

B Cells and Antibodies in the Pathogenesis of Myelin Injury in Semliki Forest Virus Encephalomyelitis

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To determine the contribution of B cells to brain myelin injury in Semliki Forest Virus (SFV) encephalomyelitis, normal C57BL/6 (B6) and B-cell-deficient (C57BL/6-tm1Cgn) B6 mice were infected with SFV. The peak of clinical disease, i.e., the time at which the greatest proportions of mice had moderate to severe clinical signs, appeared earlier in B6 mice [day 7 postinfection (pi)] than in B-cell-deficient mice (day 21 pi). By flow cytometry, no clear differences were found in the percentages of CD3⁺CD4⁺ T cells in the brains of B6 and B-cell-deficient mice. However, by day 21 pi, percentages of CD3⁺CD8⁺ T cells were greater in brains of B-cell-deficient than in those of B6 mice. On day 21 pi, percentages of CD19⁺ B cells were maximal in B6 mice, but B cells were absent in B-cell-deficient mice at all time points. Sera obtained from B6 mice showed antibody responses to SFV, to SFV E2 peptides p137-151 and p115-133, and to peptides of myelin oligodendrocyte glycoprotein p18-32 and myelin basic protein (MBP) p64-75. Sera obtained from B-cell-deficient mice showed minimal or no reactivity to SFV, E2, or myelin peptides. CNS inflammatory and PAS-positive macrophage foci were maximal on days 7–14 pi in all mice. Additionally, B6 mice had brain white matter vacuolation, whereas B-cell-deficient mice did not. These data suggest that brain infiltrating B cells and anti-myelin antibodies contribute to myelin injury in SFV encephalomyelitis. © 2000 Academic Press

INTRODUCTION

Infection of the central nervous system (CNS) by SFV, an alphatogavirus, has been used to study the pathogenesis of CNS viral infections and demyelinating disease. SFV induces an acute CNS virus infection, followed by demyelination after viral clearance. Mice infected by intraperitoneal (ip) injection with SFV develop encephalitis with demyelination that is maximal

between 14 and 21 days pi, after the immune response has cleared infectious virus from both blood and brain (1–3). Demyelination appears to be immune-mediated (4–6), and remyelination occurs by day 35 pi.

Our previous studies showed that SFV infection triggered susceptibility to experimental allergic encephalomyelitis (EAE) induced with myelin basic protein (MBP) in EAE-resistant C57BL/6 (B6) mice, suggesting that the infection enhanced autoimmune responses to this major myelin component (7). In a comparison of SFV infection in SJL and B6 mice, greater levels of IL-4 mRNA in the brains of B6 mice correlated with higher levels of virus and more severe clinical disease, whereas more efficient clearance of virus and less severe disease was observed in the absence of IL-4 mRNA (8). These data suggest that TH2 cells and antibody production which are regulated by this cytokine have major roles in the host response to the infection and to viral clearance. Our more recent studies demonstrated that mice immunized with killed SFV or with peptide 115-133 of the major SFV envelope glycoprotein E2 developed cross-reactive T cell responses to the peptides of myelin oligodendrocyte glycoprotein (MOG), MBP, and proteolipid protein (PLP), which mimic peptides of E2. Furthermore, mice immunized with either E2 115-133 or MOG 18-32 developed a late onset CNS disease with spongiform brain white matter lesions that correlated with the onset and incidence of induced serum antibody responses to the mimicked peptides (9). Therefore, prominent roles for B cells and antibodies that cross-react with myelin antigens are implicated in the pathogenesis of SFV encephalomyelitis and of CNS white matter injury.

B cell responses have also been identified as an essential factor for the severity of clinical disease and pathology in EAE in the common marmoset *Callithrix jacchus*. Autoantibodies against MOG in acute lesions of this EAE model and of acute multiple sclerosis (MS) lesions were associated with disintegration of the myelin sheaths (10–11). Therefore, in CNS disease initiated by T cell responses, antibodies against myelin may contribute to tissue injury.

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In this study, we investigated the role of B cells and anti-SFV antibodies in the pathogenesis of the myelin injury that follows acute SFV infection in the CNS of B6 mice and B-cell-deficient mice. Our findings suggest that B cells in the brains, and anti-myelin antibodies, induce CNS white matter injury following active SFV infection.

