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Antibody-Mediated Protection against Cytotoxic T-Cell Escape in Coronavirus-Induced Demyelination

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C57BL/6 (B6) mice infected with mouse hepatitis virus (MHV) strain JHM develop a clinically evident, demyelinating encephalomyelitis. Infectious virus can be isolated from the spinal cords of these mice and is invariably mutated in the immunodominant CD8 T-cell epitope recognized in this strain. We showed previously that these persistently infected mice did not mount a measurable serum anti-MHV neutralizing antibody response. Here we show that cytotoxic T-lymphocyte (CTL) escape was not detected in MHV-infected BALB/b mice (*H-2^b* haplotype), even though the same CD8 T-cell epitopes were recognized as in B6 mice. BALB/b mice had 25-fold more MHV-specific antibody-secreting cells in the central nervous system, the site of infection, than B6 mice, suggesting that local production of anti-MHV antibody contributed to this absence of CTL escape. Additionally, administration of anti-MHV neutralizing antibody to infected B6 mice suppressed the development of CTL escape mutants. These findings indicate a key role for the anti-MHV antibody response in suppressing virus replication, thereby minimizing the emergence and competitive advantage of CTL escape mutants.

The importance of CD8 cytotoxic T lymphocytes (CTL) for control of viral infections has been illustrated in multiple studies. To counter this host defense mechanism, viruses have evolved a diverse group of strategies to evade the CD8 T-cell immune response. One evasive strategy involves the generation of mutations in immunodominant CD8 T-cell epitopes, resulting in CTL escape. This mechanism of immune evasion has been identified in many persistent viral infections, including in humans infected with human immunodeficiency virus type 1 or hepatitis B virus and nonhuman primates infected with hepatitis C virus or simian immunodeficiency virus (3, 7, 14, 16). Some of the factors important for the selection of CTL escape mutants have been identified (3, 21). CTL escape is more likely when the CD8 T-cell immune response is focused on a single immunodominant epitope and when the epitope is located in a region of the virus amenable to mutation without loss of virulence.

One common theme of these studies is that CTL escape does not occur if viruses are cleared efficiently. This has been demonstrated in mice infected with lymphocytic choriomeningitis virus or influenza A virus in which CTL escape is not detected under normal circumstances but does occur if mice transgenic for a single lymphocytic choriomeningitis virus or influenza A virus T-cell receptor are infected with large amounts of virus (24, 25). The factors responsible for inefficient virus clearance at early times after infection are not well understood but are critical for understanding the process of CTL escape.

CTL escape mutants are commonly detected in C57BL/6 (B6) mice infected with the neurotropic JHM strain of mouse hepatitis virus (MHV) (21). MHV-infected rodents develop acute and chronic demyelinating diseases of the central nervous system and serve as a useful animal model for the human disease multiple sclerosis (29). Mice infected with wild-type MHV develop acute encephalitis after intranasal or intracerebral inoculation. The infection becomes nonlethal and persistent if mice are infected with attenuated virus or if they are protected from acute disease by administration of anti-MHV antibodies or T cells (29). In one model, suckling B6 mice were inoculated intranasally with MHV and nursed by dams previously immunized to MHV. Under these conditions, no suckling mice developed acute encephalitis. However, a variable fraction (40 to 90%) developed hind limb paralysis with histological evidence of a demyelinating encephalomyelitis at 3 to 8 weeks postinfection (p.i.). Mice that remained asymptomatic at 60 days p.i. rarely developed MHV-induced clinical disease (20). In each case, virus isolated from symptomatic mice was mutated in the immunodominant CD8 T-cell epitope encompassing residues 510 to 518 of the surface (S) glycoprotein (CSLWNGPHL, epitope S510), and these mutations abolished recognition by central nervous system (CNS)-derived lymphocytes in direct ex vivo cytotoxicity assays (22). Mutations were not detected in regions flanking the epitope or in the subdominant CD8 T-cell epitope encompassing residues 598 to 605 of the S protein (RCQIFANI, epitope S598). No mutations in epitope S510 were identified in virus harvested from mice with acute encephalitis (22). Epitope S510 is located in a region of the S protein that tolerates deletion and mutation (1, 19), which is likely to facilitate the selection of CTL escape mutants.

Notably, serum neutralizing anti-MHV antibody was not detectable in most mice that developed hind limb paralysis but was present in approximately 50% of mice that remained asymptomatic at 60 days p.i (9, 20). Other studies have highlighted the key role that anti-MHV antibodies have in prevent-

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ing viral recrudescence. MHV-specific antibody-secreting cells (ASC) were detected in the CNS of infected B6 mice as early as 7 days p.i (33). Infection of mice homozygous for disruption of the immunoglobulin μ chain gene or for disruption of the J_H locus resulted in normal virus clearance initially, but virus recrudescence invariably occurred in these animals (12, 13, 26). Passive infusion of anti-MHV antibody prevented the reemergence of virus, confirming the importance of antibody in this process (12, 13).

Since maternal antibody-protected infected B6 mice do not mount a detectable serum anti-MHV antibody response, an attractive possibility was that this deficiency resulted in an inability to control virus replication, facilitating the selection of CTL escape mutants. Conversely, a strong anti-MHV antibody response was predicted to reduce virus replication and, by extension, to diminish the selection of CTL escape variants.

