

EFFECT OF IMMUNOSUPPRESSION ON CHRONIC LCM VIRUS INFECTION OF MICE*

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Mice chronically infected with lymphocytic choriomeningitis (LCM)¹ virus were once thought to be immunologically tolerant to this virus (1). In 1963, Volkert transferred syngeneic immune spleen cells to mice that were chronic LCM carriers, thus ablating what was then thought to be a tolerant state. Adoptive immunization of these mice resulted in measurable titers of complement-fixing antibody to LCM viral antigens when previously there had been none (2). Associated with rising antibody titers were decreasing plasma virus titers, thus indicating that the animal's state of immunity played a role in determining the virus titer in its plasma.

In 1967, Oldstone and Dixon (3) demonstrated that mice chronically infected with LCM virus were not immunologically tolerant but instead mounted antibody responses to viral antigens after which circulating immune complexes were formed and deposited in their glomeruli. Antibody responses to LCM antigens in unmanipulated chronic carrier mice have not yet been quantitated, but it has been shown that sufficient anti-LCM antibody is produced to complex with at least 99% of the circulating LCM virus (4). Possibly, then, immune responses of chronic LCM carriers may play some role in attenuating the amount of virus carried by these mice.

The studies in this report were directed toward evaluating what effect suppression of the LCM immune response would have on the amounts of this viral antigen in tissues and infectious virus in plasma of chronic carrier mice. In addition, histological, hematological, and immunopathologic data were collected on both immunosuppressed and unmanipulated mice.

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¹ *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; I.C., intracerebrally; KLH, keyhole limpet hemocyanin; LDV, lactic dehydrogenase virus; LCM, lymphocytic choriomeningitis; PAS, *p*-aminosalicylate; PBS, phosphate-buffered saline; SAS, saturated ammonium sulfate.

Materials and Methods

Mice.—SWR/J inbred breeders were obtained from Jackson Memorial Laboratories, Bar Harbor, Me. Pregnant C3H mice were obtained from L. C. Strong Research Foundation, San Diego, Calif. 4–6-wk old Swiss-Webster mice for LCM virus titrations were obtained from Simonsen Laboratory, Gilroy, Calif.

LCM Virus.—LCM virus used was strain CA-1371 originally obtained from Dr. Wallace Rowe of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. It was passed on L cell monolayers in our laboratory, and a 10^6 LD₅₀ dose was inoculated intracerebrally (I.C.) into each newborn mouse. The method of virus titration used in this laboratory has been previously described (5).

Immunosuppressive Agents.—Cyclophosphamide was obtained from Mead Johnson and Co., Evansville, Ind., and a solution prepared by adding water to the ampoule immediately before use. The drug was injected intraperitoneally (i.p.) at a concentration of 10 mg/ml diluent, doses being administered based on each animal's weight. X-irradiation was administered via a Gammacel Irradiator (Atomic Energy of Canada, Ltd. Ottawa, Canada) using a Cesium 137 source emitting 125 R/min. Varying times of exposure were used to obtain different doses of irradiation.

Immunofluorescent-Histopathologic Techniques.—Methods used were similar to those previously described from this laboratory (4) except as noted below. Mice to be autopsied were etherized and exsanguinated by cutting the brachial vessels. Complete blood counts were done, and the serum was saved for virus titration and immunoglobulin determinations. Tissues were fixed in Bouin's solution for standard hematoxylin-eosin and *p*-aminosalicylate (PAS) stains. Additional tissue was frozen for in liquid nitrogen immunofluorescent examination. Rabbit antisera for mouse immunoglobulin, mouse third component of complement, rat fibrinogen, and mouse albumin were prepared as previously described (4). In all cases the rabbit immunoglobulin was isolated, conjugated to fluorescein isothiocyanate (FITC), and again fractionated on DEAE; the 0.05 M PO₄ or 0.1 M PO₄ fractions were used unabsorbed to stain unfixed mouse tissues. Anti-LCM antiserum was prepared from the blood of the mothers of the LCM infected neonates. The mothers were exsanguinated 28 days after their litters were born, and a 50% saturated ammonium sulfate (SAS) cut of the serum so obtained was conjugated to FITC. The conjugate was then fractionated on DEAE and the 0.1 M PO₄ fraction used for staining tissues after absorption with 6-mo to 1-yr old NZB × NZW washed kidney homogenate. This step was necessary to remove antibodies found to be present which stained mouse glomeruli having immune complexes resulting from etiologies other than chronic LCM virus infection. The absorbed anti-LCM conjugate stained only tissue from animals infected with LCM virus.