MATERIALS AND METHODS

SFV

The avirulent A774 strain of SFV with a titer of 3×10^8 plaque-forming units (PFU)/mL on BHK-21 cells (American Type Culture Collection, Rockville, MD) was used as stock virus (7). Inactivation of SFV was accomplished by UV irradiation of 1 mL (10^7 PFU) of infectious virus in Hanks' balanced salt solution (HBSS) (GIBCO/BRL, Grand Island, NY) added to 60-mm tissue culture plates (Falcon Plastics, VWR Scientific, San Francisco, CA) precoated with fetal bovine serum (FBS) (GIBCO/BRL). Plates were exposed to UV light (General Electric) at a distance of 8 cm for 20 min. After UV exposure, the virus exhibited no detectable infectivity when tested on Vero or BHK-21 cells (American Type Culture Collection).

Infection of Mice

Female, 5- to 6-week-old normal B6 and B-cell-deficient, C57BL/6-tm1Cgn, mice purchased from the Jackson Laboratory (Bar Harbor, ME) were used in all experiments. B-cell-deficient mice were housed under sterile conditions. To induce disease, groups of mice were inoculated ip with SFV (10^4 PFU) in Dulbecco's phosphate-buffered saline (D-PBS) (1 \times) (GIBCO/BRL). For all experiments, animals were randomly selected for sacrifice. Mice were anesthetized with Methoxyflurane (Pitman-Moore, Mundelein, IL), blood was collected, and then animals were sacrificed. Brains were harvested for mononuclear cell(s) isolation on days 0, 7, 10, 14, 21, and 35 pi.

Isolation of Cells From Neural Tissue

Isolation of lymphocytes from neural tissue was carried out as previously described by Irani and Griffin (12). Briefly, brains from three to five mice were sterilely removed, pooled, then gently minced through a fine mesh screen using a syringe plunger (30 mL), and collected into sterile HBSS (10 mL/brain) containing 0.05% collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN), trypsin inhibitor TLCK (0.1 μ g/mL) (Sigma Chemical Co., St. Louis, MO), DNase I (10 μ g/mL) (Sigma), and Hepes buffer, pH 7.4 (10 mM) (GIBCO/BRL). The resulting tissue slurry was mixed at room temperature for 1 h and then allowed to settle at unit gravity for 30 min to deplete any undigested

debris. The supernatant was collected, pelleted at 200g for 5 min, and resuspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS (10 mL) for each brain. Five milliliters of this suspension was carefully layered onto 10 mL of a modified density separation medium prepared by mixing various amounts of RPMI 1640 media containing 10% FBS, Hepes buffer (10 mM) (GIBCO/BRL), and gentamycin (50 μ g/mL) (GIBCO/BRL) with Ficoll-Paque (Pharmacia, Piscataway, NJ) in a 50-mL conical centrifuge tube (Falcon Plastics, VWR Scientific). Each gradient was centrifuged at 500g for 30 min, and the overlying media and interface of tissue debris were removed using a transfer pipette (SARSTEDT, USA). The entire 10 mL of gradient medium was diluted 10-fold with HBSS and centrifuged at 300g for 10 min to pellet the cells which had entered the gradient. These cells were counted (1×10^4 /brain) (trypan blue exclusion), washed twice in D-PBS or HBSS, and resuspended in D-PBS or HBSS (100 μ l). Cells were subsequently stained for flow cytometry (FACScan, S.U.N.Y. Health Science Center at Brooklyn).

Immunofluorescent Staining and FACS Analysis of Isolated Cells

To stain the lymphocytes, fluorescein (FITC)-conjugated rat monoclonal antibodies (mAbs), anti-CD45 and anti-CD3 complex, and/or phycoerythrin (PE)-conjugated rat monoclonal antibodies, anti-CD4, anti-CD8a, and anti-CD19, were added according to manufacturer's recommendation, and samples were incubated for 10 min in the dark at room temperature. All mAbs were purchased from Pharmingen (San Diego, CA). Control experiments consisted of staining with conjugated, isotype-matched irrelevant monoclonal antibodies at similar concentrations. Brains were then washed in D-PBS (GIBCO/BRL) and resuspended in D-PBS (1 mL) with 1% paraformaldehyde (Polysciences, Inc.). Single- and dual-color immunophenotyping of lymphocytes were performed on a FACScan (Becton-Dickinson) equipped with a 15-MW air-cooled 488 argon-ice laser. Forward scatter (FSC) and side scatter (SSC) signals obtained in linear mode were used to establish a gate to identify the lymphocyte population and excluded other cells and debris. A minimum of 10,000 events were collected per sample in the selected gate. The gain on the photomultiplier tube detecting fluorescence intensity was adjusted so that 99% of cells with background fluorescence staining were scored between 10^0 and 10^1 on a 4-decade log scale. Specific fluorescence was reported as the percentage of positive cells with relative fluorescence intensity scored above background (isotype-matched control mAb staining subtracted out). Data are expressed as percentages of total cells positive for expression of the specific surface marker based on the mean fluorescence intensity compared to positive and negative con-

trols. Mouse immunoglobulin isotype control, PE-conjugated mouse IgG2a (Pharmingen, San Diego, CA), was used as the negative control, while a FITC-conjugated rat anti-mouse CD45 (leukocyte common antigen, Ly-5) (Pharmingen) mAb was used as the positive control.

