# IMMUNOPATHOGENESIS OF ACUTE CENTRAL NERVOUS SYSTEM DISEASE PRODUCED BY LYMPHOCYTIC CHORIOMENINGITIS VIRUS

## I. Cyclophosphamide-Mediated Induction of the Virus-Carrier State in Adult Mice*

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Lymphocytic choriomeningitis (LCM)[1] virus takes its name from the acutely fatal central nervous system (CNS) disease which was described by Armstrong and Lillie in their original isolation and characterization of this agent (1). Subsequent work has established that LCM virus will regularly produce infection in adult mice but the occurrence of fatal choriomeningitis is influenced by the interaction of variables including route of inoculation (2, 3), strain of virus (4, 5), and age and strain of mouse (6–9). Brains of affected mice show severe infection of choroid plexus, ependyma, and leptomeninges (10, 11), and animals die with a characteristic syndrome (1, 2, 4) including a prominent convulsive diathesis, the exact mechanism of which has not been elucidated.

Evidence that acute choriomeningitis has an immunopathological basis derives from two sources: (*a*) the noncytocidal nature of LCM infection of cells in vivo as indicated by the ability of mice, infected during gestation or within 24 hr of birth, to develop and reproduce in spite of a widespread systemic infection (12, 13), and (*b*) the ability of immunosuppressive treatments to convert a potentially fatal infection into a chronic carrier state compatible with long-term survival, in spite of high virus titers in the brain or other tissues (14–16).

The present study reports the use of cyclophosphamide (CY) as a convenient and reliable method of converting potentially lethal choriomeningitis in adult mice into a nonfatal carrier infection, together with a description of the

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[1] *Abbreviations used in this paper:* CF, complement fixation; CNS, central nervous system; CY, cyclophosphamide; i.c., intracerebral; LCM, lymphocytic choriomeningitis; PBS, phosphate-buffered saline; PID, days after infection.

progression of infection which occurs in the CNS during persistence of the carrier state. A comparison is then made with the carrier state established by virus inoculation of neonatal mice, in which the distribution of virus infection in the CNS differs both qualitatively and quantitatively. Most importantly, these observations are essential for interpretation of the different response of these two types of carriers to transferred immune lymphoid cells, the subject of the next paper in this series. A preliminary overview of these studies has been presented in a recent review (17).

## *Materials and Methods*

*Animals.*—Weanling and adult male (10–12-wk-old) BALB/c mice obtained from Flow Laboratories, Dublin, Va., were used for experimental infections, and adult Swiss Webster mice from Microbiological Associates, Walkersville, Md., were used for virus titrations.

*Drug.*—CY (Cytoxan, Mead Johnson & Co., Evansville, Ind.) was diluted to give a dose of 150 mg/kg in 0.5 ml which was injected intraperitoneally. This dose produces toxic deaths in no more than 10% of mice weighing over 20 g, while the fatality rate is greater in younger mice.

*Virus.*—The E-350 strain of LCM virus was provided by Dr. Frederick Murphy (16) who originally obtained it from the American Type Culture Collection (VR 134). Stock virus was prepared by intracerebral (i.c.) inoculation of adult mice, and brains were harvested 6 days later. A clarified 20% homogenate in buffered saline was stored at −70°C. This preparation had an adult mouse i.c. titer of $10^6$ $LD_{50}$/0.03 ml, and was used at a $10^{-3}$ dilution to give a standard inoculum of about 1000 $LD_{50}$.

*Virus Titrations.*—Anesthetized mice were bled from the heart or the retroorbital venous plexus; blood was diluted into 4 volumes of cold phosphate-buffered saline (PBS) containing 0.75% bovine plasma albumin with heparin, and plasma was obtained by centrifugation. Brains were removed from perfused mice, homogenized, and prepared as clarified 20% suspensions in PBS. Decimal dilutions of plasma or brain homogenates were titrated i.c. in adult Swiss mice. Titers were calculated by the Kärber method (18) and expressed as the $LD_{50}$/0.03 ml or 0.03 g.

*Immunofluorescent Staining.*—Brains from saline-perfused mice were cut in 8-$\mu$ parasagittal sections in a cryostat, fixed in acetone for 5 min, and stored at −20°C. Fluorescein-conjugated LCM-immune serum was obtained from Dr. Roger Wilsnack (10), and used with an Evans blue counterstain as previously described (19). The specificity of this reagent was indicated by its failure to stain uninfected brains and by an approximate correlation of numbers of stainable cells with titer of infectious virus. CNS areas were graded for fluorescence according to a semiquantitative scale: trace, less than 1% of cells fluorescing; 1, 1–25%; 2, 25–50%; 3, 50–75%; and 4, 75–100%.

*Light Microscopy.*—Mice were perfused with isotonic saline followed by 1% acetic acid in 10% formalin. Paraffin sections were cut at 10 $\mu$ and stained with hematoxylin and erythrosin.

