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B-Cell-Mediated Lysis of Cells Infected with the Neurotropic JHM Strain of Mouse Hepatitis Virus

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Cells expressing the spike (S) glycoprotein of the neurotropic JHM strain (JHMV) of mouse hepatitis virus (MHV) are susceptible to lysis by B cells derived from naïve mice, including B cells from perforin-deficient mice. Cytolysis requires interaction of the virus receptor and the viral S glycoprotein, is independent of other viral-induced components, and is not a unique property of B cells. Neutralizing anti-S-protein monoclonal antibodies (mAb) and a mAb specific for the viral receptor inhibit lysis. However, cells infected with an MHV strain unable to induce cell–cell fusion are resistant to lysis and lysis of JHMV-infected cells is inhibited by an anti-S-protein nonneutralizing mAb which prevents S-protein-mediated cell fusion. These data suggest that B cells may function as antibody-independent innate immune response during JHMV infection *in vivo*.

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INTRODUCTION

Cellular components of the immune system, including natural killer (NK) cells, B cells, macrophages, and both CD4⁺ and CD8⁺ T cells, are recruited into the central nervous system (CNS) following infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) (Bergmann *et al.*, 1999; Dorries *et al.*, 1991; Williamson, 1992; Williamson *et al.*, 1991). Mice deficient in either CD4⁺ or CD8⁺ T cells exhibit enhanced mortality following infection (Williamson and Stohlman, 1990), suggesting T cells are the predominant antiviral immune effectors. However, CD4⁺ T cells appear to play a secondary role in viral clearance by enhancing CD8⁺ T cell survival or function (Stohlman *et al.*, 1998). Therefore, similar to many other viral infections (Kagi *et al.*, 1995), most interest has focused on CD8⁺ T cells as the major effectors of JHMV clearance (Stohlman *et al.*, 1999). NK cells were suggested to exhibit limited antiviral activity based on the observation that JHMV replication within the CNS of mice deficient in either CD4⁺ or CD8⁺ T cells is partially inhibited (Williamson and Stohlman, 1990). However, there is little evidence for a direct role of NK cells in reducing infectious virus (Houtman and Fleming, 1996) and, in contrast to T cells, NK cells are not retained in the CNS following viral clearance (Marten *et al.*, 2001).

Humoral immunity has also been implicated in JHMV

infection of the CNS. B cells are rapidly recruited into the CNS during acute JHMV infection (Dorries *et al.*, 1991; Williamson *et al.*, 1991; Williamson, 1992) and antiviral antibody (Ab) is present in the cerebral spinal fluid during both acute infection and viral persistence (Dorries *et al.*, 1986, 1987; Fleming *et al.*, 1983a). Suckling mice weaned on immunized dams are protected from acute encephalitis (Perlman *et al.*, 1987) and JHMV-resistant Brown Norway rats develop a more rapid neutralizing Ab response compared to susceptible Lewis rats (Dorries *et al.*, 1986, 1987), also supporting a role for humoral immunity. Passive transfer of neutralizing anti-spike (S) protein monoclonal antibody (mAb) protects mice from lethal challenge, although not all mAb with neutralizing activity exhibit this *in vivo* activity (Buchmeier *et al.*, 1984). In addition, mAb specific for other viral structural proteins, which lack neutralizing activity, also protect mice from mouse hepatitis virus (MHV) infection (Fleming *et al.*, 1989; Lecomte *et al.*, 1987; Nakanaga *et al.*, 1986; Yokomori *et al.*, 1992). However, the inability to detect JHMV-neutralizing Ab prior to clearance of the majority of infectious virus from the CNS suggested that humoral immunity played little or no role in initial JHMV clearance (Parra *et al.*, 1997, 1999; Stohlman *et al.*, 1986, 1999). Infection of mice lacking the ability to produce Ab by virtue of the absence of mature B cells (μ MT mice) demonstrated that infectious virus is initially cleared from the CNS with kinetics similar to that of wild-type (wt) mice (Lin *et al.*, 1999). These data indicated that the antiviral cellular immune response inhibits infectious virus replication in the absence of humoral immunity. How-

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ever, in contrast to wt mice, in which infectious virus is completely cleared from the CNS by approximately 2 weeks postinfection (p.i.), low levels of residual virus remained in the CNS of μ MT mice.

