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Deficits in discriminated learning remain despite clearance of long-term persistent viral infection in mice

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Mice persistently infected with lymphocytic choriomeningitis virus (LCMV) exhibit impaired learning ability. In this report, we determined whether clearance of the virus was associated with restoration of behavioral function. Neonatal Balb/cByJ mice were persistently infected with LCMV and tested as adults in a nonconditional spatial discrimination task. The presence of viral proteins in neurons was confirmed immunohistochemically and infectious virus was quantified in the blood by plaque assay. LCMV-infected adult mice made more errors in a Y-maze avoidance task compared to sham-inoculated controls. After the initial behavioral analysis, infected and control mice received a dose of cytotoxic T-lymphocytes sufficient to clear virus from these mice. Following complete clearance of the virus, mice were re-tested in the behavioral task, 5 months after the original test. No reversal of the learning deficit was seen following viral clearance; mice that had been cleared of the virus and those that remained persistently infected behaved similarly. These data indicate that persistent LCMV infection of the CNS lasting up to 7 months results in discriminated learning impairments that are not reversed by subsequent anti-viral immunocytotherapy.

Keywords: lymphocytic choriomeningitis virus; behavior; cytotoxic T lymphocytes

Introduction

The subtle neurobehavioral and cognitive sequelae of neurotropic viral infection are poorly understood. Some viral infections of the human central nervous system (CNS) with continued medical relevance include rabies, measles, herpes simplex, and human immunodeficiency virus (HIV). Infected individuals often manifest alterations in behavior that correspond to viral invasion of the CNS. While much can be gained by studying clinically affected patient populations, animal models have been invaluable for investigating the host and viral factors that contribute to neuropathogenesis and for testing potential therapeutic treatments.

One model system used to study the functional impact of viruses on the brain is lymphocytic choriomeningitis virus (LCMV). LCMV, a member of the arenavirus family (Peters *et al*, 1996) is a nonlytic virus that can result in multiple outcomes in the natural host, the mouse, predicated on the route and dose of infection and the age and immune status of the mouse. When given intracerebrally to adult immunocompetent mice, death occurs within 7 days of infection due to the generation of $CD8^+$ cytotoxic T lymphocytes (CTL) that localize to and kill infected ependymal and meningial cells. However, when inoculated into immunologically naive newborn mice, the virus establishes a lifelong persistent infection by infecting the thymus, where anti-LCMV CTL are removed by negative selection (Zinkernagel *et al*, 1978; reviewed in Borrow and Oldstone, 1997). In such persistently infected mice, LCMV infects neurons in the central nervous system, as well as cells in peripheral tissues (Fazakerley *et al*, 1991; Oldstone *et al*, 1986; Rodriguez *et al*, 1983). Infected neurons are found in the cerebral cortex, limbic system, basal ganglia and cerebellum, all areas thought to be critical for behavioral function (Rodriguez *et al*, 1983).

During persistent infection, LCMV does not cause inflammation or neuronal loss. No abnormal

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histological or morphological changes in the brain are observed (Rodriguez, 1983). Nevertheless, central nervous system dysfunction occurs. Hotchin and Seegal (1977) observed that persistently infected adult mice exhibited decreased exploration and locomotion and increased startle reactivity. More recently we have examined the impact of viral persistence on behavioral indices of avoidance learning and motor activity. Mice persistently infected with LCMV demonstrated hypoactivity during a 2 h locomotor activity test and impaired learning in a spatial discrimination task when tested as adults (Gold *et al*, 1994). LCMV-induced alterations in cholinergic system function have been indicated both behaviorally (Gold *et al*, 1994) and in an earlier *in vitro* study (Oldstone *et al*, 1977). Furthermore, a decrease in GAP-43 expression both *in vitro* and *in vivo* following persistent LCMV infection has been shown (de la Torre *et al*, 1996). Such alterations in neuronal transduction mechanisms are thought to be important for the neuronal plasticity accompanying learning and memory, and may in part underlie the observed behavioral changes.

While behavioral changes, and in particular learning impairments, would be expected with viruses that cause selective destruction of restricted regions in the brain, such as VSV (Mohammed *et al*, 1990) and encephalomyelocarditis virus-D (Yayou *et al*, 1993), other viruses like LCMV, seem capable of disrupting neuronal function without causing lysis of neurons. In another example, infection with a mixture of murine leukemia viruses does not produce lesions or necrosis yet is associated with spatial learning deficits in mice (Sei *et al*, 1992). Reductions in met-enkephalin and substance P levels in the striatum of infected mice and concomitant increases in neural concentrations of the excitotoxin quinolinic acid in these mice may be involved in the spatial learning deficits observed (Ha *et al*, 1995; Sei *et al*, 1996).

