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Antibody Is Required for Clearance of Infectious Murine Hepatitis Virus A59 from the Central Nervous System, But Not the Liver

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Intracerebral inoculation with mouse hepatitis virus strain A59 results in viral replication in the CNS and liver. To investigate whether B cells are important for controlling mouse hepatitis virus strain A59 infection, we infected muMT mice who lack membrane-bound IgM and therefore mature B lymphocytes. Infectious virus peaked and was cleared from the livers of muMT and wild-type mice. However, while virus was cleared from the CNS of wild-type mice, virus persisted in the CNS of muMT mice. To determine how B cells mediate viral clearance, we first assessed CD4+ T cell activation in the absence of B cells as APC. CD4+ T cells express wild-type levels of CD69 after infection in muMT mice. IFN-γ production in response to viral Ag in muMT mice was also normal during acute infection, but was decreased 31 days postinfection compared with that in wild-type mice. The role of Ab in viral clearance was also assessed. In wild-type mice plasma cells appeared in the CNS around the time that virus is cleared. The muMT mice that received A59-specific Ab had decreased virus, while mice with B cells deficient in Ab secretion did not clear virus from the CNS. Viral persistence was not detected in FcR or complement knockout mice. These data suggest that clearance of infectious mouse hepatitis virus strain A59 from the CNS requires Ab production and perhaps B cell support of T cells; however, virus is cleared from the liver without the involvement of Abs or B cells. The Journal of Immunology, 2001, 167: 5254–5263.

Mouse hepatitis virus strain A59 (MHV-A59) infects both the CNS and the liver in mice, thereby allowing us to investigate the differences between the ability of the immune system to clear the virus in these organs. In the first 2 wk following intracerebral or intranasal infection with MHV-A59, susceptible mice develop an acute meningoencephalitis and hepatitis. Infectious virus is cleared from the brain by 16–20 days postinfection (dpi) and from the liver by 8–10 dpi in immunocompetent mice (1). However, viral RNA persists in the CNS for at least 6 mo after infection (2). CNS demyelination is induced by the infection and peaks after virus is cleared (3, 4). How the immune system responds to and controls viral infections in the CNS is still largely unknown. T cell depletions, adoptive transfers, and the use of T cell-deficient mice have shown that T cells have an important role in preventing mortality associated with acute encephalitis and hepatitis after high dose MHV infections (4–9). However, the role of T cells in the actual clearance of virus from surviving mice is difficult to assess from the literature. Although many studies performed in T cell-deficient or depleted mice have shown a delay in viral clearance, most of these studies were not extended beyond the acute period of infection to determine whether virus was eventually cleared (4–9). One long term study showed that CD8+ T cells (β2-microglobulin knockout mice) who survived acute infection cleared infectious MHV-A59 by 30 dpi in most mice, suggesting that CD8+ T cells are not absolutely required for clearance (4). However, it is known that T and/or B cells are required for viral control. In recombinase-activating gene 1 (RAG) knockout and SCID mice who lack T and B cells, MHV is not cleared, and animals die (10).

The role of B cells in the clearance of a primary viral infection has traditionally been considered to be fairly minimal. Instead, the relatively slowly responding B cells have been studied primarily as protectors against re-establishment of a viral infection. In accord with this protective role, B cell-derived Abs protect mice from the lethal effects of high dose MHV infection when transferred near the time of infection (11–13). However, the contrast of viral clearance in CD8+ T cell-deficient mice with the lack of viral clearance in T and B cell-deficient RAG knockout mice suggests that the B cell could be an additional potent viral regulator during a primary infection. Such a role becomes more plausible when it is recognized that B cells may act through Ag presentation and consequent activation of T cells, cytokine production, cognate interactions, or the powerful mechanism of Ab production.

To investigate the possible role of B cells in viral clearance during a primary infection, we studied MHV-A59 infection in both...
the CNS and liver of mice deficient in B cells. The results demonstra-
one that B lymphocytes are required for the clearance of in-
efectious MHV-A59 from the CNS, but surprisingly are not re-
quired for clearance from the liver. These results support the novel
collection that the requirement for B cells in clearance of a viral
infection can be organ specific and thereby also strongly support
the growing awareness that different immune mechanisms may be
important in different tissues (14–16). In addition, our data con-
tribute to the small, but growing, body of evidence that B cells can
be required for clearance of primary viral infections from the CNS
in T cell–competent mice (17–19).

