidneys. Batches of 8 to 10 mice were treated with organ extracts from congenitally infected or normal mice as described in "Materials and Methods". Even though the result was negative with regard to suppression of viral antigen in both spleens and kidneys as shown by individual antigen titration against specific guinea pig immune serum, the serological tests carried out with serum pools from the various batches of treated mice are listed in Table 6 because they permit a comparison of results obtained by direct and indirect CF. Both tests were made with undiluted murine sera. Ten different pools of normal mouse serum were included as negative controls. The positive control serum was obtained from non-tolerant

Table 6

Direct and indirect CF tests with undiluted serum pools from congenitally infected mice treated with organ extracts from 2 to 4 week-old virus carriers or normal mice of the same age

Serum pools			Direct CF					Indirect CF					
No.	Donors		LCM antigen		normal antigen		no antigen	guinea pig serum					corrected means
	No. of mice	treated with extracts of						immune				normal	
								C' units					
			1:2	1:16	1:2	1:16		1.25	1.5	1.75	2	1.25	
1	8	infectious kidney, day 14	87*	100	100	100	100	12	25	62	87	100	39†
1a	8	normal kidney, day 14	94	100	100	100	100	6	25	37	50	100	22
2	8	infectious kidney, day 21	94	100	94	100	100	0	6	12	25	100	3
2a	8	normal kidney, day 21	75	94	100	100	100	50	75	87	94	100	69
3	8	infectious kidney, day 28	94	100	100	100	100	0	6	37	50	100	15
3a	8	normal kidney, day 28	94	100	100	100	100	5	8	12	37	100	8
I	10	infectious kidney and spleen	6	50	94	100	100	12	25	50	62	100	29
Ia	10	normal kidney and spleen	12	62	94	100	100	25	37	62	75	100	42
II	10	infectious kidney and spleen	100	100	100	100	100	6	12	50	62	100	25
IIa	10	normal kidney and spleen	12	50	87	94	100	12	25	50	62	100	29
N 1								0	0	0	12	100	
N 2	N 1 - N 10 =							0	0	6	25	100	
N 3	negative controls							0	0	0	12	100	
N 4								0	0	0	12	100	
N 5	(normal mice of different		100 % in all tubes					0	0	0	6	100	
N 6	age groups ranging from							0	0	6	12	100	
N 7	1 to 11 months)							0	0	6	12	100	
N 8								0	0	0	0	100	
N 9								0	0	0	10	100	
N 10								0	0	0	12	100	
MIS	positive control		0	0	100	100	100	87	94	100	100	100	

* Figures indicate estimated percentage of hemolysis

\+ Differences (%) between mean percentages of hemolysis in tubes 1 to 4 with sera under test and in corresponding tubes with control serum N 2

immunized adult mice. It did not fix complement directly in the indirect test as shown by the complete hemolysis in the tube containing normal instead of immune guinea pig serum.

As can be seen in Table 6, the results of direct and indirect CF tests with the same sera were not in good agreement. According to the adopted method of evaluation, 8 serum pools from treated mice (Nos. 1, 1 a, 2 a, 3 and I through II a) were positive in indirect CF but only 5 pools in the direct test (Nos. 1, 2 a, I, I a and II a). Moreover, there was no strict parallelism with respect to the relative intensity of the reactions. In the direct test several sera, notably No. II a, crossreacted slightly with normal spleen extract. Contrary to expectation, mice treated with infectious organ extracts on the average produced less antibody than those inoculated with normal extracts.

The normal control sera were all clearly negative in direct CF but did not behave strictly alike in the indirect test. Some reactions indicate very slight non-specific neutralization of antigen, which was most marked with serum N2 and least with N 8.

Pool N2 was therefore used as negative control in subsequent indirect CF tests.