*Complement-Fixing (CF) Antibody.*—Sera were tested for CF antibody in a microtiter test (20), using LCM antigen at an optimum dilution of 1:40 (obtained from Microbiological Associates, Inc., Bethesda, Md.) and 5 $CH_{50}$. Any anticomplementary activity present in sera was successfully removed by incubating equal volumes of serum, saline, and fresh ovalbumin for 30 min at 45°C.

## RESULTS

## *Drug-Induced Adult LCM Carriers*

*Protection from Acute LCM Disease by Cyclophosphamide.*—All adult BALB/c mice inoculated i.c. with 1000 $LD_{50}$ of LCM virus developed acute LCM

within 6–8 days. Fig. 1 illustrates survival after a single intraperitoneal (i.p.) dose of CY, 150 mg/kg, given 3 days after LCM virus. More than 90% of mice were spared from acute LCM. After recovering from the temporary toxicity caused by the drug (weight and hair loss), they remained asymptomatic for at least 6 months. A small number of mice (less than 10%) which received LCM followed by CY developed a fatal convulsive diathesis from 1 to 2 wk after infection. These animals appeared to have "escaped" from the immunosuppressive effect of CY, and the presence of acute choriomeningitis was confirmed histologically. There were no deaths in mice which received CY only.

*Timing of Immunosuppression.*—To permanently protect the greatest number of mice from acutely fatal LCM disease, the most propitious time to give CY

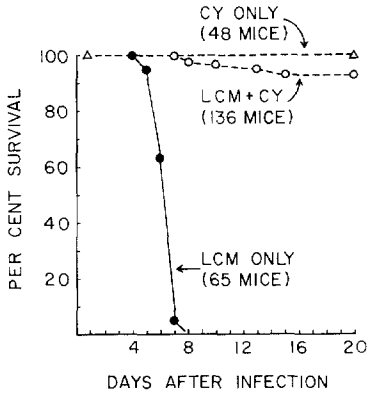


FIG. 1. Survival after i.c. inoculation of 1000 $LD_{50}$ of LCM virus in 12-wk-old BALB/c mice, and the effect of a single i.p. inoculation of CY, 150 mg/kg, 3 days after virus.

was 3–5 days after virus. If drug administration was delayed until 6–7 days after virus inoculation all mice died and survival time was not prolonged.

If a single CY dose of 150 mg/kg on day 3 was followed by doses of 50 mg/kg on days 8, 13, 18, and 23, effective immunosuppression occurred in all mice, and there were no deaths up to 21 days after infection. However, many mice failed to survive this drug schedule, and died 20–25 days after infection, so that 40 day survival was less than 50%.

*Neuropathological Observations.*—Mice given virus only were sacrificed daily to follow the development of CNS lesions. Pathologic changes, except for infrequent mild vasculitis, were confirmed to choroid plexus, ependyma, and meninges. The most severe inflammation was regularly seen in the choroid plexus of all ventricles. It first appeared about 48 hr before death (4 or 5 days after infection) in the area of the tenia choroidea and then spread to the rest of the plexus. The early infiltrate was composed primarily of large mononuclear cells with fine chromatin granules and nuclear indentation. In addition, a few polymorphonuclear leukocytes were seen. 2 days later, in moribund mice the