Synthesis of Peptides

SFV-E2 has been reported to be the target for antibody responses in SFV-infected mice (13), and the E2 peptides 115-133 (IQDTRNAVRASRIQYHHDP) and 137-151 (GREKFTIRPHYGKEI) were selected for testing antibody responses. The myelin peptides MBP 64-75 (THYGSLPQKSQH) and MOG 18-32 (DEAELPSRISPGKNA), which have sequence homology to these peptides and are targets of anti-E2 cross-reactive antibodies (9), were also tested. Peptides were synthesized by an automated peptide synthesizer at the Biochemistry Laboratory of Johns Hopkins University, School of Hygiene and Public Health. Synthetic peptides were analyzed by HPLC and purified, and their compositions were confirmed by amino acid analysis. Human fibrinopeptide B (FB) 1-14 (EGVNDNEEGFF-SAR) (Sigma) was used as the negative control.

Enzyme-Linked Immunosorbent Assay (ELISA)

For the semiquantitative determination of antibody responses in sera, mice were anesthetized and then bled and sacrificed on days 0, 7, 10, 14, 21, and 35 pi. Blood samples from two to three mice of the same group were pooled to obtain a sufficient volume of sera. Flat-bottomed immunolon-2 microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with 2 μ g/well of UV-irradiated SFV protein or 2 μ g/well of each of the following peptides: E2 115-133, E2 137-151, MOG 18-32, MBP 64-75, or control peptide, human FB 1-14 (9). ELISAs were performed as previously described (9). The optical densities (OD) of the wells were read by a microtiter plate reader (Molecular Devices) using a 450- and/or 490-nm measurement filter. All specimens were analyzed in duplicate and averaged using SOFTmax Windows Version 2.3 software (Molecular Devices).

Histopathology

Brains were obtained from mice 0, 7, 10, 14, 21, and 35 days pi and fixed in 10% Formalin. Sections 6 μ m thick were cut and stained with hematoxylin and eosin-luxol fast blue stain. Numbers of inflammatory foci (>20 perivascular mononuclear cells) were counted in meninges and parenchyma in each section containing the entire mouse brain. Each brain sample was also scored as to the extent of diffuse vacuolation in the cerebellar or brain stem white matter as follows: 0 = no vacuolation; 1 = rare, scattered vacuoles; 2 = scattered

and some clusters of vacuoles, 3 = numerous clusters of vacuoles. Histologic evaluation was performed by an observer blinded to the presence or the absence of clinical signs in the animals. The significance of differences in numbers of inflammatory foci, incidence of vacuolation, and vacuolation scores were determined by the Students's *t* and Fisher tests.

Viral Titration

The course and clearance of SFV infection within the brains were monitored by measuring the dilution of virus which killed 50% of cells in culture (TCID₅₀), as previously described (14). Briefly, brains from one to two SFV-infected B6 and B-cell-deficient mice were sterilely removed from the skulls at each time point pi. Ten percent (w/v) brain D-PBS suspensions were obtained by homogenization and passing through a 1-mL syringe. Serial dilutions of supernatants were made and 100 μ l was added in quadruplicates to monolayers of BHK-21 cells (American Type Culture Collection). Medium without brain homogenates (normal cell control) and stock virus control were set up at the same time. After a 48-h incubation at 37°C in 5% CO₂-humidified air, cells were observed and fixed with formaldehyde (10%) (Polysciences, Inc., Warrington, PA) for 20 min., washed in D-PBS, and then stained with toluidine blue O (1%) (Fisher Scientific, Springfield, NJ) for 3–5 min. The plates were analyzed, and results were expressed as mean TCID₅₀ content of quadruplicate wells.

