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Analysis of Antibody Responses to Predominant Linear Epitopes of Theiler's Murine Encephalomyelitis Virus

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Using synthetic peptides, we have defined the major linear antibody epitopes of Theiler's murine encephalomyelitis virus (TMEV), i.e., A1A ($VP1_{12-25}$), A1Ba ($VP1_{146-160}$), A1Cb ($VP1_{262-276}$), A2A ($VP2_{2-16}$), A2B ($VP2_{165-179}$), and A3A ($VP3_{24-37}$). A time course study with either pooled or individual sera indicates that susceptible SJL mice intracerebrally infected with TMEV strongly and selectively recognize the A1Cb epitope of VP1, compared with resistant BALB/c or C57BL/6 mice, which broadly recognize most of the epitopes on the different capsid proteins. However, antibodies from SJL mice subcutaneously immunized with TMEV recognize primarily A1Ba, A1Cb, and A2A epitopes. A similar predominant recognition of the A1Cb epitope was found with antibodies from the cerebrospinal fluid of intracerebrally virus-infected SJL mice. Interestingly, a substantial level of antibodies against the A1Cb epitope in virus-infected SJL mice is of the immunoglobulin G2a subclass, in contrast to an undetectable level of this immunoglobulin G subclass in virus-immunized SJL mice. The level of in vitro viral neutralization by antibodies did not correlate with the clinical signs. Antibodies to A1Cb, A2A, and A2B were able to neutralize viral plaque formation in vitro, while antibodies to A3A, A1A, and A1Ba were not.

Either the DA strain or the BeAn strain of Theiler's murine encephalomyelitis virus (TMEV) induces chronic, immune-mediated demyelination when intracerebrally inoculated into susceptible mouse strains (27, 29, 32, 45, 53, 54). TMEV, like other picornaviruses, has four structural capsid proteins (VP1, VP2, VP3, and VP4) assembled as an icosahedral structure (18, 33, 42, 44). The clinical signs of TMEV-induced demyelination include a spastic waddling gait, extensor spasms, and incontinence (29, 31). The histopathology of the virally induced demyelination consists of mononuclear cell infiltration and myelin sheath damage limited to the white matter (11, 27, 29). In addition, many immunological and genetic parameters associated with susceptibility to this disease parallel those of human multiple sclerosis (1, 10, 15, 22, 23, 32, 45, 51), and thus this system is considered to be one of the best infectious animal models for multiple sclerosis.

Antibody epitopes which are involved in neutralization of viral infectivity have previously been investigated (15, 39, 48, 58, 59), although no attempt has been made to compare the antibody levels of mouse strains susceptible to viral demyelination with the levels of strains resistant to it. These studies have suggested that the major neutralizing epitopes reside on VP1 and that these epitopes may also be involved in the virally induced pathogenesis observed in studies of escape mutants of TMEV (48, 59). Diseases induced by picornaviruses, including poliovirus, foot-and-mouth disease virus, and Theiler's virus, can be effectively prevented by preimmunization with either killed or attenuated viruses (9, 40, 50); the protection is mainly due to the production of virus-neutralizing antibodies to VP1 epitopes (2, 4, 58). In addition, studies with chimeric viruses constructed from the neurovirulent GDVII strain and demyelinating DA or BeAn strains of the TO (Theiler's original isolates) subgroup have suggested that a region including VP1 plays a role in viral persistence and demyelination (16, 35, 52). This is particularly important because viral persistence in the central nervous system (CNS) appears to be prerequisite for susceptibility to viral pathogenesis of demyelination.

Recently, we prepared a battery of fusion proteins in the λgt11 expression system, which contain overlapping areas of the entire capsid proteins derived from the TMEV BeAn strain (8, 24). Utilizing these recombinant λgt11 clones, we identified six major areas (13 to 26 amino acid residues) recognized by antibodies from resistant C57BL/6 and BALB/c mice or susceptible SJL mice. Interestingly, the epitopes recognized by SJL mice are different from those recognized by resistant C57BL/6 or BALB/c mice, especially when the antibodies are obtained from mice intracerebrally (i.c.) infected with live TMEV BeAn. Although the initial epitope analysis using recombinant λgt11 clones was informative, this system has several inherent disadvantages for further analyses. For example, quantity and purity of the fusion proteins are difficult to control. In addition, the large (120-kDa) β-galactosidase portion of the fusion proteins may have an influence on the presentation of epitopes.

