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Antiviral Antibodies Are Necessary To Prevent Cytotoxic T-Lymphocyte Escape in Mice Infected with a Coronavirus▽

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Mutation within virus-derived CD8 T-cell epitopes can effectively abrogate cytotoxic T-lymphocyte (CTL) recognition and impede virus clearance in infected hosts. These so-called "CTL escape variant viruses" are commonly selected during persistent infections and are associated with rapid disease progression and increased disease severity. Herein, we tested whether antiviral antibody-mediated suppression of virus replication and subsequent virus clearance were necessary for preventing CTL escape in coronavirus-infected mice. We found that compared to wild-type mice, B-cell-deficient mice did not efficiently clear infectious virus, uniformly developed clinical disease, and harbored CTL escape variant viruses. These data directly demonstrate a critical role for antiviral antibody in protecting from the selective outgrowth of CTL escape variant viruses.

Viral evasion of the cytotoxic CD8 T-cell (CTL) response, which is important for the clearance of virus-infected cells, is a critical element in the establishment of persistent or chronic infections. Viruses that cause persistent infections can effectively evade the CTL response by preventing major histocompatibility complex (MHC) class I/peptide presentation in infected cells or by undergoing mutations that diminish or eliminate recognition by CTLs ("CTL escape"). While mutations often occur in the epitope itself, diminishing binding to the MHC class I molecule or to the T-cell receptor (TCR), mutations can also occur in residues flanking epitopes, thereby hindering processing (11, 39, 44). CTL escape is described most commonly in the context of chronic infections such as those caused by human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and human hepatitis C virus (HCV) (reviewed by Goulder et al. [15] and Bowen et al. [5]). However, all of these infections occur in humans or nonhuman primates and are very difficult or impossible to manipulate experimentally. Consequently, the host- and virus-specific factors that contribute to the selection of CTL escape variant viruses have been inferred from the human or simian infections but not experimentally proven. For example, it is generally believed that mutations in CTL epitopes will occur if these changes do not significantly modify virus fitness and do not result in a novel T response to the mutated epitope (reviewed in reference 24). Similarly, an antiviral immune response that depends primarily (if not solely) on the CTL response would be prone to escape. By extension, a CTL response accompanied by a robust antiviral antibody response would effectively control the virus, minimize the opportunity for CTL escape, and consequently decrease clinical disease. Conversely, the failure to mount an effective neutralizing antibody response, as seen in HIV- or HCV-infected patients, would predispose to viral persistence (7, 38, 41).

Mice infected with the neurotropic coronavirus, mouse hepatitis virus strain JHM (JHMV), serve as a useful surrogate for analyzing many aspects of CTL escape in humans. In this instance, suckling C57BL/6 (B6; *H-2*^{b} MHC haplotype) mice are infected with JHMV and nursed by dams previously immunized to the virus. Mice are protected from acute encephalitis but later develop hindlimb paralysis/paresis (HLP) and chronic demyelination. Virus can be isolated from the brains and spinal cords of infected mice and, in nearly all instances, is mutated in the immunodominant CTL epitope recognized in these mice. BALB/c mice (*H-2*^{d} MHC haplotype) do not show the same phenomena; suckling mice nursed by JHMV-immune dams do not develop chronic disease or show evidence of CTL escape. The immunodominant CD8 T-cell response in *H-2*^{b} mice is directed at a CD8 T-cell epitope that is in a region of the spike (S) glycoprotein (residues 510 to 518 [CSLWNGPH L]; epitope S510) that tolerates mutation while the L^d-restricted epitope recognized in BALB/c mice is located in a conserved region of the nucleocapsid (N) protein (residues 318 to 326 [APTAGAFFF]; epitope N318). However, JHMV infection of suckling BALB/b (*H-2*^{b}) mice, differing from BALB/c only at the MHC locus, also did not result in chronic demyelination or CTL escape (10).

We have demonstrated that the presence of anti-JHMV antibodies correlates with protection from CTL escape in these infected BALB/b and BALB/c mice. Anti-JHMV antibody-secreting cells (ASC) can be detected in significantly greater numbers in the infected BALB/c and BALB/b central nervous system (CNS) but not the B6 CNS, suggesting that a potent antivirus antibody response at the site of infection could prevent CTL escape. However, BALB and B6 mice differ in many aspects besides this difference in numbers of ASC in the CNS, such as bias towards Th1 or Th2 cellular immune responses, unique patterns of cytokine production, and differential susceptibility to microbial infection (40). Thus, the relationship between anti-JHMV ASC recruitment to the CNS and protec-

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tion from CTL escape cannot be attributed to this observation alone.

Here, we took a genetic approach to prove more directly that the antibody response, not differences in genetic background between BALB and B6 mice, was critical for protection against CTL escape. We engineered BALB/b mice that contained a genetic deletion at the J_H locus and therefore were unable to mount an anti-JHMV antibody response. In these mice, which differ from BALB/b mice only in their lack of a B-cell response, chronic demyelination and CTL escape were detected in all animals, indicating that CTL escape is facilitated by a suboptimal or absent anti-JHMV antibody response.