Following initial, but incomplete, clearance, virus replication rapidly increased within the CNS of μ MT mice (Lin *et al.*, 1999). Viral reactivation within the CNS of μ MT mice is prevented by the passive transfer of polyclonal antiviral Ab, but not nonspecific Ab (Lin *et al.*, 1999), suggesting that humoral immunity is critical for maintaining viral persistence. However, the mechanism(s) which contributes to the prevention of JHMV reactivation within the CNS of wt mice is not clear. The absence of both B cells and Ab in μ MT mice leaves open the question of whether B cells play an Ab-independent role in either suppressing virus replication during acute infection or limiting subsequent CNS virus reactivation. A potential Ab-independent role of B cells would be masked by the neutralization of infectious virus via passive transfer of polyclonal Ab (Lin *et al.*, 1999). Although Ab appears to be a critical component in establishing and/or maintaining CNS viral persistence (Lin *et al.*, 1999), the rapid influx of B cells into the CNS (Dorries *et al.*, 1991; Williamson, 1992; Williamson *et al.*, 1991) prior to detection of neutralizing antiviral Ab (Parra *et al.*, 1999; Stohlman *et al.*, 1986, 1999) suggests a possible Ab-independent role during JHMV pathogenesis.

The observation that splenic B cells from naïve mice interact *in vitro* with cells infected with the A59 strain of MHV (MHV-A59), resulting in lysis of both the virus-infected cell and the B cell (Holmes *et al.*, 1986; Nishioka and Welsh, 1993; Welsh *et al.*, 1986; Wysocka *et al.*, 1989), suggests a potential for B cells in suppressing viral replication. Lysis is inhibited by polyclonal neutralizing Ab (Coutelier *et al.*, 1994), suggesting a role of the viral S protein, which is the sole viral target of neutralizing JHMV Ab (Collins *et al.*, 1982; Fleming *et al.*, 1983b). Furthermore, analysis of the cellular distribution of the MHV receptor (MHV-R), the ligand for the viral S protein, demonstrated that B cells, and to a far lesser extent macrophages, but not T cells, express MHV-R (Coutelier *et al.*, 1994). MHV-R is a murine membrane glycoprotein of the biliary glycoprotein group within the immunoglobulin superfamily, designated CEACAM1 (Beauchemin *et al.*, 1999). These data suggest that interactions between the S protein, expressed on the surface of infected cells, and the MHV-R expressed by B cells results in cytolysis. Following interaction of the S protein and MHV-R the cytoplasmic contents of the two cell types are mixed and the MHV-A59-infected cells undergo apoptosis and lysis (Wysocka *et al.*, 1989; Nishioka and Welsh, 1993). In addition, cytolysis is independent of interleukin (IL)-1, tumor necrosis factor (TNF)- β , Fas/FasL interactions, or TNF- α (Nishioka and Welsh, 1993). Consistent with these observations neither Fas/FasL interactions nor TNF appears to contribute to either JHMV clearance or patho-

genesis (Parra *et al.*, 2000; Stohlman *et al.*, 1995). Together these data suggested that cytolysis is mediated via a fusion-dependent process.

Due to the rapid accumulation of B cells in the CNS of infected mice (Dorries *et al.*, 1991; Williamson, 1992; Williamson *et al.*, 1991) prior to the detection of neutralizing Ab (Parra *et al.*, 1997, 1999; Stohlman *et al.*, 1986, 1999), these studies were initiated to determine if JHMV-infected cells were susceptible to B-cell-mediated lysis. The data demonstrate that cytolysis occurs following interaction with cells expressing the JHMV S protein, that the interaction(s) leading to lysis is independent of other viral proteins or MHV-induced effects on the host cell, and that cytolysis occurs via a perforin-independent process dependent upon cell-cell fusion. These results suggest the potential for S protein-MHV-R interactions to contribute to either JHMV clearance from the CNS or prevention of JHMV reactivation.

RESULTS

MHV-R expression and B-cell-mediated cytolysis

Expression of MHV-R on B cells derived from the spleens of naïve C57BL/6 (H-2^b), BALB/c (H-2^d), and SJL (H-2^s) mice was examined by flow cytometry using anti-CD19 to detect B cells and mAb CC-1 to detect MHV-R expression. In C57BL/6 mice approximately 70% of cells analyzed were CD19⁺ and of this population >92% expressed MHV-R (Fig. 1). Analysis of cells from BALB/c mice showed approximately 62% CD19⁺ cells of which >92% expressed MHV-R (Fig. 1). In contrast to B cells derived from C57BL/6 and BALB/c mice, which express MHV-R (Dveksler *et al.*, 1993; Yokomori and Lai, 1992), <1% of the CD19⁺ cells derived from SJL mice expressed MHV-R detected by mAb CC-1 (Fig. 1). SJL mice are relatively resistant to JHMV infection (Stohlman and Frelinger, 1978) and express only the CEACAM1b isoforms of the MHV-R in contrast to the CEACAM1a isoforms expressed by the susceptible C57BL/6 and BALB/c mouse strains (Dveksler *et al.*, 1993; Yokomori and Lai, 1992).