Immunoglobulin Determinations.—Radial immunodiffusion techniques were used as previously described by Mancini et al. (6). Murine immunoglobulin purified by Pevikon block electrophoresis was used as a standard and also as immunogen to raise the rabbit antiserum used.

Precipitation of LCM Virus-Immunoglobulin Complexes.—The procedure used was a modification of that previously reported by Notkins et al. (7) in working with lactic dehydrogenase virus (LDV) infection of mice. Infectious serum was used fresh or after extended storage at -70°C . To 50 λ of infectious mouse serum was added 10 λ of ^{125}I trace-labeled purified mouse immunoglobulin. Then 200 λ of 56 $^{\circ}\text{C}$ heat inactivated rabbit antiserum to the 50% SAS cut of normal mouse serum was added. The mixture was incubated for 15 min at 37 $^{\circ}\text{C}$ and 30 min at 4 $^{\circ}\text{C}$. It was next centrifuged for 30 min at 3,000 rpm in an International PR-6 centrifuge and a 200 λ aliquot of supernatant removed. The aliquot of supernatant was added to an additional 200 λ of the precipitating rabbit antiserum and a second incubation of 15 min at 4 $^{\circ}\text{C}$, etc. carried out. The supernatant from the second centrifugation was then analyzed for remaining ^{125}I counts. If the procedure used had removed 99% of the ^{125}I counts, the supernatant was

tested for infectivity by serial 10-fold dilutions injected I.C. into Swiss-Webster mice as described above. Some mouse sera tested had immunoglobulin concentrations that exceeded the precipitating capacity of the rabbit antiserum as used above. These were diluted 1:2 in phosphate-buffered saline (PBS) and then carried through the procedures as before. Controls included: (a) Attempts to precipitate LCM virus grown on L cells which had no immunoglobulin present on its surface. No measurable precipitation of infectivity occurred when this virus was first mixed with heat inactivated normal mouse serum and carried through the above procedure. (b) Use of rabbit antiserum to mouse albumin as the precipitating antiserum. No loss of infectivity occurred.

RESULTS

Prevention of Immune Response to LCM Virus.—12 normal 6-wk old SWR/J male mice were given 4 weekly doses of 300 R. Several hours after the fourth dose of irradiation, 10^6 LD₅₀ of LCM virus was administered I.C. Weekly doses of x-irradiation of 300 R were continued for 6–8 wk more at which time the mice were sacrificed.

The virus titer in plasma from these mice at the time of sacrifice was $10^{3.7 \pm 0.3}$ LD₅₀/0.03 ml and circulating immunoglobulin-virus complexes were not demonstrated in any of four mice examined. Immunoglobulin levels were 0.4 mg/ml with a range of 0.2 mg/ml to 0.6 mg/ml (normal 2.3 ± 0.4 mg/ml). There was no histologic evidence of acute inflammatory disease characteristic of acute LCM infection. As expected of mice that had been infected for only 6–8 wk, no histopathologic evidence of chronic glomerular disease was noted. Immunofluorescent examination of each mouse's kidney, liver, and brain showed a distribution of LCM antigen similar to that in neonatally infected mice without associated Ig and C and no immune deposits in glomeruli.