Relative sensitivity of direct and indirect CF

Sera of individual virus carriers in different age groups were tested comparatively by direct and by indirect CF. The results are summarized in Table 7 which shows that the percentage of positive reactors again rose with increasing age. There were no sera from tolerant mice older than 7 1/2 months available at the time of this test. On the whole, the indirect test detected more positive animals (31 per cent) than the direct CF (10 per cent).

It is noteworthy that it has often not been possible to demonstrate antibodies by either method in sera of mice with marked kidney lesions. Of 4 individual sera. from such animals among those listed in Table 7 one was positive and 1 suspicious in indirect CF, while all 4 sera were negative in the direct test. A similar result was obtained with 5 pools each containing 4 to 12 individual sera from mice with kidney disease. Only one of them was positive in indirect CF. In the direct test 2 pools showed trace reactions only with both LCM and normal spleen extracts, the others being negative.

Tumor incidence in congenitally infected mice

Tumors seen in NMRI mice obviously all originated from cells of the reticulo-endothelial system. Epithelial tumors were missing. In the batch of 82 mice observed for one year, 66 carrying strain "W" and 16 "WCC", 11

Table 7

Summary of results obtained with sera from congenitally infected mice tested individually by direct and by indirect CF

Serum donors		Direct CF		Indirect CF		Comparison		
Age span (days)	No.	positive	trace reactions	positive	questionable slight reactions	pos. in both tests	pos. in DCF only	pos. in ICF only
28 - 90	15	0	0	1 (68 days)	0	0	0	1
91 - 180	26	5	2	10	3	2	3	8
181 - 229	10	0	0	5	3	0	0	5
Totals	51	5	2	16	6	2	3	14

Table 8
Tumor formation in congenitally infected mice

Group	Virus strain	Tumor incidence				Tumor age (days) in definite cases		Observation period
		definite cases		suspicious cases		lower limit	average	
		No.	%	No.	%			
A	W	12 / 66 (6)	18	5 / 66 (4)	8	135	239	1 year
	WCC	0 / 16	0	0 / 16	0	-	-	1 year
	LCM - free controls	7 / 49 (1)	14	4 / 49 (0)	8	99	284	1 year
B (miscellaneous mice from various experiments)	W	3 / 20 (0)	15	1 / 20 (0)	5	186	265	varying between 137 and 389 days; average 200 days
	WCC	0 / 16	0	0 / 17	0	-	-	varying between 135 and 311 days; average 202 days

in brackets: No. of animals showing glomerulonephritis besides changes suggestive of leucosis

definite and 6 suspicious (early?) cases of leucosis were recorded, all in mice infected with strain "W". Among 49 LCM-free controls 7 definite and 4 suspicious cases were found. In mice from other experiments well within the tumor age (see Table 8) 3 more cases and 1 suspicious animal were encountered, all in carriers of "W" virus, none in those infected with "WCC".

Macroscopically, the tumors found in NMRI mice differed from Gross-type leukemia as seen in AKR mice. The blood picture presented no striking abnormalities even when spleen and lymph nodes were very much enlarged. In the majority of the cases the spleen showed an increase in size, in one animal reaching 10 times its normal weight, in others an increase of 2 to 7 times. The color of the spleen usually was dark chocolate in contrast to its purplish-grayish tinge in AKR leukemia. The consistency of swollen spleens and lymph nodes was more solid and the growth rate of the tumors much less rapid in NMRI mice. Swelling of spleen and lymph nodes did often not go parallel. Animals with very large spleens sometimes had only moderately enlarged lymph nodes and vice versa. Peripheral (submaxillary, subscapular, axillary and inguinal) lymph nodes were more frequently involved than those located in the pleural and peritoneal cavities. In the control group a fatal thymic tumor was noted, and 3 cases of moderate to marked enlargement of the thymus, suggestive of early stages of thymic sarcomas, were seen in both the LCM-infected and normal groups.

In some tumor-bearing or supicious mice, kidney lesions suggestive of glomerulonephritis were recorded besides the tumorous changes as indicated in Table 8. One animal in the normal group showed multiple small, round tumors in the kidneys.