Materials and Methods

Mice

All mice were female unless bred on site or otherwise noted. nu/nu mice lack cell surface IgM and therefore lack mature B lymphocytes. C57BL/6 mice, nu/nu mice completely backcrossed onto the C57BL/6 background (B6.129S2-Igh-6), and RAG1 knockout mice (B6.129S7-Rag2<sup>1<sup>tem1</sup><sup>mom</sup>) on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). The absence of B cells was verified by flow cytometric analysis of splenocytes labeled with FITC-labeled anti-B220 or PE-labeled anti-CD19 (BD Pharmingen, San Diego, CA). FcεRIγ knockouts on C57BL/6 background (C57BL/6J-TmAtc-KO-Fcer1g), FcγRIIb knockouts on C57BL/6 background (C57BL/6N-Tac-Fcgr2b), and C57BL/6 controls for FcR knockout mice were purchased from Taconic Farms (Germantown, NY). FcγRIII and FcγRIIIa knockouts on C57BL/6 background ((C57BL/6 <sup>129</sup>-KO)Fcer1g-KO)Fcg2r), C57BL/6 × 129 controls, and female and male C57BL/16 mice were also purchased from Taconic Farms. Male β2-
microglobulin mice on C57BL/6 × 129 background were bred at Univer-
sity of Pennsylvania (Philadelphia, PA) and used at 4–6 wk of age. Other strains of mice were received at 3 wk of age, allowed to acclimate for 1 wk, and infected at 4 wk of age unless otherwise noted. JHd and mtg-transgenic (Tg) mice were derived at Yale University (New Haven, CT) and bred at University of Pennsylvania. JHd mice have a deletion of the Ig H chain J segments and one D segment (20). These mice have been backcrossed onto C57BL/16 for eight generations. The mlm-Mt-g mice are transgenic for a re-
combined H chain on the JHd background. The Vh186.2- bearing H chain VDJ segment from a canonical anti-(4-hydroxy-3-nitro-phenyl)-specific Ab linked to the H chain intrinsic enhancer plus Cml–4 (IgM) constant region exons has been introduced to these mice. The mlm-Mt-g mice lack the Ig secretion signal, resulting in a lack of Ab secretion, but the rescue of normal B cell development. The switch region is also largely deleted, re-
ducing the functionality of trans-isotype switching (21, 22). Heterozygous ani-
mals were identified by PCR of tail DNA using primers for Vh186.2 (TGCTCTTTTGGCATCAGAC-3′ and TGAGGAGACTGTGAGAGTG-
3′), C3-deficient mice backcrossed 10 generations onto C57BL/6 were do-
nated by Dr. J. Lambrix (University of Pennsylvania) with the permission of H. Colten (23). All mice were housed under pathogen-free conditions.

Virus, viral infection, and viral titers

MHV-A59 virus obtained from Dr. L. Sturman was grown in enriched DMEM with 10% FCS on confluent l2 or 17C1-1 cells for 16–28 h and harvested from the cells by three freeze/thaw cycles.

Mice were infected with live virus at 4 wk of age unless otherwise specified. Twenty microtiter of virus in 0.75% BSA (fraction V; Sigma, St. Louis, MO) in PBS was injected through a 26-gauge needle intracere-
brally into the left hemisphere of mice anesthetized with methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL) or isoflurane (Abbott Laborato-
ries, Chicago, IL). As a control, mice were infected with 20 μl uninfected 17C1-1 or L2 cell lysate. Mice were infected with 50 PFU unless otherwise noted. To prevent RAG1 knockout mice from dying during the acute stage of infection, an injection of hyperimmune serum raised against MHV-A59 in C57BL/6 was given i.p. at the time of infection.3 For intrahepatic in-
fections, 500 PFU were given in two injections of 25 μl each, one just below the right cranial rib cage and slanted slightly cranially and the other below the xiphoid process of the sternum. Previous work in our laboratory demonstrated that when such injections were made with India ink, the ink successfully entered the liver. Viral titers were determined by plaque assay

3 A. E. Matthews, E. Lavi, S. R. Weiss, and Y. Paterson. Neither B cells or T cells are required for central nervous system demyelination in mice persistently infected with MHV-A59. Submitted for publication.

on L2 cells (24). Brain (left hemisphere or whole brain), spinal cord, and liver samples from metaphane- or carbon monoxide-ethanized, sterile PBS-
perfused mice were collected in sterile gel saline (140 mM NaCl, 0.3 mM CaCl<sub>2</sub>, 0.84 mM MgCl<sub>2</sub>, 19 mM boric acid, 0.13 mM sodium borate, and 0.17% gelatin) and homogenized using nylon mesh bags (Teto, Elmsford, NY).

Isolation of splenocytes and CNS lymphocytes

Spleens, brains, and/or spinal cords were removed from mice after killing by cervical dislocation or carbon dioxide overdose. When CNS tissue was collected, mice were first perfused with sterile PBS to prevent contamina-
tion with blood lymphocytes. Organs were homogenized in nylon mesh bags. Lymphocytes were purified from brains and spinal cords by resus-
pending homogenate in 30% Percoll and spinning at 1300 × g for 30 min. Cells in the pellet were washed twice and used. Before splenocytes were used, RBC were removed by suspension in Tri-aminommonium chloride (0.017 M Tris and 0.75% NH<sub>4</sub>Cl, pH 7.2).

Flow analysis of T cells

Spleen and CNS lymphocytes were treated with Caltag PE-anti-CD4 (3 × 10<sup>4</sup> cells; South San Francisco, CA) and BD PharMingen FITC-anti-
CD69 (1 μl/10<sup>5</sup> cells) Abs for 20–60 min and then washed twice with 5% horse serum in PBS. Cells were fixed in 2% paraformaldehyde (pH 7.2–
7.3), and data were acquired within 6 days.

ELISAs and ELISPOTS

For IFN-γ sandwich ELISAs, plates were coated with R46-2-A2 anti-IFN-γ (5 μg/ml). After blocking with 5% horse serum in PBS, cell supernatants were incubated as serial 1/4 dilutions for 1 h at 37°C. Rabbit anti-IFN-γ (1/3000–5000, 1 h, 37°C; a gift from the laboratory of Dr. P. Scott) was used as the detection Ab, which was then bound by peroxidase-labeled anti-rabbit Ig (1/3000, 30 min, 37°C; Jackson ImmunoResearch Laborato-
ries, West Grove, PA). ABTS (Kirkegaard & Perry Laboratories, Ga-
thersburg, MD) was added as the substrate, and absorbance was read 10–20 min later at 405 nm using a microplate reader (Molecular Devices, Menlo Park, CA).