In a second experiment 20 SWR/J male mice were given a LD₅₀ x-irradiation dose of 700 R, a dose shown to suppress the primary immune response to keyhole limpet hemocyanin (KLH) for at least 1 mo, and infected with 10^6 LD₅₀ of LCM virus I.C. 36 h later. No further irradiation was given. 10 of the mice died within 2 wk. Plasma from the remaining 10 mice had virus titers of $10^{3.5 \pm 0.2}$ LD₅₀/0.03 ml 2 mo after infection. This value was the same 4 mo after infection at which time the mice were sacrificed. At this time immunoglobulin was complexed to virus in their plasma indicating that the mice had recovered from the radiation and were synthesizing anti-LCM antibody. After 99% of the plasma immunoglobulin had been precipitated, virus titers in the plasma decreased two logs. The average concentration of serum immunoglobulin was within normal limits, 2.1 mg/ml with a range of 1.9 mg/ml to 2.4 mg/ml. Heavy PAS positive glomerular deposits were seen on histopathological examination, and immunofluorescence showed marked glomerular deposition of murine immunoglobulin and complement.

Suppression of an Established Immune Response.—This portion of the study utilized SWR/J and C3H mice infected neonatally with 10^6 LD₅₀ of LCM virus I.C. Immunosuppression by both x-irradiation and cyclophosphamide

injections began when the mice were 2-mo old and continued weekly for the duration of the experiments.

In Table I are shown the virus titers of C3H mice which underwent immunosuppressive procedures for 1 mo. The dose of x-irradiation was 150 R per week, the largest amount of radiation tolerated by uninfected mice. The dose of cyclophosphamide was 50 mg/kg body weight per week, a dose shown to suppress a primary response to KLH in preliminary investigations. As can be seen, the virus titers increased by about $10^{0.5}$ for control-, irradiated-, and cyclophosphamide-treated animals. Thus the experimental manipulations had no apparent effect for the short time the mice were studied.

During the course of the experiments, the C3H mice neonatally infected with LCM virus became ill, and they died after only 4–6 wk of treatment. We found that these mice had become pancytopenic and hypogammaglobulinemic

TABLE I

Virus Titers of LCM Carrier C3H Mouse Plasma; Log₁₀ SW Mouse LD₅₀/0.03 ml I. C.

Age of mouse	Controls	150 R/wk	Cytosan
<i>mo</i>			<i>50 mg/kg per wk i. p.</i>
2	3.4 ± 0.4 8 mice	3.3 ± 0.4 11 mice	3.3 ± 0.5 12 mice
3	3.7 ± 0.4 8 mice	3.8 ± 0.4 6 mice	4.0 ± 0.3 8 mice

as a result of bone marrow aplasia in response to only 150 R per week of irradiation. Autopsies showed aplasia of all lymphoid organs but no apparent alteration in the glomerulonephritis which was very mild at the age of the mice studied. Because of the extreme radiosensitivity of the LCM-infected C3H mice in which only six doses of x-irradiation were invariably fatal, these studies were discontinued.

The SWR/J mouse proved to tolerate immunosuppression better, thus allowing extended observations. In Table II are shown the virus titers of immunosuppressed SWR/J mice neonatally infected with LCM virus and the doses of immunosuppressant are indicated. No significant change in virus titer was noted for the various doses of suppressants used. Immunoglobulin complexed to virus was present in the plasma of four out of four mice tested in the 300 R/wk irradiated group after 5 wk of treatment indicating an anti-LCM response persisted. In the group given the larger dose of cyclophosphamide, five out of eight mice tested after 4 mo of immunosuppression had no detectable immunoglobulin complexed to virus.

The degree of immunoglobulin depression resulting from the larger doses of immunosuppressant in SWR/J mice is shown in Fig. 1. As can be seen, the serum immunoglobulin level was progressively depressed over a 6 mo period

TABLE II
Virus Titers of LCM Carrier SWR/J Mouse Plasma; Log₁₀ SW Mouse LD₅₀/0.03 ml I. C.

Age of mouse	Untreated controls	Cytoxan	Cytoxan	Irradiation	Irradiation
<i>mo</i>		50 mg/kg per wk	70 mg/kg per wk	150 R/wk	300 R/wk
2	4.0 ± 0.3	4.0 ± 0.3	4.1 ± 0.4	4.0 ± 0.3	4.0 ± 0.3
3	4.0 ± 0.3	3.5 ± 0.1			4.4 ± 0.6
4	3.9 ± 0.5	4.0 ± 0.2	3.7 ± 0.4	4.3 ± 0.3	
5	4.1 ± 0.4				
6	4.1 ± 0.2	3.9 ± 0.2	4.3 ± 0.6		
7	3.7 ± 0.2				
8	3.5 ± 0.2	3.9 ± 0.3	3.7 ± 0.4		
10	3.7 ± 0.3	3.7 ± 0.2			