Differences noted between LCM strains "W" and "WCC"

Apart from higher pathogenicity of "WCC" for newborn mice and their different behavior in the kidneys of weanlings as shown in Fig. 1, both strains differed in other respects. In spite of an equally high or greater antigen content of the kidneys in mice 5 to 12 months old, the incidence of kidney disease in WCC-infected animals was significantly lower than in mice carry-

ing "W" virus (22 vs. 43 per cent, considering all cases in different experiments). Leucosis was not observed in 32 animals infected with "WCC" but occurred at a rate of 17 per cent in 86 carriers of strain "W", not counting some presumptive early cases (7 per cent). CF antigen was demonstrable in the brains of adult mice infected congenitally with either strain but its titer was on the average 3 times higher in animals carrying "WCC" virus and, perhaps as a consequence thereof, signs of involvement of the central nervous system were more frequent in "WCC"-infected mice.

There is evidence suggesting that some of the aberrant characteristics of "WCC", presumably acquired by serial ic passage in adult mice, can be reversed by natural vertical passage. Thus, in the course of only 3 or 4 such passages, this virus lost much of its cerebral pathogenicity for newborn mice and, after 8 generations, it caused marked peritoneal and pleural exudation following intraperitoneal inoculation in adult animals, a property which it did not have when the mother to embryo passages were started. It now resembles in this respect its sister strain "WCP" maintained by peritoneal passage in mature mice (18).

Discussion

The pathogenicity of LCM virus for mouse embryos, which is still under debate (8), has a bearing on both the problem of immunological tolerance (2,9) and the question of a possible toxic effect of LCM virus. From the figures on litter sizes given in Tables 1 and 2 one may conclude that the unmodified strain "W" is practically harmless for the sexual organs of the parents as well as for embryos, whereas a moderate pathogenic effect of modified "WCC" virus has become evident. This has been substantiated by increasing reproductive inefficiency of "WCC"-infected mice in later generations.

In view of the rough parallelism between infectivity and CF antigen titers in congenitally infected mice (20,7) it was decided to use the CF test for the demonstration of viral antigen in organs, especially spleen and kidneys.

The curves shown in Fig. 1 indicate a basic difference between spleen and kidneys with respect to fluctuation of the antigen content in young congenital carriers. A tentative explanation is that in the kidneys of embryos and young baby mice the formation of viral antigen occurs in cycles with different timing in different litters. Unpublished data on variation of the CF antigen titer in whole embryos of different weight point in the same direction.

The failure to demonstrate in the kidneys of 14 to 28 day-old carriers an extractable factor capable of inhibiting the progressive accumulation of viral antigen in the kidneys with increasing age is not surprising in view of what is thus far known on virus-host (19) and virus-cell interaction (4) in murine LCM.

On the basis of the results presented in Figs. 1 and 2 the possibility was considered that the continuous increase of the renal antigen content up to a high level, possibly in combination with a toxic effect on the glomeruli, might be primarily responsible for the glomerulonephritis occurring in many congenitally infected NMRI mice later in life. Initially, a toxic effect of the virus seemed unlikely in view of the missing or very low pathogenicity of strain "W" for embryos which were known to harbor much infectious virus and CF antigen. Nevertheless, a comparison of the antigen titers of the kidneys at birth with those in adult mice showing nephritis revealed a considerable difference. On the basis of this result a toxic effect in older mice could be not ruled out and the possibility existed that the antigen content in embryos might lie below the threshold of toxicity.

The lower incidence of glomerulonephritis in congenital carriers of strain "WCC" in spite of their high antigen content of the kidneys is not necessarily an argument against a toxic effect of LCM virus in high concentration, since "WCC" is a modified strain differing in several respects from natural virus.