Since neurons are not destroyed by LCMV infection, the ability to clear virus from the host with anti-viral immunocytotherapy can be exploited for studying the relationship between viruses and behavior. Viral clearance is mediated by virus-specific CTL which are restricted by H-2 molecules of the mouse major histocompatibility complex (MHC). Viral nucleic acid, proteins, and infectious virus particles are cleared completely upon adoptive transfer of MHC-matched anti-viral CTL, although the timing and mode of clearance appears to be different within the CNS than for other infected tissues (Oldstone *et al*, 1986; Tishon *et al*, 1993). Infectious virus and viral proteins are cleared from the liver, lungs, spleen, and blood, usually by 30 days after adoptive transfer of LCMV-specific H-2 restricted CTL. In contrast, viral presence in the kidney and brain requires approximately 120 days for complete clearance following CTL inoculation.

The present study was designed to address whether learning deficits in mice neonatally infected with LCMV would persist after the virus was cleared from the infected animals in adulthood. In this model, neurons were infected for maximally 7 months duration. Performance in a spatial discrimination task, considered to be a test of reference memory (Beninger *et al*, 1986; Feeser *et al*, 1987), was assessed in persistently infected and uninfected adult mice and then again following viral clearance treatments in these same mice. Our results show that spatial discrimination deficits associated with long-term viral infection persist in the absence of the original offending viral antigen.

Results

Body weight, gender, and mortality

The relative timing of the experimental procedures is illustrated in Figure 1. The mice infected with LCMV showed a typical pattern of early weight loss associated with the effects of the virus compared to sham-inoculated controls (Hotchin, 1962; Oldstone *et al*, 1985). In this study, the initial body weight difference at 5 weeks of age between the two groups was approximately 40%. In adulthood, the LCMV-infected mice began to gain weight at a rate similar to that of the control mice, although they never reached control levels (data not shown). This was particularly true in female mice.

A cohort of 85 mice were trained in the Y-maze, consisting of 43 males (n=29 Sham- and n=14 LCMV-inoculated) and 42 females (n=22 Sham- and n=20 LCMV-inoculated). Following the irradiation/viral clearance procedure, 14 mice died, 13 of the 14 were from the LCMV group. The final cohort that was re-tested on the Y-maze consisted of 71 mice distributed as follows: 38 male mice (n=28 Sham- and n=10 LCMV-inoculated), and 33 female mice: (n=22 Sham- and n=11 LCMV-inoculated).

The behavioral results were examined to determine if a gender difference existed in any of the groups. There were no gender differences in the incorrect responses for any of the group comparisons either during initial testing ($F<1.0$) or following clearance treatment ($F<1.0$), therefore the data presented are collapsed across this factor.

Y-maze testing

The results of the initial Y-maze testing of mice at 10–12 weeks of age are shown in Figure 2. LCMV-

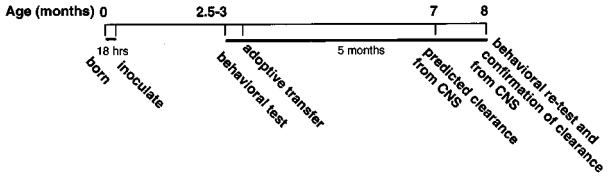

Figure 1 Timeline of experimental protocol.

infected mice exhibited significantly worse performance in the nonconditional spatial discrimination task when compared with sham-inoculated controls. Incorrect responses were analyzed using a 2 factor (infection, day) ANOVA with repeated measures on the within subjects factor, day. LCMV-infected mice committed significantly more incorrect responses than uninfected control mice ($F(1,83)=41.2$, $P<0.0001$) and there was a significant group by day interaction ($F(5,415)=3.9$, $P<0.01$) indicating a different rate of learning across days between groups. This deficit in acquisition of the discrimination was reflected in a significantly greater number of incorrect responses on days 2–5 in the LCMV-infected group (simple main effects). In addition, the repeated measures factor across days was also significant for incorrect responses ($F(5,415)=40.7$, $P<0.001$), indicating that both groups demonstrated reduced errors over time. These data were summarized briefly in a prior publication (de la Torre *et al*, 1996).

Mice then received irradiation and adoptive transfer with either CTL or vehicle (Modified Eagle's Media; MEM) and approximately 5 months later were re-tested on the Y-maze. The number of incorrect responses in persistently infected mice (LCMV-MEM) was compared with mice that received adoptive immunotherapy to clear the virus (LCMV-CTL) and similarly treated sham-inoculated controls (Sham-MEM and Sham-CTL) shown in Figure 3. Data were analyzed using a 3 factor mixed ANOVA with 2 between subjects factors (infection, clearance) and repeated measures on the within subjects factor, day. Following clearance treatment, there was no significant group by test day interaction, indicating that the rate of learning across the 6 days was not dependent on group, therefore for graphical presentation the total number of incorrect responses across test days 1–6 have been summed. For comparison purposes, the sum total of incorrect responses for initial testing prior to the clearance treatments (data shown in Figure 2) has been included on the left side of Figure 3. As for the initial test data, following clearance treatment there was a marked difference in the total number of incorrect responses, or amount of learning, between groups indicated by a significant main effect of infection ($F(1,67)=7.03$, $P=0.01$). Thus, mice infected with LCMV (LCMV-MEM and LCMV-CTL groups) displayed an increased number of incorrect responses compared to Sham-inoculated mice (Sham-MEM and Sham-CTL groups). However, there was no main effect of clearance and no clearance by infection interaction, indicating that the clearance treatment had no effect on performance within groups. Mice that were infected with LCMV and given CTLs (LCMV-CTL) performed similarly to infected mice which remained persistently infected (LCMV-MEM). Likewise, sham-inoculated animals receiving either MEM or CTL transfer did not differ in re-acquisition performance.