To determine if cells infected with JHMV were susceptible to B-cell-mediated lysis, spleen cells were prepared from naïve C57BL/6, BALB/c, and SJL mice and compared for their ability to lyse J774.1 (H-2^d) cells infected with either JHMV or MHV-A59. Consistent with the expression of MHV-R, cells derived from both C57BL/6 and BALB/c mice lysed cells infected with either JHMV or MHV-A59, but not uninfected cells (Fig. 2). Therefore, cytolysis is independent of differences in the S proteins of these two viruses. The MHV-A59 S protein contains a 142-amino-acid deletion relative to the S protein of JHMV although there is 90% amino acid homology over the remainder of the protein (Parker *et al.*, 1989). No difference in lysis was observed comparing adherent-cell-depleted spleen cells from naïve BALB/c mice to a

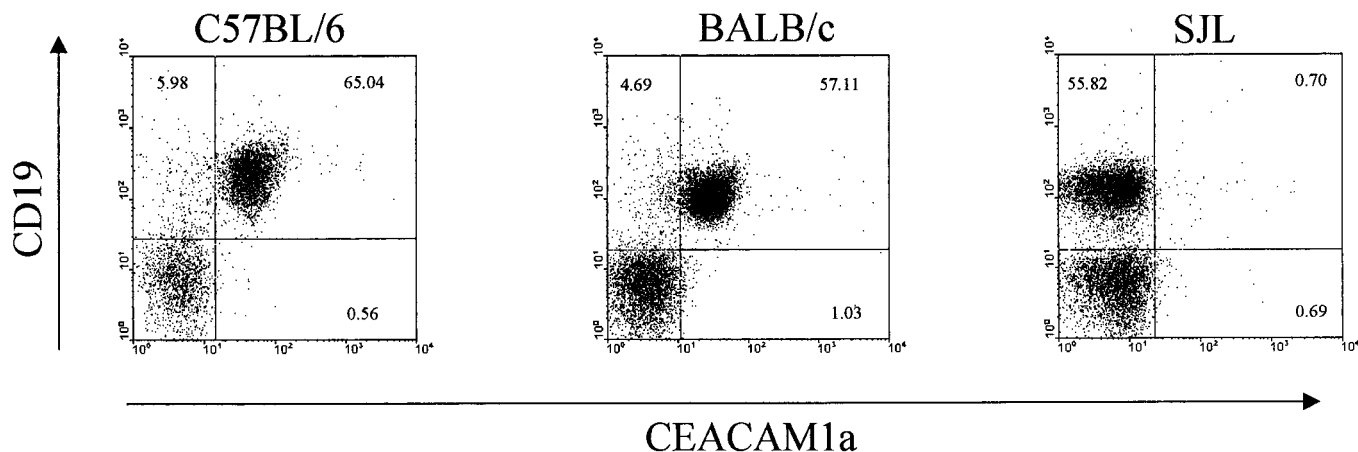


FIG. 1. MHV-R expression on CD19⁺ splenic B cells derived from C57BL/6, BALB/c, and SJL mice. Adherent-cell-depleted spleen cells were analyzed by flow cytometry for CD19 expression using a FITC-conjugated mAb. MHV-R expression was detected using a biotinylated mAb (CC-1) and PE-conjugated streptavidin.

splenic population containing >90% CD19⁺ cells depleted of T cells and CD11b⁺ cells by negative selection using Ab-coated magnetic beads (data not shown). Addition of 1 μ g/ml anti-MHV-R mAb CC-1 inhibited lysis of JHMV-infected cells (data not shown), similar to previous results with MHV-A59-infected cells (Coutelier *et al.*, 1994), confirming a requirement for MHV-R-S protein interactions. No lysis was detected with cells coated with a peptide encompassing the immunodominant L^d-restricted CD8⁺ T cell epitope (Bergmann *et al.*, 1993), confirming the naïve status of the donor BALB/c mice (data not shown). Finally, similar results were obtained with spleen cells derived from either naïve C57BL/6 or BALB/c mice and IC-21 (H-2^b) cells infected with either JHMV or MHV-A59 (data not shown). These data demonstrate that B cells from naïve mice expressing the MHV-R are able to lyse cells infected with JHMV via a major histocompatibility complex-independent mechanism.