RESULTS

Development of Clinical Disease in SFV-Infected B6 and B-Cell-Deficient Mice

Infection of B6 and B-cell-deficient mice ip with SFV produced three types of clinical outcomes on days 7–10 pi: (1) weakness, ruffled fur, and weight loss, from which the mice may recover or progress to (2) permanent paralysis and (3) death. Mice were observed daily for clinical manifestations of SFV and were scored on a scale of 0–VI as follows: 0 = no abnormality, I = mild hind limb weakness (some difficulty righting themselves when turned on their back), II = moderate hind limb weakness (as in I), sometimes associated with floppy tail, III = weakness of hind limbs accompanied by some forelimb weakness, sometimes more marked on one limb or one side, but not complete paralysis, IV = hind limb paresis accompanied by mild forelimb weakness, V = paralysis of hind limbs, associated with moderate forelimb weakness, and VI = quadraplegia, moribund. It should be noted, that stages I and II are weakness primarily due to systemic viral effects, and stages II–VI are mild and severe paralysis due to CNS immunopathology (7). No mice exhibited any clinical signs of infection on day 0. The peak of clinical disease,

TABLE 1
Clinical Stages of SFV-Infected B6 and B-Cell-Deficient Mice (BCD)

Mice	Day after infection	No. mice ^a	No. sacrificed ^a	Number of mice at stages of infection ^b						No. dead	
				0	I	II	III	IV	V		VI
B6	0	30	8	30	0	0	0	0	0	0	0
	7	22	6	0	5	3	4	2	2	3	3
	14	13	5	0	4	2	2	2	2	0	1
	21	7	4	3	3	1	0	0	0	0	0
	35	3	3	3	0	0	0	0	0	0	0
BCD	0	20	4	20	0	0	0	0	0	0	0
	7	16	4	2	6	3	2	1	1	0	1
	10	11	3	1	1	4	1	2	1	1	0
	14	8	2	0	0	2	3	2	1	0	0
	21	6	3	0	0	1	4	1	0	0	0
	35	3	3	0	0	1	2	0	0	0	0

^a The discrepancies in numbers of mice at each stage after day 7 are due to the number of mice sacrificed for histological and virological studies.

^b Stages I and II represent temporary general weakness attributable mainly to effects of viral infection. Stages III–VI are neurological signs, i.e., mild to severe paralysis, attributable to immunopathology.

i.e., the time at which the greatest proportions of mice were in clinical stages III–VI, appeared earlier in B6 mice (day 7 pi) than in B-cell-deficient mice (day 21 pi), and by day 35 pi, no new mice exhibited clinical signs (Table 1).

Distributions of Lymphocyte Subpopulations in Brains of SFV-Infected B6 and B-Cell-Deficient Mice

Relative proportions of lymphocyte subpopulations in mononuclear cells isolated from brains of SFV-infected B6 and B-cell-deficient mice were determined on days 0, 7, 10, 21, and 35 pi. The results of one representative experiment are described below and illustrated in Fig. 1.

CD3⁺CD4⁺. Prior to infection (day 0), the percentages of CD3⁺CD4⁺ T cells were 7 and 4%, in brains of both SFV-infected normal B6 and B-cell-deficient mice, respectively. The percentages of CD3⁺CD4⁺ T cells in SFV-infected normal mice rose to 22% on day 7 pi, remained unchanged through day 21 pi, and then decreased to 9% on day 35 pi (Fig. 1A). The percentages of CD3⁺CD4⁺ T cells in brains of B-cell-deficient mice increased to 11% on day 7 pi, increased slightly to 16% on day 10 pi, remained unchanged through day 21 pi, and decreased slightly to 13% by day 35 pi (Fig. 1A).

CD3⁺CD8⁺. The percentages of CD3⁺CD8⁺ T cells were 12 and 6% in brains of SFV-infected normal B6 and B-cell-deficient mice on day 0. The percentages of CD3⁺CD8⁺ T cells in SFV-infected normal mice rose to 32% on day 7 pi, increased to 25% on day 21 pi, and decreased to 9% on day 35 pi (Fig. 1B). In contrast, the

percentages of CD3⁺CD8⁺ T cells in brains of B-cell-deficient mice were 21% on day 7 pi, substantially increased to 49% on day 21 pi, and decreased to 32% by day 35 pi (Fig. 1B).

CD19⁺. The percentages of CD19⁺ B cells in brains of SFV-infected normal B6 mice were 10% on day 0, increased to 18% on day 7 pi, decreased to 11% on day 10 pi, increased substantially to 78% on day 21 pi, and then decreased to 6% on day 35 pi (Fig. 1C). In contrast, the percentages of CD19⁺ B cells were low in B-cell-deficient mice (1%) and remained unchanged throughout the course of the experiment (Fig. 1C).

ELISA

Serum antibodies to UV-inactivated SFV and SFV E2 and myelin peptides. Sera obtained from SFV-infected B6 mice reacted strongly (OD \geq 1.0) to UV-SFV and E2 137-151 beginning on day 7 pi, and reactivity remained high until day 35 pi. In contrast, sera obtained from infected B-cell-deficient mice showed minimal increases in reactivity to UV-SFV and E2 137-151 over the course of infection. Reactivities to E2 115-133 increased in the B6 mice but were absent in the B-cell-deficient mice (Table 2).