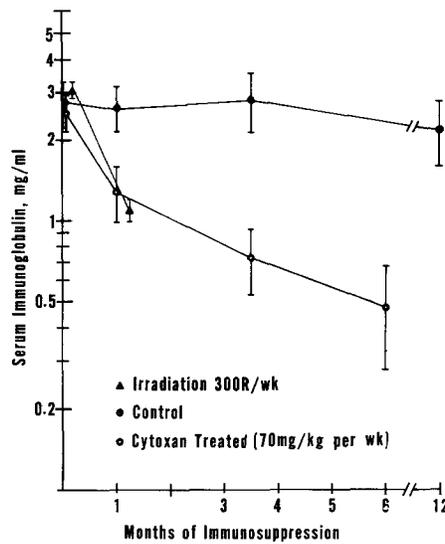


FIG. 1. Effect of immunosuppression on serum immunoglobulin levels of SWR/J mice chronically infected with LCM virus.

when cyclophosphamide was used. The mice given 300 R/wk of irradiation also had decreases in serum immunoglobulin levels but the procedure was soon lethal, ending further observations.

The glomerulonephritis of untreated SWR/J mice chronically infected with LCM virus is illustrated in the series of pictures *A-E* (Fig. 2) that shows progressive increases in the amount of PAS positive glomerular deposits at 2, 4, 6, 8, and 10 mo. For comparison, the glomerulus in *F* is from an 8-mo old mouse treated with cyclophosphamide for 6 mo. 24 of 26 mice treated with 70 mg cyclophosphamide per kilogram body weight had no glomerular disease. The two mice that developed glomerular disease while being given cyclophos-