In order to confirm and extend our earlier studies with λgt11 fusion proteins, we prepared a series of synthetic peptides including the six major antibody epitope areas of TMEV previously identified (24). In this paper, we now further define the major linear antibody epitopes within 15 amino acid residues. In addition, relative antibody levels specific for each antibody epitope during the course of TMEV infection and postimmunization have been examined to assess the differences in antibody epitope recognition. Subcutaneous (s.c.) immunizations prior to viral infection prevent development of the disease after subsequent infection with live virus, while

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similar immunizations given after viral infection exacerbate the viral pathogenesis of demyelination (9), suggesting that timing may be more important than the level of the immune response. Our results indicate that the antibody response to A1Cb, the C-terminal epitope of VP1, which exhibits a strong virus-neutralizing activity in vitro, is more elevated in susceptible SJL mice with clinical symptoms than in resistant BALB/c or C57BL/6 mice. Although the relationship between virally induced demyelination and the particularly strong antibody response to this epitope is not yet clear, the potential involvement of antibodies to this viral epitope in the pathogenesis of immune-mediated demyelination is here discussed with respect to the immunoglobulin (Ig) isotypes and the capacity for in vitro viral neutralization.

MATERIALS AND METHODS

Animals. Inbred mouse strains (C57BL/6, BALB/c, and SJL/J) were purchased from either the Jackson Laboratories, Bar Harbor, Maine, or the National Cancer Institute.

Viruses. The BeAn 8386 strain of TMEV was propagated in BHK-21 cells grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 7.5% donor calf serum and purified by isopycnic centrifugation on Cs_2SO_4 gradients as previously described (9).

Synthetic peptides. The synthetic peptides representing the amino acid residues of various antibody epitopes of TMEV were prepared by using the RaMPS system (DuPont Co., Wilmington, Del.) with 9-fluorenylmethyloxycarbonyl reagents. A major single peptide (>95%) was present in each of the peptide preparations according to the reverse-phase high-pressure liquid chromatography analyses.

Injection of mice with TMEV. For i.c. inoculation of virus, 1.3×10^6 PFU of virus in 30 μl was administered into the right cerebral hemisphere of mice anesthetized with methoxyflurane. This inoculum consistently induced neurological signs in susceptible mouse strains (8).

Assessment of demyelinating disease. Clinical symptoms of demyelination in TMEV-infected mice were examined by observation of mice. Mice were allowed to walk on a polyethylene (Dynalab) walking board and were observed for symptoms which included a waddling gait, extensor spasms, paralysis, loss of the righting reflex, incontinence, and/or hunched posture.

Immunization of mice with TMEV and peptide conjugates. Mice were injected s.c. with 100 μl (25 μg) of a 1:1 mixture of UV-inactivated TMEV BeAn (~ 2,000 ergs/mm^2/min for 30 min) and complete Freund's adjuvant. Follow-up injections were given 10 days apart for a total of three injections. These injections were the same as described above except that incomplete Freund's adjuvant was used instead of complete adjuvant. Cerebrospinal fluid was obtained from mice i.c. infected with live virus or s.c. immunized with UV-inactivated virus according to the method of Fleming et al. (14). Pooled cerebrospinal fluid was used for enzyme-linked immunosorbent assay (ELISA) after examination for blood contamination, which was less than 1 part in 10,000 as determined by using microscopic criteria.

Antibodies against synthetic peptides were similarly prepared. The synthetic peptides (1 mg) were conjugated to keyhole limpet hemocyanin (15 mg) with 0.1% glutaraldehyde. After extensive dialyses, the peptide conjugates (100 to 150 μg per mouse), emulsified in Freund's adjuvants, were injected into separate groups of mice as described above.

Rabbit antibodies to TMEV were prepared by repeated immunizations of a New Zealand White rabbit with UV-inactivated TMEV BeAn 8386 as described previously (8). The initial immunization was performed by s.c. injections of virus (200 μg) in complete Freund's adjuvant, and the subsequent booster injections were made in incomplete Freund's adjuvant. The antisera from rabbits immunized with individual viral capsid proteins isolated by electroelution from sodium dodecyl sulfate (SDS)-polyacrylamide gels exhibited their respective monospecificity in a Western blot (immunoblot) assay.