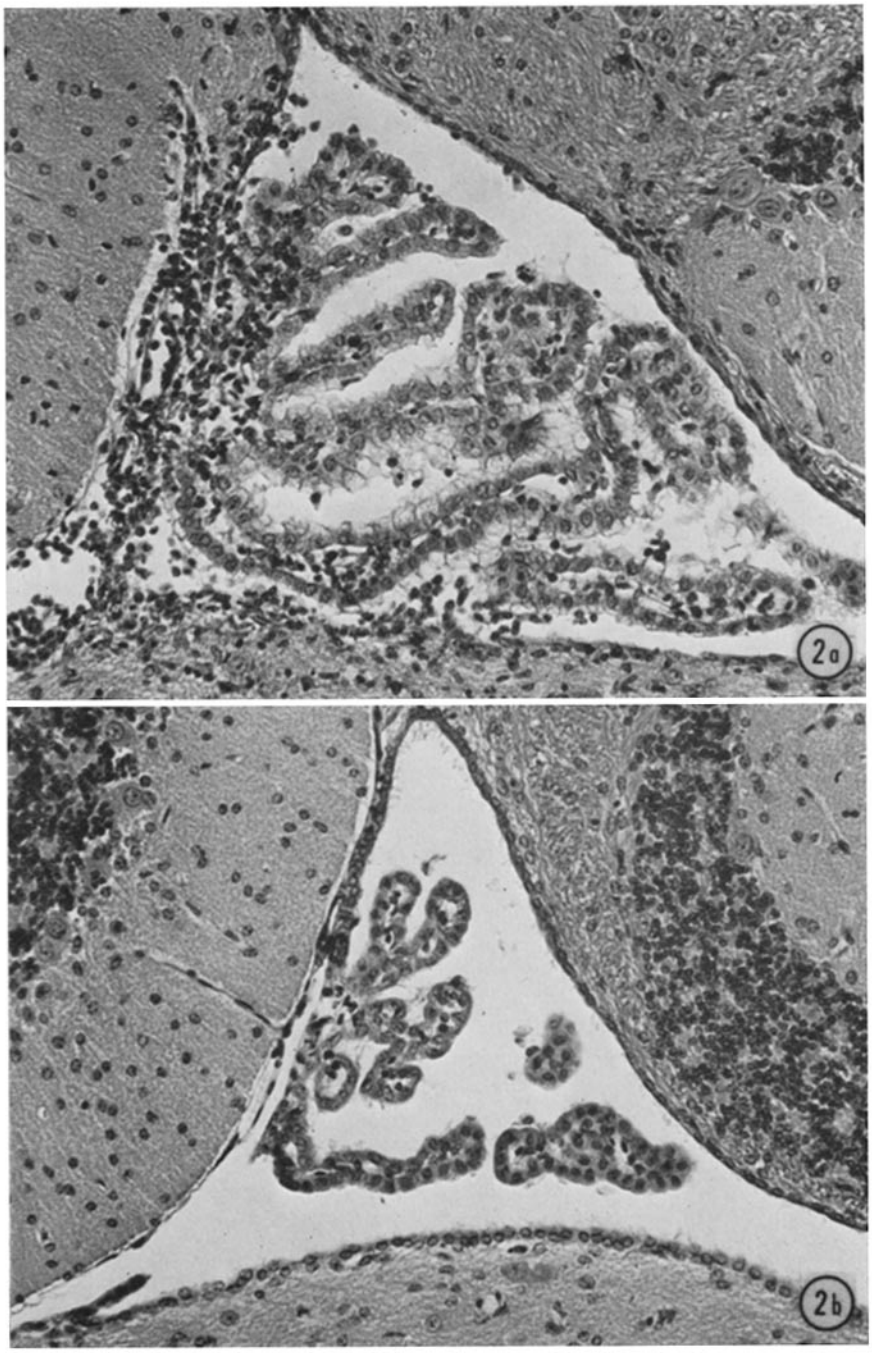


FIG. 2. (*a*) Choroid plexus of fourth ventricle from a mouse inoculated i.c. with LCM virus and perfused when moribund 6 days later. Hematoxylin and erythrosin. × 225. (*b*) Similar view of choroid plexus from a mouse inoculated with virus, given CY (150 mg/kg) 3 days later, and asymptomatic when perfused 10 days after virus injection. × 225.

inflammatory lesion (Fig. 2 *a*) was composed mostly of small and large mononuclear cells, but polymorphonuclear leukocytes were more common.

Adult mice receiving LCM virus followed by CY did not develop histologic evidence of acute choriomeningitis (Fig. 2 *b*) except for the less than 10% of mice which died 7–14 days later of acute LCM disease. CNS lesions in these animals were similar to those in mice receiving virus only.

*Virologic Studies.*—The titers of virus in brain and blood obtained at serial intervals after i.c. inoculation of adult mice are shown in Table I. The brain

TABLE I

*Titers of Virus in Brain and Blood of Adult Male BALB/c Mice Inoculated Intracerebrally with LCM Virus with and without CY**

| Specimen | CY | Days after infection | No. of pools | $Log_{10}$ titer per 0.03 ml or 0.03 g | |
|---|---|---|---|---|---|
| | | | | Median | Range |
| Brain | − | 1 | 1 | <0.5 | — |
| | − | 3 | 1 | 5.0 | — |
| | − | 5 | 3 | 5.7 | 5.3–6.0 |
| | − | 6 | 8 | 5.8 | 5.7–6.2 |
| | − | 7 | 2 | 5.6 | 5.2–6.0 |
| | + | 5–15 | 9 | 6.0 | 5.3–6.9 |
| | + | 17–32 | 8 | 5.0 | 4.6–6.0 |
| | + | 131–220 | 2 | 3.0 | 2.8–3.3 |
| Plasma | − | 1 | 1 | <0.5 | — |
| | − | 3 | 4 | 0.6 | <0.5–0.7 |
| | − | 5 | 4 | 0.7 | <0.5–0.8 |
| | − | 6 | 3 | 0.8 | 0.8–1.3 |
| | − | 7 | 3 | <0.5 | <0.5–<0.5 |
| | + | 7 | 2 | 1.1 | 0.8–1.5 |
| | + | 9–15 | 4 | 2.4 | 1.8–3.2 |
| | + | 17–32 | 3 | 1.5 | 1.0–3.2 |
| | + | 131–220 | 2 | 3.9 | 3.8–4.0 |

* Specimens from two mice were combined for each pool. Mice received a single dose of CY on days 3 or 5 or multiple doses on days 3, 8, 13, 18, and 23 after infection. Virus titers were similar regardless of CY schedule.

was heavily infected by day 3 and peak virus titers were reached within 5 days after inoculation. Minimal viremia was present from days 3 through 6 but had disappeared by day 7 in mice which received virus only.