S protein expression is sufficient for cytotoxicity

To determine if cytotoxicity requires only an interaction between the S protein and the MHV-R, or whether an-

other MHV-encoded viral component expressed during infection is required, lysis of cells infected with recombinant vaccinia virus (rVV) expressing either the JHMV S protein or the MHV-A59 S protein were compared to JHMV-infected cells. Cells expressing only the S proteins encoded by JHMV or MHV-A59 were lysed at levels comparable to those of JHMV infected cells (Fig. 3). No cytolysis was detected using cells infected with a rVV expressing β -galactosidase. These data demonstrate that expression of the MHV S protein alone is sufficient to initiate cytolysis and that no other MHV-encoded protein or viral-induced alteration in the host cells is required for B-cell-mediated cytolysis.

S protein fusion-dependent B cell cytotoxicity

B cells were tested for the ability to lyse cells infected with the nonfusogenic MHV-2 strain (Tsai *et al.*, 1999), which utilizes the identical MHV-R (Chen *et al.*, 1997), to confirm a requirement for S-protein-mediated cell-cell fusion (Wysocka *et al.*, 1989). In contrast to cells infected with either fusogenic JHMV or MHV-A59, cells infected with MHV-2 were resistant to B-cell-mediated lysis (Fig.

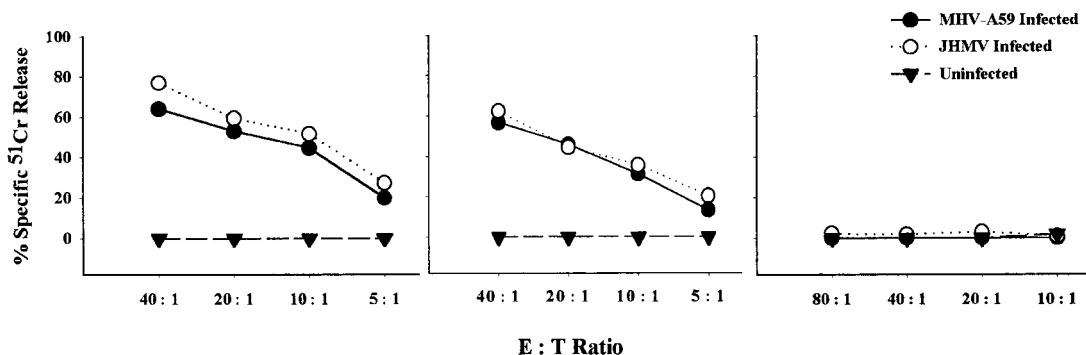


FIG. 2. JHMV-infected cells are susceptible to B-cell-mediated lysis. Spleen effectors were prepared from naïve C57BL/6, BALB/c, or SJL mice as described under Materials and Methods. J774.1 cells were infected with JHMV or MHV-A59 for 6 h prior to assay at a m.o.i. of 5. Spleen cells at various effector to target (E:T) ratios were tested for cytotoxicity using a 4-h ⁵¹Cr release assay. Data shown are representative of four experiments.

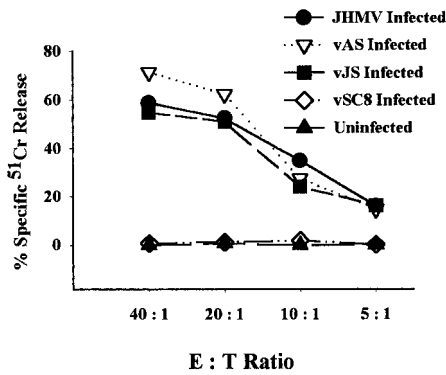


FIG. 3. MHV S protein expression is sufficient for cytolysis. J774.1 cells were infected with rVV expressing the S proteins derived from the JHMV (vJS) and MHV-A59 (vAS) or a control rVV (vSC8) for 6 h at an m.o.i. of 5 prior to labeling. Control cells were infected with JHMV 6 h prior to labeling. Splenic effector cells were derived from naïve BALB/c mice. Specific ⁵¹Cr release was determined after 4 h incubation at 37°C. Data shown are representative of three experiments.