Immunodot assay. Nitrocellulose papers were placed on a Whatman 3MM paper presoaked with phosphate-buffered saline (PBS). Either TMEV or synthetic peptides with or without conjugation to bovine serum albumin (BSA) (0.1 μg in 1 μl) were then applied to the filter paper. The synthetic peptides (1 mg) were conjugated to BSA (5 mg) with 0.1% glutaraldehyde and were extensively dialyzed. The rates of conjugation to BSA were similar for individual peptides, as analyzed on a molecular weight basis by using SDS gel electrophoresis. After a brief incubation (2 to 5 min), the filters were blocked in 1% BLOTTO and incubated with a 1:250 to 1:10,000 dilution of either normal control sera or immune sera. Unless otherwise indicated, a pool of sera was prepared by adding equal volumes of individual sera from each experimental group. These filters were washed and then incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Promega). The color reaction of the enzyme was developed by adding a mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (21). The level of IgG subclasses specific for TMEV epitopes was similarly assessed with appropriate biotin-labeled, goat anti-mouse IgG subclass reagents (CalTag) followed by strepavidin conjugated with alkaline phosphatase.

Western analysis. Precleared culture supernatants from TMEV-infected BHK-21 cells were dissolved in 2% SDS-5% 2-mercaptoethanol, and the individual polypeptides were then separated by 12% polyacrylamide gel electrophoresis according to the method of Laemmli (26) with a vertical-slab gel apparatus. The separated polypeptides in the acrylamide gel were transferred onto nitrocellulose paper in Tris glycine-methanol buffer, pH 8.3 (56), with a Bio-Rad electroblotting apparatus. The nitrocellulose paper was preincubated with 1% BLOTTO and was subsequently incubated with appropriately diluted antibodies, followed by alkaline phosphatase-conjugated goat anti-mouse IgG. Finally, the colorimetric reaction of alkaline phosphatase was developed by adding the substrates as described above.

ELISA for detection of TMEV antigens. Antibodies specific for viral epitopes were measured by an adaptation (8) of the indirect ELISA as described previously (12). Briefly, 0.3 μg of either total virus or individual peptide-BSA conjugate was used to coat microtiter plates. A BSA solution (0.3 μg) was also used to coat the plates, to serve as a negative control. Unless otherwise stated, twofold serial dilutions of sera starting from a 1:100 ($2^0 \times 100$) dilution were reacted with the antigens on the microtiter plates and then with goat anti-mouse secondary antibody conjugated with alkaline phosphatase. After the plates were washed, substrate (*p*-nitrophenyl phosphate) for the enzyme was added, and the enzyme reaction was colorimetrically measured by an ELISA reader at 410 nm. The antibody titers of ELISA represent $\log_2 \times 100$.

Viral neutralization assay. The abilities of the antibodies specific for TMEV and its individual epitopes to neutralize viral infection in vitro were assessed by measuring the inhibition of viral plaque formation as described previously (32). Various dilutions of antisera and control normal serum were added to monolayers of BHK cells in 60-mm petri dishes and were incubated for 30 min prior to the addition of infectious

TABLE 1. Synthetic peptides used and their reactivities to antibodies

Peptide	Position	Reactivity[a]	
		SJL	C57BL/6
A1A	12 NDDASVDFVAEPVK 25	−	+
A1Ba	146 RWAPTGAPADVTDQL 160	+	+
A1Bb	153 PADVTDQLIGYTPSL 167	−	−
A1Ca	251 TLFFPWPTPTTTKIN 265	−	−
A1Cb	262 TKINADNPVPILELE 276	+	+
A2A	2 QNTEEMENLSDRVAS 16	+	+
A2B	165 TGYRYDSRTGFFATN 179	+	+
A2c	250 QPVNPVFNGLRHETVIAQ 267	−	−
A3A	24 PIYGKTISTPSDYM 37	+	±
A3c	215 GDDFTLRMPISPTKWVPQ 232	−	−

[a] 1:300 dilutions of hyperimmune sera from either SJL or C57BL/6 mice that had been s.c. immunized three times with UV-inactivated TMEV.

TMEV (150 to 200 PFU in 500 μl of DMEM). After an additional incubation with TMEV for 45 min, a layer of agar in DMEM was gently poured onto the virus-infected cells and incubated for 5 days at 33°C. The number of viral plaques was then counted following a viable staining of the cell layers with 0.1% neutral red in PBS. Reduction of the PFU by 50% in the presence of antibodies compared with normal control sera was considered a positive result for viral neutralization.