In CY-induced LCM carrier mice, virus titers in the brain persisted at a level similar to that seen in the moribund animal for at least 30 days after infection, and a small number of specimens examined up to 7 months after infection yielded considerable virus titers. Viremia was regularly present in immunosuppressed mice in plasmas obtained from 7 days to 1 yr after infection, at a higher titer than the trace levels seen in nonsuppressed animals.

*CF Antibody.*—No antibody was found in the sera of mice dying of acute

LCM, or those of drug-induced virus carriers (tested up to 30 days after initiation of infection), or of neonatal carriers (tested up to 45 days after infection).

*Distribution of Virus in the Brain.*—Table II compares the distribution of viral antigen in different areas of the brains from mice inoculated i.c. with LCM virus and examined as adults by direct immunofluorescent staining.

TABLE II

*Distribution and Severity of Infection in the CNS of Mice Inoculated Intracerebrally with LCM Virus**

| CNS area | LCM only | LCM + CY | | Neonatal LCM | |
|---|---|---|---|---|---|
| | PID 6–8 | PID 7–12 | PID 23–32 | PID 7 | PID 56–75 |
| Choroid plexus and ependyma | 4‡ | 3 | 3 | 3 | 1 |
| Leptomeninges | t | t | t | 2 | t |
| Endothelium | 0 | 0 | 0 | t | t |
| Cerebral cortex | 0 | t | t | 2 | 2 |
| Olfactory area | t | 1 | 1 | 3 | 3 |
| Hippocampus, pyramidal cells | 0 | 0 | t | 2 | 2 |
| Hippocampus, dentate gyrus | 0 | 0 | t | 3 | 3 |
| Lenticulostriate area | 0 | t | 1 | 2 | 2 |
| Subventricular plate | | | | 3 | |
| Diencephalon | t | t | 1 | 2 | 2 |
| Midbrain | 0 | 0 | t | 2 | 1 |
| Pons and medulla | 0 | 0 | t | 2 | 1 |
| Cerebellum, Purkinje cells | 0 | 0 | 0 | t | 2 |
| Cerebellum, granule cells | 0 | 0 | t | 3 | t |
| Cerebellum, molecular layer | 0 | t | 2 | | t |
| Cerebellum, white matter | 0 | 0 | t | t | t |

* LCM only: inoculated as adults. LCM + CY: inoculated as adults and given CY, 150 mg/kg, on day 3 after infection (PID 3). Neonatal LCM: inoculated within 24 hr of birth. PID: days from infection to sacrifice. Choroid plexus represents composite for lateral and fourth ventricles. Olfactory area includes olfactory bulb and paleocortex.

‡ Proportion of cells infected based on immunofluorescent staining: t, less than 1%; 1, 1–25%; 2, 25–50%; 3, 50–75%; 4, 75–100%. Each column represents median of one to four sections examined on each of 5–10 mice. In almost all instances range was no greater than plus or minus one step on grading scale.

Mice which received LCM virus only and were sacrificed when moribund 6–8 days later demonstrated extensive viral antigen in the choroid plexus and ependyma (Fig. 3 *a*). Virus was seen in the leptomeninges of some animals (Fig. 4 *a*), and trace amounts were often seen in the olfactory area and diencephalon. Other CNS structures were virtually devoid of viral antigen.

In adult mice inoculated i.c. with virus and then immunosuppressed with CY, fluorescent viral antigen not only persisted in choroid plexus, ependyma, and