4A). To confirm both the requirement for S protein expression and cell-cell fusion, cytolysis of cells infected with the fusogenic JHMV was carried out in the presence of anti-S-protein mAb. mAb J.2.6 neutralizes JHMV and inhibits S-protein-mediated fusion, while mAb J.7.5 exhibits neutralizing activity but does not inhibit fusion (Fleming *et al.*, 1983b). Both mAb inhibited B-cell-mediated cytolysis (Fig. 4B), consistent with a requirement for S protein-MHV-R interaction. B-cell-induced lysis of JHMV-infected cells was also inhibited by anti-S-protein mAb J.2.5 (Fig. 4B), which prevents cell-cell fusion but lacks neutralizing activity (Fleming *et al.*, 1983b). By contrast mAb J.3.3, specific for the JHMV N protein (Fleming *et al.*, 1983b), which recognizes an epitope expressed on the surface of infected cells (Kyuwa *et al.*, 1994), did not

inhibit cytolysis (Fig. 4B) nor did two additional isotype control mAb specific for irrelevant proteins (data not shown). These data suggest that cytolysis requires both S protein-MHV-R interactions and the induction of cell-cell fusion (Wysocka *et al.*, 1989). Finally, spleen cells derived from $P^{-/-}$ mice lysed both JHMV- and MHV-A59-infected cells (Fig. 4C), consistent with a mechanism dependent on cell-cell fusion and independent of perforin and other potential cytolytic pathways, i.e., Fas/FasL interactions, TNF- α , TNF- β , and IL-1 (Nishioka and Welsh, 1993).

MHV-R expression is sufficient for cytolysis

To determine if lysis mediated by interactions of the MHV S protein and MHV-R expressed by B cells is dependent upon a unique property of B cells, HeLa-MHVR, HeLa cells transfected with the gene encoding MHV-R (Gallagher, 1996), were examined for lysis of JHMV-infected cells. Expression of MHV-R on HeLa-MHVR cells was compared to the expression on CD19⁺ B cells by flow cytometry. Although HeLa-MHVR cells showed a bimodal distribution of MHV-R expression, the average mean fluorescence intensity of MHV-R expression is similar to, or exceeds, the expression on cytolytic CD19⁺ B cells derived from BALB/c and C57BL/6 mice (Fig. 5A). Due to the large size of HeLa cells compared to splenic B cells, HeLa-MHVR and control HeLa-tTA cells were added to the cytolytic assays at 20% the number of added nonadherent spleen cells. Even though added at reduced effector numbers, lysis of JHMV-infected cells occurred following the addition of HeLa cells expressing MHV-R, but not control HeLa cells (HeLa-tTA) (Fig. 5B). Inhibiting S protein and MHV-R interactions via anti-MHV-R mAb (1 μ g/ml) completely abolished cytolysis