To obtain cell supernatants for ELISAs, 3 × 10<sup>4</sup> lymphocytes purified from brain and spinal cord were incubated with 3 × 10<sup>4</sup> naive syngeneic splenocytes as a source of APC in a 96-well plate. Naive syngeneic spleno-
cytes alone produced undetectable or naive CNS lymphocyte levels of IFN-γ. UV-irradiated virus (no viral plaques detected by viral titer) at a multiplicity of infection of 10, based on the original titer, was added as a source of Ag. L2 or 17clone-1 lysate was added to some wells to serve as the negative control. Supernatants were collected 72 h later, spun down to remove cellular debris, and frozen at −70°C until use.

To detect secreted Abs by ELISA, serum was collected from A59-infec-
ted mice. Plates were coated with virus (5.5 × 10<sup>3</sup> PFU/ml in DMEM-
10), incubated with 5% horse serum in PBS, detected with Vector biotin-
ylated anti-murine IgG (1/2000; Vector, Burlingame, CA) and then
streptavidin (30 min, 37°C), which was then bound with alkaline phosphatase-labeled goat anti-murine IgG (1/2000; Vector, Burlingame, CA) and
then streptavidin-peroxidase (1/2000; Jackson ImmunoResearch Laborato-
ries), and visualized with ABTS. The Vector Ab cross-reacts with murine IgM.

For IFN-γ ELISPOTs, 96-well plates were coated and blocked as de-
scribed above. Cells were added in triplicate in 2-fold dilutions and in-
cubated overnight without moving. Rabbit anti-IFN-γ was used as de-
scribed above and detected by goat alkaline phosphatase-labeled anti-rabbit Ig (30 min, 37°C. Jackson ImmunoResearch Laboratories). Spots were visualized by incubating overnight with 5-bromo-4-chloro-3-indolylphosph-
ate (Sigma) in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma) with 0.05% agarose (FMC Bioproducts, Rockland, ME) at room temperature in the dark.

For plasma cell ELISPOTs, plates were coated with Sigma HRP-labeled goat anti-mouse Fab (1/200, 4°C overnight), then blocked with 5% horse serum in PBS (1 h, 37°C). Pooled brain and spinal cord lymphocytes from four or five mice were added in triplicate in 2-fold dilutions starting with 10<sup>5</sup> cells and incubated for 19–24 h without moving. Secreted mouse Ab were detected with Vector HRP-labeled goat anti-mouse IgG (H and L) (1/1000, 1 h, 37°C), which was then bound with alkaline phosphatase-labeled streptavidin (30 min, 37°C). Spots were visualized as described for IFN-γ ELISPOTs. Wells were washed with PBS and/or 0.05% Tween in PBS between reagents.

Sequencing the S gene

Virus from the brains of each of four mMT mice with brain titers >10<sup>5.5</sup>
PFU/g collected at 16, 30, or 45 dpi was grown overnight on L2 cells. Total
RNA was purified from each flask using an RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized using a Superscript kit (Life Technologies, Grand Island, NY). PCR was then performed using multiple different primers, and the sequence was determined (CHOP Sequencing Facility, Children’s Hospital of Philadelphia, Philadelphia, PA). The sequences obtained were compared with wild-type A59 sequence (24) using MacVector and Clustal.

**Immunohistochemistry**

Brains and spinal cords were removed from mice perfused with PBS and were placed in 10% formalin for 24–48 h. Each was cut transversely into six pieces. Tissue processing, paraffin embedding, and sectioning were performed at the Wistar Histology Core Facility. Paraffin-embedded tissues were stained with BD Pharmingen anti- syndecan (1/500). Sigma goat anti-mouse Ab isotypes (1/100), or Vector biotinylated goat anti-mouse IgG (1/200) Abs. Goat serum or goat anti-gp70 Abs were used as negative controls. These Abs were detected by the UltraProbe Universal kit secondary Ab (Biomedia, Foster City, CA) after first blocking with 5% mouse serum in PBS or adding 5% mouse serum to the secondary Ab. Abs were visualized with methyl red. Tissues were counterstained with hematoxylin.

**Histology for demyelination**

Spinal cords were processed as described for immunohistochemistry and were stained with Luxol Fast Blue for myelin. Demyelination was quantified by separating each section into quadrants by eye and counting how many quadrants had evidence of demyelination per mouse.

**Immune serum production and serum transfer**

Female C57BL/6 mice were infected i.p. with 10^5 PFU MHV-A59 in PBS, then boosted with 10^6 PFU 3 wk later. After another 3 wk mice were euthanized by CO_2 overdose. Blood was collected from the left ventricle while simultaneously perfusing through the right ventricle with sterile PBS. Blood was allowed to clot overnight at 4°C. Serum Abs were purified by ammonium sulfate precipitation and dialyzed against PBS. The neutralizing titer 50% (NT50) of serum Ab was determined by incubating serial dilutions of serum with 500 PFU/ml MHV-A59 in DMEM-10 for 30 min at 37°C. NT50 was calculated as the dilution at which plaque numbers were reduced by 50%. For serum transfer, 200 μl anti-MHV serum Ab (NT50 of 1/5000), age-matched naive serum, or sterile PBS was given i.p. at 13, 15, 17, and 19 days postinfection. This volume contains the equivalent of one-fifth of the neutralizing activity found in one mouse 3 wk after i.p. infection (data not shown).

**Statistics**

The ANOVA t test was used to determine whether differences between two groups were statistically significant. Significance was assigned to p < 0.05. Viral titer calculations were performed using values transformed to log_{10}.