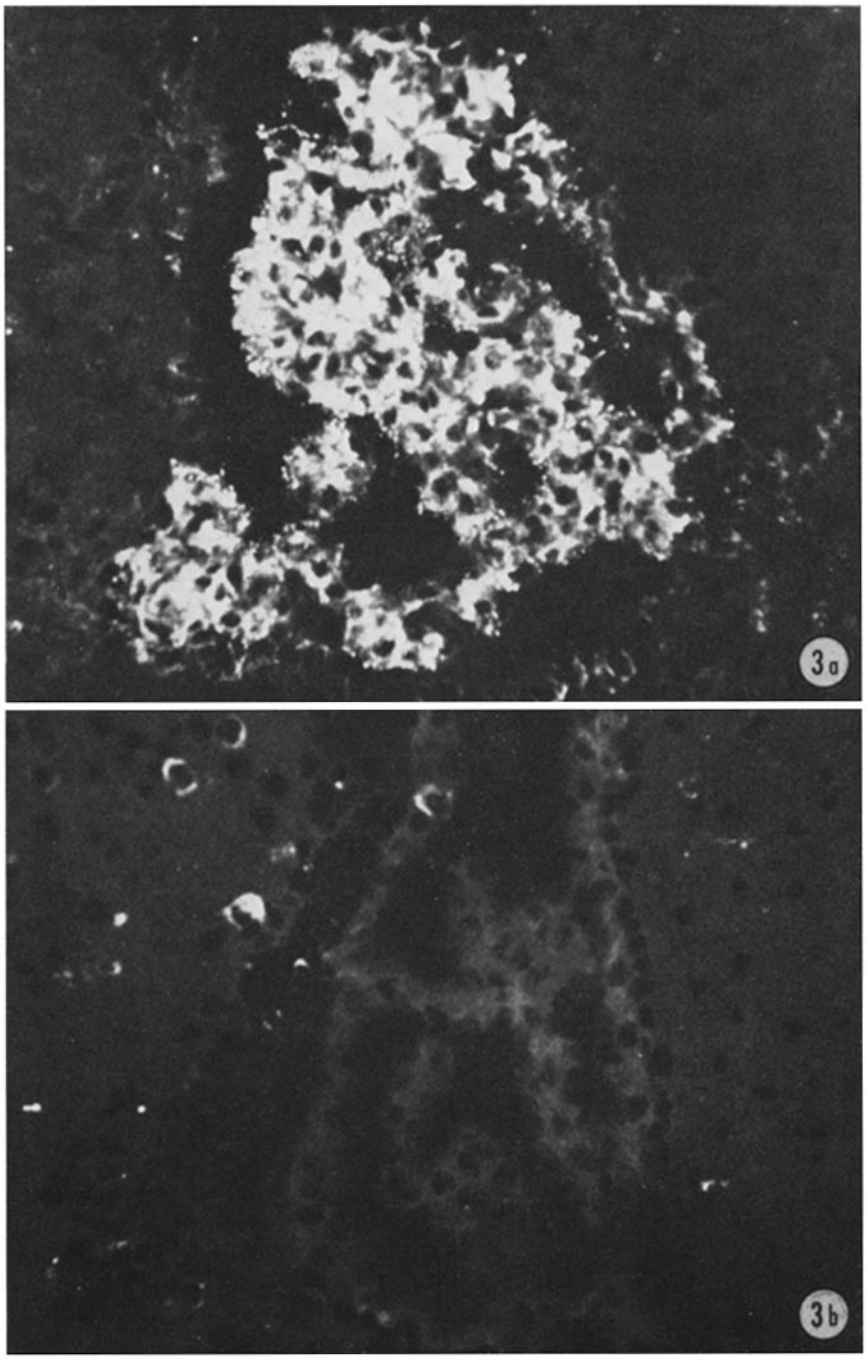


FIG. 3. (*a*) Heavily infected choroid plexus from an adult mouse inoculated i.c. with LCM virus and perfused when moribund 6 days later. Anti-LCM immunofluorescent stain. × 280. (*b*) Similar view to show limited infection of choroid plexus in an LCM-carrier mouse inoculated within 24 hr of birth and killed at age 8 wk. × 280.