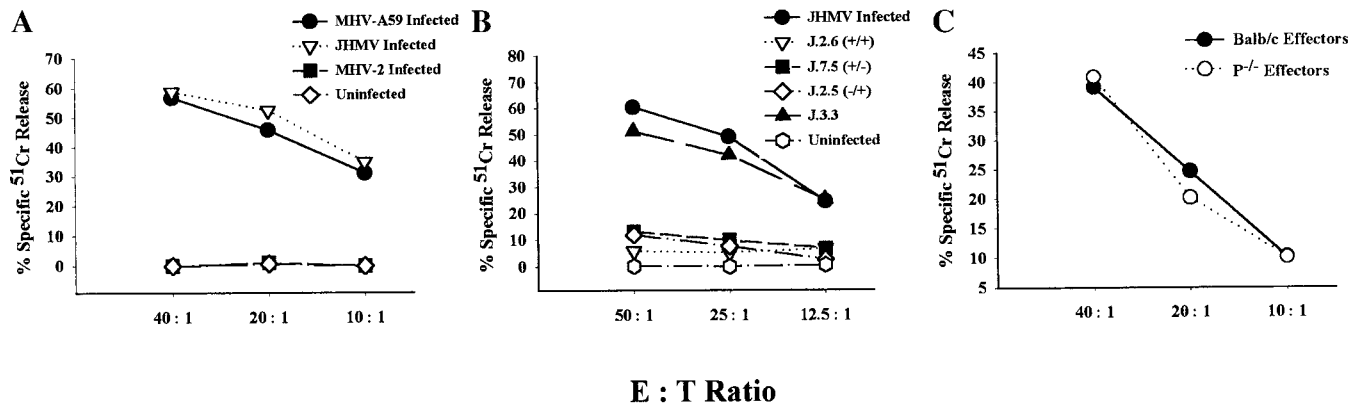


FIG. 4. Cytolysis requires cell-cell fusion. (A) J774.1 cells were infected with the nonfusogenic MHV-2 strain and the fusogenic JHMV and MHV-A59 strains of MHV at an m.o.i. of 5 and tested for susceptibility to B-cell-mediated lysis at 6 h p.i. Effectors from the spleens of BALB/c mice were incubated with the virus-infected cells at various ratios. (B) Spleen cells from BALB/c mice were tested for lysis of JHMV-infected cells in the presence of various JHMV-specific mAb [J.2.6 anti-S-protein mAb with both neutralization and fusion inhibition (+/+) activities, J.7.5 anti-S-protein mAb with neutralization activity only (+/-), J.2.5 anti-S-protein mAb with fusion inhibition activity only (-/+), and J.3.3 anti-N-protein mAb]. The mAb were added at the initiation of the assay at final concentrations of 30–50 μ g/ml. (C) B cells from $P^{-/-}$ mice were examined for their ability to lyse cells infected with JHMV. J774.1 cells were infected with JHMV at an m.o.i. of 5 mixed with effectors derived from the spleens of BALB/c and $P^{-/-}$ mice at indicated E:T ratios. The percentage ⁵¹Cr release was determined after 4 h incubation at 37°C. Data shown are representative of three or four experiments.

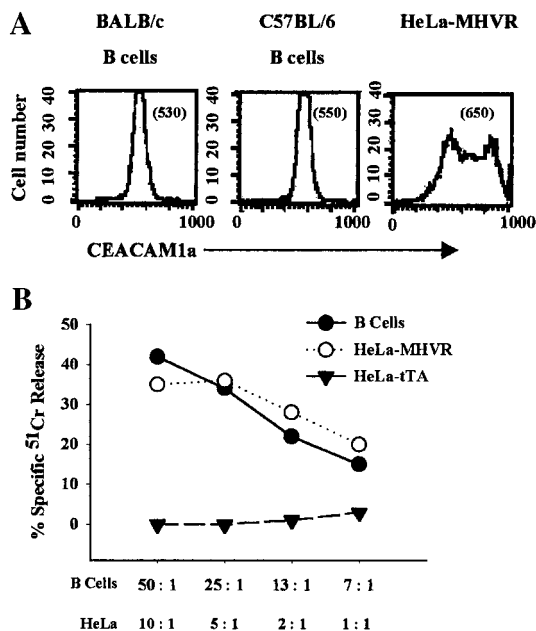


FIG. 5. B cells are not required for cytolysis. (A) Surface MHV-R expression on HeLa-MHVR cells compared to CD19⁺ B cells derived from BALB/c and C57BL/6 mice. MHV-R expression was determined by flow cytometry using biotinylated anti-MHV-R CC-1 mAb and PE-conjugated streptavidin. Mean fluorescence intensity for each population is shown in parentheses. (B) Lysis of JHMV-infected cells mediated by HeLa-MHVR cells compared to BALB/c-derived splenic B cells. J774.1 cells were infected with JHMV at an m.o.i. of 5. Nonadherent spleen cells were added at various E:T ratios starting at 50:1. HeLa-MHVR and control HeLa-tTA were added at E:T ratios starting at 10:1. Specific ⁵¹Cr release was determined after 4 h incubation at 37°C. Data are representative of three experiments.

mediated by HeLa-MHVR cells (data not shown). These data demonstrate that cytolysis resulting from interactions between the S protein and the cells expressing high levels of the MHV-R is not a unique property restricted to B cells.

DISCUSSION

To begin to explore a potential Ab-independent role for B cells in the pathogenesis of CNS infection, JHMV-infected cells were examined for susceptibility to B-cell-mediated lysis, similar to cells infected with MHV-A59 (Holmes *et al.*, 1986). The data demonstrate that cells infected with JHMV are susceptible to lysis. These data show that expression of only the viral-encoded S protein is required. Therefore, no other MHV-encoded protein or alteration in host cell metabolism (Kyuwa *et al.*, 1994) participates in B-cell-mediated cytolysis. Lysis is prevented by both neutralizing anti-JHMV S protein mAb and mAb CC-1 specific for the isoforms of the CEACAM1a allele of the MHV-R which blocks MHV infection *in vitro* and *in vivo* (Dveksler *et al.*, 1993; Coutelier *et al.*, 1994; Smith *et al.*, 1991). By contrast, neither mAb specific for other viral proteins nor isotype-matched mAb with other

specificities inhibited lysis. The absence of a role for Fas/FasL interactions, TNF- α , TNF- β , or IL-1 (Nishioka and Welsh, 1993) in lysis; the ability of B cells derived from P^{-/-} mice to lyse JHMV-infected cells; and the inhibition of lysis by an anti-S-protein mAb which inhibits fusion, but does not neutralize, are all consistent with a fusion-dependent process (Wysocka *et al.*, 1989). However, the mechanisms leading to a loss of membrane permeability and induction of apoptosis (Nishioka and Welsh, 1993) following interaction between the S protein and the MHV-R are not clear.