MATERIALS AND METHODS

Virus. Wild-type MHV was purified and titered as previously described (20). The attenuated J2.2-V-1 strain of MHV was kindly provided by John Fleming (University of Wisconsin, Madison).

Animals. Specific-pathogen-free B6 and BALB/c mice were obtained from the National Cancer Institute (Bethesda, Md.). C.B10H2^b (BALB/b) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). To obtain mice persistently infected with MHV, suckling mice were infected intranasally at day 10 of age with 6×10^6 PFU of MHV in 10 μl and nursed by dams previously immunized to MHV as described previously (20). In some experiments, asymptomatic maternal antibody-protected mice were immunized with MHV in complete Freund's adjuvant at 41 days p.i., followed by immunization in incomplete Freund's adjuvant at 49 and 56 days p.i.

Determination of anti-MHV antibody titers. The virus neutralization titer of mouse sera was determined by a constant virus-variable serum plaque reduction assay (20). Blood was obtained at weekly intervals from infected mice beginning 7 days p.i. and continuing until 60 days p.i. or until harvest if they developed hindlimb paralysis.

SRBC assays. We immunized 28-day-old MHV-infected or uninfected mice intraperitoneally with 0.7% sheep red blood cells (SRBC) (Colorado Serum Company, Denver, Colo.) in 250 μl of phosphate-buffered saline (PBS). Eight days postimmunization, spleens were harvested and stained with fluorescein isothiocyanate-conjugated peanut agglutinin and Texas red-conjugated anti-B220 antibodies (28). Spleen samples were analyzed on a FACS Vantage (Becton Dickinson, Mountain View, Calif.).

Elispot assays. Elispot assays were conducted as previously described (26). Briefly, multiscreen 96-well plates (Millipore, Bedford, Mass.) were coated overnight with either 500,000 PFU of MHV, for quantifying MHV-specific ASC, or with 500 ng of rabbit anti-mouse IgG plus IgM (Jackson Immunoresearch, West Grove, Pa.). After wells were blocked with 2% bovine albumin in minimal essential medium, various dilutions of lymphocytes prepared from bone marrow, CNS, or spleen were placed into these wells and incubated at 37°C for 4 h. After washing, biotinylated goat anti-mouse IgG plus IgM (Jackson Immunoresearch) in PBS with 10% fetal bovine serum was added to each well for 1 h. Spots were developed with streptavidin-peroxidase (Jackson Immunoresearch) and diaminobenzidine (Sigma). Spots were visualized and counted with a Wolfe stereoscopic microscope.

Flow cytometry and antibodies for flow cytometry. CNS-derived lymphocytes were prepared as previously described (22). In brief, brains and spinal cords were homogenized to a single-cell suspension, and lipids were removed with a 30% Percoll (Pharmacia, Uppsala, Sweden) gradient. Lymphocytes were isolated with Lympholyte-M (Cedarlane Laboratories, Homby, Ontario, Canada). Fluorescein isothiocyanate-conjugated anti-CD19, phycoerythrin-conjugated anti-kappa, fluorescein isothiocyanate-conjugated peanut agglutinin, cyanin-conjugated anti-B220, and biotinylated anti-Fas and anti-IgM antibodies were prepared as previously described (28).

Anti-MHV antibody administration. Individual litters of suckling mice were infected with MHV as described above. Half of each litter was given a cocktail of the anti-MHV neutralizing antibodies 5A13.5 (72 μg) and 5B19.2 (11 μg) in 250 μl of PBS (a gift of M. Buchmeier, The Scripps Institution, La Jolla, Calif.) by intraperitoneal inoculation. This quantity of antibody was based on a previous determination of the amount of antibody required to protect mice from acute encephalitis (31). Control mice received equal amounts of isotype-matched antibodies in 250 μl of PBS or PBS only. Antibodies were administered at 17, 22, 27, and 34 days of age (7, 12, 17, and 24 days p.i.).

RNA sequence analysis. Total RNA was harvested with Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) from the CNS of mice either when they became symptomatic or at day 60 p.i. The region encompassing the S510 epitope was sequenced by reverse transcription-PCR as previously described (22).

RESULTS

B6 mice persistently infected with MHV have a specific defect in anti-MHV antibody production. To confirm our previous results, we measured serial serum antibody titers in 62 MHV-infected suckling B6 mice nursed by MHV-immune dams. Maternally derived antibody decays with a half-life of 5 to 6 days and is only absorbed by the neonatal mouse until 17 days after birth (17). None of these mice developed acute encephalitis, but 90% (56 of 62) developed hind limb paralysis with histological evidence of chronic demyelination several weeks p.i. At the time that these mice developed hind limb paralysis, 43 had neutralizing titers of less than 1:100, 4 had neutralizing titers of greater than 1:400, and 9 had titers of between 1:100 and 1:400. Titers of anti-MHV antibody declined after weaning (Fig. 1A and B), suggesting that the suckling mice had mounted a minimal antibody response to MHV even though they were persistently infected with the virus (20, 22). The same pattern of results was obtained when neutralizing titers were measured by plaque assay (Fig. 1A and B) or total anti-MHV titers were measured by enzyme-linked immunosorbent assay (data not shown).