MATERIALS AND METHODS

Viruses. JHMV was prepared and its titer determined as described previously (29). The strain of JHMV (also called JHMV.IA) (27) used in these studies is highly neurovirulent, with a 50% lethal dose of 1.0 PFU (28).

Mice. C57BL/6 mice were purchased from The National Cancer Institute, Frederick, MD, and BALB/b mice were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB/c J_HD mice were purchased from Taconic (Hudson, NY). To generate BALB/b J_HD mice, the two mouse strains were interbred and genomic DNA from tails of F_1 mice was screened for the disrupted J_H locus by PCR, while the expression of H-2D^b and H-2D^d on peripheral blood leukocytes was assessed by flow cytometry. Primers (5′→3′) amplifying the native J_H locus were the forward primer CCCCACCATCACAGACCTTT and reverse primer ACCTTGCACCAGTCAGAGAC, and those amplifying the disrupted J_H locus were the forward primer AAGTAGCCGGATCAAGCGTAT and reverse primer GATGGATTGCACGCAGGTTCT. Antibodies used to screen for expression of MHC class I were fluorescein isothiocyanate (FITC)-conjugated anti-H-2D^b (monoclonal antibody [MAb] KH95) and phycoerythrin (PE)-conjugated anti-H-2D^d (MAb 34-2-12), both obtained from BD Pharmingen (San Diego, CA). All mice were housed in accordance with guidelines and protocols established by the University of Iowa Animal Care Unit.

Infection of mice. Dams were passively immunized 7 days after delivery via intraperitoneal injection of a cocktail of anti-JHMV neutralizing MAbs (5A13.5 and 5B19.2 [gifts from M. Buchmeier, The Scripps Research Institute, La Jolla, CA]). At 10 days of age, suckling mice were intranasally infected with 4×10^4 PFU of JHMV.

Intracellular cytokine staining. Mononuclear cells were harvested from the brains of suckling mice 7 or 10 days postinfection (p.i.). Peptides corresponding to immunodominant CD8 (S510) or CD4 (epitope M134, spanning residues 134 to 147 of the transmembrane [M] protein) T-cell epitopes were used at final concentrations of 1 and 5 μM, respectively. Cells were incubated with peptide and antigen-presenting cells (EL-4 for CD8 and CHB3 for CD4) for 5.5 h at 37°C. Cells were washed, incubated in blocking buffer containing anti-FcγRIII/II MAB (clone 2.4G2) and subsequently stained with FITC-conjugated anti-CD8α (Ly2; clone 53-6.7) or FITC–anti-CD4 (L3T4; clone GK1.5) MAb on ice for 1 h. After washing, cells were permeabilized, fixed, and stained intracellularly with PE-conjugated anti-gamma interferon (IFN-γ) MAb on ice for 1 h. Cells were analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Data sets were analyzed using FlowJo software (Tree Star, Inc, Ashland, OR). All antibodies and reagents were purchased from BD Pharmingen (San Diego, CA).

CD107a/CD107b degranulation assay. Mononuclear cells were harvested from the brains of BALB/b and BALB/b J_HD suckling mice 7 days p.i. and incubated for 5.5 h in the presence of monensin, S510 peptide, antigen-presenting (EL-4) cells, and FITC–anti-CD107a and FITC–anti-CD107b or isotype control antibodies (gifts from Steven Varga, University of Iowa). Cells were then surface stained with PE–Cy7–anti-CD8α followed by intracellular staining with PE–anti-IFN-γ. Relative CD107a/b expression was examined after gating on CD8$^+$ IFN-γ$^+$ cells.

In vitro cytotoxicity assay. Mononuclear cells (effectors) were harvested from the brains of BALB/b and BALB/b J_HD suckling mice 7 days p.i. and analyzed in direct ex vivo cytotoxicity assays as previously described (6). Briefly, effector cells were cocultured for 4 h with ^{51}Cr-labeled, S510 peptide-pulsed EL-4 cells (targets) at the indicated ratios. To determine actual effector input, aliquots of mononuclear cells were stained with FITC–anti-CD8α and PE-conjugated D^b/S510 tetramers (NIH tetramer facility). The percent specific release was defined as 100 × [(experimental release − spontaneous release)/(total release for detergent treated − spontaneous release)]. Maximum spontaneous release was <10% in all experiments.

ELIspot assays. Determination of numbers of ASC was performed essentially as described previously (10). Briefly, total mononuclear cells were isolated from the brains of infected suckling mice at 7, 10, and 14 days p.i. Serial dilutions of cells from each animal were incubated for 4 h on Multiscreen plates (Millipore, Bedford, MA) that had been previously coated with whole JHMV virus, to detect virus-specific ASC, or coated with rabbit anti-mouse IgG plus IgM (anti-IgG/IgM) antibody (Jackson ImmunoResearch, West Grove, PA). Virus-specific and total antibodies were detected with biotinylated donkey anti-mouse IgG/IgM antibody (Jackson ImmunoResearch, West Grove, PA). Wells were subsequently incubated with horseradish peroxidase-conjugated streptavidin and washed, and spots were developed with diaminobenzidine (Sigma, St. Louis, MO). Spots were counted by direct visualization with a stereomicroscope.