The majority of B cells express MHV-R and are therefore capable of mediating the lysis and/or apoptosis of JHMV-infected cells expressing the S protein on their surface. B220⁺, surface Ig⁺ (Williamson, 1992; Williamson *et al.*, 1991), and CD19⁺ (Marten *et al.*, 2001) B cells are rapidly recruited into the CNS following JHMV infection. These data suggest that B-cell-mediated lysis and/or apoptosis may occur within the CNS. However, the clearance of infectious JHMV from the CNS during acute infection of μ MT mice, which lack B cells and therefore are unable to secrete Ab, is similar to that of wt mice (Lin *et al.*, 1999). These data suggest that B cells and/or Ab provide a minor contribution to immune-mediated viral clearance during acute infection. Although these data are not consistent with an Ab-independent B-cell-mediated mechanism, μ MT mice are unable to completely eliminate infectious virus from the CNS (Lin *et al.*, 1999) under conditions under which CD8⁺ T cell perforin-mediated cytolysis is down regulated (Bergmann *et al.*, 1999). In addition, JHMV reactivates within the CNS of μ MT mice following the initial T-cell-mediated reduction in infectious virus (Lin *et al.*, 1999). The ability of passively transferred polyclonal Ab containing neutralizing activity to suppress reactivation is consistent with a role of anti-JHMV Ab in maintaining viral persistence (Lin *et al.*, 1999). Nevertheless, B cells may contribute to clearance of infectious virus during acute infection, resulting in suppression of virus reactivation, or play an active role in preventing virus reactivation prior to the induction of anti-S-protein Ab. The potential ability of B cells to contribute to the complete suppression of infectious virus and/or prevention of viral recrudescence may be masked via passive Ab transfer (Lin *et al.*, 1999).

In wt mice induction of anti-S-protein Ab with either neutralizing or fusion-inhibiting activities, or accumulation of B cells in the CNS secreting Ab with these functions, would inhibit a potential Ab-independent B-cell-dependent mechanism of viral suppression. This suggests that during infection, an Ab-independent mechanism could contribute to viral clearance only early, i.e., during the loss of perforin-mediated cytolysis by CNS-infiltrating virus-specific CD8⁺ T cells (Bergmann *et al.*, 1999) but prior to the induction of anti-S-protein Ab with neutralizing or fusion-inhibiting activities. Consistent with this hypothesis, virus is cleared from the CNS of

$P^{-/-}$ mice (Lin *et al.*, 1997). MHV-R⁺ B cells that are rapidly recruited into the CNS during acute infection (Dorries *et al.*, 1991; Williamson, 1992; Williamson *et al.*, 1991) are presumably not virus specific based on the absence of detectable serum neutralizing antiviral Ab (Parra *et al.*, 1997, 1999; Stohlman *et al.*, 1986, 1999). However, it is not clear how rapidly B cells secreting virus-specific anti-S-protein Ab are induced in the periphery or how rapidly they infiltrate the CNS following JHMV infection. These data suggest that MHV-R⁺ B cells may indeed contribute to JHMV clearance in an Ab-independent manner. B cells may also sequester virus following S protein–MHV-R interactions, thus effectively limiting virus spread in addition to an Ab-independent mechanism of virus suppression. Finally, B cell–S protein interactions lead to the lysis of both the B cell and the virus-infected cell (Wysocka *et al.*, 1989), suggesting that this interaction may contribute to the observations that serum neutralizing Ab are not detectable until the majority of infectious virus, and presumably infected cells expressing the S protein, has been eliminated (Lin *et al.*, 1997; Parra *et al.*, 1997, 1999; Stohlman *et al.*, 1986).