Viral titers

All plaque assays, performed to measure viral titer in the serum, consistently demonstrated that virus was present in the mice that had been injected with LCMV and virus was absent in the sham-inoculated controls. Mice which received the anti-viral CTL

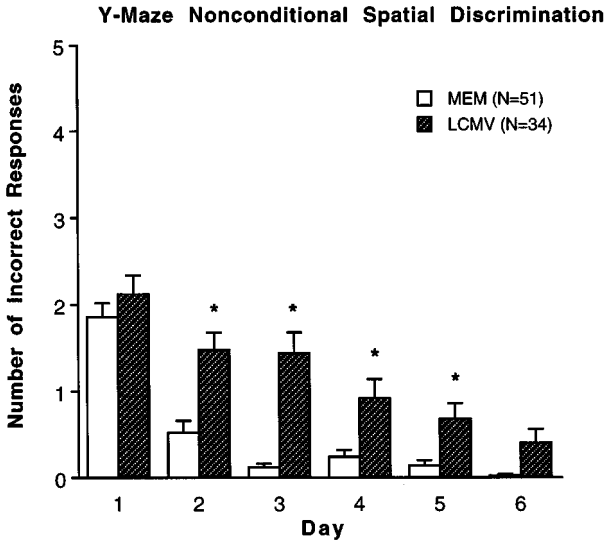

Figure 2 Initial Y-maze test. Discriminated avoidance learning in sham-inoculated (Sham) and virus-inoculated (LCMV) mice. The mean+s.e.m. numbers of incorrect responses are shown during 5 trials/day/6 days. *$P<0.05$, Significant effect of LCMV compared to Sham controls.

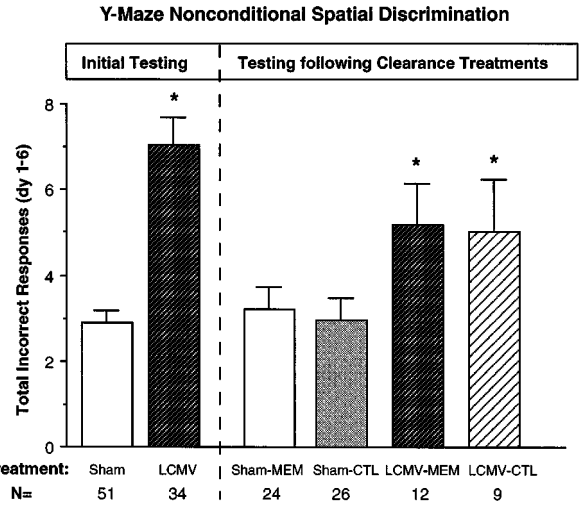

Figure 3 Post-clearance treatment Y-maze test. Discriminated avoidance learning in sham-inoculated (Sham) and infected (LCMV) mice that received adoptive transfer with either CTL or vehicle (MEM). The total numbers of incorrect responses collapsed over 5 trials/day/6 days of testing (mean+s.e.m.) are shown. The total numbers of incorrect responses for the initial test are included for comparison purposes. *$P<0.05$, Significant effect of LCMV compared to Sham controls.

immunotherapy had no detectable infectious virus by 2 months post adoptive transfer, even at the lowest dilution of serum, indicating that virus had been cleared from the sera of these animals. Samples from mice which had performed particularly well or poorly on the behavioral test were examined for a correspondence with viral load. Representative animals from each of these groups and their pre- and post-CTL transfer viral titers are shown in Table 1. Despite the typical variability of viral load in persistently infected mice, no trends between viral titers and Y-maze performance were noted.

Immunohistochemistry

The absence of infectious virus in the serum does not unequivocally prove that virus has been cleared from the CNS, particularly given the disparity in clearance rates from the CNS versus other organs (Oldstone *et al*, 1986). Therefore, we stained brain sections from representative LCMV-infected mice and adoptive-transfer recipients with a monoclonal antibody to LCMV nucleoprotein (NP) to determine how much virus, if any, remained in the presumably 'cleared' mice. The results are shown in Figure 4. In Panel A, staining within the caudate of a persistently infected mouse can be detected, demonstrating that persistently infected mice have detectable viral antigen present in the CNS. Likewise, persistently infected mice injected with vehicle (B) show immunoreactivity in the caudate four months

Table 1 Plaque assay results: Viral load in serum samples from LCMV-infected mice before and after adoptive transfer