Virus sequencing. Total RNA was isolated from the brains of chronically infected mice and reverse transcribed to cDNA as previously described (31). Using the resulting cDNA as template, a 1,055-bp region of the spike glycoprotein encompassing epitope S510 was amplified by PCR. The primers (5′→3′) used were forward primer AACCCCTCGTCTTGGAATAGGAGGTATGG and reverse primer CCTACGGATTGAACGCTATCATTGACTAAC. PCR products were sequenced directly by the University of Iowa DNA Core. Results were analyzed and recorded with Sequencher software (Gene Codes, Ann Arbor, MI).

Statistical analyses. Significance was determined using a nonpaired, two-sided Student's *t* test or chi-square test, where indicated.

RESULTS

The magnitude and kinetics of ASC accumulation in the CNS of BALB/b mice correlate with protection from CTL escape. We previously demonstrated that there is a marked reduction in the accumulation of virus-specific ASC, but not of CD19$^+$ IgM^{lo} B cells, at days 21 to 30 p.i. in the CNS of antibody-protected, infected B6 mice relative to BALB/b mice, and this correlated with the development of clinical disease and CTL escape (10). Since CTL escape is detected as early as 10 days p.i., we next extended these observations to earlier times after infection by examining ASC recruitment to the CNS at days 7, 10, and 14 p.i. We found that there were 35 to 85% fewer virus-specific and total (IgG plus IgM) ASC in the CNS of CTL escape-susceptible B6 mice relative to CTL escape-resistant BALB/b mice at each time point examined (Fig. 1A to C). These data show that anti-JHMV ASC are detected at very early times p.i. in BALB/b mice and could contribute to the suppression of CTL escape.

Survival of suckling mice correlates with the dose of passively administered antibody. To directly examine the role of the antiviral antibody response in protection from CTL escape, we next engineered B-cell-deficient BALB mice that carry genes that encode the appropriate MHC restriction for the S510 epitope (*H-2D^b*). To do this, B-cell-deficient (J_HD) BALB/c (*H-2^d*) mice were interbred with BALB/b (*H-2^b*) mice to generate BALB/b J_HD mice. The MHC haplotype and J_H gene disruption were verified with flow cytometric and PCR analyses as described in Materials and Methods. As our system of persistent JHMV infection and CTL escape requires maternal antibody to protect suckling mice from an otherwise lethal acute encephalitis, we passively immunized BALB/b J_HD and control BALB/b dams with a cocktail of anti-JHMV antibodies. To ensure that litters were not overprotected and to increase the likelihood of developing persistent infection of infected suckling mice, we titrated a dose of anti-JHMV antibody passively administered to dams that resulted in survival of between 50 and 65% of JHMV-infected suckling pups. We

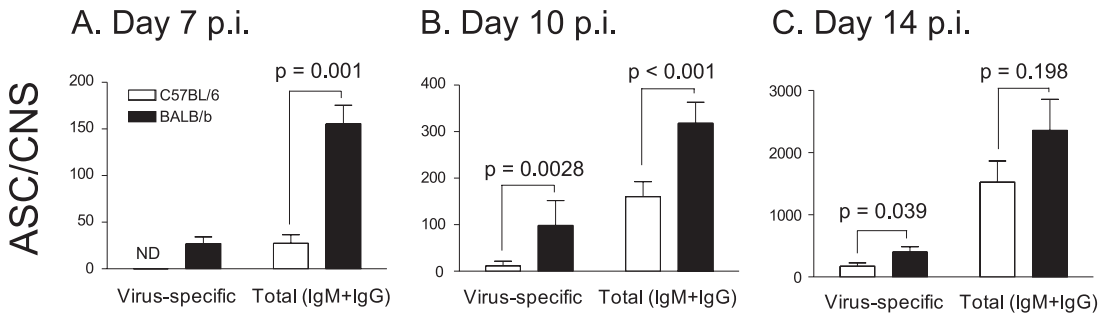

FIG. 1. Kinetics of ASC recruitment to the CNS of antibody-protected, JHMV-infected suckling B6 and BALB/b mice. B6 and BALB/b dams were passively immunized with anti-JHMV neutralizing antibody cocktail at 7 days postpartum as described in Materials and Methods. Three days later, suckling mice were infected with 4×10^4 PFU of JHMV. At 7 (A), 10 (B), and 14 (C) days p.i., mononuclear cells were harvested from the brains of suckling mice and the absolute numbers of virus-specific (left bars) and total IgG-plus-IgM (right bars) ASC were determined as described in Materials and Methods. Data represent the mean ± standard error for six to eight B6 and BALB/b suckling mice derived from at least two independent litters at each time point. Nonpaired, two-sided *t* tests were used for statistical analyses. ND, none detected.