Sera obtained from SFV-infected B6 mice reacted strongly (OD >1.0) with MOG 18-32 peptide beginning on day 10 and reactivity remained high until day 35 pi. These sera also reacted with MBP 64-75 peptide, with the highest reactivity observed on day 14 pi. In contrast, sera obtained from B-cell-deficient mice did not react with MOG 18-32 or MBP 64-75 over the course of

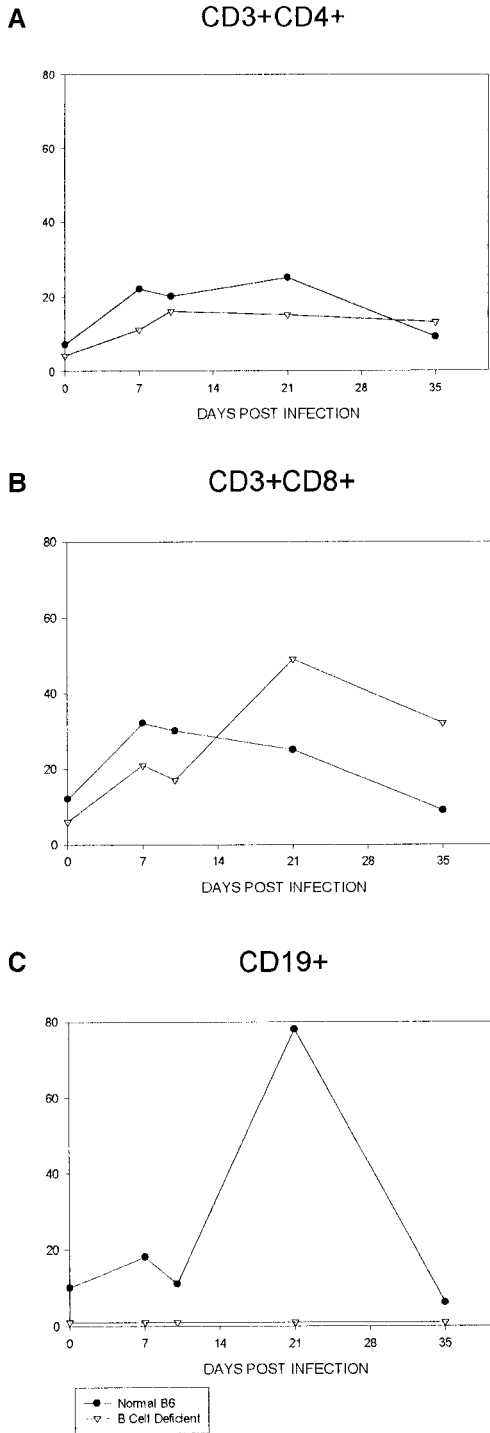


FIG. 1. Flow cytometric data obtained during SFV infection. Percentages of CD3⁺CD4⁺ (A), CD3⁺CD8⁺ (B), and CD19⁺ (C) lymphocytes isolated from brain throughout the course of infection in normal and in B-cell-deficient mice. Data are representative of one experiment and are expressed as percentages of total lymphocytes.

infection (Table 3). Sera obtained from SFV-infected B6 and B-cell-deficient mice also did not react with the negative control, human FB peptide 1-14 (Table 3).

TABLE 2
Anti-SFV and E2 Peptide Antibodies in Sera of SFV-Infected Mice^a

Antigen/mice	Antibody responses (OD value) on days pi					
	0	7	10	14	21	35
UV-SFV^b						
B6	0.962	1.881	nt	2.031	2.028	2.039
BCD	0.913	0.912	0.995	1.008	0.934	0.240
E2 137-151						
B6	0.819	1.428	1.740	1.421	1.573	1.132
BCD	0.343	0.526	0.721	0.670	0.760	0.289
E2 115-133						
B6	0.234	0.550	0.864	0.633	0.874	0.871
BCD	0.063	0.016	0.014	0.062	0.046	0.013

Note. nt, not tested.

^a Sera were obtained from freshly bled normal (B6) and B-cell-deficient (BCD) B6 mice (2-3/group pooled), and antibody levels were determined by ELISA. Data are expressed as optical densities (O.D.) minus background.

^b UV-SFV, ultraviolet-inactivated Semliki Forest Virus.