**Results**

**Infectious MHV-A59 persists in the CNS, but not the liver, in B cell-deficient mice**

To determine whether MHV strain A59 could be cleared in the absence of B cells, we infected B cell-deficient muMT and wild-type C57BL/6 mice intracerebrally (i.c.) with 50 PFU MHV-A59 and assessed viral titers at various time points after infection. As shown in Fig. 1A, virus initially replicated in the brain with the same kinetics in both B cell-deficient and wild-type mice. Viral titers peaked at 6 dpi and decreased by 10 dpi. It has been shown that T cells are important for early control of viral titers (25), and it is likely that T cells are responsible for this decrease in viral titers. A dramatic difference in viral kinetics is observed after day 10. C57BL/6 mice have cleared infectious virus by day 16. In contrast, most muMT mice do not clear virus at all. Virus persisted in muMT mice as late as 148 dpi (10^5.9 PFU/g). The viral titers in some persistently infected mice reached levels similar to or greater than those present at the peak of acute infection. Viral titers in the spinal cord were similar to those in the brain, although the peak during acute disease was on day 10 rather than day 6 (Fig. 1B). In addition, a small number of mice (n = 2) had late demyelinating lesions in the CNS in the absence of infectious virus. Since demyelination is induced by MHV infection, these data suggest that some mice can clear productive infections even in the absence of B cells. However, our results demonstrate that in most cases B cells are required for clearance of infectious MHV-A59 from the CNS.

Unlike many other strains of MHV, MHV-A59 infects the liver as well as the CNS after i.c. infection. To determine whether virus clearance in peripheral tissue also requires B cells, the livers of infected mice were titrated (Fig. 2B). In contrast to the results from the CNS (Fig. 1A), B cell-deficient mice clear virus from the liver as efficiently as wild-type mice. Therefore, the requirement for B cells to clear infectious MHV-A59 depends on the organ infected. Despite the persistence of virus in the CNS of muMT mice, and therefore the presence of a reservoir of virus, infectious virus was detected in the liver of only 1 of 36 mice collected after 6 dpi (10^3.1 PFU/g at 30 dpi). In this experiment the liver was inoculated indirectly by movement of the virus from the CNS, whereas the CNS was inoculated directly. Thus, it is possible that if the virus is introduced directly into the liver of muMT mice, the local immune response may not be sufficient to clear infection. To address this issue we directly injected 500 PFU MHV-A59 into the livers of muMT and C57BL/6 mice. Infectious virus was still cleared with normal kinetics from the liver by B cell-deficient mice after intrahepatic immunization (Fig. 2C). Another possible simple explanation for the absence of persistent virus in the liver is that the environment of the liver does not permit viral persistence. However, in other strains of immunocompromised mice MHV-A59 does persist in the liver after i.c. injection. We injected Rag1 knockout mice (n = 7) i.c. with 10 PFU MHV-A59 and detected an average of 10^4.4 ± 2.1 PFU at 22–33 dpi. We also found persistent virus (10^5.6 ± 1.75 PFU) in β2-microglobulin knockout mice (n = 2) 11 days after i.c. injection. In addition, attempts to reinfect the livers of muMT mice 10 dpi with a very high dose of MHV-A59 (2 × 10^7 PFU) given intrahepatically did not result in detectable virus 3 days later. Taken together, these results strongly suggest that the absence of virus in the liver is due to local immunity.

![FIGURE 1. Viral titers in the brain and spinal cord.](http://www.jimmunol.org/fig/f1.jpg)
MHV-A59 infection can occur acutely, usually during the first 17 days, after which clinical signs slowly disappear (26). High levels of infectious virus during acute infection can precede death. The discovery of viral persistence plus the observation of late deaths after acute infection in muMT mice led us to question whether mice can survive a persistent viral infection in the absence of B cells. To answer this question, we infected mice with various doses of MHV-A59 i.c. and observed these mice for >4 mo. Strikingly, no matter what infectious dose was used, lethality was 88–100% (Fig. 3). The lower the infectious dose, however, the later the average time of death. This result may reflect a higher Ag load and increased rate of activation-induced cell death of T cells. Alternatively, it may reflect lethal effects of demyelination, as demyelination has been observed to increase with increasing infectious doses (27).

Brains and livers were harvested from these mice when they were moribund or postmortem to determine the cause of death. CNS demyelination was widespread in all animals examined (average of 77% of spinal cord quadrants demyelinating in 10 mice, with half of those mice demonstrating 100% spinal cord quadrant involvement). Infectious virus was undetectable in the livers, but viral titers in the brains of these animals were undetectable. There are five mice per time point. Error bars represent the SEM.

In immunocompetent C57BL/6 mice, deaths related to high dose MHV-A59 infection occur acutely, usually during the first 17 days, after which clinical signs slowly disappear (26). High levels of infectious virus during acute infection can precede death. The discovery of viral persistence plus the observation of late deaths after acute infection in muMT mice led us to question whether mice can survive a persistent viral infection in the absence of B cells. To answer this question, we infected mice with various doses of MHV-A59 i.c. and observed these mice for >4 mo. Strikingly, no matter what infectious dose was used, lethality was 88–100% (Fig. 3). The lower the infectious dose, however, the later the average time of death. This result may reflect a higher Ag load and increased rate of activation-induced cell death of T cells. Alternatively, it may reflect lethal effects of demyelination, as demyelination has been observed to increase with increasing infectious doses (27).

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**T cell activity in B cell-deficient mice**

B cells can serve as APC to activate T cells. Despite the presence of macrophages and dendritic cells, which also serve as APC, CD4+ T cell responses to some Ags are suboptimal in the absence of B cells (28–30). No acute CD8+ T cell defects have been identified in B cell-deficient mice (29, 31, 32). To elucidate whether the CD4+ T cell response was activated normally in response to MHV-A59 in the absence of B cells, we isolated lymphocytes from the CNS of muMT and wild-type C57BL/6 mice and fluorescently labeled them for both the Th cell marker CD4 and the activation marker CD69. Seven days after infection a similar increase in the percentage of CNS CD4+ cells that displayed activation markers was observed in both strains of mice (Fig. 4A). When the numbers of CD4+CD69+ cells were calculated, a slight decrease in activated CD4+ T cells was observed in muMT mice compared with wild-type mice, but the difference was not statistically significant (Fig. 4B).