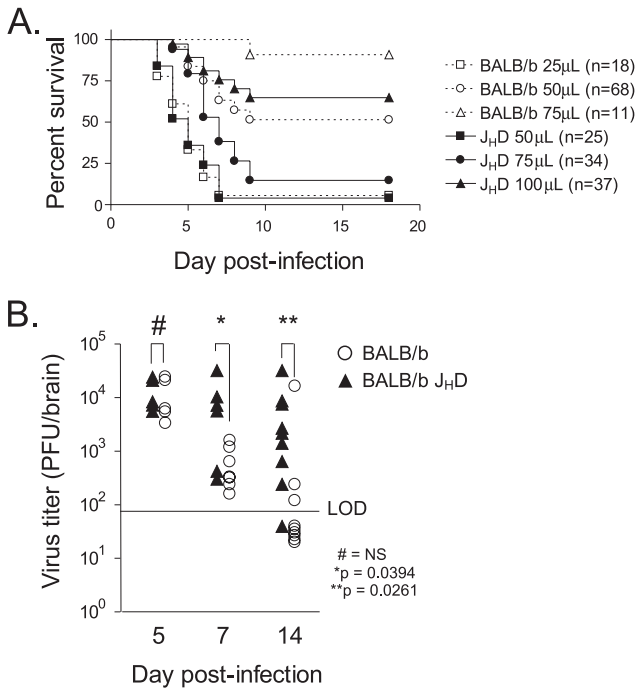

FIG. 2. Survival of virus-induced acute encephalitis and virus titers in antibody-protected, JHMV-infected suckling BALB/b and BALB/b J_HD mice. (A) Survival analysis of suckling mice nursed by dams that were passively immunized with graded doses of neutralizing antibody cocktail. BALB/b and BALB/b J_HD dams were passively immunized at day 7 postpartum; suckling mice were infected with 4×10^4 PFU of JHMV 3 days later (day 10 postnatal) and monitored for survival (days 3 to 18 p.i.). Results for each graded dose of neutralizing antibody are derived from between 3 and 13 litters from multiple independent BALB/b and BALB/b J_HD dams. Numbers in parentheses indicate total numbers of pups infected in each group. (B) BALB/b and BALB/b J_HD suckling mice were infected with 4×10^4 PFU of JHMV. At the indicated day p.i., brains were aseptically harvested, homogenized in sterile PBS, and clarified by centrifugation. Supernatants were collected, and the titer of infectious virus was determined on HeLa-MHVR cells (HeLa cells stably transfected with mouse hepatitis virus [MHV] receptor) as described previously (29). Symbols on the graph represent individual mice assayed from multiple independent litters at each time point. The limit of detection (LOD) for the assay is 80 PFU/brain. Nonpaired, two-sided *t* tests were used for statistical analyses. NS, not statistically significant.

found that in order to achieve nearly equivalent survival of suckling mice, passive immunization of B-cell-deficient BALB/b J_HD dams required twofold more antibody than B-cell-sufficient BALB/b mice (Fig. 2A and Table 1). Thus, for all subsequent studies, BALB/b dams were immunized with 50 μl of an equal mixture of neutralizing antibodies (neutralizing titers of 1:45,000 and 1:2,700 for 5A13.5 and 5B19.2, respectively), while BALB/b J_HD dams were immunized with 100 μl of the antibody cocktail. In addition to determining the appropriate suboptimal dose of passive antibody, these results also suggest a potential role for maternally derived or endogenous natural antibody in protection of suckling mice from acute JHMV infection.

Delayed virus clearance in BALB/b J_HD mice. Since inefficient virus clearance has been postulated to contribute to the outgrowth of CTL escape variant viruses, we first assayed the brains of BALB/b and BALB/b J_HD suckling mice harvested at days 5, 7, and 14 p.i. for infectious virus. We found no difference in infectious virus burdens between BALB/b and BALB/b J_HD suckling mice at day 5 p.i. However, at all later time points examined, there was a marked persistence of infectious virus in the B-cell-deficient mice (Fig. 2B). This is in contrast to B-cell-sufficient BALB/b mice, which generally cleared infectious virus to levels below the limit of detection by day 14.

Sustained virus-specific CTL responses in BALB/b J_HD mice. Since the sustained virus burden described above for BALB/b J_HD mice could result from impaired cell-mediated responses, we next determined whether CTL responses were normal in maternal antibody-protected BALB/b J_HD mice. Of note, CTL responses have been reported to be normal in J_HD mice infected with an attenuated strain of JHMV (36). For this purpose, we examined the magnitude of antiviral T-cell responses to immunodominant JHMV-specific CD8 and CD4 T-cell epitopes on days 7 and 10 p.i. as described in Materials and Methods. Both BALB/b and BALB/b J_HD suckling mice exhibited similar frequencies and numbers of virus-specific CD8 and CD4 T cells at day 7 p.i. (Fig. 3A to F). However, by day 10 p.i., BALB/b J_HD mice exhibited a >2-fold increase in the total number of inflammatory mononuclear cells in the JHMV-infected CNS (data not shown), which translated into a 2.5-fold increase in absolute numbers of total and epitope S510-specific CD8 cells compared to BALB/b mice (Fig. 3D

TABLE 1. Incidence of clinical disease[a] and development of CTL escape[b] in BALB/b and BALB/b J_HD mice