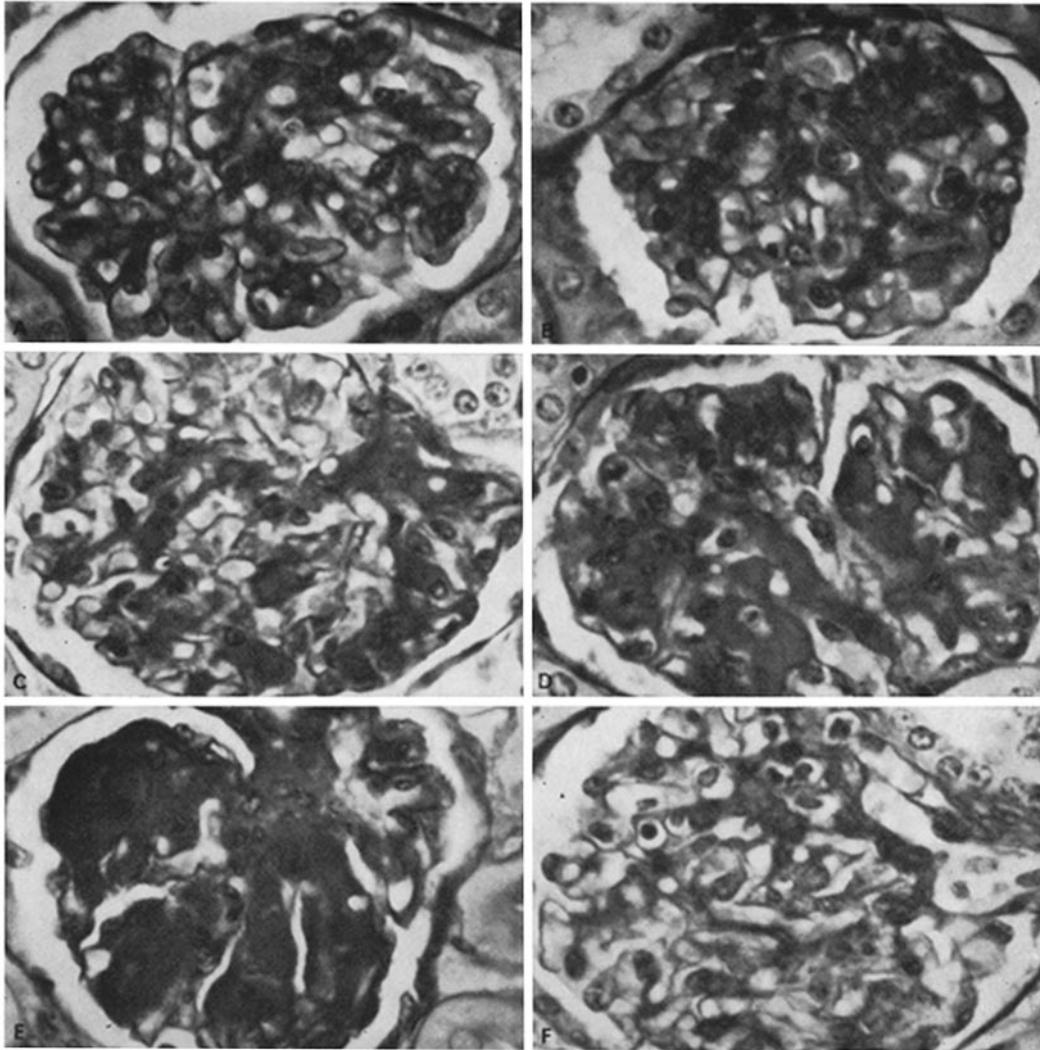


FIG. 2. Series of glomeruli from SWR/J mice infected with LCM virus for (A) 2 mo, (B) 4 mo, (C) 6 mo, (D) 8 mo, and (E) 10 mo. Progressive deposition of glomerular PAS positive material is shown. In (F) is glomerulus from 8-mo old mouse treated with cyclophosphamide for 6 mo showing absence of PAS positive material. ($\times 400$, PAS)

phamide were lymphoid depressed as measured by depressed serum immunoglobulin levels and decreased peripheral white blood cell counts. One of these two mice was tested for immunoglobulin complexed to LCM virus and complexes were present indicating a persistent anti-LCM response.

Fig. 3 shows representative immunofluorescent studies of glomeruli from cyclophosphamide treated and control 8-mo old SWR/J mice chronically