These data demonstrate that MHV-R⁺ B cells from susceptible mice lyse JHMV-infected cells via a fusion-dependent mechanism. The rapid recruitment of MHV-R⁺ B cells into the infected CNS may constitute an innate immune response contributing to the reduction of infectious CNS virus and prevent virus reactivation, either via a lytic interaction or via the sequestration of infectious virus. An interaction between MHV-R⁺ B cells and JHMV-infected cells also provides a plausible mechanism to explain the delayed appearance of JHMV-neutralizing Ab until after the majority of infectious virus has been cleared from the CNS by a vigorous cell-mediated immune response.

MATERIALS AND METHODS

Mice

BALB/c, C57BL/6, and perforin-deficient ($P^{-/-}$) mice on the C57BL/6 genetic background (C57BL/6-*Pfp^{tm1Sadz}*) were purchased from The Jackson Laboratory (Bar Harbor, ME). SJL mice were purchased from the National Institutes of Health (Frederick, MD). Mice were purchased at 6 weeks of age and all mice were used between 7 and 14 weeks of age. $P^{-/-}$ mice were maintained locally in a sterile environment by intercrossing. No age- or gender-dependent differences were noted.

Viruses

Plaque-purified populations of the MHV strains JHMV, MHV-A59, and MHV-2 were propagated and plaque assayed on DBT cell monolayers as described previously (Bergmann *et al.*, 1993). rVV expressing the S protein derived from JHMV, the S protein derived from MHV-A59,

and *Escherichia coli* β -galactosidase were propagated in HeLa cells and plaque assayed on monolayers of BSC-1 cells as previously described (Stohlman *et al.*, 1993).

Effector cell preparation and cell lines

Single-cell suspensions from spleens of naïve mice were prepared in RPMI medium. Following red blood cell lysis using Gey's solution, the cells were suspended in RPMI medium containing 2% fetal calf serum (FCS) and incubated on plastic petri plates at 37°C for 1 h to remove adherent cells. The resulting nonadherent populations contained 50 to 60% CD19⁺ B cells as determined by flow cytometry (see below). These macrophage-depleted populations were used in most experiments as cytolytic effectors. HeLa cells expressing the MHV-R, designated HeLa–MHVR, and a control line, designated HeLa–tTA (Gallagher, 1996), were kindly provided by Dr. Thomas Gallagher (Loyola University Medical Center, Chicago, IL). HeLa cells were grown in Dulbecco's modified MEM (DMEM) containing 10% FCS.

Flow cytometry

Splenocyte samples were blocked at 4°C for 15 min with anti-CD16/CD32 (mAb 2.4G2; PharMingen, San Diego, CA) and a mixture of equal amounts of goat serum, human serum, and horse serum was added to a final concentration of 10% prior to staining. Cells were washed with PBS containing 0.1% bovine serum albumin prior to staining of B cells with fluorescein isothiocyanate-conjugated anti-CD19 (mAb ID3; PharMingen) at 4°C for 15 to 20 min. MHV-R expression was examined by incubation with biotinylated mAb CC-1, which detects CEACAM1a but not CEACAM1b (Williams *et al.*, 1990), and visualized with phycoerythrin-conjugated streptavidin (PharMingen). Samples were analyzed on a FACStar flow cytometer (Becton–Dickinson, Mountain View, CA).

Target cells

The J774.1 (H-2^d) and IC-21 (H-2^b) cell lines were propagated in DMEM containing 10% FCS. Cells were plated at a concentration of 1×10^6 in 100-mm plates for 8 h prior to infection with MHV at a m.o.i. of 1 to 5. MHV-infected cells were incubated for 6 h at 37°C prior to use. J774.1 cells were infected with rVV at an m.o.i. of 5 to 10 and were incubated for 10 h at 37°C prior to use.

Cytotoxic assays

Infected and uninfected cells (1×10^6) were labeled at 37°C for 1 h with 60 μ Ci of Na⁵¹CrO₄ (NEN Life Sciences, Boston, MA) and washed three times prior to use. Labeled cells in 96-well microtiter plates (1×10^4 /well) were mixed with varying numbers of effector cells to achieve the desired effector/target ratios. Some cytolytic

assays were performed in the presence of anti-JHMV-specific mAb J.2.5, J.2.6, J.7.5 (anti-S-protein), and J.3.3 (anti-nucleocapsid protein) (Fleming *et al.*, 1983b), prepared either as serum-free culture supernatants or as ascities, or anti-MHV-R mAb (CC-1). After 4 h incubation at 37°C, 0.1 ml was removed and counted in a gamma counter. Data are presented as percentage specific ⁵¹Cr release = 100 × (experimental cpm – spontaneous cpm)/(maximum release cpm – spontaneous cpm). Spontaneous release in all assays was ≤20% of total.

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