Studies with lymphocytic choriomeningitis virus (LCMV) infection in muMT mice suggest that even when normal levels of activation are detected, CD4+ T cells can be defective in IFN-γ production, especially at late time points after stimulation (33). We thus used ELISPOT analysis to determine the number of IFN-γ-secreting cells directly ex vivo. Analysis of unstimulated ex vivo CNS lymphocytes showed that muMT mice had statistically similar numbers of IFN-γ-secreting cells as wild-type mice 10 and 31 dpi (Fig. 5A). The ELISA system allowed us to quantify total IFN-γ production as well as responsiveness to viral Ag in vitro. Again, IFN-γ production in the absence of restimulation was similar at both time points, even after 3 days in culture (data not shown). However, IFN-γ production by muMT mice in response to UV-irradiated MHV-A59, while similar to that in wild-type mice at 10 dpi, was significantly decreased at 31 dpi (p = 0.0072; Fig. 5B). Since inactivated virus is preferentially processed for presentation to CD4+ T cells, these data suggest that there may be a defect in the ability of CD4+ T cells to produce IFN-γ late after infection.

Epitope escape, or mutation of a T cell epitope that prevents T cell recognition, and subsequent viral persistence have been demonstrated in mice infected while young and receiving passive immunity against MHV by suckling on immune dams. These mice often do not develop Ab responses to MHV, and in this sense resemble muMT mice (34, 35). All identified CD4+ and CD8+ T cell epitopes of MHV-A59 in C57BL/6 mice are located in the
spike (S) protein, although most of these epitopes were identified using another wild-type viral strain, MHV-JHM. Although MHV-A59 lacks the CD8 epitope S510-518 present in the S protein of the poorly hepatotropic MHV-JHM, it possesses epitope S598-605. The S gene also contains the three known CD4 epitopes (S329-343, 358-372, and 408-422) (36). RT-PCR was used to determine the RNA sequences of the S genes from pools of virus isolated from four muMT mice with high titers of persistent virus. The S gene sequences from all four mice were identical with wild-type sequences (data not shown). In contrast to the situation in neonates receiving passive immunity from their mothers, persistent virus in B cell-deficient mice shows no sign of epitope escape in the S Ag.

Role of Ab in clearance of MHV from the CNS

Although the blood-brain barrier becomes permeable to Abs during inflammation, after the resolution of inflammation the blood-brain barrier regains its ability to block the entry of Abs while allowing the entry of activated lymphocytes (37, 38). Therefore, we hypothesized that a strong and long-lasting Ab response in the CNS requires the presence of Ab-secreting cells (ASC) within the CNS. We analyzed CNS tissues for the presence of plasma ASC by immunohistochemistry using anti-syndecan-1 and anti-Ig. Anti-syndecan-1 binds to a cell surface proteoglycan that recognizes cell matrix proteins. Syndecan-1 is specifically found on epithelial tissues, immature B cells located in the bone marrow, and ASC (39). Staining with anti-syndecan-1 revealed no positive cells during acute infection (Fig. 6A). Syndecan-1-positive plasma cells did appear by 16 dpi and were still present 30 dpi (Fig. 6, B and C). Plasma cells were found much more frequently in the spinal cord than in the brain (data not shown). Staining sequential sections with anti-Ig isotype and anti-syndecan-1 verified that these cells contained Ab (Fig. 6, D–F). ASC were predominantly IgG2a or IgG2b positive, with occasional IgG1-positive cells (data not shown). An ELISPOT assay to detect ASC further demonstrated active Ab secretion from CNS lymphocytes at 16 dpi (data not shown). We conclude that functional plasma cells appear in the CNS of wild-type mice, consistent with a role for Ab in contributing to and maintaining viral clearance.

To determine whether the normal serum Abs produced in response to MHV-A59 are capable of controlling CNS viral titers, we passively transferred such serum Abs into infected muMT mice. When immune serum was administered 13, 15, 17, and 19 dpi, a highly significant drop in viral titers at 21 dpi was observed compared with that in mice that received naive serum or PBS (p = 0.0072, by t test). Fifty percent of animals treated with immune serum had no detectable virus. We conclude that Ab alone, in the absence of any other B cell function, is capable of controlling and even clearing infectious MHV-A59 from the CNS.

However, such data cannot tell us whether inducible levels of Ab are actually an important factor for clearance during natural progression of an infection. It is possible that during infection B cells are critical for some other reason, such as for cytokine production or cognate interactions with other cells. To conclusively differentiate between these two possibilities, we employed the mlgM-Tg mouse, which has B cells that cannot secrete Ab (21, 22). Abs containing the Tg H chain are specific for 4-hydroxy-3-nitro-phenyl when they also contain endogenous L chains. When they contain endogenous κ L chains, they have variable specificities and can generate Abs specific for self Ags, various proteins (L. Hannum and M. J. Shlomchik, unpublished observations), and viral Ags (A. Whitmore and M. J. Shlomchik, unpublished observations). Secondary lymphoid structure, including the presence of follicular dendritic cells, is normal in these mice (22). Abs containing the Tg H chain are capable of controlling and even clearing infectious MHV-A59 from the CNS.