Mouse strain (n)[c]	Antibody dose (μl)[d]	% Survival (no. surviving/total)	% Symptomatic (no. symptomatic/total)	% CTL escape (no. with escape/total)	
				Symptomatic	Asymptomatic
BALB/b (18)	25	5.6 (1/18)	0 (0/1)	NA[e]	0 (0/1)
BALB/b (68)	50	51.5 (35/68)	5.7 (2/35)	100 (2/2)	9 (3/33)
BALB/b (11)	75	100 (11/11)	0 (0/11)	NA	0 (0/2)[f]
BALB/b J_HD (25)	50	8 (2/25)	100 (2/2)	100 (2/2)	NA
BALB/b J_HD (34)	75	14.7 (5/34)	100 (5/5)	100 (5/5)	NA
BALB/b J_HD (37)	100	64.8 (24/37)	100 (24/24)	100 (24/24)	NA

[a] Incidence of clinical disease (HLP) in BALB/b versus BALB/b J_HD mice (nursed on dams receiving 50 or 100 μl of antibodies, respectively). $\chi^2 = 51.35$; $P < 0.001$.
[b] Prevalence of CTL escape detected in BALB/b versus BALB/b J_HD mice (pooled symptomatic and asymptomatic mice nursed on dams receiving 50 or 100 μl of antibodies, respectively). $\chi^2 = 32.44$; $P < 0.001$.
[c] n = number of suckling mice examined for each group.
[d] Dose of anti-JHMV neutralizing antibody cocktail administered to dams.
[e] NA, not applicable.
[f] Viral RNA could only be amplified from 2/11 asymptomatic mice.

and E). Although IFN-γ production by virus-specific CD8 T cells correlated well with D^b/S510 tetramer staining, we further evaluated the functional capacity of CD8 T cells from BALB/b and BALB/b J_HD mice. First, we examined epitope-specific CD8 T cells for their ability to translocate CD107a and CD107b to the cell surface, as a marker for degranulation (4). We found no difference in the proportions of CD107a/b$^+$ epitope S510-specific CD8 T cells between BALB/b and BALB/b J_HD mice 7 days p.i. (Fig. 3G; BALB/b, 79% ± 2.2%, and BALB/b J_HD, 77% ± 3.2% [$P = 0.598$]). As an additional measure of CD8 T-cell function, we performed direct ex vivo cytotoxicity assays. CTLs from the B-cell-deficient BALB J_HD mice were as least as cytolytic as those harvested from BALB/b mice (Fig. 3H).

Together, these results show that there is no defect in the anti-JHMV CTL response in BALB/b J_HD mice. Moreover, these data indicate CTL responses are sustained or CTL recruitment is prolonged compared to that in the B-cell-sufficient BALB/b strain, probably due to continued antigenic stimulation (i.e., delayed kinetics of virus clearance). Based on these results, as well as the observed sustained virus burden (Fig. 2B), we predicted that the incidence of clinical disease and prevalence of CTL escape variant viruses would be increased in persistently infected, maternal antibody-protected BALB/b J_HD mice.

BALB/b J_HD mice uniformly develop clinical disease and harbor CTL escape variant virus. To determine whether the frequency of CTL escape at epitope S510 was increased in the absence of an endogenous anti-JHMV antibody response, we next examined the incidence of clinical disease and assayed for the presence of CTL escape variant viruses in BALB/b and BALB/b J_HD suckling mice that were equivalently protected from acute encephalitis. Although similar numbers of suckling BALB/b and BALB/b J_HD mice survived the acute infection (Fig. 2A), only 5.7% (2/35) of surviving BALB/b mice developed HLP between days 18 and 60 p.i. (Fig. 4 and Table 1). In marked contrast, 100% of BALB/b J_HD mice developed clinical disease (Table 1; $\chi^2 = 51.25$ and $P < 0.001$ for development of HLP in BALB/b versus BALB/b J_HD mice). Moreover, BALB/b J_HD mice developed clinical disease sooner than BALB/b mice (median time to HLP was 30 days for J_HD versus 54 days for wild-type mice). In line with studies of B6 mice (10, 21, 31, 32), virus mutated in epitope S510 was detected in each BALB/b and BALB/b J_HD mouse that developed clinical disease. Among all mice surviving the acute infection, the incidence of CTL escape was significantly greater in BALB/b J_HD mice than in BALB/b mice ($\chi^2 = 32.44$; $P < 0.001$). Of note, all epitope S510 variant sequences detected in this study (Table 2) have been shown to abrogate or diminish recognition by S510-specific CTL (30).

CTL escape virus was detected in 9% (3/33) of asymptomatic BALB/b mice (Fig. 4 and Table 2), lower than the 20 to 30% observed in optimally protected asymptomatic B6 mice (>98% protection from acute encephalitis) (21, 31, 32). These data further suggest that BALB/b mice are relatively resistant to the selection of JHMV CTL escape variants, even when the experimental system is heavily skewed towards establishment of persistent JHMV infection (i.e., protection afforded by maternal antibody is suboptimal). As a composite, our data indicate that an endogenous antiviral antibody response is critically important to protect from the selective outgrowth of CTL escape variant viruses.