Mouse#	*Transferred*	*Pre-transfer*	*5 months post-transfer*
Worst performance			
26.1	MEM	5.5×10^3	1.6×10^5
34.3	MEM	2.2×10^5	5.6×10^4
34.2	MEM	1.5×10^5	1.7×10^5
25.3	MEM	1.4×10^4	1.5×10^5
Best performance			
25.2	MEM	1.0×10^4	1.8×10^5
34.1	MEM	6.6×10^5	2.1×10^5
10.1	MEM	7.2×10^4	1.2×10^4
26.2	MEM	2.3×10^4	2.4×10^5
Worst performance			
29.2	CTL	1.3×10^4	0
30.4	CTL	9.7×10^4	0
30.3	CTL	3.0×10^4	0
Best performance			
12.1	CTL	7.2×10^4	0
12.3	CTL	2.1×10^4	0
29.1	CTL	7.8×10^3	0

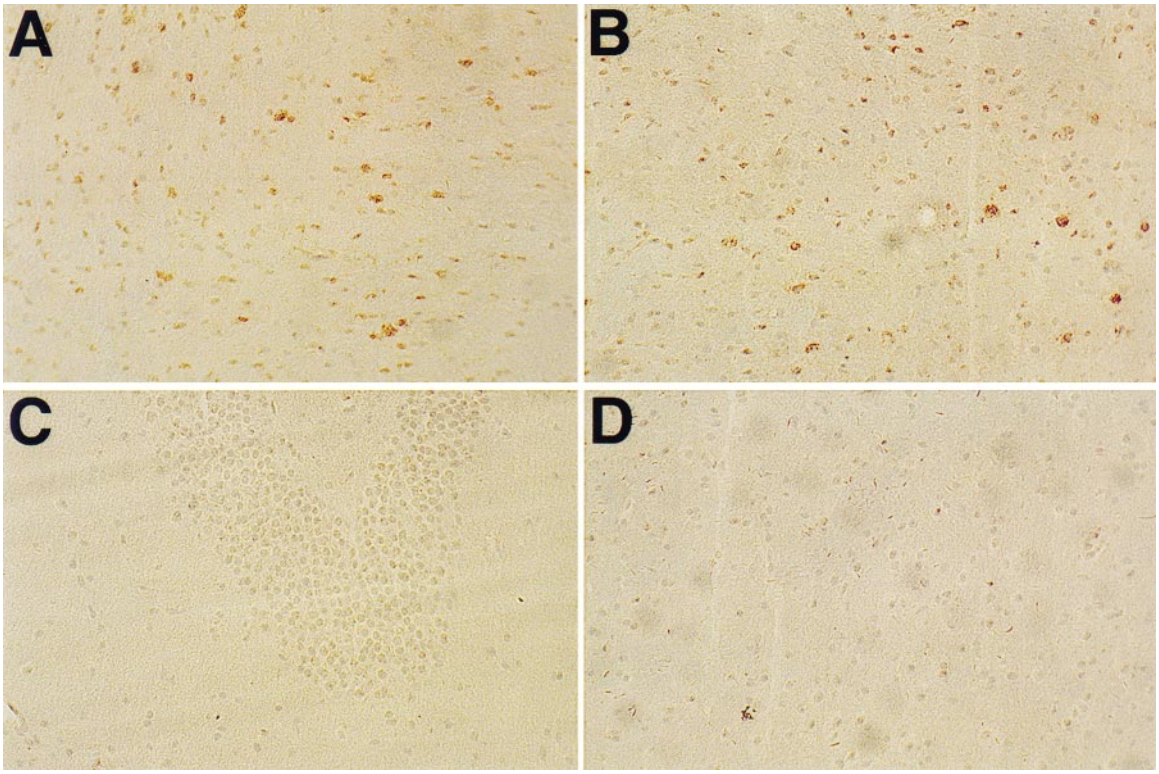

Figure 4 Immunostained brain sections from persistently infected mice or from mice which had received the adoptive-transfer 5 months previously. (A) shows significant staining within the caudate of a persistently infected mouse. Similarly, in (B) persistently infected mice injected with vehicle demonstrate significant immunoreactivity, indicating the presence of viral antigen within these brains. In contrast, sections from persistently infected mice given the anti-viral CTL immunotherapy show no staining for viral antigen as seen in the cerebellum (C) and the caudate (D).

following infection. In contrast, representative sections from either the cerebellum (C) or the caudate (D) of persistently infected mice given the anti-viral CTL immunotherapy do not stain for viral antigen, indicating that the adoptive-transfer with anti-viral CTL had efficiently cleared virus from these mice.