FIGURE 5. IFN-γ secretion from CNS lymphocytes in muMT (Igh6) and C57BL/6 mice. Brain and spinal cord lymphocytes were purified from infected muMT and C57BL/6 mice 10 and 31 dpi. A, Lymphocytes were analyzed for the number of cells present that secreted IFN-γ ex vivo by ELISPOT. There was no statistically significant difference between muMT and C57BL/6 mice by t test analysis. B, In separate experiments CNS lymphocytes were set up in cell cultures for 72 h. IFN-γ production in response to UV-irradiated MHV-A59 was measured by ELISA. IFN-γ secretion by cells collected from naive mice of both strains was below the limit of detection in most samples. A statistically significant difference was detected between muMT and C57BL/6 mice (p = 0.0072, by t test), which is symbolized by the asterisk in the graph. There were five to eight mice per group. This experiment was performed three times with similar results. Error bars represent the SEM.
the mlgM-Tg mice we used JhD and C.B-17 mice. Since this genetic background supports a different type of immune response from that of muMT (40, 41), we tested whether infectious virus persists in these B cell-deficient mice. As in muMT mice, while infectious virus is completely cleared from the livers of JhD mice and the CNS and livers of wild-type C.B-17 mice, it is not cleared from the CNS of JhD mice (Table I). Therefore, our earlier observation that B cells are required for viral clearance from the CNS, but not the liver, is not limited to one specific genetically restricted immune response but, rather, holds true in different genetic backgrounds.

After i.c. infection of JhD and mlgM-Tg mice, virus persisted in the CNS, but not the liver, unlike in control C.B-17 mice (Table I). With the low infectious dose (10 PFU) used some mice had no detectable virus. Similar frequencies of JhD and mlgM-Tg mice had persistent virus. The CNS viral titers were also very similar in JhD and mlgM-Tg brains. These data suggest that secreted Ab rather than other B cell functions are required for control of virus. Ab can act through several different pathways besides direct binding and neutralization of virus. Two of these pathways use FcR, which bind the constant region of the Ab molecule. The process of opsonization uses FcRs to mediate the uptake of Ab-coated viruses by macrophages. Ab-dependent cell cytotoxicity (ADCC) by NK cells is triggered through FcR signaling. There are three FcRs that recognize IgG, the predominant general isotype of Ab made after MHV infection: FcRγI, II, and III. To determine whether opsonization and/or ADCC are important for MHV clearance, we infected FcR knockout mice. FcRγ subchain knockout mice (which lack FcRγI, FcRγIII, and FcReI) lack the FcRs necessary for ADCC and also lack opsonization activity, although one of the three well-known FcRs that mediates opsonization (FcRγII) is still present. No infectious virus was detectable 30 dpi (Table II). Although in vitro work suggested that opsonization could not occur in FcRγ subchain knockout mice (42), we wanted to make sure that the capacity for opsonization was absent in vivo. Therefore, we also used FcR double knockouts that lack all three FcRγs. These animals are available on the 129×B6 background, so 129×B6 mice were used as controls. Again, virus was cleared from the CNS by 30 dpi (Table II). A very small number of FcRγII knockout mice was also obtained and infected, and no persistent virus was detected 30 dpi (data not shown).

Ab can also activate the complement cascade, resulting in humoral elements that have multiple functions, including the formation of pores on the surface of cells to which Ab binds. C3 knockout mice, which cannot activate the complement cascade, do not have persistent infectious virus at 30 dpi (Table II), demonstrating that Ab-mediated clearance of MHV does not require a functional complement pathway.

**FIGURE 6.** Detection of syndecan-1, IgG plasma cells in the CNS. Formalin-fixed, paraffin-embedded spinal cord sections from C57BL/6 mice taken at various time points after i.c. infection with 50 PFU MHV-A59 were labeled with Abs to syndecan-1, a plasma cell marker. Ab was visualized with methyl red. Shown are sections taken at 10 dpi (A), 16 dpi (B), and 30 dpi (C; magnification, ×400). Spinal cord tissue from mice mock-infected with cell lysates had no positive cells and looked like those in A (data not shown). Sequential spinal cord sections from a 30 dpi C57BL/6 mouse were stained with an isotype control (D), anti-syndecan-1 (E), or anti-IgG2a (F; magnification, ×200), confirming that Ab-positive cells are syndecan positive.

**FIGURE 7.** Brain viral titers after passive transfer of serum Abs against MHV-A59. Brain viral titers were assessed by viral plaque assay 21 dpi. PBS, naive serum, or hyperimmune serum was administered i.p. on days 13, 15, 17, and 19 postinfection. For calculations, mice with undetectable PFU were assigned the value of 100 (2 log10), because that is the threshold of detection. Circles represent individual animals.
Discussion

Our results show that B cells are required for the clearance of a primary MHV-A59 infection in the CNS. After an initial and transient decrease in viral titers between 6 and 10–16 dpi, which is probably mediated by T cells and possibly NK cells (25), viral titers in B cell-deficient mice increase and persist. Our data join the growing body of evidence that B cells, which classically have been considered to be protective against reinfection, can be vitally important for the control of primary infections. Similar patterns, with initial control of pathogen but lack of clearance, have been demonstrated for infection with MHV strain JHM as well as LCMV, HSV-1, Plasmodium, and malaria (18, 43–46). Semliki Forest virus clearance is delayed several weeks in the absence of B cells (47). In addition, Sindbis virus is cleared from the CNS of immunodeficient mice by transferred B cells, but not T cells (19), and vesicular stomatitis virus is acutely lethal in B cell-deficient mice (48). Collectively, these results suggest that B cells are required for clearance of some primary viral infections, although the mechanisms of B cell activity may differ depending on the pathogen.