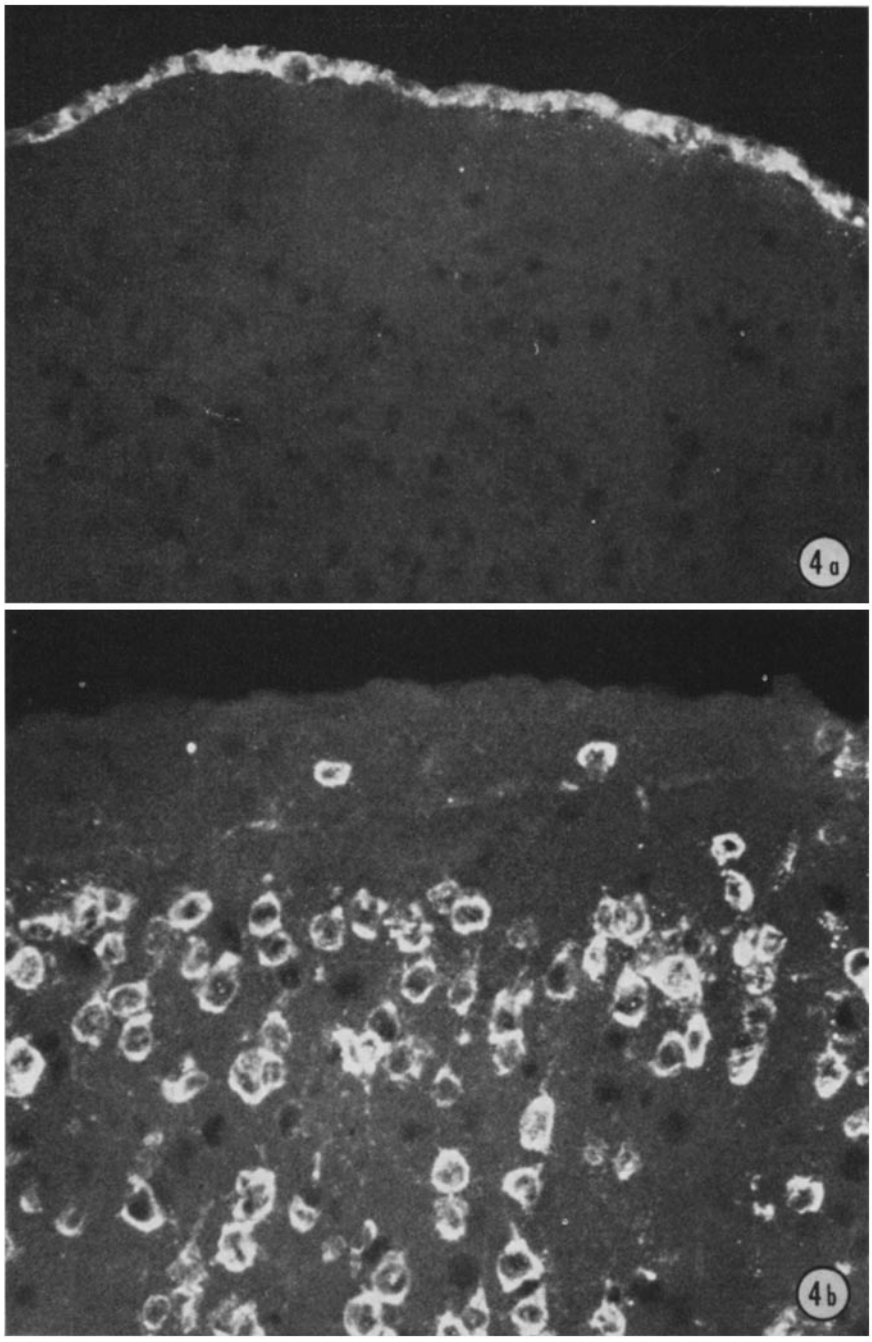


FIG. 4. (*a*) Infection of leptomeninges of cerebral cortex from an adult mouse inoculated i.c. with LCM virus and perfused when moribund 6 days later. Anti-LCM immunofluorescent stain. × 280. (*b*) Similar view of cerebral cortex to show absence of leptomeningeal infection and marked infection of cortical neurons in an LCM-carrier mouse inoculated within 24 hr of birth and killed at age 8 wk. × 280.