However, the toxicity hypothesis cannot explain the elevation of the serum globulin level, and the massive accumulation of IgG (or IgM) in the kidneys of nephritic mice (9, 6, 13) believed to be due to deposits of antigen-antibody complexes in the glomeruli (9). Final proof that the immunoglobulin in such kidneys actually represents LCM-specific antibody is, as far as we are aware, still lacking. Successful elution of CF antibody from diseased kidneys reported from one laboratory (9) was not confirmed by another using the same technique (3, p. 38).

It should be mentioned in this connection that kidney extracts from old normal mice are often strongly anticomplementary when mixtures of extract and complement are held at 3 °C for 21 hours. When incubated at 37° for 30 minutes, this a.c. activity is much less marked or missing. Kidney extracts from normal young mice did not show this effect at low temperature. Since immune sera from mice fix complement in presence of specific antigen much better in the cold than at 37°, the a.c. effect of kidney extracts from old mice appears to be due to an immunological reaction by antigen-antibody complexes with complement involving other antigens with which the animals had to cope. Consequently, the immunoglobulin found in kidneys of LCM-infected mice may not only be LCM antibody.

Indirect evidence for a participation of antibodies in the pathogenesis of glomerulonephritis was obtained in studies on the age distribution of kidney disease and of serum antibodies demonstrable by direct CF as shown in Fig. 3, where the antibody curve roughly parallels the broken line for exudative cases of nephritis. Both curves indicate a decline in the age span between 9 and 12 months. A similar age distribution of antibody-carrying mice with a maximum at 4 to 8 months was found in indirect CF tests (1).

Nevertheless, certain experimental results are not easily reconcilable with the antigen-antibody complex theory, for instance, the fact that there was no parallelism whatever between glomerulonephritis and serum antibodies in individual animals. This is not evident in Fig. 3 where arithmetical means are recorded. In a number of individual cases relatively strong reactions were obtained in indirect CF tests and the kidneys contained much antigen but failed to show any sign of disease. Since antibodies produced by congenital carriers have a distinct neutralizing effect on viral antigen at least in vitro as shown by positive indirect CF tests, it was surprising to find in nephritic kidneys in the presence of large amounts of immunoglobulin such high antigen titers as indicated in Fig. 2.

The significance of non-specific cross-reactions with normal spleen extract as observed in direct CF tests is at present not clear. Their percentage rose with increasing age and was higher in mice with kidney lesions than in those without. Further work will have to show whether or not they are due to autoantibodies reacting with a "new" antigen (virus-host complex?) for which the animals are not tolerant. The presence of such a system would not invalidate the tolerance theory. Another possibility is that antibodies directed against a leucosis antigen presumably present in some NMRI mice are involved.

Swelling of the suprarenal lymph nodes and their complete or partial depletion of viral antigen as noted in mice with glomerulonephritis as well as

the significance of infiltrating lymphocytes in the kidneys of virus carriers also require further study.

From data given in Tables 6 and 7 it can be concluded that indirect CF is superior to the direct test in sensitivity. However, it does not bring to light the non-specific reactions just mentioned since the indicator serum from guinea pigs used in the indirect test is LCM-specific. The weak reactions generally obtained with both tests have a parallel in the low-titer antibodies demonstrable by immunofluorescence in serums of congenital carriers older than 3 months (3, p. 32).

As in previous experiments in which congenitally infected animals were observed for many months, cases of leucosis appeared in both infected and normal batches of mice. In contrast to these earlier observations, the numerical incidence and the malignancy of the tumors did not differ significantly in the two groups of NMRI mice. A number of animals showed glomerulonephritis in addition to changes suggestive of leucosis.

Possible correlations between chronic LCM infection, tumor incidence and frequency of glomerulonephritis will be discussed in the following paper.

Summary

The pathogenesis of "late onset disease" with glomerulonephritis as a dominating syndrome was studied in random-bred white NMRI mice infected congenitally either with unmodified LCM virus (strain "W"), which is practically non-pathogenic for mouse embryos, or with the modified and more pathogenic strain "WCC".