RESULTS

Verification of the antibody epitopes with synthetic peptides. Our earlier studies, using recombinant λgt11 clones (8, 24), have indicated that the number of major conformation-independent, linear antibody epitopes of TMEV is restricted; six such epitopes have been identified and localized on individual capsid proteins. In order to verify and to further define the boundary of these antibody epitopes, we have generated a series of synthetic peptides corresponding to the epitope regions previously determined with recombinant λgt11 clones. The immunodot assay indicates that the synthetic peptides (A1A, A2A, A2B, and A3A) corresponding to the antibody epitope regions are indeed recognized by anti-TMEV antibodies (Table 1). Hyperimmune sera were used for the initial identification of linear antibodies, since we have shown previously that such immunization induces high levels of antibodies to a broad range of linear antibody epitopes (24). The areas of A1B ($VP1_{146-167}$) and A1C ($VP1_{251-276}$) epitopes previously determined by recombinant phages have been further defined with two overlapping peptides for each epitope area: A1Ba ($VP1_{146-160}$) and A1Bb ($VP1_{153-167}$) for the A1B area and A1Ca ($VP1_{251-265}$) and A1Cb ($VP1_{262-276}$) for the A1C area. The results indicate that the antibody reactivities to the A1B and A1C epitopes reflect those of A1Ba and A1Cb, respectively. In addition, a synthetic peptide representing A3A ($VP3_{24-37}$), which is 6 amino acids smaller than the initially identified epitope, displayed antibody reactivity. Since our initial recombinant λgt11 clones failed to cover the C-terminal regions of VP2 and VP3, we have also included synthetic peptides reflecting these regions, i.e., $VP2_{250-267}$ and $VP3_{215-232}$. However, these regions do not appear to be recognized by anti-TMEV antibodies. Table 1 displays the list of synthetic peptides used and their reactivities with antibodies from SJL and C57BL/6 mice hyperimmune to TMEV. These results with synthetic peptides are consistent with our initial epitope study with the λgt11 system and further define the major linear antibody epitope regions within 15 amino acid residues.

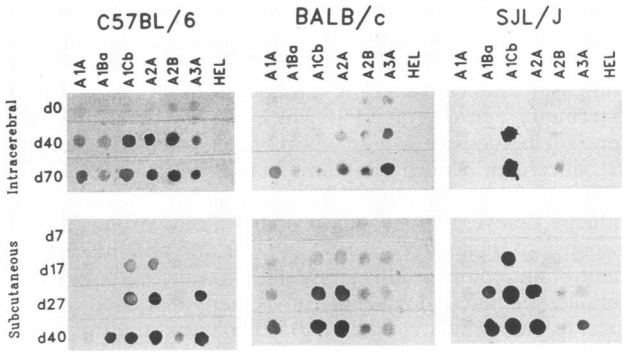

FIG. 1. Immunodot assay demonstrating relative reactivities of pooled sera from mice i.c. infected or s.c. immunized with TMEV to individual linear antibody epitopes. Preimmune sera and sera bled at day 40 and day 70 after i.c. infection with live TMEV (1:200) were tested for the analysis of epitope reactivity. Sera bled at day 7, day 17, day 27, and day 40 after the initial s.c. immunization (1:2,000) were also tested. HEL_{47-61} corresponding to hen egg lysozyme residues of amino acids 47 to 61 was used as a control peptide.

Analysis of epitope recognition following infection or immunization with TMEV. In order to assess the level of antibody responses to individual epitopes following viral infection or immunization, five mice of each strain (SJL, BALB/c, and C57BL/6) were i.c. infected with TMEV, and sera from the mice were collected at days 0, 10, 20, 40, and 70. All five susceptible SJL mice developed a typical TMEV-induced demyelinating disease 30 to 35 days after i.c. inoculation of virus, and none of the resistant strains exhibited clinical signs. An additional three mice of each strain were s.c. immunized with UV-inactivated TMEV in complete Freund's adjuvant for the first immunization at day 0 and for the subsequent immunizations at days 10 and 20 in incomplete Freund's adjuvant. The mice were bled at days 7, 17, 27, and 40. These sera were tested for epitope recognition in order to compare the differences in the specific antibody levels among these mouse strains at different time points after viral infection or immunization.