DISCUSSION

Numerous reports indicate that antiviral antibodies are necessary to protect the host from either acute virus infection or recrudescence during persistent infection despite the presence of high-magnitude endogenous CTL responses (19, 20, 22, 43). Indeed, prior studies indicate that antibody is important in preventing virus recrudescence in JHMV-infected adult mice (3, 36). However, our results are the first to establish a critical link between antiviral antibody and protection from CTL escape. CTL escape occurs because CD8 T cells in BALB/b J_HD suckling mice are the primary effector arm of the immune response, and the absence of antibody accentuates CD8 T-cell-driven immune pressure on the virus. The result of this intense immune pressure is the selective outgrowth of viruses that have acquired nonsynonymous mutations in the immunodominant S510 CD8 T-cell epitope.

In the present study, we demonstrate directly the key role that antiviral antibodies have in suppressing replication and contributing to clearance of infectious virus, thus minimizing the opportunity for selection of CTL escape variant viruses. In the absence of anti-JHMV antibodies, virus clearance is mediated primarily by CD8 T cells; consistent with this, anti-

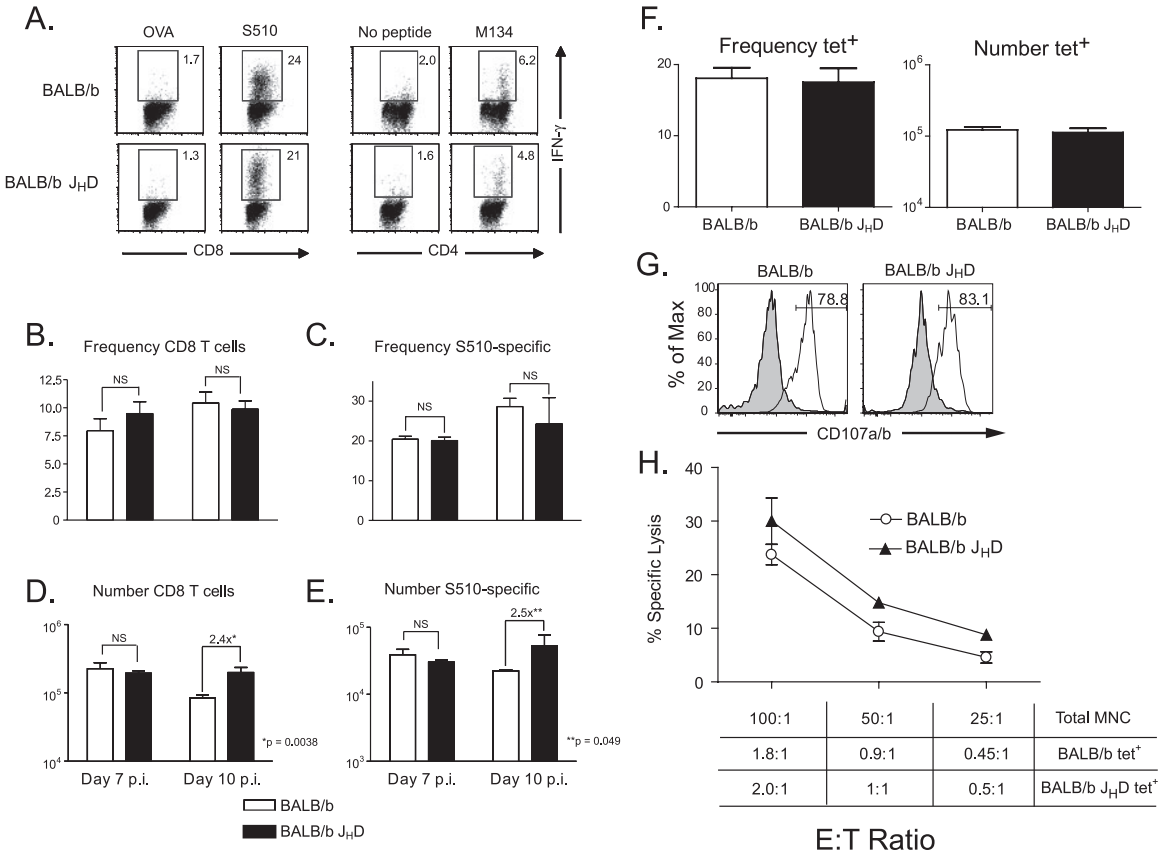

FIG. 3. Antiviral T-cell responses in antibody-protected, JHMV-infected BALB/b and BALB/b J_HD suckling mice. (A) Flow cytometric dot plots demonstrating the frequency of virus-specific T cells in the CNS of antibody-protected, JHMV-infected BALB/b and BALB/b J_HD suckling mice at 7 days p.i. Numbers indicate the fraction of $CD8^+$ T cells specific for the immunodominant S510 epitope (left panels) or $CD4^+$ T cells specific for the immunodominant M134 epitope (right panels). Aliquots of cells were also cultured with an irrelevant peptide ($OVA_{257–264}$) for CD8 T cells, or no peptide for CD4 T cells, to establish background IFN-γ staining. Summaries of the frequencies (B and C) and absolute numbers (D and E) of total and virus-specific $CD8^+$ T cells on days 7 and 10 p.i. are shown. Data represent the mean ± standard error for four to eight BALB/b and BALB/b J_HD suckling mice at each time point. Nonpaired, two-sided *t* tests were used for statistical analyses. NS, not statistically significant. (F) Frequency and number of D^b/S510 tetramer-positive CD8 T cells detected in BALB/b and BALB/b J_HD mice 7 days p.i. Four mice per strain were used in these analyses. (G) CD107a and CD107b surface expression by activated, epitope-specific CD8 T cells from BALB/b and BALB/b J_HD mice. Shown are histograms representing the proportion of $CD8^+$ IFN-γ^+ T cells expressing surface CD107a/b. Shaded plots represent staining by isotype control antibodies. Data are representative of analyses performed on four mice from each strain. (H) CNS-derived mononuclear cells were harvested from JHMV-infected suckling BALB/b and BALB/b J_HD mice 7 days p.i. Ex vivo cytolytic activity was assayed at the indicated effector-to-target cell (E:T) ratios using EL-4 target cells coated with 1 μM of S510 peptide and calculated as described in Materials and Methods. E:T ratios are displayed as either total populations or percentage of epitope S510-specific cells as determined by D^b/S510 tetramer staining. Four mice per strain were used in these analyses.