In congenital carriers the CF antigen titer of the spleen rose about 4-fold during the first 2 weeks of life and then remained at a high level for at least one year, whereas the kidney titer dropped considerably during the first month, especially in mice infected with strain "W", and then increased gradually to reach a high plateau similar to that of the spleen at 4 to 5 months.

It was not possible to demonstrate in kidney extracts from 2 to 4 week-old carriers of strain "W" a substance capable of inhibiting the continuous accumulation of viral antigen in the kidneys.

Glomerulonephritis occurred at a rate of roughly 50 % in infected mice and 4 % in normal controls observed for 1 year. The incidence of the more acute form characterized by exudation was highest in the age span between 3 and 9 months. Thereafter the course became more chronic.

The average antigen content of the kidneys was greater in animals showing acute nephritis than in mice of similar age without macroscopic kidney lesions, thus indicating a correlation between nephritis and amount of viral antigen present in the kidneys.

CF antibodies with a similar age distribution to that of acute kidney disease were demonstrable in sera from congenital carriers with a frequency of 6—20 % depending on the age of the donors, but there was no correlation between serum antibodies and nephritis in individual animals. In general, positive reactions were rather weak and indicative of a greatly impaired capacity for antibody synthesis in the serum donors. The sera of some mice, most of them older than 6 months, reacted non-specifically with normal spleen extract. The antigen involved in such reactions is still unknown.

The indirect complement-fixation method proved more sensitive than the direct test but was unable to detect the non-specific reactions just mentioned.

In carriers of strain "W" observed for 1 year reticuloendothelial tumors (leucosis) occurred at a rate of 18 %, whereas such tumors were missing in

animals infected with "WCC". The tumor incidence in LCM-free controls was 14 %.

Results pertaining to the pathogenesis of glomerulonephritis are discussed in the light of the prevailing theory that this syndrome is caused by antigen-antibody complexes formed in the blood and deposited in the kidneys.

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Zusammenfassung

Beobachtungen über „Späterkrankungen" und Tumorauftreten bei unterschiedlichen, congenital mit LCM-Virus infizierten Labormäusestämmen

I. Versuche mit MRI-Mäusen

Die Pathogenese von Späterkrankungen mit Glomerulonephritis als vorherrschendem Syndrom wurde bei nicht ingezüchteten weißen NMRI-Mäusen untersucht, die entweder mit dem unveränderten, für Mäuseembryonen praktisch apathogenen LCM-Virusstamm „W" oder mit dem modifizierten, pathogeneren Stamm „WCC" kongenital infiziert waren.

Bei solchen Tieren stieg der Gehalt der Milz an komplementbindendem Antigen in den ersten beiden Lebenswochen etwa 4fach an und verblieb mindestens 1 Jahr lang auf diesem hohen Niveau, während der Nierentiter, besonders bei Trägern des Stammes „W", im Laufe des ersten Monats beträchtlich abfiel. Danach erfolgte ein allmählicher Wiederanstieg, bis im Alter von 4—5 Monaten ein Plateau in etwa gleicher Höhe wie bei der Milz erreicht wurde.

Es gelang nicht, in Nierenextrakten von 2—4 Wochen alten, mit dem Stamm „W" infizierten Mäusen eine Substanz nachzuweisen, welche die kontinuierliche Anschoppung von Virusantigen in den Nieren zu verhindern vermochte.

Während einer einjährigen Beobachtungszeit erkrankten rund 50 % der infizierten Tiere und 4 % der normalen Kontrollen an Glomerulonephritis. Die mehr akuten, mit Exsudation einhergehenden Fälle waren am häufigsten in der Altersspanne von 3—9 Monaten. Danach wurde der Verlauf mehr und mehr chronisch.

Der mittlere Antigengehalt der Nieren war höher bei Mäusen mit akuter Nephritis als bei etwa gleichaltrigen Tieren ohne makroskopisch erkennbare Nierenveränderungen, was auf eine Beziehung zwischen der Nierenschädigung und der in den Nieren vorhandenen Antigenmenge schließen läßt.