This defect in anti-MHV antibody response may represent a general effect on B-cell maturation, a specific effect on production of anti-MHV antibody, or a more general inhibition of antibody production. To determine whether infection of neonatal mice compromised the B-cell compartment, control and infected mice were analyzed at day 21 p.i. by multicolor flow cytometry. Bone marrow suspensions were stained with anti-B220 (CD45R), anti-heat stable antigen (CD24), and either anti-CD43, anti-BP-1 (aminopeptidase A), or anti-IgM antibodies. These combinations allowed assessment of the pro-B-cell, pre-B-cell, and immature B-cell developmental stages. Infected mice demonstrated normal levels of all bone marrow subsets, indicating that B-cell lymphopoiesis was unaffected (data not shown). Splenocytes were also analyzed in a three-color protocol to examine the status of peripheral B-cell subsets by staining with anti-B220, anti-CD21, and either anti-IgD or anti-CD1 antibodies. These combinations allow enumeration of follicular, marginal zone, and immature B cells in the spleen. Consistent with the bone marrow analysis, splenic B-cell subset composition was comparable between control and infected mice (data not shown).

To determine whether MHV-infected mice could mount an antibody response to other antigens, germinal center formation was measured after administration of sheep red blood cells at 18 days p.i., when mice were 4 weeks old. Mice were examined 8 days post-SRBC challenge, the peak of the germinal center response (28). The frequency of germinal center B cells in MHV-infected mice was the same as in uninfected age-matched controls (Fig. 2A and B). In the absence of erythrocyte immunization, MHV-infected mice (Fig. 2C) and naïve

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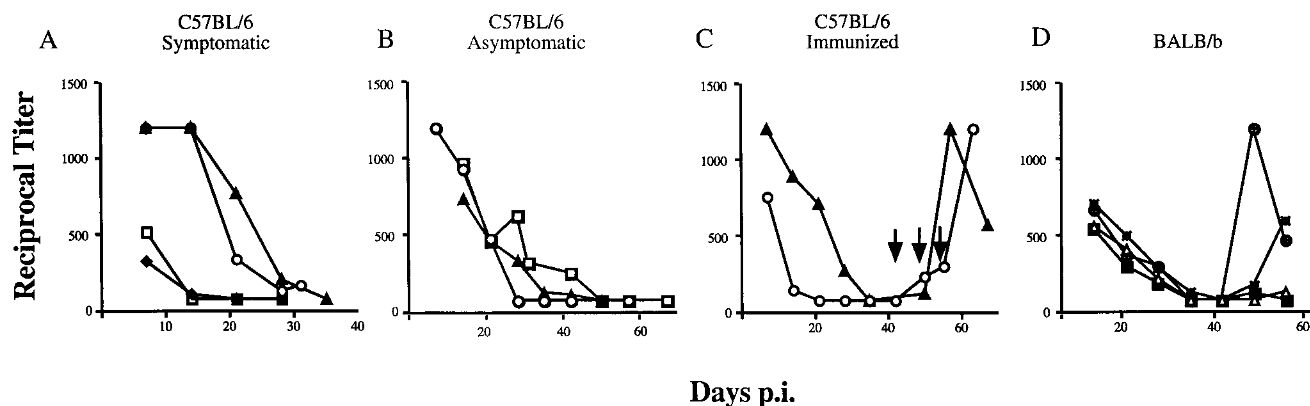

FIG. 1. Antibody titers in MHV-infected B6 and BALB/b mice. (A and B) Serum neutralizing antibody titers were measured from samples serially drawn from maternal antibody-protected MHV-infected B6 mice. Although initial titers were variable, they invariably declined after passive transfer of maternal antibody ceased at day 17 of life (17). Four different mice that developed hind limb paralysis are shown in panel A, whereas three mice that remained asymptomatic are shown in panel B. Each symbol indicates a single mouse. These data are representative of the 56 symptomatic and 6 asymptomatic mice analyzed. (C) To determine whether this decline in antibody titers reflected an inability to mount an antibody response to MHV, asymptomatic B6 mice were immunized with MHV at days 41, 49, and 56 p.i. These mice mounted a robust neutralizing antibody response to MHV, as measured by neutralizing titer. An additional 16 mice, not shown, were immunized and analyzed, with similar results. (D) Serial serum neutralizing titers from four maternal antibody-protected BALB/b mice were measured as described in the legend to panel A.

mice (data not shown) exhibited the same low background of germinal center formation.

Although this experiment demonstrated no difference in germinal center formation in MHV-infected mice compared to controls, it remained possible that antibody production to a non-MHV antigen would be impaired. To address this possibility, we immunized three MHV-infected and six naïve mice with ovalbumin. Asymptomatic MHV-infected mice were able to mount an antibody response to ovalbumin at levels comparable to those seen in MHV-naïve B6 mice, as measured by enzyme-linked immunosorbent assay (data not shown).

These results suggested that any effect of MHV in this model was specific for synthesis of anti-MHV antibodies at early times p.i. This could occur by deleting or blocking the maturation of MHV-specific B cells. To distinguish between these possibilities, asymptomatic mice were immunized with MHV beginning at 41 days p.i. As shown in Fig. 1C, this resulted in a robust antibody response to MHV, equivalent to that observed in dams actively immunized with infectious virus (4). Therefore, infection with MHV did not result in permanent deletion of MHV-specific B cells from the repertoire.