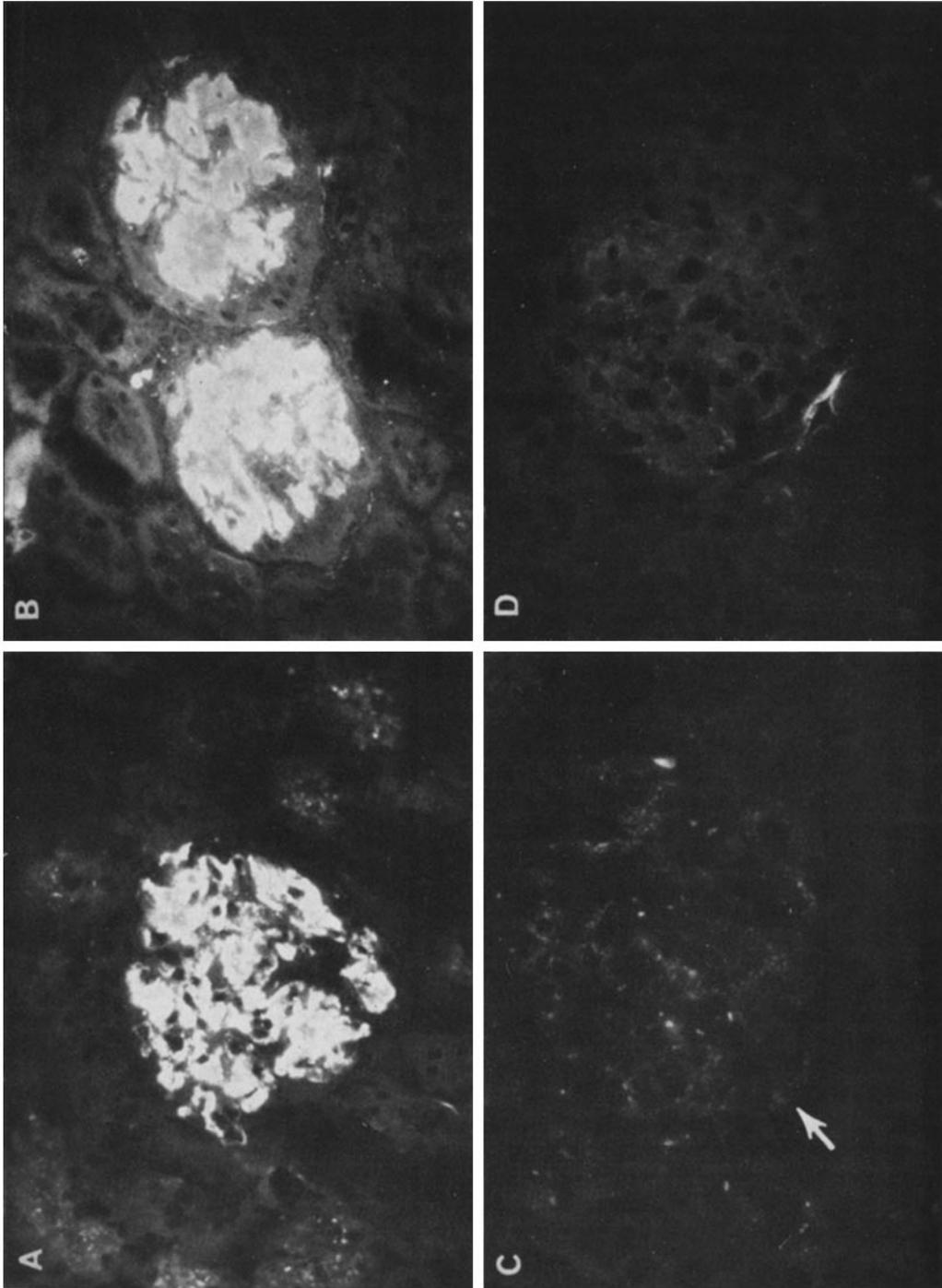


FIG. 3. Immunofluorescent micrographs of glomeruli from SWR/J mice chronically infected with LCM virus for 8 mo. (A) Rabbit antimouse Ig on control and (B) rabbit antimouse C3 on control show heavy immune deposits. (C) Antimouse Ig (arrow indicates margin of glomerulus) and (D) antimouse C3 on glomeruli from mice treated with cytoxin for 6 mo show only traces of immunoglobulin and no complement. (X 240)

infected with LCM virus. As described in the legend for Fig. 3, immunoglobulin and complement deposits found in control mice were prevented by cyclophosphamide administration to the experimental group. 24 of 26 cyclophosphamide-treated mice had no or very little immune complex deposition in their glomeruli.

Frozen sections of liver, kidney, and brain stained with a fluorescein conjugated anti-LCM antiserum were examined to assess the amount of LCM antigen; marked variation in the amount of antigen occurred from one mouse to the next in all groups. However, on the average, control and immunosuppressed groups had comparable amounts and distribution of tissue antigen. Shown in Fig. 4 is the distribution of LCM antigen in tubules of a kidney section. The percent of tubules on a given section which were positive for LCM antigen varied from 2% to 30%. No group of mice had a percent of positive tubules statistically different from other groups. Thus, as with the virus titers in plasma, immunosuppression did not increase quantities of immunohistochemically detectable LCM virus antigen in tissues. There was no difference between the small amount of LCM viral antigen deposited in the glomeruli of control and immunosuppressed mice. Microscopic sections of liver, spleen, brain, kidney, lung, heart, bone marrow, thymus, and a portion of intestine were scanned for the presence of tumors. A total of 140 uninfected and 225 LCM-infected mice were examined. One case of lymphocytic leukemia was