Although B cells are crucial for MHV-A59 clearance from the CNS, they are not required for clearance of MHV-A59 from the liver, whether the virus is injected i.c. or intrahepatically, suggesting a novel organ-specific dependency for B cells. In addition, we show in this study that virus can persist in the livers of CD8\(^{+}\) T cell-deficient \(\beta_{2-}\)microglobulin knockout mice and T and B cell-deficient RAG1\(^{-/-}\) mice. Therefore, the liver is not inherently resistant to MHV-A59 persistence, and the immune response is implicated to account for the difference observed between the liver and the CNS in the absence of B cells. Interestingly, other viruses that require B cells for clearance, including Sindbis, vesicular stomatitis virus, HSV-1, Semliki Forest virus, and LCMV, also infect the CNS (18, 19, 47–49). The CNS environment may directly favor a dependency on B cells, perhaps by down-regulation of T lymphocyte function. After MHV-JHM infection, CD8\(^{+}\) T cells persisted in the CNS and maintained their ability to produce IFN-\(\gamma\), but they gradually lost cytotoxic activity (50). In another system, T cells responding to Sindbis virus infection in the CNS down-regulated IL-2 production and lost their ability to proliferate after entering the brain (51). Transfer of LCMV-primed lymphocytes to mice chronically infected with LCMV resulted in much slower clearance of infectious virus from the CNS than from peripheral organs such as spleen, liver, and lung. After clearance, LCMV Ags persisted in the CNS, but not peripheral organs (52, 53). Immune function may be altered by immunosuppressive compounds found in the CNS, such as TGF-\(\beta\), IL-10, and gangliosides (54, 55). In contrast, the liver is exquisitely accessible to lytic CD8\(^{+}\) T cells. When hepatitis B-specific CD8\(^{+}\) T cell lines were transferred to transgenic mice that express hepatitis B Ags in many organs, they caused pathological changes only in the liver (56). The liver also acts as a sink for activated T cells (57) and a home for NK cells with high lytic potential (58). While the specific cells that are required for clearance of neurotropic MHV from the liver have not been identified, CD8\(^{+}\) T cells, CD4\(^{+}\) T cells, and NK cells are important for early control of viral titers (59–61). Persistence of infectious virus in the liver and clearance from the liver also occur in B cell-deficient mice on the C.B-17 background, demonstrating that this organ-specific pattern is not restricted to mice of one genetic background.

We speculate that there is a meaningful correlation between the almost nonexistent regenerative capacity of the neuronal component of the CNS and the requirement for nonlytic Ab function, which may preserve CNS function while controlling viral infection. In contrast to the CNS, the liver has high accessibility to, and microenvironmental support of, physically destructive lytic or apoptosis-inducing T cells and no requirement for B cells to clear MHV-A59. Because the liver has a very high capacity for regeneration, ill effects of immune-mediated destruction in pursuit of a pathogen are less likely to be long-lasting. In addition, it is interesting to hypothesize that the presence of small and therefore vulnerable areas with unique functions within the brain may also have favored the development of a CNS environment that selectively supports relatively benign Ab responses rather than lytic mechanisms of immune control. The longer that such lytic mechanisms are relied on in the CNS, the greater the chance of destruction of a region critical for normal organism function. Again, the liver provides an excellent contrast, in that the hepatocytes form a remarkably homogeneous population. Vigorous destruction of a small part of this organ could be a very effective and practical way to prevent the spread of a pathogen without sacrificing much in the way of organism function.

At low and high doses of infection, most B cell-deficient mice eventually die with high viral titers in the CNS. We conclude that while immune cells in the absence of B cells can prevent the type of rapid death observed in the absence of T cells (4, 5, 10), they are unable to control the infection well enough over a long period of time to allow survival. The decrease in IFN-\(\gamma\) responsiveness at

**Table I.** Viral persistence and CNS viral titers 30 dpi in mice with various B cell defects

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Description</th>
<th>Total No. of Mice</th>
<th>% Positive for Virus*</th>
<th>PFU/g in Brain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.B-17(^{+})</td>
<td>Wild type</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>JhD</td>
<td>B cell knockout</td>
<td>24</td>
<td>63%</td>
<td>5.6</td>
</tr>
<tr>
<td>mlgM-Tg</td>
<td>B cell knockout, Ab</td>
<td>13</td>
<td>69%</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* Mice were infected with 10 pfu intracranially due to the high susceptibility of the transgenic mice to MHV-A59 induced mortality (data not shown). Brains were collected 31 dpi and viral titers were measured. All mice are on C.B-17 genetic background.

* Livers of all mice were negative for infectious virus.

**Table II.** Absence of infectious virus 30 dpi in immunodeficient mice lacking FcRs or complement

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>No. of Mice</th>
<th>PFU/g in Brain</th>
</tr>
</thead>
</table>

\footnotesize{a} Background of this strain is (C57BL/6 \(\times\) 129). All other strains are on C57BL/6 background.

\footnotesize{b} Animals were infected with 50 PFU of MHV-A59 i.c. C3-deficient mice and their six C57BL/6 controls were infected at 7–8 wk of age. All other mice were infected at 4 wk of age. All livers were negative for infectious virus at 30 dpi.
late time points could possibly play a role in the late deaths. IFN-γ is important for the clearance of infectious virus from oligodendrocytes in the CNS, and, while present, it may help animals resist lethally high levels of viral replication in the absence of B cells (62).