MHV-specific ASC were present at low levels in the CNS of maternal antibody-protected B6 mice. In B6 mice infected with an attenuated variant of MHV, cells producing anti-MHV antibody were detected in the CNS even after infectious virus could no longer be identified. Their presence at the site of

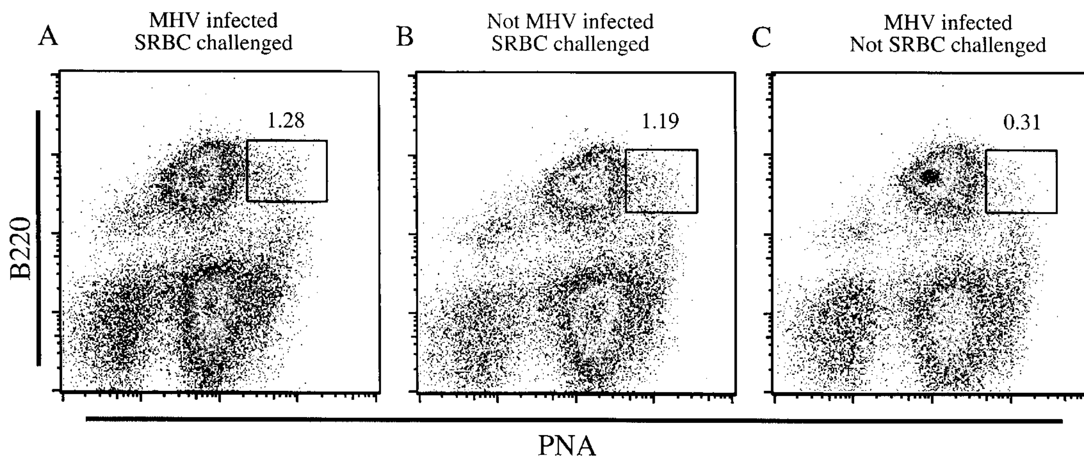

FIG. 2. Germinal center reactions in MHV-infected mice. Maternal antibody-protected B6 mice at day 18 p.i. (28 days of age) and age-matched uninfected B6 mice were inoculated intraperitoneally with sheep erythrocytes as described in Materials and Methods. Germinal center formation at 8 days p.i., as measured by $B220^{+}$, high peanut agglutinin (PNA^{hi}) cells, was equivalent in MHV-infected (A) and uninfected (B) mice challenged with sheep erythrocytes. (C) Germinal center formation in MHV-infected mice not immunized with sheep erythrocytes was minimal and equivalent to that detected in untreated naïve mice (not shown). These data are representative of six individual experiments.

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TABLE 1. ASC in the CNS[a]

Strain	Days p.i. (no. of mice analyzed)	No. of ASC	
		MHV specific	Total
B6	21–25 (5)	49 ± 24	923 ± 602
	26–30 (3)	88 ± 76	1,699 ± 1,429
	34–45 (5)	37 ± 17	461 ± 249
	>60 (7)	14 ± 12	197 ± 146
BALB/c	14 (4)	408 ± 75*	558 ± 111
	21–25 (5)	2,420 ± 945*	4,997 ± 1,702
BALB/b	14 (4)	350 ± 63*	671 ± 140
	21 (4)	1,242 ± 142*	3,058 ± 1,101

[a] *, significantly different from maternal antibody-protected B6 mice at all times p.i. At days 21 to 25 and 34 to 45 p.i., $P \leq 0.05$; days 26 to 30 and 60+, $P \leq 0.01$. Values are means plus standard errors of the means.

TABLE 2. ASC in the spleen[a]

Strain	Days p.i. (no. of mice analyzed)	No. of ASC	
		MHV specific	Total (10^3)
B6	21–25 (5)	2,430 ± 1,600	86.1 ± 47.5
	26–30 (3)	2,700 ± 246	165.0 ± 21.8
	34–45 (5)	5,060 ± 1,700	71.6 ± 18.2
	>60 (7)	9,870 ± 2,900	334.5 ± 60.3
BALB/c	14 (4)	1,538 ± 202	64.1 ± 7.12
	21–25 (5)	9,007 ± 3,356	101.4 ± 57.2
BALB/b	14 (4)	1,008 ± 148	48.2 ± 5.5
	21 (4)	5,392 ± 2,566	51.5 ± 19.0

[a] Values are means plus or minus standard errors of the means.

infection may have contributed to suppression of virus replication (26). To determine if there was an effect on anti-MHV antibody production within the CNS in B6 mice protected by maternal antibody, we used Elispot assays to detect plasma cells secreting anti-MHV IgG and IgM antibodies. The total number of immunoglobulin-secreting cells was determined with anti-IgG and anti-IgM antibodies together as capture antibodies.

Infected B6 mice failed to mount a significant antibody response to MHV within the CNS at all times. Asymptomatic mice had, on average, 49 ± 24 ASC in the CNS at 21 to 25 days p.i., and this number did not increase significantly at any time p.i. (Table 1; Fig. 3A). Additionally, there was only a modest influx of non-MHV-specific ASC (923 ± 602 cells at 21 to 25 days p.i.). By contrast, many antibody-secreting cells specific for MHV could be found in the spleen and bone marrow of infected B6 mice at 21 to 25 days p.i. On average, B6 mice had 2,430 ± 1,600 MHV-specific ASC in the spleen (Table 2), and 0.0047% of bone marrow cells were specific for MHV (data not shown). The number of ASC in both of these organs rose with time p.i. Furthermore, analysis of nine mice with hindlimb paralysis also revealed similar numbers of MHV-specific ASC in the CNS and spleen (days 26 to 30 p.i., CNS: 85 ± 32, spleen: 2,978 ± 885) as in asymptomatic mice (Tables 1 and 2).