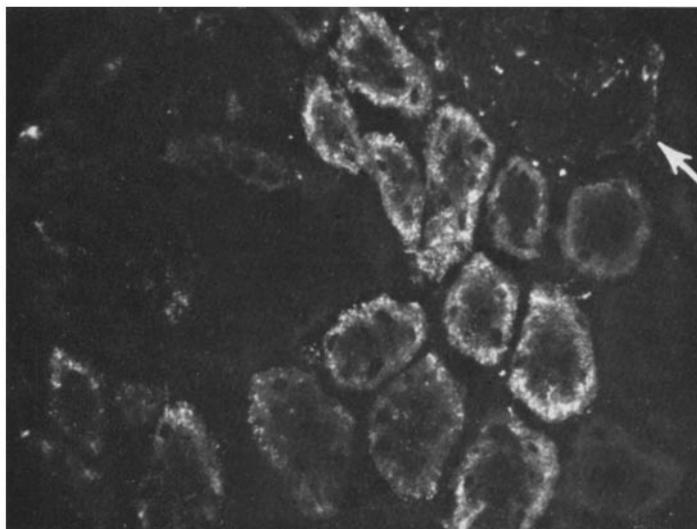


FIG. 4. LCM antigen in the tubules and a glomerulus (arrow) of kidney from a 6-mo old SWR/J mouse chronically infected with LCM virus. Antigen positive tubules appeared in groups such as shown here; most tubules were antigen negative in both control and immunosuppressed mice. Little antigen was seen in glomeruli and immunosuppressive manipulations did not alter this. ($\times 240$)

found in a 10-mo old control SWR/J mouse. Two pulmonary adenomas were found in uninfected control SWR/J mice; none were found in LCM-infected mice. Neoplasms were not seen in any of the cyclophosphamide treated or x-irradiated mice of the SWR/J strain. Neoplasms were not seen in any of the control or immunosuppressed C3H mice.

DISCUSSION

Our studies were directed toward assessing the role played by the immune response in determining the titer of virus in plasma and the amount of viral antigen in tissues of mice acutely and chronically infected with LCM virus. This problem was approached by using two methods: the first was to prevent primary immune responses in adult mice by irradiation with 4 weekly doses of 300 R preceding infection with LCM virus. As noted, the amount of virus that irradiated mice continued to carry was no different from that carried by neonatally infected nonsuppressed mice of the same age. No acute disease and no glomerular immune deposits were seen, and immunoglobulin complexed to circulating virus was not demonstrated, indicating that the humoral immune response to the virus had been prevented. An alternative method of inducing the LCM carrier state in adult mice was infecting mice 36 h after a single dose of 700 R x-irradiation. This treatment did not prevent the late development of glomerulonephritis, and immunoglobulin complexed circulating virus was present 2 mo after the carrier state was induced indicating a recovery of antibody formation.

The second method of assessing the role of the immune response in chronic LCM infection was to immunosuppress neonatally-infected mice after they reached 2 mo of age. These mice already had circulating immunoglobulin complexed virus and immune deposits in their glomeruli. Thus, the task was to ablate an established immune response, a situation more applicable to therapy of disease in progress. Here x-irradiation in maximally tolerated doses was ineffective in suppressing antiviral antibody formation as indicated by the persistence of immunoglobulin-virus complexes in the circulation, although glomerular accumulation of immune complexes was prevented. By contrast, weekly doses of cyclophosphamide given over a prolonged period were effective in clearing the circulation of immunoglobulin complexed to virus in some cases. Prolonged cyclophosphamide administration was also effective in preventing glomerular immune complex deposition in over 90% of the mice treated. However, neither x-irradiation nor cyclophosphamide altered the amount of virus carried by these mice.

It has not been possible to establish the potential roles of humoral and cellular immune responses on virus levels in chronic LCM infection. In our experiments it is clear that the levels of antibody achieved in the infected animals alone is not a potent regulator of LCM levels since their suppression caused no change in LCM level. In addition, whatever cellular immunosuppression

was achieved by the x-ray and cyclophosphamide doses employed also did not affect LCM levels. If one assesses cellular immune function by skin graft survival, cyclophosphamide is not an impressive cellular immunosuppressant (8). The effect of x-irradiation on cellular immune mechanisms depends on a number of variables as discussed by Taliaferro et al. (9). The work of Uhr and Scharff (10) indicates that established cellular immunity is not ablated by subsequent sublethal irradiation. It is thus probable that some degree of cellular immunity persisted in our irradiated mice chronically infected with LCM.