Histopathology

No mice had histologic abnormalities on day 0 prior to infection. As in previous studies (8), beginning on day 7 pi, the brains of both normal B6 and B-cell-deficient mice had perivascular mononuclear cell infiltrates and microglial nodules (Figs. 2A-2D). Numbers of focal inflammatory lesions at each time point postinfection were similar in the two groups of mice (Table 4) and numbers of lesions with PAS-positive macrophages (Figs. 2A-2D) were also not significantly different (numbers not shown). The inflammatory response was maximal from days 10-14 pi and diminished after that time point in both groups. The B6 mice addition-

TABLE 3
Anti-MOG 18-32, MBP 64-75, and Fibrinopeptide B (FB) 1-14 Antibodies in Sera of SFV-Infected Mice^a

Antigen/mice	Antibody responses (OD value) on days pi					
	0	7	10	14	21	35
MOG 18-32						
B6	0.237	0.709	1.113	1.043	1.033	1.155
BCD	0.000	0.000	0.013	0.000	0.000	0.000
MBP 64-75						
B6	0.182	0.182	0.264	0.936	0.266	0.758
BCD	0.036	0.004	0.010	0.020	0.024	0.007
FB 1-14						
B6	0.051	0.077	0.060	0.063	0.073	0.111
BCD	0.005	0.034	0.003	0.016	0.007	0.001

^a Sera were obtained from freshly bled normal (B6) and B-cell-deficient B6 mice (2-3/group pooled), and antibody levels were determined by ELISA. Data are expressed as optical densities (OD) minus background.