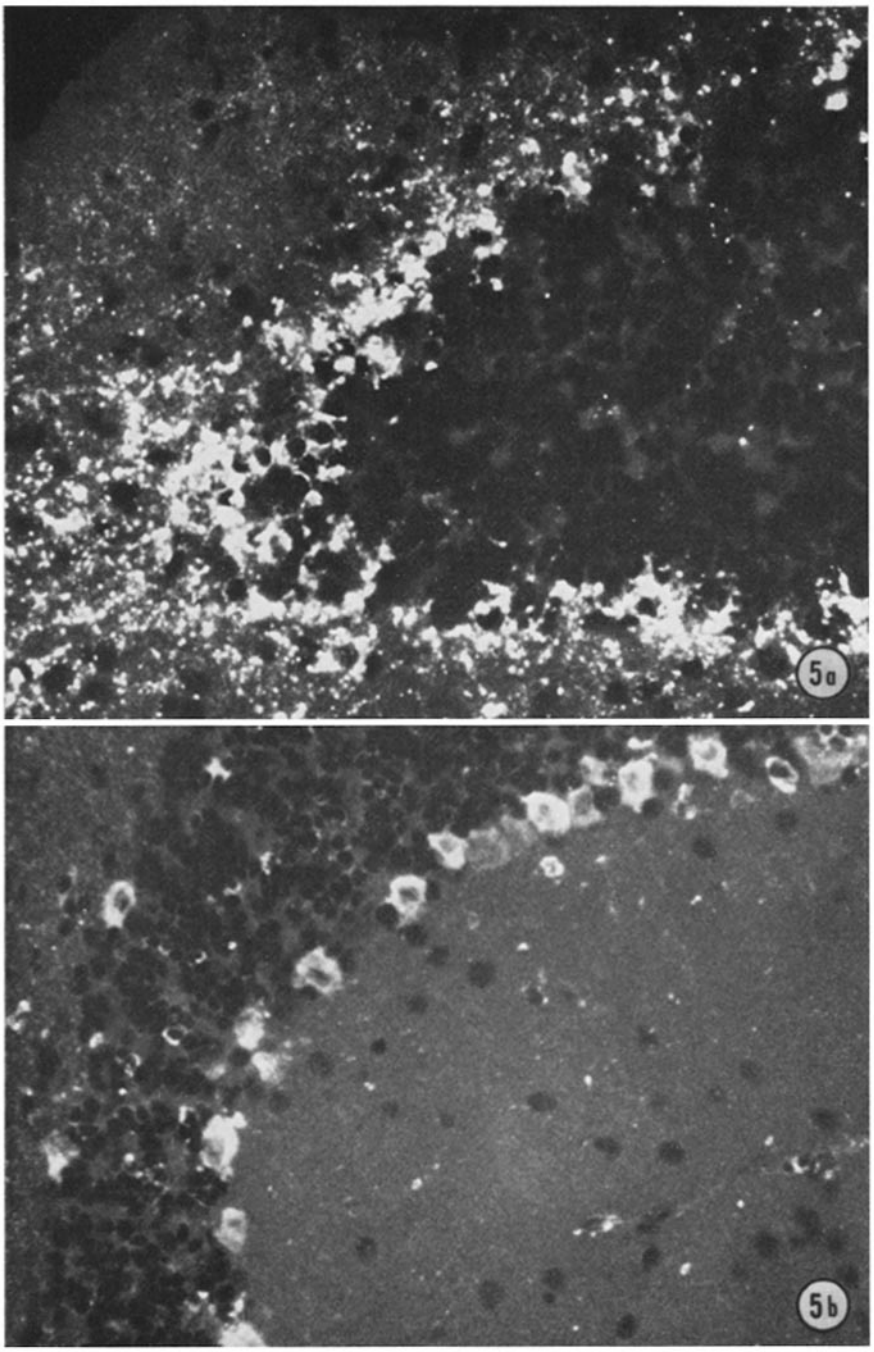


FIG. 5. (*a*) Cerebellar folium showing infection, probably of basket cells, in inner half of molecular layer, from an adult mouse inoculated i.c. with LCM virus (day 0), given a single inoculation of CY (150 mg/kg) on day 3, and apparently normal when perfused on day 24. Anti-LCM immunofluorescent stain. × 280. (*b*) Similar view to show infection of Purkinje cells in an LCM-carrier mouse inoculated within 24 hr of birth and killed at age 8 wk. × 280.

meninges, but gradually extended into the brain parenchyma. After 10–12 days, antigen was most frequently seen in areas (cerebral cortex, lenticulostriate, diencephalon) contiguous to infected membranes, and with time (24–32 days after inoculation) it appeared in many parenchymal areas. Of particular note was the specific cerebellar distribution of viral antigen, most of which was confined to the inner molecular layer adjacent to Purkinje cells with some extension into the outer molecular layer (Fig. 5 *a*). This suggests an association of viral antigen with processes of Bergmann glia, basket cells, or both (21). The Purkinje cells themselves showed minimal evidence of infection; a few were stained in brains of mice with persistent infections of several months duration. Trace amounts of antigen were seen in cerebellar white matter and granule cells. Viral antigen in the brain parenchyma was usually of a finely granular character, contrasting with its appearance in neonatal carriers.

### *Neonatally Inoculated Carriers*

For comparison with the CY-induced carriers, the distribution of viral antigen in the CNS of neonatally inoculated carriers was also examined. Mice were inoculated i.c. within 24 hr of birth and about 80% survived after a transient growth-retarding illness (6) during the first 3 wk of life. As young adults (56–75 days of age) they were neurologically asymptomatic and free of histological evidence of choriomeningitis.

*Viral Antigen in Brain.*—When examined as adults, neonatally established carriers had brain virus titers similar to those in drug-induced carriers, but the appearance and distribution of antigen were strikingly different. There was only a limited infection of choroid plexus (Fig. 3 *b*) and of leptomeninges (Fig. 4 *b*). However, parenchymal structures were far more heavily infected than in drug-induced carriers (Table II). Also, infected cells had a diffuse and heavy burden of antigen throughout the perikaryal cytoplasm, with little apparent extension into dendrites or axons (Figs. 4 *b* and 5 *b*). Another marked distinction between the two kinds of carrier mice was the severe infection of Purkinje cells in animals inoculated at birth (Fig. 5 *b*), with very little viral antigen in other parts of the cerebellum (Table II).

When mice were examined at intervals after neonatal inoculation, the pattern of infection seen in the adult appeared to be established within 1 wk. However, there were several important exceptions. Antigen appeared slowly in Purkinje cells, so that very few were stained 7 days after infection and this proportion gradually increased as the animal matured. In contrast, choroid plexus, leptomeninges, and granule cells were heavily infected 7 days after inoculation with a subsequent marked decrease in the number of antigen-positive cells (Table II).

## DISCUSSION

*Virus Strain.*—These experiments were conducted with a highly neuroadapted strain of LCM virus which normally has little ability to replicate in

extraneural tissues of adult mice. This accounts for the minimal viremia in non-suppressed mice (5), and could influence the response to immunosuppression (13, 14, 22) as well as the distribution of infection in the brain (13).