The level of predominance of antibody epitopes was tested by immunodot assay using peptide-BSA conjugates (Fig. 1). The level of antibodies to A1A increased noticeably in both C57BL/6 and BALB/c mice i.c. infected with TMEV compared with that in virus-infected SJL mice. However, a major population of antibodies from susceptible SJL mice infected with live TMEV appeared to react with the A1Cb epitope. In contrast, antibodies from resistant C57BL/6 mice similarly i.c. infected with TMEV strongly recognized A1Cb and the other epitopes as well, including A1A, A2A, A2B, and A3A. The pattern of predominant antibody response to a single viral epitope, A1Cb, appears to be unique in susceptible SJL mice i.c. infected with virus, since the antibodies produced by the same mouse strain immunized with UV-inactivated virus strongly recognize additional epitopes, including A1Ba, A2A, and A3A.

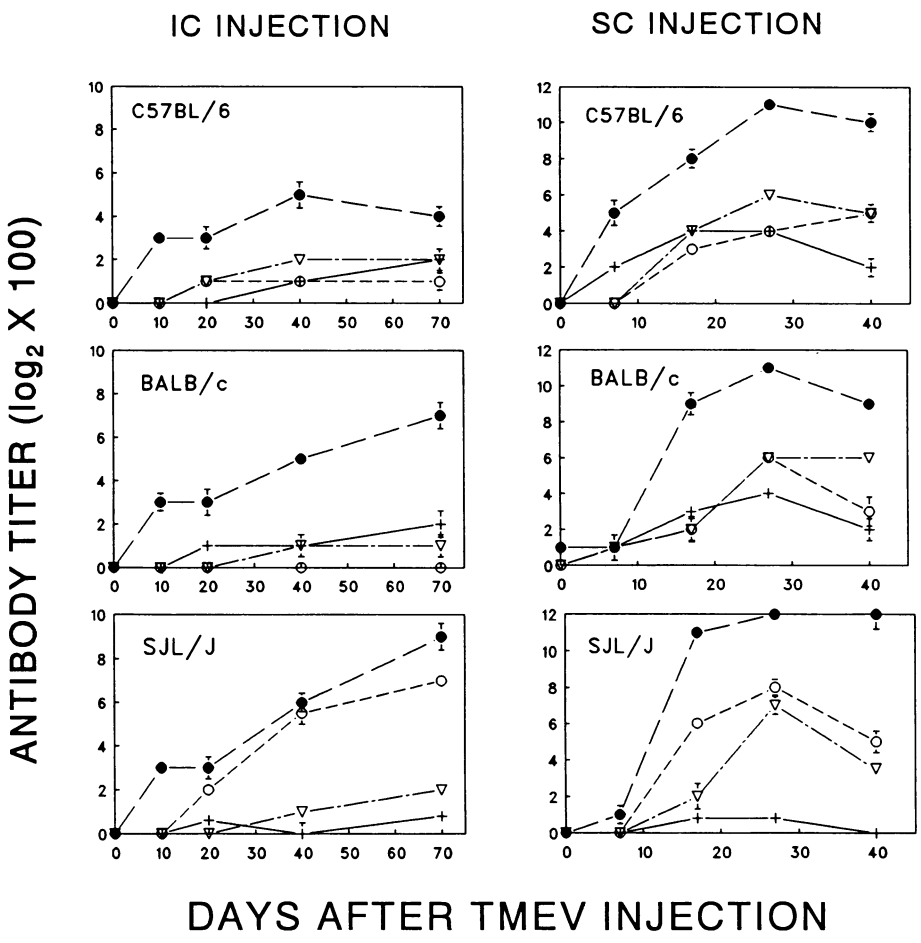

FIG. 2. Titers of pooled sera from C57BL/6, BALB/c, and SJL mice either i.c. infected or s.c. immunized with TMEV to various antibody epitopes. Sera were obtained either from mice 70 days after i.c. infection with live TMEV or from mice 40 days after the initial immunization (two additional booster injections after 10 days each) with UV-inactivated TMEV. A twofold serial dilution starting at 1:100 was tested for reactivity of the sera to the peptides or purified TMEV. The symbols used in this figure represent titers of antibodies as follows: ●, TMEV; +, A1A; ○, A1Cb; and ▽, A2A. The vertical lines indicate standard errors of triplicate determinations.