Komplementbindende Antikörper wurden in 6—20 % der untersuchten Seren von kongenital infizierten Mäusen verschiedener Altersstufen nachgewiesen, wobei die Altersverteilung der positiven Reagenten der der akuten Nephritisfälle ähnelte. Dagegen bestand keine Korrelation zwischen Serumantikörpern und Nephritis bei Einzelmäusen. Positive Reaktionen waren im allgemeinen schwach und ließen auf eine stark verminderte Fähigkeit zur Antikörpersynthese bei den Serumspendern schließen. Die Seren von manchen, zumeist über 6 Monate alten Mäusen reagierten unspezifisch mit normalem Milzextrakt. Das hierfür verantwortliche Antigen ist noch unbekannt.

Die indirekte Komplementbindungsmethode war empfindlicher als der direkte Test, vermochte jedoch die eben erwähnten unspezifischen Reaktionen nicht zu erfassen.

Bei 18 % der mit dem Stamm „W" infizierten, 1 Jahr lang beobachteten Mäuse wurden retikuloendotheliale Tumoren (Leukose) festgestellt, während „WCC"-infizierte Tiere keine Tumorbildung erkennen ließen. Bei LCM-freien Kontrollen betrug die Tumorrate 14 %.

Die Befunde zur Pathogenese der Glomerulonephritis werden im Lichte der heute vorherrschenden Theorie diskutiert, daß dieses Syndrom durch Antigen-Antikörperkomplexe verursacht wird, die im Blut gebildet und in den Nieren abgelagert werden.

Résumé

Observations sur des «maladies retardées» et l'apparition de tumeurs chez différentes souches de souris de laboratoire infectées congénitalement avec un virus LCM

On a recherché la pathogénèse des «maladies retardées» avec une glomerulonéphrite comme syndrome prédominant chez des souris blanches NMRI non consanguines qui furent infectées congénitalement soit avec la souche virale LCM «W» non modifiée et pratiquement non pathogène pour les embryons de souris, soit avec la souche pathogène «WCC».

Le taux de l'antigène fixant le complément de la rate augmenta chez ces animaux d'environ quatre fois dans les deux premières semaines de vie pour rester durant au moins une année à ce niveau élevé, alors que le titre antigénique rénal tomba nettement durant le premier mois, en particulier chez les porteurs de la souche «W». Une remontée graduelle du titre s'en suivit pour atteindre un niveau sensiblement égal à celui de la rate vers l'âge de 4—5 mois. Il n'a pas été possible de mettre en évidence dans des extraits de reins de souris âgées de 2—4 semaines et infectées avec la souche «W», une substance capable d'empêcher le stockage continuel de l'antigène viral dans les reins.

50 % des animaux infectés et 4 % des animaux de contrôle normaux présentèrent une glomerulonéphrite durant le temps d'observation d'une année. Les cas les plus aigus avec exsudation se situèrent entre les âges de 3 à 9 mois. Les cas devinrent ensuite de plus en plus chroniques. Le taux antigénique moyen des reins fut plus élevé chez les souris atteintes de néphrite aiguë que chez les animaux du même âge environ ne présentant aucune lésion rénale macroscopiquement reconnaissable, ce qui permet de faire un rapprochement entre la lésion rénale et la quantité d'antigène présente dans les reins. Des anticorps fixant le complément furent trouvés dans 6—20 % des sérums examinés chez des souris d'âges différents, infectées congénitalement; la répartition par âge des réagissantes positives ressemblait à celle des néphrites aiguës. Il n'y a pas eu par contre de corrélation entre les anticorps sériques et les néphrites chez les souris prises séparément.