Discussion

The purpose of the present study was to determine whether clearance of persistent LCMV infection from neurons would result in improved performance in a Y-maze conditional discrimination procedure compared with mice that remained persistently infected. First we demonstrated that persistent LCMV infection resulted in learning deficits in Balb/c mice, extending our initial observation of behavioral dysfunction (Gold *et al*, 1994) to a second strain of mouse. Learning deficits were measured by an increased number of incorrect responses over 6 days of testing in a discriminated avoidance Y-maze task when tested at 3 months and again at 8 months of age. These results are consistent with other reports of behavioral deficits observed in virally infected rodents (Dittrich *et al*, 1989; Gold *et al*, 1994; Hotchin and Seegal, 1978; McFarland and Hotchin, 1983; Mohammed *et al*, 1991; Sei *et al*, 1992). Second, we determined that clearance of the virus was not associated with restoration of behavioral function, consistent with what has been shown for VSV (Mohammed *et al*, 1991) and HSV (Beers *et al*, 1995) in studies using rats. Thus, mice persistently infected with LCMV show learning deficits that correlate with a history of infection but that do not require continuous viral infection.

In the present work a short-term memory paradigm was used to test trial-independent memory processes (reference memory). Since a mild footshock was used to motivate acquisition of the spatial discrimination in the Y-maze, it is possible that performance factors could influence these results. However, alterations in motivational factors do not readily account for the differential avoidance performance. Previously we have shown that persistent LCMV infection does not significantly affect shock sensitivity thresholds in persistently infected male DBA/2J mice, and that learning deficits were evident in these mice independent of body weight (Gold *et al*, 1994). Motivation also does not seem to be related to body weight differences in the present study as no gender differences were observed in Y-maze performance. Therefore, the deficits in discrimination performance reported here are not likely to be due to altered somatosensory functioning.

The present results along with other earlier observations (Hotchin and Seegal, 1977, 1978; Gold *et al*, 1994) of behavioral disruptions associated with persistent LCMV infection are particularly interesting as persistent LCMV infection has historically been characterized by an absence of morphological abnormalities in the CNS (Rodriguez *et al*, 1983; Fazakerley *et al*, 1991). A disruption of homeostasis of various physiologic systems (immune, endocrine, exocrine) is thought to occur in the absence of tissue destruction and for the CNS, neurochemical and functional alterations without concomitant morphologic injury have been proposed. The existence of more sensitive, recently available analytical tools has revealed modest pathological changes in the persistently infected CNS including an increase in glial fibrillary acidic protein (GFAP) mRNA and GFAP-positive astroglial cells compared to uninfected controls (de la Torre *et al*, 1996). Furthermore, the reduction of GAP-43 immunoreactivity in the hippocampus in persistently infected mice directly correlates with the degree of astrogliosis and inversely correlates with levels of LCMV nucleoprotein expression. Alterations in synaptic plasticity suggested by the GAP-43 results may be related to changes in neuronal transduction systems that could underlie the functional changes associated with persistent LCMV infection.

Immune-mediated viral clearance from the brain, documented by either Northern blot or *in situ* hybridization, takes approximately 4 months following adoptive transfer of haplotype-matched, anti-viral CTL (Oldstone *et al*, 1986; Tishon *et al*, 1993). In the present study, the behavioral deficit remained in both persistently infected mice and mice from which virus was cleared. The CTL-mediated viral clearance was confirmed by both plaque assay and immunostaining within the brain. Hence virus infection of neurons for maximally 7 months duration can be terminated by CTL but the learning deficits are not reversible. Immunotherapy by adoptive transfer of CTL can produce a transient mild to moderate illness during the period of CTL-mediated clearance of virus in peripheral tissues (5–15 days; Rall *et al*, 1995). Following this initial period, most mice recover completely and become indistinguishable from uninfected control mice. In the present study, the adoptive transfer procedure resulted in considerable mortality predominantly in the LCMV-inoculated group. Why some persistently infected mice die and others survive has not been addressed but remains an interesting question for further study. The clearance of virus in the absence of cytolysis suggests that CTL may clear virus from neurons through the release of cytokines, primarily interferon-γ (Oldstone *et al*, 1986; Tishon *et al*, 1995). If so, perhaps the sustained expression of certain cytokines within the brain parenchyma explains the long-term learning deficits in these mice.

Alternatively, gliosis (astrocytosis; Oldstone *et al*, 1986) is associated with CTL immunotherapy,

raising the possibility that uninfected resident brain cells may contribute to the alteration of behavioral function. While some glial cell activation is evident in both persistently infected mice (de la Torre *et al*, 1996), and in mice receiving adoptive CTL transfers (Oldstone *et al*, 1986), we do not attribute the learning deficit reported here to this astrocytosis, since other mouse models with prominent astrocytosis such as young heterozygous IL-6 transgenic mice and HIV-1 gp120 transgenic mice are not impaired in the same task (Heyser *et al*, 1997; IL Campbell and LH Gold, unpublished data).