These low numbers of MHV-specific ASC in the CNS did not reflect an age-related inability to mount an antibody response to the virus. For these experiments, we inoculated 4- and 6-week-old naïve B6 mice with an attenuated strain of MHV because naïve B6 mice all die by 7 days after infection with wild-type virus. This attenuated virus is cleared by 12 to 15 days p.i. in B6 mice (12). We detected, on average, 725 MHV-

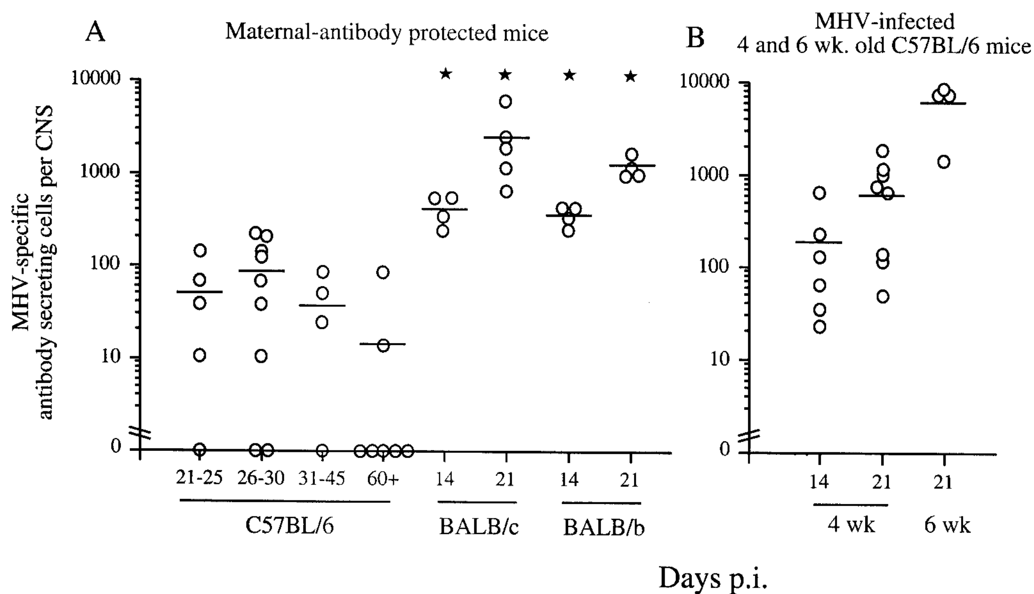

FIG. 3. ASC in the CNS of MHV-infected mice. We quantified the number of anti-MHV ASC in the CNS of B6, BALB/c, and BALB/b mice. (A) Maternal antibody-protected BALB/b and BALB/c mice had 10- to 20-fold more MHV-specific ASC in the CNS than B6 mice at day 21 p.i. *, significantly more anti-MHV ASC were detected in the CNS of infected BALB/b or BALB/c mice than B6 mice at all times p.i. ($P < 0.05$). (B) Naïve B6 mice infected with MHV were capable of mounting an antiviral antibody response. Four- and six-week-old B6 mice were infected with attenuated MHV and analyzed 14 and 21 days p.i. These mice mounted significantly greater anti-MHV responses within the CNS than their maternal antibody-protected counterparts at the same age.

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specific ASC in the CNS of mice inoculated at 4 weeks and harvested at day 21 p.i. This was 10- to 20-fold greater than the number found in maternal antibody-protected mice harvested at a similar age (34 to 45 days p.i. [44 to 55 days of age], Fig. 3B). Likewise, there were 400-fold more MHV-specific ASC in the CNS of naive B6 mice inoculated at 6 weeks of age and examined at 21 days p.i. than in maternal antibody-protected mice at 60 days p.i.(70 days of age). The numbers of MHV-specific ASC in the spleen and bone marrow of maternal antibody-protected mice at 34 to 45 days p.i. were, by contrast, approximately the same as in age-matched mice inoculated with attenuated virus (data not shown). Thus, the diminished accumulation of cells secreting anti-MHV antibody was largely limited to the CNS, the site of infection.

MHV-specific ASC were detected at higher levels in the CNS and spleen of maternal antibody-protected infected BALB/c and BALB/b mice compared to B6 mice. Unlike suckling B6 mice, BALB/c mice nursed by dams immunized to MHV are protected from acute encephalitis and never develop hind limb paralysis at later times after infection (4). CTL escape mutants have never been identified in these mice (data not shown). Consistent with a role for anti-MHV antibody in protection from late-onset clinical illness and CTL escape, large numbers of anti-MHV ASC were detected in the CNS of maternal antibody-protected BALB/c mice. Cells were detected as early as 14 days p.i. and increased fourfold in the next 7 to 10 days (Table 1, Fig. 3A). At 21 to 25 days p.i., BALB/c mice had 50-fold more MHV-specific ASC in the CNS than did B6 mice at the same time point.