JHMV CTL responses are sustained in infected BALB/b J_HD mice, while they diminish in BALB/b mice (Fig. 3D and E). In the absence of an anti-JHMV antibody response, CTL escape at epitope S510 is detected in all mice (Tables 1 and 2 and Fig. 4). This high rate of CTL escape is facilitated by the location of epitope S510 in a region of the S glycoprotein that tolerates mutations and deletions; mutations in CD8 T-cell epitopes are not selected if they impair virus viability. In HIV-infected humans and SIV-infected macaques, some CTL mutations attenuate the virus and their selection requires coevolution with a second compensatory mutation (13, 33), whereas others are selected only at late stages of the disease process when the controlling immune response is impaired (2, 14). However, the epitope S510 variants selected in vivo are as fit as wild-type virus when assayed in tissue culture cells or in mice (unpublished data).

Dandekar et al. (10) and this study show that the most striking differences between infected BALB and B6 mice are in the numbers of anti-JHMV ASC at the site of virus replication (the CNS for JHMV), with greater numbers of cells present in both BALB/b and BALB/c mice. In the study by Dandekar et al., no maternal antibody-protected suckling BALB/b mice developed clinical disease or evidence of CTL escape. In that study, 100% of suckling BALB/b mice survived the acute phase of infection, suggesting transmission of an optimal or supraoptimal level of protective maternal antibody. This likely resulted in a lower virus burden prior to the development of an endogenous anti-JHMV antibody response, thereby diminishing the likelihood of CTL escape. In the present study, we skewed our experimental system towards establishment of high virus burdens in BALB/b suckling mice by titrating a suboptimal dose of neutralizing antibody administered to nursing BALB/b dams.

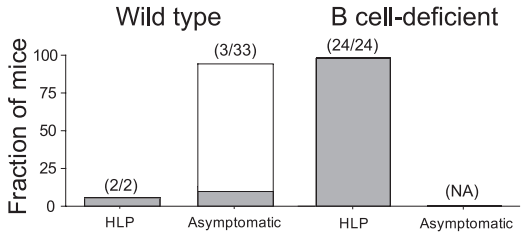

FIG. 4. Prevalence of clinical disease and development of CTL escape in BALB/b and BALB/b J_HD mice that survive acute infection. Thirty-five BALB/b and 24 BALB/b J_HD survivors of acute encephalitis were monitored for clinical signs of HLP. Total RNA from brains and spinal cords was harvested from symptomatic mice at the time of disease onset and asymptomatic mice at day 60 p.i. and analyzed for the presence of mutations in epitope S510. Bars represent the proportions of wild-type BALB/b and B-cell-deficient BALB/b J_HD mice that were symptomatic (HLP) or asymptomatic. Shaded bars and numbers in parentheses indicate the proportion of mice within each group that harbored CTL escape variant viruses. $\chi^2 = 51.35$ and $P < 0.001$ for incidence of clinical disease in BALB/b versus BALB/b J_HD mice. $\chi^2 = 32.44$ and $P < 0.001$ for prevalence of CTL escape detected in BALB/b versus BALB/b J_HD mice (pooled symptomatic and asymptomatic mice). NA, not applicable.