The persistently disrupted learning despite viral clearance suggests an irreversible deficit that is not dependent on continuous viremia. Whether the behavioral results are consequent to developmental changes associated with neonatal infection or permanent dysfunction associated with long-term infection of neurons remains unknown. However, our results suggest that clearance of virus from the CNS after long-term neuronal infection does not necessarily restore normal brain function. Models in which CNS clearance can be achieved over a shorter time frame will be useful for examining this question further. Recently, Rall and Oldstone (1995) used transgenic technology to express an MHC class I molecule in CNS neurons, since normal neurons lack these molecules and can therefore not be recognized by the CTL T cell receptor. In that report, LCMV was cleared from neurons within 15–30 days, an average of 3–4 months earlier than in neurons without MHC expression. Since the length of time for viral clearance from the CNS is significantly reduced, it will be of interest to study these mice to determine whether the behavioral deficit is reversible after a shorter period of neuronal infection. Alternatively, if recovery of function occurs over a longer time scale, it is possible that behavioral improvements might become evident at later times following CNS clearance.

The demonstration that a behavioral deficit resulting from a long-term persistent viral infection can endure has important implications for understanding the pathophysiological consequences of viral insult in the brain. This is of interest considering recent neurodevelopmental theories regarding several mental disorders (Weinberger and Lipska, 1995). In particular, it is thought that early cortical maldevelopment may lead to dysfunctional connectivity during brain maturation that can result in abnormalities in brain physiology and cognitive function. It will be important to determine whether LCMV has cumulative effects on neurons; if so, perhaps a short early viral exposure followed by clearance would restore behavioral function. Alternatively, a threshold may exist whereby neonatal infection and presence during the period of maximal neuronal growth is sufficient to result in a permanent deficit.

Materials and methods

Subjects

BALB/cByJ mice (TSRI Breeding facility) were mated and the offspring were injected intracranially within 18 h of birth with either 10 μl LCMV (Armstrong strain clone 53b, 1×10^5 pfu/ml) or sham-inoculated with 10 μl vehicle (Modified Eagle's Media; MEM). Pups from one litter were all injected with the same agent and were then returned to their cage and housed with their birth mothers. Cages were Plexiglas ($28 \times 17 \times 11.5$ cm) with sawdust bedding which was changed weekly. The animals were housed in a temperature-controlled environment (Biosafety Level 2) with a normal 12 h light:dark cycle (6 a.m.:6 p.m.) and had access to food and water *ad libitum*.

At 4–5 weeks of age, pups were weaned from their birth mothers. At this time, pups were also weighed and bled from the orbital sinus under Metofane anesthesia. The amount of infectious virus in sera was quantitated by plaque assay. Mice were group housed four per cage with same-sex littermates. Occasionally, pups from litters born on the same date were combined in a cage. Mice were weighed monthly.

Initial discrimination testing

When the mice were between 10 and 12 weeks of age, they were trained in a nonconditional spatial discrimination task. Mice were allowed to explore the apparatus for 5 min on the day preceding the start of spatial discrimination testing. The testing apparatus was a Y-shaped maze, consisting of three Plexiglas arms (25.4 cm long $\times$ 8.3 cm high) with interior sloping walls and a floor lined with metal, and covered by a dark translucent Plexiglas lid. The end compartment of each arm could be separated from the rest of the arm by a guillotine style door. A shock generator delivered footshock (Parameters: AC 60 Hz, non-scrambled, 400 μA) to the metal lined floors and sides of the maze.

Spatial discrimination testing commenced by placing the mouse into the end of one arm, called the start arm. The door was closed so that the mouse was confined to the end compartment of the start arm for 30 s. On the initial trial, once the mouse moved into the end of one arm of the maze, the door was closed, and that arm was considered as the preferred arm. The mouse was then returned to the start compartment. In the following trials, mice were given 10 s after the door had been opened to move into the arm opposite to the initial preference (designated safe arm) to avoid a footshock (30 s maximum footshock duration). If the mouse chose the safe arm after the onset of the shock, an escape response was recorded. An avoidance response

was distinguished by the mouse choosing to move into the safe arm *prior* to the onset of the shock. Incorrect responses (errors) were defined as the mouse moving into the preferred arm. Following such a response the mouse was then placed into the end section of the safe arm for 30 s with the door closed. This procedure was followed for a total of five trials each day for 6 days.

The testing was done in the presence of masking noise during the light phase of the light/dark cycle. A 40 W bulb centered 17 inches above the Y-maze in a darkened room created a dim light inside the maze with the lids closed, but allowed the experimenter to observe the movement of the mice. The maze was thoroughly cleaned between mice to eliminate odor cues.

Adoptive transfer and clearance
Within 2–3 weeks following Y-maze testing, the mice underwent an adoptive-transfer procedure which employed a combined treatment of irradiation and injection of cytotoxic T-lymphocytes (CTL) or vehicle (MEM). The CTL were prepared from the spleens of syngeneic mice which had been injected i.p. with 2×10^5 pfu/ml LCMV. One to two months later, these donor mice were sacrificed and their spleen cells were harvested. Erythrocytes were lysed by treatment with 0.83% NH_4Cl solution, and single-cell suspensions were prepared in MEM medium. Cells were counted and the percentage of viable lymphocytes was determined by trypan blue exclusion.