Unlike epitope S510, the single immunodominant CD8 T-cell epitope recognized in BALB/c mice is located in the nucleocapsid (N) protein (APTAGAFFF, encompassing residues 318 to 326, N318), a protein that is highly conserved among different mouse hepatitis virus strains (2, 18). This constraint would be likely to prevent the emergence of CTL escape mutations in MHV-infected BALB/c mice. To address more directly the role of anti-MHV antibodies in preventing CTL escape, we infected BALB/b mice ($H\text{-}2^b$ restricted) with MHV. In preliminary experiments, we determined that MHV-infected BALB/b mice mounted a CD8 T-cell response to epitope S510 that was identical to that seen in B6 mice (data not shown).

With the Elispot method, we detected similar numbers of MHV-specific ASC in the infected BALB/b CNS at days 14 and 21 p.i., as in the CNS of infected BALB/c mice (e.g., day 21, BALB/b: 1,242 ± 142; BALB/c: 2,040 ± 945; Table 1, Fig. 3). We also quantified the number of MHV-specific and total ASC in both the spleen (Table 2) and the bone marrow (data not shown). We did not find significant differences in the frequency of MHV-specific ASC in the bone marrow between B6 and BALB/c or BALB/b mice. However, BALB/c and BALB/b mice had 2- to 4-fold more MHV-specific ASC at 21 to 25 days p.i. in the spleen (2,430 ± 1,600 (B6, $n = 5$) versus 9,010 ± 3,360 (BALB/c, $n = 5$) versus 5,392 ± 2,566 (BALB/b, $n = 4$). Collectively, these data suggest that maternal antibody-protected MHV-infected B6 mice have a diminished ability to accumulate MHV-specific ASC in the spleen and CNS when compared to infected BALB/c or BALB/b mice. Of note, BALB/b mice, like B6 mice, did not have a measurable endogenous serum neutralizing antibody response prior to 49 days p.i (Fig. 1D), suggesting that the CNS response was most critical in suppressing virus persistence.

CTL escape variants were not selected in maternal antibody-protected BALB/b mice. Next, we determined whether CTL escape mutants were detected in MHV-infected BALB/b mice, reasoning that the robust anti-MHV antibody response in the CNS might be protective. To analyze virus for the presence of mutations in epitope S510, RNA was harvested from the spinal cords of 19 BALB/b mice harvested between 52 and 62 days p.i. No mutations in epitope S510 were detected in any of these mice. These data demonstrated that CTL escape mutations did not arise in the setting of a robust anti-MHV antibody response in the spleen and CNS of infected BALB/b mice.

Similar numbers of B cells were detected in the CNS of infected B6, BALB/c, and BALB/b mice. $B220^+$ B cells were detected in the CNS of maternal antibody-protected B6 mice with hind limb paralysis (15). Furthermore, CNS-localized B cells have been implicated in the pathogenesis of MHV (26). To determine whether there was also a difference in the influx of B cells into the CNS between B6 and BALB/c or BALB/b mice, we analyzed the number and phenotype of B cells in the MHV-infected CNS. Cells in the CNS of B6, BALB/c, and BALB/b mice had the phenotype of nonactivated mature B cells ($CD19^+$, IgM^{low}, $kappa^+$). They were also $Fas/CD95^-$, demonstrating that they were not involved in germinal center reactions (Fig. 4). These cells were present in the CNS of B6, BALB/c and BALB/b mice at approximately the same frequency and absolute number.

Passive immunization with anti-MHV antibody protected B6 mice from clinical disease and diminished the frequency of CTL escape. These results suggested that a critical factor in the development of CTL escape variants and clinical disease in B6 mice was a deficiency in anti-MHV antibody production. To address directly whether we could compensate for the putative defect in anti-MHV antibody production in B6 mice, with consequent prevention of CTL escape and clinical disease, we administered a cocktail of two anti-MHV neutralizing antibodies to half of the mice in individual litters, as described in Materials and Methods. Both of these antibodies were directed against epitopes within the S protein and are likely to represent only a fraction of the response observed in MHV-infected B6, BALB/c, or BALB/b mice. Control mice received a cocktail of irrelevant antibodies or PBS only.

Clinical disease was prevented in all 16 mice that received anti-MHV antibodies (Fig. 5). By contrast, of the mice that received irrelevant antibodies or PBS, 9 of 18 developed hind limb paralysis ($P < 0.005$; 6 of 12 receiving PBS and 3 of 6 receiving irrelevant antibodies). In our previous experiments, we determined that mice rarely developed clinical disease if they were asymptomatic at 60 days p.i. To control for a delay in disease onset resulting from administration of anti-MHV antibody, some mice were observed for an additional 40 days. These mice remained asymptomatic.