Our data agree with and extend the results of others. Sharon and Pollard (11) administered cyclophosphamide to LCM-infected Haas strain gnotobiotic mice. They stated that there was no change in virus titers over a 5-mo period although neither quantitation of virus titers nor demonstration of the effectiveness of immunosuppression was presented. Our data include quantitative determinations of total serum Ig suppression in cyclophosphamide and also x-ray treated mice (Fig. 1). In addition, we were able to show that LCM-specific antibody complexed to the circulating virus could be suppressed without altering the plasma virus titer. In 1956, Rowe (12) first noted that virus titers in adult mice given single doses of x-irradiation before LCM infection remained unaltered from control nonirradiated acutely infected mice. Hotchin and Weigand (13) made similar observations. While a single dose of irradiation at the time of LCM infection of an adult mouse may be life saving, our data show that a subsequent anti-LCM humoral immune response does develop just as in the neonatally-infected mice. We found that continued weekly irradiation could prevent the humoral response and yet not alter the plasma virus titers. Recently, Brojorgansen et al. administered a single dose of x-irradiation to C3H mice chronically infected with LCM virus. Virus titers in plasma were unchanged in spite of excessive mortality resulting from stem cell lesions after doses of x-irradiation that were sublethal for uninfected mice (14). In confirmation, we found that C3H mice chronically infected with LCM virus developed bone marrow aplasia and died after irradiation doses tolerated by SWR/J mice. Sharon has reported similar radiation sensitivity in Haas strain gnotobiotic mice chronically infected with LCM virus (15).

Effects of cyclophosphamide on various chronic viral infections have been investigated with different results. In chronic LDV infection of mice, Dubey et al. (16) observed increased virus titers in plasma after 2 mo of weekly cyclophosphamide administration, although the mice underwent no obvious ill effects. Cheema et al. (17) noted a log increase in virus titer of mink infected with Aleutian disease and treated with cyclophosphamide; glomerular immune complex deposition which occurs in unmanipulated mink infected with Aleutian disease virus was prevented. Thus, in these two chronic viral infections the quantities of virus carried by the hosts apparently were increased by immunosuppression in contrast to the observations with LCM.

There is speculation in the literature that some chronic glomerulonephritis in humans are caused by chronic viral infections (18, 19). Although cyclophosphamide has not been proven generally beneficial to patients with chronic immune complex glomerulonephritis (20-27), experimental results of this and other reports (11, 17, 28) may encourage further trials.

Prolonged immunosuppressive therapy of the disease occurring in (NZB \times NZW) F_1 hybrids may cause increased incidence of neoplasia in the females. Walker and Bole reported malignancies of various types in 6 out of 10 mice given daily injections of 8 mg of cyclophosphamide per kilogram body weight for 36-64 wk (29). Russell and Hicks reported a 29% incidence of neoplasms of various types in 76 mice given long term cyclophosphamide treatment (28). This contrasts sharply with the total absence of neoplasia in our immunosuppressed SWR/J mice chronically infected with LCM virus. The absence of tumors in our SWR/J mice treated for 6 mo may be explained by differences inherent in the mouse strains or by the shorter period of treatment of our mice.

SUMMARY

C3H mice chronically infected with LCM virus were found to be lethally affected by small doses of immunosuppression which caused bone marrow aplasia but had no effect on the amount of virus carried by the mouse. Humoral immune response of SWR/J mice to acute LCM infection was found to be totally suppressed by repeated single doses of 300 R/wk with no alteration in the level of virus carried by the mouse. In contrast, the established anti-LCM humoral immune response encountered in mice chronically infected with LCM virus was not suppressed by the same irradiation procedure. Over half of the chronic LCM carrier SWR/J mice treated with cyclophosphamide for 6 mo had total anti-LCM humoral immunosuppression, but showed no change in the level of virus carried. The glomerulonephritis which occurs in chronic LCM carrier mice was prevented by cyclophosphamide treatment in 90% of the mice. The humoral immune response which occurs in chronic LCM carrier mice appears to play no role in controlling the amount of virus carried by the mouse. Suppression of the LCM immune response by cyclophosphamide does prevent the development of glomerulonephritis in these mice.

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