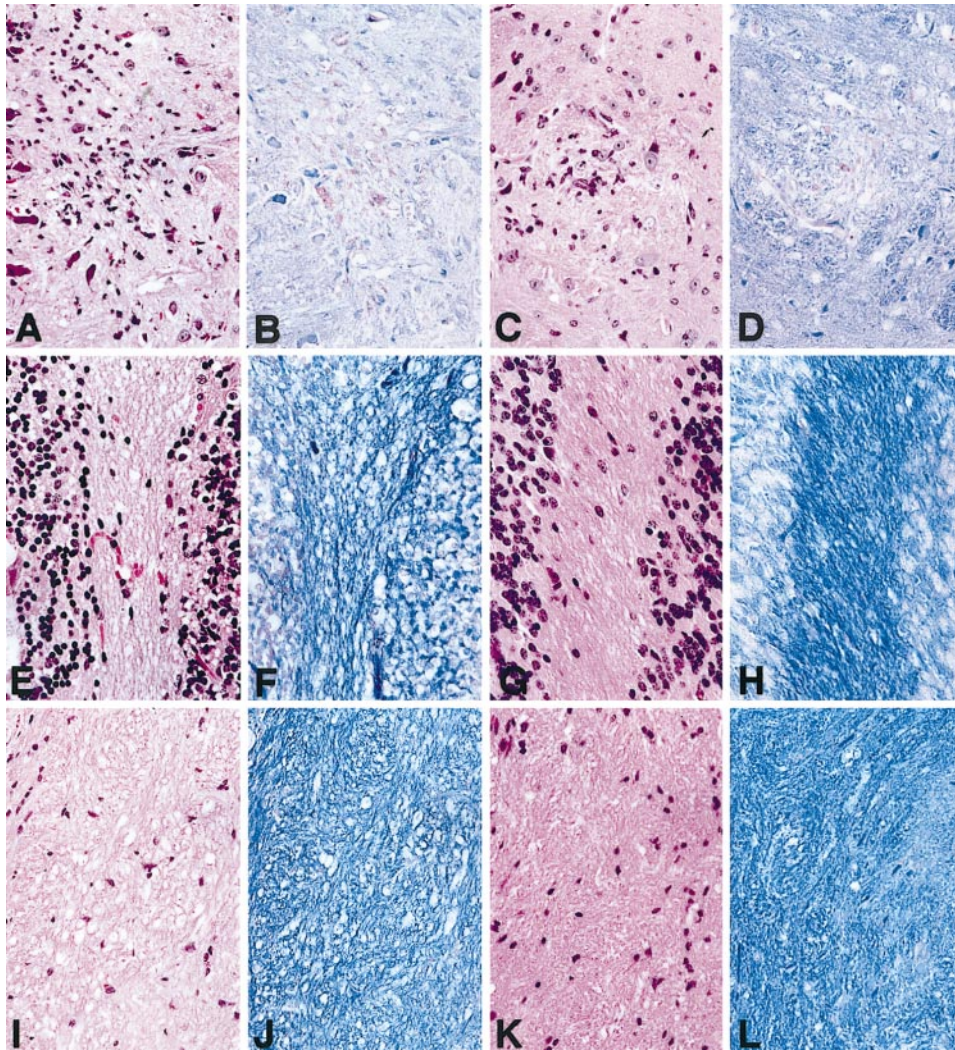


FIG. 2. Paraffin sections of brains of SFV-infected B6 and B-cell-deficient mice. The mice have the same extent of CNS inflammation and microglial reaction at each time point but only B6 mice show diffuse white matter vacuolation (spongiosis). A, C, E, G, I, and K are stained with hematoxylin and eosin; B, D, F, H, J, and L are stained with luxol fast blue PAS. (A–D) Day 14 postinfection. Microglial nodules (clusters of nuclei in the center of each field) in brain stem of B6 (A, B) and B-cell-deficient (C, D) mice. Pink PAS-positive foamy macrophages in B and D indicate focal tissue breakdown and phagocytosis. Original magnification, 391 \times . (E–H) Day 14 postinfection. White matter vacuolation without inflammation or myelin phagocytosis (PAS-positive macrophages) in the cerebellum of a normal mouse (vacuolation score = 3) (E, F), but not in the cerebellum of a B-cell-deficient mouse (vacuolation score = 0) (G, H). Original magnification, 391 \times . (I–L) Day 21 postinfection. Vacuolation without myelin phagocytosis in brain stem white matter of a B6 mouse (vacuolation score = 3) (I, J), but not in the brain stem white matter of a B-cell-deficient mouse (vacuolation score = 0) (K, L). Original magnification, 488 \times .

ally showed more diffuse white matter vacuolation beginning on day 7 and persisting thereafter (Figs. 2E, 2F, 2I, and 2J). These lesions were not inflammatory and were not associated with astrogliosis or detectable axonal injury, as assessed in representative sections stained by immunoperoxidase for glial fibrillary acidic protein and by Bielschowsky preparation, respectively (not shown). No similar white matter vacuolation was seen in the B-cell-deficient mice (Figs. 2G, 2H, 2K, and 2L), and the vacuolation scores in B-cell-deficient mice were less than those in the B6 mice from days 4 to 21 (Table 4).

Viral Titrations

In the SFV-infected B6 mice, viral titers were maximal on day 6 pi (10^{-5}), decreased on day 7 pi (10^{-2}), and became undetectable thereafter ($<10^{-2}$). In the B-cell-deficient mice, viral titers were high on day 7 pi (10^{-5}), remained high (10^{-4}) until day 21 pi, and then became undetectable thereafter.

DISCUSSION

The present study investigated the involvement of B cells in the pathogenesis of SFV-induced CNS injury.

TABLE 4
Histopathology^a

Day post infection	No. brain inflammatory foci		White matter vacuolation scores	
	B6	B cell deficient	B6	B cell deficient
0-2	0, 0, 1	0	0, 0, 0	0
4-7	0, 3, 17, 22	69	1, 2, 2, 2	0
10-14	1, 2, 10, 11, 14	16, 47	1, 2, 2, 2, 3	1, 0
21	0, 0, 2, 8	10	1, 3, 3, 3	1
35	ND	5	ND	0
Vacuolation score (days 4-21)	—	—	2.1 ± 0.2*	0.5 ± 0.3*

^a Inflammation and vacuolation scoring is described under Materials and Methods. Each number is either total foci or vacuolation score in a single section of the entire brain of an individual mouse; ND, not done.

* Mean ± SE, $P < 0.01$.

In SFV-infected B6 mice, paralytic disease was severe and CNS viral titers were maximal on day 6 pi and decreased substantially on day 7 pi. In contrast, the B-cell-deficient mice had a later onset of a less severe clinical disease, despite the fact that their CNS viral titers were much higher than those of B6 mice on day 7 pi and remained high for a minimum of 2 weeks. This indicates an immunopathological mechanism related to B cells for the induction of disease that may be impaired in the B-cell-deficient mice. Similar to the SFV-infected mice, herpes simplex virus-1 (HSV-1)-infected B-cell-deficient mice also failed to clear virus from the brain, but unlike the SFV-infected mice, the clinical disease and CNS inflammatory responses in HSV-1-infected B-cell-deficient mice were greater than those in wild-type B6 mice (15). The reasons for these differences are unclear, but might relate to a difference in cytopathic effects of HSV-1 compared with SFV. Since the B-cell-deficient mice might have other immunologic abnormalities, e.g., of antigen presentation or NK activity, it is also possible that these may affect their responses to the SFV infection.