One function of B cells, shared with macrophages and dendritic cells, is the presentation of foreign Ags in the proper context for T cell recognition. In B cell-deficient mice, CD4⁺ T cell function varies depending on the Ag inducing the response, indicating that some Ags are more dependent on presentation by B cells than others (28–30). Indeed, while B cell-deficient mice have a normal CD4⁺ T cell proliferative or activation response to human IgG and LCMV (30, 33), they have a substandard response to pigeon cytochrome c and Chlamydia trachomatis (28, 63). Measuring the induction of the activation marker CD69 on CD4⁺ T cells in muMT and wild-type mice after MHV-A59 infection revealed no dependency upon B cells for CD4⁺ T cell activation. Another possible defect was suggested in a study with LCMV, which demonstrated that even with normal activation, CD4⁺ T cells stimulated in the absence of B cells may have deficiencies in IFN-γ production at late time points (33). Interestingly, despite the differences in the presence of infectious virus, muMT and wild-type mice produced similar levels of IFN-γ ex vivo or in vitro without additional viral Ag both early and late after infection, suggesting no defect. Similarly, with MHV-JHM infection in B cell-deficient mice, the same amount of IFN-γ mRNA was present in the CNS of muMT mice as in wild-type mice 10 dpi, and actually elevated amounts were present in muMT mice at 35 dpi (43). However, we demonstrated that at 31 dpi, IFN-γ production in response to viral Ag was significantly decreased in muMT mice. These data suggest that IFN-γ secretion by CD4⁺ T cells in response to a viral Ag is defective at late time points after infection in B cell-deficient mice. Whether this defect is directly due to the absence of B cell help or is a response to persistent infectious virus is unclear.

Although CD8⁺ T cell activation and memory responses are reported to be normal in B cell knockout mice (29, 31, 32), CD8⁺ T cell exhaustion has been reported in LCMV-infected B cell knockout mice (18, 32). However, most CD8⁺ T cell-deficient mice that survive acute infection successfully clear MHV-A59 (5). Also, CD8⁺ T cell lytic activity normally decreases in the CNS after MHV infection (50). Therefore, any defect in the CD8⁺ T cell compartment induced by the absence of B cells is unlikely to account for persistence of infectious virus (5). In addition, when CD8⁺ T cell numbers at 30 dpi were assessed by flow cytometry, the percentage and numbers were actually higher in muMT than C57BL/6 mice, the opposite of the result expected if exhaustion were occurring (n = 3/group; 54 ± 0.46% CD8⁺ cells in muMT vs 39 ± 1.62% CD8⁺ cells in C57BL/6 mice, p = 0.0001; 3.4 ± 0.9 × 10⁵ CD8⁺ cells in muMT vs 1.6 ± 0.3 × 10⁵ CD8⁺ T cells in C57BL/6 mice, p = 0.0039).

Sequence of the S gene of persistent virus revealed no evidence of CD8⁺ T cell epitope escape. A similar lack of epitope mutations was found in the immunodominant epitope S510-518 in MHV-JHM isolated from B cell-deficient mice (43).

Although Ag presentation is one function of B cells, they are specialized for the production of Abs. Serum Ab transfer into B cell-deficient mice demonstrated that Ab has the potential to control infectious virus. Tg (mlGm-Tg) mice that cannot secrete Ab but have cell surface Ab, however, were not able to control infectious virus. Their B cells should be competent to internalize Ag by receptor-mediated endocytosis for subsequent processing and presentation to T cells. These B cells should also be activated by Ag to secrete cytokines and up-regulate any cell surface molecules that might be involved in cell-cell interactions. Therefore, cell-associated functions are insufficient for control of virus, and Ab secretion is implicated as the main B cell effector function that is critical for control of virus in the CNS.

It is well established that Ab can function by directly binding and neutralizing virus, opsonizing it to facilitate phagocytosis, binding cell surface viral proteins on infected cells and stimulating ADCC, or activating the complement cascade. Using various knockout mice, we have demonstrated that neither opsonization, ADCC, nor complement is required for viral clearance. Although Ab responses in the absence of complement are often impaired, the ability of C3-deficient mice to clear MHV implies the presence of Abs. A strong Ab response can be generated in the absence of complement when a replicating virus is used as the Ag (64). These data imply that the role of Ab in viral clearance is to neutralize virus, although it is also possible that either the complement or the FeR pathway alone is sufficient for Ab-mediated viral clearance. Alternatively, work with measles and Sindbis viruses suggests that Ab may also affect viral replication by altering viral gene expression and affecting normal host cell function (65, 66).

The importance of Abs for viral clearance correlates with the appearance of B cells devoted to Ab production in the CNS of wild-type mice by 16 dpi, although early Ab effects may also be mediated by peripherally produced Ab, which can be detected as early as 7–10 dpi (26, 67, 68). ASC are also found in the brain after Sindbis infection or direct Ag infusion into the CNS (38, 69). In mice persistently infected with LCMV, plasma cells were often concentrated in the brain as well as the kidney and spleen, but were much less common in the liver (70). The identification of plasma cells in the CNS correlates with the observation that there are often higher titers of Ab in the cerebrospinal fluid than in the blood after CNS infections (71). The CNS seems to be an organ where plasma cells can permanently reside, perhaps partially as a response to the ability of viral Ag and genomes to persist in this location (72). This ability is important, since the blood-brain barrier is believed to be able to prevent the entry of peripheral Abs (37). After peripheral infections, it has been suggested that plasma cells predominantly localize to the bone marrow following clearance of infection (73). It will be difficult to determine whether plasma cells maintain virus at undetectable levels or actually clear infectious virus, as plasma cells are resistant to radiation and chemotherapeutic methods of immunosuppression.

We have shown that Abs are required for clearance of MHV-A59 from the CNS, but not the liver. Not only can Abs play a critical role in the clearance of a primary infection, the role of Abs is dependent on the organ infected. While Ab production is crucial for B cell-mediated viral clearance of MHV-A59, B cells may also help to maintain normal IFN-γ production by CD4⁺ T cells. We suggest that Abs act neither through FcRs nor the complement cascade but, rather, act to clear virus by direct neutralization of virus and/or altering viral replication intracellularly.

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References