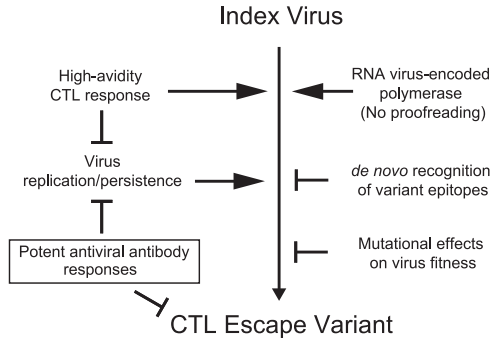

FIG. 5. Overview of the mechanistic correlates of CTL escape. T-bars and arrows represent factors that counter or contribute to CTL escape, respectively. High-avidity CTL responses are critically important for virus clearance but also contribute to the selection of CTL escape variant viruses when only one or a few immunodominant epitopes are targeted. Potent antiviral antibody responses, in combination with high-avidity CTL responses, decrease the likelihood of CTL escape by limiting virus replication and/or persistence. In addition, antiviral antibody responses may also directly limit the spread of CTL escape variant viruses that may arise in vivo. When antiviral antibody responses are suboptimal or absent, the high-avidity CTL response may not effectively control virus replication or limit persistence. In this instance, the adaptive immune response that is focused solely on immunodominant CTL epitopes may more readily select for CTL escape variant viruses. Additional factors that influence the selection and emergence of CTL escape variant viruses include de novo CTL responses, which may suppress the replication of viruses encoding variant epitopes, as well as altered virus fitness, which may limit the replicative capacity, tropism, or spread of CTL escape variant viruses.

In this instance, ~50% of suckling mice survived acute JHMV infection, yet only 5.7% (2/35) of the survivors developed clinical disease associated with CTL escape (Fig. 2A and 4 and Table 1). Thus, even in a setting where mice are suboptimally protected, the emergence of a rapid and effective endogenous antibody response suppresses virus replication and minimizes CTL escape. Supporting this conclusion, when we titrated a dose of maternally derived antibody to BALB/b J_HD mice that resulted in equivalent protection from acute disease, 100% (24/24) developed clinical disease and subsequent CTL escape (Fig. 4 and Table 1).

CTL escape occurs in the context of CD8 T-cell epitopes, such as epitope S510, that elicit CTL responses of high functional avidity (21, 25, 26). While some B-cell-deficient mice, such as μMT mice, have abnormalities in their T-cell responses (3), anti-JHMV CD8 T-cell responses are normal in JHMV-infected J_HD mice, when measured by cytokine expression (Fig. 3) or by tetramer S510 staining and cytolytic activity (36). Furthermore, the selection of CTL escape viruses in all infected BALB/b J_HD mice further shows the efficacy of the CD8 T-cell response; an impaired response would not exert sufficient immune pressure to facilitate their selection. Indeed, studies suggest that CTL escape most commonly occurs in the presence of a CTL response exhibiting high selective pressure (15). Others have reported decreased CD8 T-cell diversity in J_HD mice (1, 17), but in those studies, no differences in CD8 T-cell function were observed. Of note, the epitope S510-specific CTL response is polyclonal but is functionally monospecific, since recognition is diminished or abrogated by single-amino-acid changes in TCR contact residues (30). Thus, the extent of TCR diversity, even if less in J_HD mice, is unlikely to affect our results because a polyclonal S510-specific CTL response in B6 mice is unable to prevent the selective outgrowth of CTL escape variant viruses.

TABLE 2. Epitope S510 mutations detected in antibody-protected, JHMV-infected BALB/b and BALB/b J_HD mice

Mouse group	Mutation detected in mice[a]:	
	Symptomatic	Asymptomatic
Wild type, BALB/b	W513R	Deletion of entire epitope
	G515R	G515E N514S
B-cell-deficient, BALB/b J_HD	Deletion of entire epitope (7) W513R (3) N514D (3) ΔSL (2) N514S (2) N514K (2) L512R L512F P516L G515R ΔLWN	NA

[a] All mutations identified in this study have been shown to abrogate or diminish recognition by S510-specific CTL (30). Values in parentheses indicate the number of mice in which the mutation was detected. NA, not applicable.

Antiviral antibody responses are likely to limit the selective outgrowth of CTL escape variant viruses during both acute and chronic stages in the infection (Fig. 5). First, efficient antiviral antibody responses during the acute phase lessen the immune pressure exerted by CTL by limiting replication, persistence, and spread. CTL escape variants are often detected in humans and chimpanzees infected with HCV, HIV, or SIV (9, 12, 37, 42, 45), infections that induce weak or delayed neutralizing antibody responses (7, 8, 16, 38). Similarly, kinetics of virus

clearance are slower in JHMV-infected BALB/b J_HD mice than in BALB/b mice (Fig. 2B), supporting a role for antiviral antibody in the initial stage of the disease. Second, a robust antiviral neutralizing antibody response prevents high-level replication of CTL escape variants, once they are selected in the persistently infected host. This is illustrated in our study by the poorly controlled growth of CTL escape variants in JHMV-infected BALB/b J_HD mice (Fig. 4 and Tables 1 and 2). In HIV-infected patients, a diminished CD4 T-cell response has the same effect at late stages of disease and facilitates the outgrowth of CTL escape viruses (reviewed in reference 23).

While the selection of CTL escape variant viruses is influenced by the magnitude and functional avidity of CTL responses and is more common when virus clearance is delayed (34, 35), our results are the first to directly establish a link between antiviral antibody responses, diminished virus clearance, and the selection of CTL escape variant viruses. These results have implications for the rational design of vaccines, by providing support for efforts to develop vaccines that induce robust T- and B-cell responses to viruses, such as HIV-1 and HCV, in which CTL escape is a common problem (e.g., see reference 18), thereby minimizing the factors that contribute to selection of these viruses.

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