Both control and LCMV-infected recipient mice received a 450 rad dose of irradiation to kill endogenous splenocytes and make room for the adoptively transferred cells, and the following day, the groups were divided in half and injected i.p. with 0.2 ml of either CTL (5×10^7 cells) or vehicle (MEM). About 2 months following this procedure, all mice were bled from the orbital sinus and plaque assays were performed to quantify infectious virus in the blood. At the time of sacrifice, blood samples were collected and plaqued, and the brains were frozen on dry ice for analysis and immuno-staining.

Repeat Y-maze testing
Approximately 4–5 months following clearance of LCMV from the blood, the mice were re-examined in the Y-maze, following the same procedure as described earlier. Mice were again habituated to the maze for 5 min, and on the subsequent day were allowed to reestablish an arm preference. The discrimination testing was then repeated under identical circumstances as the initial testing.

Plaque assays
In mice persistently infected with LCMV, titers of circulating virus were quantitated by culture of sera from these mice on permissive cells. Blood was collected from mice at different points during the course of the study. The blood samples were centrifuged, and sera were frozen and stored at -70°C until the time of the plaque assay. Sub-confluent (70–80%) monolayers of Vero cells, plated in 6-well tissue culture dishes (Costar, Cambridge, MA) were incubated at 37°C with serial tenfold dilutions of the serum samples for one hour with frequent rotation. Following incubation, the inocula were removed and a 0.5% agarose/1 × MEM+7% fetal bovine serum molten media was added to the monolayers and allowed to solidify. Following a 6 day incubation period, monolayers were fixed with 10% formaldehyde in PBS for at least 1 h. Agarose overlays were removed and the monolayers were stained with 0.1% crystal violet in PBS. The maximum number of countable plaques was calculated, and total number of plaque-forming units (PFUs) per ml of serum was determined.

Immunohistochemistry
To identify whether viral antigen remained in the mice receiving the adoptive immunotherapy, immunohistochemistry using a NP-specific monoclonal antibody was performed on acetone-fixed, cryostat-cut sections of brain tissue. Persistently infected mice at birth given either the anti-viral CTL therapy or vehicle (MEM), were sacrificed by halothane inhalation. The brains from these animals were removed and quick frozen in a dry ice/isopentane bath and stored at -70°C prior to sectioning. At least three mice per group were analyzed.

Horizontal sections (10 μM) were cut from each brain and transferred to SuperFrost Plus microscope slides (Fisher). Sections were post-fixed in ice-cold acetone for 15 min and allowed to warm to room temperature slowly. The fixed sections were rehydrated in 1 × PBS/2% fetal bovine serum (FBS), which was used for all subsequent washes and antibody dilutions. Sections were then incubated in a humidified chamber for 3 h with antibody 1.1.3, a murine monoclonal antibody raised against the LCMV-NP (ascites, diluted 1 : 500). After incubation, the primary antibody was removed and the sections were washed 3 × 5 min in the PBS/FBS buffer. A peroxidase-conjugated secondary (sheep anti-mouse, Serotec) was reacted with the sections at a dilution of 1 : 200, for 1.5 h. Sections were washed again and subsequently incubated with the chromagen diaminobenzidene (Sigma; 0.5 mg/ml in 0.1 M Tris, pH 7.2) in the presence of 0.03% hydrogen peroxide for 20 min. Sections were lightly counterstained with hematoxylin and photographed. To ensure specificity of staining, brain sections from all mice were also reacted in the absence of primary antibody.

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References

Beers DR, Henkel J, Kesner RP, Stroop WG (1995). Spatial recognition memory deficits without notable CNS pathology in rats following herpes simplex encephalitis. *J Neurological Sci* **131:** 119–127.

Beninger RJ, Jhamandas K, Boegman RJ, El-Defrawy SR (1986). Effects of scopolamine and unilateral lesions of the basal forebrain on T-maze spatial discrimination and alternation in rats. *Pharm Biochem Behav* **24:** 1353–1360.

Borrow P, Oldstone MBA (1997). Lymphocytic choriomeningitis virus. In: *Viral Pathogenesis*. Nathanson N (ed) Lippincott-Raven Publishers: Philadelphia, pp. 593–627.

de la Torre JC, Mallory M, Brot M, Gold L, Koob G, Oldstone MBA, Masliah E (1996). Viral persistence in neurons alters synaptic plasticity and cognitive functions without destruction of brain cells. *Virology* **220:** 508–515.

Dittrich W, Bode L, Ludwig H, Kao J, Schneider K (1989). Learning deficiencies in borna disease virus-infected but clinically healthy rats. *Biological Psychiatry* **26:** 818–828.

Fazakerley JK, Southern P, Bloom FE, Buchmeier MJ (1991). High resolution in situ hybridization to determine the cellular distribution of lymphocytic choriomeningitis virus RNA in the tissues of persistently infected mice: Relevance to arenavirus disease and mechanisms of viral persistance. *J General Virology* **72:** 1611–1625.

Feeser HR, Raskin LA (1987). Effects of neonatal dopamine depletion on spatial ability during ontogeny. *Behav Neurosci* **101:** 812–818.