Figure 2 displays a time course determination of ELISA titers of antibodies against the A1A, A1Cb, and A2A epitopes after i.c. infection or s.c. immunization with TMEV. The antibody titer against TMEV in C57BL/6 mice infected with virus reached a maximal level at day 40 and slightly declined at day 70. The levels of antibodies against all the antibody epitopes also reached maxima between 40 and 70 days after viral infection. In contrast, levels of antibody against TMEV in BALB/c and SJL mice continuously increased until 70 days after viral infection. In SJL mice, the level of antibodies against the A1Cb epitope increased markedly (2^7), whereas levels in both C57BL/6 and BALB/c mice remained low ($<2^1$). Antibodies to the linear epitopes peaked similarly at day 27, and the levels of antibodies against the individual epitopes were similar among all the mouse strains except SJL; SJL mice displayed a low level of antibodies to A1A. Interestingly, A2A appears to be a predominant epitope in all three mouse strains, and A1Cb seems to be a predominant epitope in only SJL mice.

Comparison of IgG subclasses reactive to A1Cb in SJL mice either infected or immunized with TMEV. Previously, it has been shown that either IgG2a (4, 7, 43, 49) or IgG2b (46) represents the major IgG subclass involved in recognition of the whole TMEV virion, especially in susceptible mice. In order to determine the relative levels of different IgG subclasses involved in the recognition of the A1Cb epitope compared with that of intact virus, we have used a modified immunodot assay employing polyclonal antibodies specific for murine IgG subclasses and IgM (Fig. 3). The results clearly indicate that substantial levels of anti-A1Cb antibodies from virus-infected mice belong to the IgG1, IgG2a, and especially the IgG2b subclasses. This distribution of IgG subclasses reactive to A1Cb appears to be somewhat skewed from that of antibodies reactive to whole virus: the levels of IgG1 and IgG2a antibodies reactive to whole virus are relatively higher than those reactive to A1Cb. Interestingly, anti-A1Cb antibodies from s.c. hyperimmunized mice did not include the IgG2a subclass, although the levels of IgG1 and IgG2b antibodies reactive to A1Cb are comparable to those of i.c. virus-infected mice. It is noteworthy that the IgG2a subclass is also lacking among antibodies reactive to the other linear epitopes (e.g., A1Ba and A2A) in SJL mice immunized with UV-inactivated virus. The lack of IgG2a anti-A1Cb antibodies in the immunized mice does not appear to reflect the lack of IgG2a antivirus antibodies. Therefore, it appears that immunizations with UV-inactivated virus result in a marked skewing against

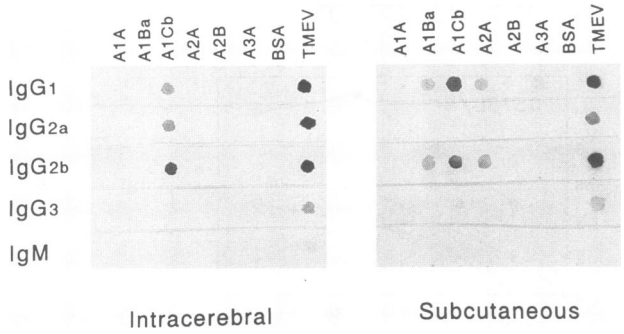

FIG. 3. Comparison of IgG subclasses of antibodies specific for individual antibody epitopes and intact virus produced in susceptible SJL mice either i.c. infected with live virus or s.c. immunized with UV-inactivated virus. A modified immunodot assay was used to analyze IgM antibodies and the subclasses of IgG antibodies as described in Materials and Methods.

this IgG subclass of antibodies recognizing the A1Cb epitope. Since the mice immunized with UV-inactivated TMEV are protected from TMEV-induced demyelinating disease (9) and the level of antibodies to this A1Cb epitope is exceptionally high in virus-infected mice showing clinical symptoms (Fig. 1 and 2), the presence of IgG2a anti-A1Cb antibodies in virus-infected susceptible mice may be associated with the manifestation of TMEV-induced demyelinating disease.