We analyzed CNS-derived viral RNA from two of the nine symptomatic control mice for the presence of CTL escape mutations (Table 3). Mutations in epitope S510 were detected in both mice (wild-type: CSLWNGPHL; CTL escape mutants: CSL*R*NGPHL; CSLW*S*GPHL), consistent with our previous reports showing that CTL escape mutants were detected in all

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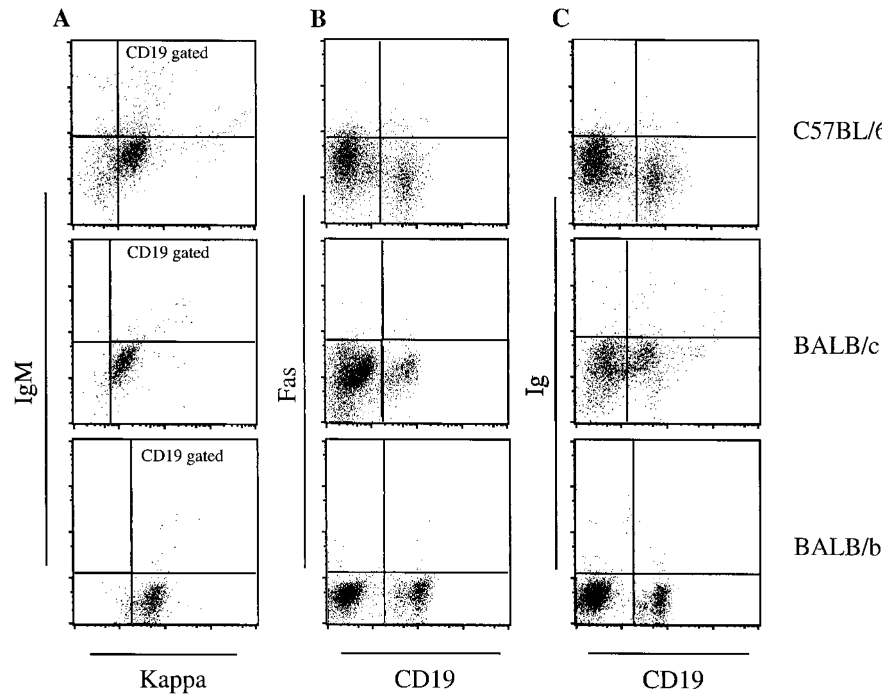

FIG. 4. Phenotype of B cells in the CNS of MHV-infected mice. Cells were harvested from the CNS of MHV-infected mice at 21 days p.i. We stained these cells with antibodies to the B-cell-restricted proteins CD19, IgM, kappa light chain, and Fas. B cells constituted approximately 20% of the mononuclear infiltrate. (A) In all three strains, infiltrating B cells exhibited an IgM^{low}, kappa-positive phenotype. (B) Fas expression is associated with B cells undergoing a germinal center reaction, and the CNS-derived $CD19^{+}$ cells were Fas negative. Three mice of all strains were analyzed. (C) Gates were drawn based on an isotype control.

symptomatic mice (21). To determine whether treatment with anti-MHV antibody completely prevented the selection of CTL escape mutants, we analyzed 13 asymptomatic mice (8 treated with anti-MHV antibody and 5 control mice) for the presence of mutations in epitope S510 at day 60 p.i. Of the eight mice treated with anti-MHV antibody, a CTL escape variant was predominant in one mouse and represented a minority of sequences in two others. Similarly, CTL escape variants were present in two control mice (Table 3). These results showed that exogenously delivered anti-MHV antibody prevented clinical disease, which was uniformly associated with the selection of CTL escape mutations, and thereby reduced the level of CTL escape to the lower levels observed in asymptomatic mice.

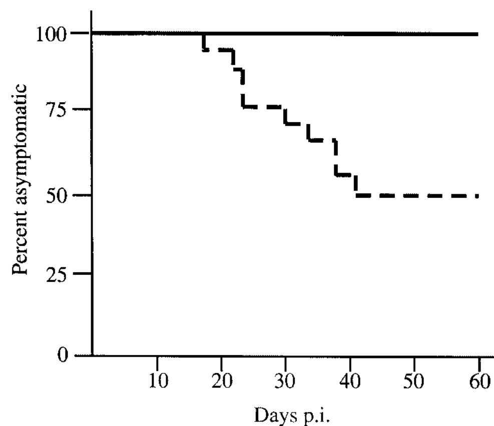

FIG. 5. Anti-MHV antibody prevented disease associated with CTL escape in B6 mice. We treated individual mice within litters with either a cocktail of anti-MHV neutralizing antibodies or with PBS or irrelevant antibody, as described in Materials and Methods. Mice were examined daily for the development of hind limb paralysis. Sixteen mice were treated with anti-MHV antibody (solid line), and 18 mice were treated with PBS or irrelevant antibodies (dashed line). The frequencies of symptoms in each limb are shown.

DISCUSSION

Our results demonstrate a suboptimal anti-MHV antibody response in maternal antibody-protected MHV-infected B6 mice as measured by serum antibody titers (Fig. 1) and by assay for ASC in the spleen and CNS (Tables 1 and 2). This defect was specific for anti-MHV ASC because these B6 mice had normal numbers of total immunoglobulin-producing cells in the spleen and bone marrow (Table 2 and data not shown) and

TABLE 3. CTL escape does not occur in MHV-infected BALB/b mice and is reduced by antibody treatment of B6 mice

Strain	Treatment	CTL escape (no. of mice/total)		
		Symptomatic	Asymptomatic	Total
B6	Anti-MHV	0/0	4/16	4/16[a]
B6	Control	9/9	2/9	11/18
BALB/b	None	0/2	0/17	0/19[b]

[a] This fraction is less than that of B6 control mice. $P < 0.05$.
[b] This fraction is less than that of either other group. $P \leq 0.01$.

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had a normal germinal center reaction to an unrelated antigen, SRBC (Fig. 2). This defect in the antibody response to MHV is likely to result from direct suppression of the response by maternal antibodies (reviewed in reference 8). Antibody-mediated suppression is observed in humans upon passive transfer of anti-Rh factor antibodies to Rh^- mothers and in animals exogenously immunized with heterologous erythrocytes in the presence of antierythrocyte antibody.