Gold LH, Brot MD, Polis I, Schroeder R, Tishon A, de la Torre JC, Oldstone MBA, Koob GF (1994). Behavioral effects of persistent lymphocytic choriomeningitis virus infection in mice. *Behav Neural Biol* **62:** 100–109.

Ha J-H, Sei Y, Basile AS (1995). Striatal met-enkephalin and substance P levels are decreased in mice infected with the LP-BM5 murine leukemia virus. *J Neurochem* **64:** 1896–1898.

Heyser CJ, Masliah E, Samimi A, Campbell IL, Gold LH (1997). Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. *Proc Natl Acad Sci* **94:** 1500–1505.

Hotchin J (1962). The biology of lymphocytic choriomeningitis infection: virus-induced immune disease. Cold Spring Harbor Symp Quant Biol **27:** 479–499.

Hotchin J, Seegal R (1977). Virus-induced behavioral alteration of mice. *Science* **196:** 671–674.

Hotchin J, Seegal R (1978). Alterations in behavior resulting from persistent lymphocytic choriomeningitis virus infection. In Birth Defects: Original Article Series. XIV. The National Foundation: pp. 171–178.

McFarland DJ, Hotchin J (1983). Host genetics and the behavioral sequelae to herpes encephalitis in mice. *Physiology and Behavior* **30:** 881–884.

Mohammed AKH, Maehlen J, Magnusson O, Fonnum F, Kristensson K (1991). Persistent changes in behavior and brain serotonin during ageing in rats subjected to infant nasal virus infection. *Neurobiology of Aging* **13:** 83–87.

Mohammed AKH, Magnusson O, Maehlen J, Fonnum F, Norrby E, Schultzberg M, Kristensson K (1990). Behavioural deficits and serotonin depletion in adult rats after transient infant nasal viral infection. *Neuroscience* **35:** 355–363.

Oldstone MBA, Ahmed R, Buchmeier MJ, Blount P, Tishon A (1985). Perturbation of differentiated functions during viral infection in vivo. I. Relationship of lymphocytic choriomeningitis virus and host strains to growth hormone deficiency. *Virology* **142:** 158–174.

Oldstone MBA, Blount P, Southern PJ, Lampert PW (1986). Cytoimmunotherapy for persistent virus infection reveals a unique clearance pattern from the central nervous system. *Nature* **321:** 239–243.

Oldstone MBA, Holmstoen J, Welsh RM, Jr (1977). Alterations of acetylcholine enzymes in neuroblastoma cells persistently infected with lymphocytic choriomeningitis virus. *J Cellular Physiol* **91:** 459–472.

Peters CJ, Buchmeier M, Rollin PE, Ksiazek TG (1996). Arenaviruses. In: *Fields Virology*. Fields BN, Knipe DM, Howley PM *et al*. (eds.). Lippincott-Raven Publishers: Philadelphia, pp 1521–1551.

Rall GF, Mucke L, Oldstone MBA (1995). Consequences of cytotoxic T lymphocyte interaction with major histocompatibility complex class I-expressing neurons in vivo. *J Exp Med* **182:** 1201–1212.

Rodriguez M, Buchmeier MJ, Oldstone MBA, Lampert PW (1983). Ultrastructural localization of viral antigens in the CNS of mice persistently infected with lymphocytic choriomeningitis virus (LCMV). *Am J Pathol* **110:** 95–100.

Sei Y, Arora PK, Skolnick P, Paul IA (1992). Spatial learning impairment in a murine model of AIDS. *FASEB J* **6:** 3008–3013.

Sei Y, Paul IA, Saito K, Layar R, Harltey JW, Morse HC, Skolnick P, Heyes MP (1996). Quinolinic acid levels in a murine retrovirus-induced immunodeficiency syndrome. *J Neurochem* **66:** 296–302.

Tishon A, Eddleston M, de la Torre JC, Oldstone MBA (1993). Cytotoxic T lymphocytes cleanse viral gene products from individually infected neurons and lymphocytes in mice persistently infected with lymphocytic choriomeningitis virus. *Virology* **197:** 463–467.

Tishon A, Lewicki H, Rall G, von Herrath M, Oldstone MBA (1995). An essential role for type 1 interferon-γ in terminating persistent viral infection. *Virology* **212:** 244–250.

Weinberger DR, Lipska BK (1995). Cortical maldevelopment, anti-psychotic drugs and schizophrenia: a search for common ground. *Schizophrenia Res* **16:** 87–110.

Yayou K, Takeda M, Tsubone H, Sugano S, Doi K (1993). The disturbance of water-maze task performance in mice with EMC-D virus infection. *J Vet Med Sci* **55:** 341–342.

Zinkernagel R, Callahan G, Althage A, Cooper S, Klein P, Klein J (1978). On the thymus in the differentiation of 'H-2 self-recognition' by T cells: Evidence for dual recognition? *J Exp Med* **147:** 882–896.