Les réactions positives furent en général faibles et laissèrent penser à une capacité diminuée dans la synthèse des anticorps de la part des animaux examinés. Les sérums de plusieurs souris, âgées pour le plupart de 6 mois, réagirent de façon non spécifique à un extrait de rate normale. L'antigène responsable est encore inconnu. La méthode de fixation du complément indirecte fut plus sensible que la directe mais n'a cependant pas permis de comprendre les réactions non spécifiques évoquées.

18 % des souris infectées avec la souche «W» et observées durant une années présentèrent des tumeurs réticuloendothéliales (leucose), alors qu'au-

cune formation tumorale n'est apparue chez des animaux infectés avec «WCC». Le taux des tumeurs chez des contrôles sans LCM s'éleva à 14 %.

On discute les résultats concernant la pathogénèse de la glomerulonéphrite à la lumière de la théorie actuellement prédominante qui dit que ce syndrome est provoqué par des complexes antigènes-anticorps qui sont élaborés dans le sang et stockés dans les reins.

Resumen

Observaciones sobre "enfermedades tardías" y aparición de tumores en varias estirpes de ratones de laboratorio, infectados por vía congénita con virus LCM

I. Experimentos con ratones NMRI

Se estudió la patogenia de enfermedades tardías con glomérulonefritis como síndrome prevaleciente en ratones NMRI blancos no consanguíneos, los cuales se infectaron por vía congénita bien con la estirpe LCM "W" no modificada, prácticamente apatógena para embriones de ratona, o bien con la estirpe "WCC" modificada, más patógena.

En semejantes animales aumentó unas 4 veces el contenido del bazo en antígeno fijador del complemento en las dos semanas primeras, permaneciendo a este nivel elevado al menos durante un año, mientras que el título renal, sobre todo en portadores de la estirpe "W", descendía un nuevo ascenso gradual para alcanzar a la edad de 4—5 meses una meseta de altura casi similar a la del bazo.

No se logró identificar ninguna substancia en los extractos renales de ratones de 2—4 semanas de edad infectados con la cepa "W", la cual fuese capaz de impedir la acumulación continua de antígeno virósico en los riñones.

Durante el periodo de observación de un año enfermaron de glomérulonefritis alrededor del 50 % de los animales infectados y el 4 % de los testigos normales. Los casos más agudos, acompañados por exudaciones, eran más frecuentes en el periodo de edad comprendido entre 3 y 9 meses. Después se tornó el curso más y más crónico.

El contenido medio en antígeno de los riñones era mayor en ratones con nefritis aguda que en animales de edad equivalente sin lesiones renales reconocibles macroscópicamente, lo que hace suponer existe una relación entre la lesión renal y la cantidad de antígeno presente en los riñones.

Anticuerpos fijadores del complemento se identificaron en 6—20 % de los sueros examinados de ratones de diversas edades, infectados por vía congénita, asemejándose la distribución por edades de los reaccionantes positivos a la de los casos de nefritis aguda. Sin embargo, no existía ninguna correlación entre los anticuerpos séricos y la nefritis en los ratones individuales. Las reacciones positivas solían ser débiles y permitían inferir una capacidad muy reducida de síntesis de anticuerpos en los donadores de suero. Los sueros sanguíneos de algunos ratones, mayores casi siempre que 6 meses de edad, reaccionaban inespecíficamente con extracto normal de bazo. Permanece desconocido todavía el antígeno responsable de esto.

El método indirecto de fijación del complemento era más sensible que la prueba directa, aunque no era capaz de abarcar las reacciones inespecíficas que acabamos de mencionar.

En el 18 % de los ratones infectados con la cepa "W", observados durante todo un año, se apreciaron tumores retículoendoteliales (leucosis), mientras que los animales infectados con "WCC" no evidenciaban formación tumoral alguna. En los testigos libres de LCM, la tasa de tumores era del orden de 14 %.

Los hallazgos relativos a la patogenia de la glomérulonefritis se discuten a la luz de la teoría que predomina hoy en día, según la cual es originado este síndrome por complejos antígeno-anticuerpos que se forman en la sangre y son depositados en los riñones.

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