Accumulating data suggest that the mechanism of antibody-mediated immune suppression involves epitope masking by the exogenous antibody (8). Fcγ receptor interactions do not appear to play a role in this suppression, at least in the context of exposure to noninfectious antigens, as Fcγ receptor-deficient mice (10) or mice treated with antigen-specific $(Fab')_2$ also demonstrate normal antibody suppression (32). This suppression is specific for the antigen in question and requires high concentrations of high-affinity antibody (8). Previous studies also showed that antibody had to be administered nearly concurrently with antigen in order for IgG-mediated suppression of the antibody response to occur. In maternal antibody-protected B6 mice, anti-MHV antibody is transferred transplacentally and as soon as nursing begins. Because virus is not introduced until day 10 p.i., ample antibody is present in the mice at the time of infection to prevent the development of an endogenous antibody response.

Antibody-mediated suppression cannot account for the differences in antibody response that we observed between B6 and BALB/c or BALB/b mice. A robust response to MHV was detected in all dams immunized to the virus, and similar amounts of anti-MHV antibody were transmitted to suckling mice (Fig. 1) (4). Furthermore, suppression of the antibody response in B6 mice was not due to differences in the quantity or affinity of the maternally derived antibody because B6 mice nursed by BALB/c dams developed hind limb paralysis and chronic demyelination, whereas BALB/c mice nursed by B6 dams did not (23). Rather, the results suggest that the primary mechanism by which MHV-infected BALB/b mice prevented the emergence of CTL escape mutations was the development of a robust anti-MHV antibody response in the CNS. Although the number of MHV-specific splenic ASC was only modestly greater in BALB/b than B6 mice (Table 2), this difference may have also contributed to the prevention of CTL escape in BALB/b mice by increasing levels of circulating antibody. It is not known why the anti-MHV antibody response in B6 mice was less than in BALB/b or BALB/c mice. BALB mice are considered to mount a prototypic Th2 response to some antigens, whereas the response in B6 mice tends to be Th1-like (30). This may contribute to the differences in antibody production that we detected.

The conclusion that a defect in accumulation of ASC in MHV-infected B6 mice in the presence of maternal antibody facilitates virus persistence is supported by previous studies of MHV-infected B10.A(18R) mice (congenic C57BL/10 mice in which the D^b locus was replaced with the D^dL^d loci) (4). These mice are essentially equivalent to C57BL/10 mice except that the CD8 T-cell response is H-2L^d restricted and directed against epitope N318. A small fraction of maternal antibody-protected MHV-infected B10.A(18R) mice develop a demyelinating encephalomyelitis with clinical disease manifested by hind limb paralysis. Consistent with the location of epitope N318 in a conserved region of the N protein (18), however, no mutations in this epitope were detected in RNA or virus harvested from symptomatic mice with hind limb paralysis. In addition, no mutations were identified in epitope S598 (H-2K^b restricted), also recognized by CD8 T cells in these mice. Thus, the B6 or B10 background, presumably by facilitating a suboptimal anti-MHV antibody response, predisposed mice to virus persistence and clinical disease, even in the absence of CTL escape. The importance of CTL escape was highlighted, however, by the diminished frequency of clinical disease observed in B10.A(18R) mice compared to that in B10 mice (16% versus 45%) (4).

A local antibody response has been implicated previously in regulating virus persistence in the CNS of mice and rats infected with MHV and other neurotropic viruses (26, 27, 34). MHV-infected Brown Norway rats, which develop a robust intrathecal anti-MHV antibody response, rarely developed clinical disease or histological evidence of significant amounts of demyelination. In contrast, Lewis rats, which did not develop an anti-MHV antibody response in the CNS, developed a subacute demyelinating encephalomyelitis (27). These results suggested that local production of anti-MHV antibody was critical for control of the infection in MHV-infected rats.

We also demonstrated that passive transfer of anti-MHV antibody to infected B6 mice prevented clinical disease and reduced the frequency of CTL escape mutations to that observed in asymptomatic mice examined at 60 days p.i. in our previous studies (22, 23). This suggested that the role of antibody was to limit viral replication and spread and therefore decrease the competitive advantage of CTL escape mutants. Anti-MHV antibody produced in the CNS would presumably limit virus replication more effectively than antibody present in the serum, consistent with the observation that CTL escape mutants emerged in anti-MHV antibody-treated B6 mice but not infected BALB/b mice. These results may also be relevant for understanding CTL escape in other persistent infections. In humans and chimpanzees infected with hepatitis C virus or humans infected with human immunodeficiency virus type 1, a strong, although not necessarily neutralizing, antivirus antibody response is detected (5, 6). Our results suggest that the presence of sufficiently high levels of antiviral neutralizing antibody at the sites of infection at early times p.i. would suppress virus replication, thereby limiting the selection of CTL escape mutants and their competitive advantage, if they still emerge.

It has been suggested that a weak CD8 T-cell response, by diminishing immune pressure on the virus, subsequently allows the development of antibody escape variants (11). Our results, indicating that a potent antiviral antibody response prevented CTL escape and clinical disease, suggest that the obverse is also true: a weak antibody response facilitates CD8 T-cell escape.

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