The viral titration results suggest that B cells and antibodies are the likely mechanism for SFV clearance in normal B6 mice. However, the greater proportions of CD3⁺CD8⁺ T cells in the brains of the B-cell-deficient mice, compared with normal B6 mice, on day 21 pi (Fig. 1B) suggest that a compensatory mechanism, such as cytotoxic T lymphocytes (CTLs), mediates viral clearance at this later time point in the B-cell-deficient mice. In lymphocytic choriomeningitis virus (LCMV) infection of B-cell-deficient mice, Homann *et al.* (16) suggested that slower clearance of virus from the B-cell-deficient mice might relate to deficiencies in CD4⁺ or CD8⁺ T cell functions (16). Since there were no clear differences in the extent of CD3⁺CD4⁺ cell infiltration in the brains of B6 and B-cell-deficient mice, the role of CD8⁺ T cells in SFV clearance becomes even more apparent. Studies by Asano and Ahmed, 1996 (17), also

demonstrated the expansion of CD8⁺ CTL following LCMV infection in B-cell-deficient mice (17). However, these CD8⁺ CTL died rapidly, indicating that B cells may be important for the delivery of anti-apoptotic signals through B7-1, B7-2, and/or CD40 (17).

B cells have the ability to contribute to the development of autoimmune disease. It has been suggested that B cell activation and antibody responses are necessary for the full development of MS and EAE (10, 18-20). Recent studies by Genain *et al.* identified autoantibodies against MOG within acute lesions of human MS and *C. jacchus* EAE, in which they seemed to be directly responsible for the disintegration of the myelin sheaths. These authors concluded that in diseases initiated by T cell responses, antibodies against essential antigens of the target organ may be required for the development of irreversible CNS tissue damage (11). Similarly, our histopathological studies revealed the presence of white matter injury in active SFV infection, which correlated with systemic antibody responses to myelin peptides.

Earlier findings in our laboratory have suggested that antibody responses to an epitope of SFV that exhibit molecular mimicry with a peptide of MOG contribute to the demyelinating lesions induced following SFV infection (9). The role of antibody was especially evident in SFV peptide E2-115-129-immunized mice, in which the later onset of clinical disease coincided with antibody production (9). In the present study we found that increased percentages of CD19⁺ B cells were detected in CNS of SFV-infected B6 mice on day 21 pi. In contrast, CD19⁺ B cells were not obtained from the CNS or spleens (data not shown) of B-cell-deficient mice at any time point. In addition, SFV-infected normal B6 mice made anti-SFV and anti-E2, anti-MOG, and anti-MBP peptide antibody responses, while B-cell-deficient mice displayed much lower or no responses to these antigens. These results are consistent with induction of cross-reactive anti-myelin anti-

body responses in active SFV infection. It has been shown that B-cell- and antibody-deficient RAG-1^{-/-} mice are also susceptible to EAE adoptively transferred by MOG-35-55-specific T cells (21). This finding is in agreement with studies by Hjelmstrom *et al.* (22), who found that B cells and antibodies are not necessary for full clinical disease in MOG-induced EAE. These data suggest that B cells or their products may play a role in immune regulation in EAE. B cells could also influence cytokine production and immune deviation or regulation and selection of functional T cell repertoires (22).

Our histopathology results showed that SFV-infected B6 and B-cell-deficient mice had similar numbers of inflammatory focal lesions with PAS-positive macrophages. However, SFV-infected B6 mice showed widespread vacuolation in the white matter, while B-cell-deficient mice showed a lesser degree of vacuolation. Previous studies by electron microscopy have shown that diffuse white matter injury in SFV infection is characterized by a spongy degeneration of myelin identical to that observed in EAE and MS (6). In this study, we have also shown that systemic antibody responses to previously described mimicked myelin epitopes correlate with brain white matter injury in active SFV infection. The CNS inflammatory response to SFV would allow serum antibodies to enter the CNS, but there likely is intrathecal antibody production as well, as described by Knopf *et al.* (23). However, despite the closeness of the demonstrated correlation between antibody responses and development of white matter vacuolation, a direct mechanism of action of antibodies in spongiform white matter injury remains to be proven. Our findings are in agreement with studies by Genain *et al.* (10, 11), Schlusener *et al.* (24), and Linington *et al.* (25), which showed enhanced demyelination in EAE with anti-MOG mAb, and with those of Litzenberger *et al.* (26), who demonstrated inflammation-dependent, MOG antibody-induced injury.

In summary, our findings show that SFV-infected B-cell-deficient mice had (1) a milder and more delayed onset of clinical disease and persistent viral titers in their CNS, (2) no antibody responses to SFV E2 antigens and myelin peptides, and (3) significantly less white matter vacuolation. These studies suggest that B cells infiltrating the CNS, as well as anti-myelin antibodies, may contribute to the pathogenesis of CNS white matter injury following SFV infection. Similar responses may contribute to CNS myelin injury in MS.

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