*Induction of Carrier State by Immunosuppression of Adult Mice.*—The use of a single dose of CY to produce LCM virus-carrier infections of mice is probably one of the most convenient and efficient methods available. In earlier studies, Haas and coworkers (23, 24) reported that the folic acid antagonist, methotrexate, produced only partial and transient suppression of LCM, perhaps because of the use of a suboptimal drug schedule. Hannover Larsen (15), however, found that seven repeated doses of methotrexate administered over the first 14 days after virus injection protected all mice against LCM induced by the viscerotropic Traub strain of virus which kills only 70–80% of mice after i.c. infection.

Hotchin (14) reported that, when induced by X-irradiation, the carrier state was characterized by persistence of virus titers in brain at levels similar to those seen in moribund mice injected with virus only. Our findings in CY-treated mice were similar and the apparently constant titers did not reflect the continued spread of infection in the CNS as revealed by immunofluorescent staining.

Under the conditions of these experiments, mice receiving virus only had minimal levels of viremia which were suppressed by 7 days after infection when animals were dying of an acute immunopathological process, while viremia was regularly present on day 7 in CY-treated mice. This underlines the dual role which the immune response can play in LCM, simultaneously inhibiting spread of infection and mediating disease (4, 25).

*Comparison of CNS Infections in Adult and Neonatal Virus Carriers.*—Infection in the adult carrier induced by CY is initially concentrated in membranes and gradually spreads to contiguous parenchyma, suggesting an age-determined low level of susceptibility of neurons and glia. It seems reasonable to presume that, in some parts of the CNS, unstained cells represent areas to which the infection has not yet extended. Differences in susceptibility between different types of neural cells may account for an unusual concentration of antigen in basket cell processes in the cerebellum without corresponding infection of juxtaposed Purkinje cells.

Neonatally infected animals, even when examined as adults, identical in age with the drug-induced carrier, differ in several respects. Inoculation shortly after birth results in an infection which involves many more cells. There is also a much heavier diffuse accumulation of antigen in the cytoplasm of individual cells, suggesting that there may be a difference at the cellular level in the nature of the persistent infection.

Although the neonatal carrier exhibits more widespread infection in most areas of the CNS, there are several exceptions, notably the minimal infection of choroid plexus and ependyma, an observation previously reported by Mims

(13) and by Oldstone and Dixon (26). One of the most obscure aspects of the carrier state in neonatally infected mice is the status of cells which carry little or no stainable antigen, yet are often contiguous with heavily stained cells. Superficially, it seems plausible that these unstained cells are those to which the infection has not yet extended. However, sequential observations indicate that these cells have been exposed to infectious virus over a long period, and during development there is actually a reduction in antigen-positive cells in choroid plexus, leptomeninges, and the internal granule cell layer. Furthermore, a comparison of the two types of carriers also indicates that antigen-negative cells are not innately resistant, since certain cells are unstained in one type of carrier only.

Mims and Subrahmanyan (27) developed considerable evidence that antigen-negative cells resisted superinfection with homologous virus and that this resistance could not be attributed to interferon production by antigen-postive cells. More recently Hotchin (28) has described a similar situation in tissue cultures persistently infected with LCM virus in which viral antigen disappears from infected cells. He has suggested that this "shutdown" phenomenon represents an abortive replicative cycle of infection with an interference-producing virus subpopulation present in laboratory strains of LCM virus.

The distribution of antigen in the CNS, although enigmatic, undoubtedly plays a critical role in the pathophysiology of acute choriomeningitis. Thus, adult mice dying with classical choroiditis have heavy infections of the choroid plexus. Furthermore, a strikingly similar fatal choroiditis occurs in drug-induced carriers with extensive choroidal infections after transfer of immune lymphoid cells, while neonatal carriers, with minimal choroidal infection, fail to develop acute CNS symptoms. These different responses to adoptive immunization are the subject of the following paper.

## SUMMARY

A single dose of 150 mg/g of cyclophosphamide (CY), given 3 days after intracerebral (i.c.) inoculation of lymphocytic choriomeningitis (LCM) virus, protected over 90% of adult BALB/c mice against acutely fatal choriomeningitis. Surviving mice became persistently infected carriers, with high virus titers in blood and brain.

Immunofluorescent examination of the brain showed that in CY-induced carriers infection was initially confined to the choroid plexus, ependyma, and leptomeninges, but over the next 30 days gradually spread to the neural parenchyma, most notably to the molecular layer of the cerebellum. By contrast, LCM virus-carrier mice produced by neonatal virus injection and examined as adults, showed a much less marked infection of choroid plexus and much more widespread infection of parenchyma, with a different distribution among brain nuclei, including heavy infection of the Purkinje cells of the cerebellum.

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