Level of predominant antibody to the A1Cb epitope in cerebrospinal fluid from virus-infected SJL mice. We have further examined whether the predominant recognition of the A1Cb epitope by serum antibodies from virus-infected mice (Fig. 1 and 2) is also shown by antibodies locally produced in the CNS (Table 2). The results clearly show that the only detectable antibodies to viral epitopes were against A1Cb. The titer of antibody against A1Cb is higher than that of antibody against TMEV, and this may reflect a reduced accessibility of

TABLE 2. Comparison of antibody reactivities in cerebrospinal fluid from susceptible and resistant mice toward virus and peptide conjugates[a]

Mouse strain and antibody source	ELISA ($\log_2 \times 100$) reactivity to:						
	A1A	A1Ba	A1Cb	A2A	A2B	A3A	TMEV
SJL (i.c.)							
CSF	1	<1	5	<1	<1	<1	2
Serum	2	1	5	<1	1	1	7
SJL (s.c.)							
CSF	1	<1	<1	<1	<1	<1	<1
Serum	1	4	5	3	<1	2	12
C57BL/6 (i.c.)							
CSF	<1	<1	<1	<1	<1	<1	<1
Serum	4	2	1	2	2	2	7
BALB/c (i.c.)							
CSF	<1	<1	<1	<1	<1	<1	<1
Serum	2	<1	<1	<1	1	1	6

[a] ELISA titers of pooled cerebrospinal fluids (CSF) from three mice in each group i.c. inoculated with live TMEV or s.c. hyperimmunized with UV-inactivated TMEV were determined by using plates coated with BSA, peptide-BSA, or purified TMEV. A serial dilution of cerebrospinal fluid was made starting from 1:200 for the titration of specific antibodies. Matching sera from the same mice were similarly tested for antibody titers. The background reactivities of all the reagents against BSA-coated wells were <1.

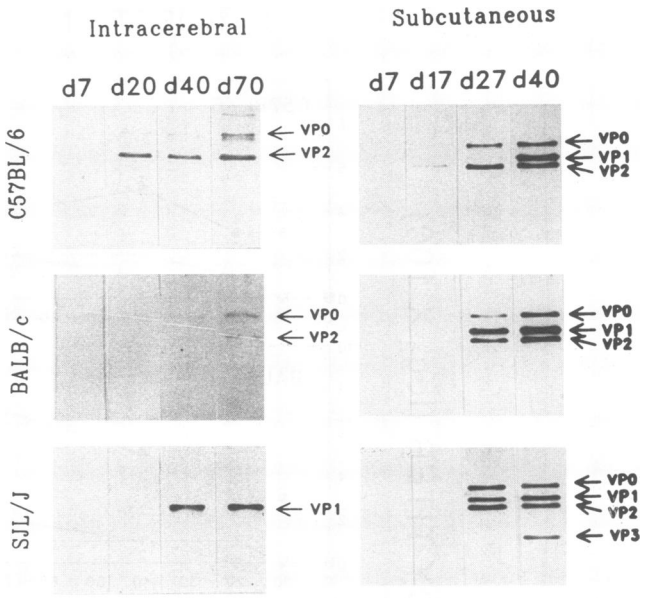

FIG. 4. Western blot analysis of reactivities of pooled sera from either i.c. infected or s.c. immunized mice to virus-infected BHK-21 culture supernatants. The relative reactivities of these antisera to individual TMEV capsid proteins were demonstrated by using 1:200 dilutions for sera from i.c. infected mice and 1:1,000 dilutions for sera from s.c. immunized mice.

this particular epitope when assayed with intact virus. This is not likely due to an insufficient quantity of virus coated onto the plates, since the same procedure can detect anti-TMEV antibodies at greater than $1:10^5$ dilutions of sera from hyperimmune mice. Cerebrospinal fluids from resistant C57BL/6 or BALB/c mice similarly inoculated with live virus did not exhibit such a reactivity toward either the A1Cb epitope or TMEV. In addition, the level of cerebrospinal antibody did not reflect the level of serum antibody against viral epitopes. For example, a pool of cerebrospinal fluids from SJL mice, which had been subcutaneously hyperimmunized with UV-inactivated TMEV and produced a higher level of serum antibody against TMEV (ELISA titer of 12 versus 10) than that of i.c. virus-infected SJL mice, failed to react with either the linear antibody epitopes or intact TMEV. Therefore, the predominance of A1Cb recognition by antibodies in cerebrospinal fluids of virus-infected mice is likely to represent the antibody population resident in the CNS of virus-infected and clinically affected mice.

Differential antibody recognition of virus capsid proteins following viral infection. In order to determine whether the antibody reactivities to individual linear antibody epitopes represent all the major epitopes of capsid proteins, pooled sera from mice either i.c. infected or s.c. immunized with TMEV were tested for their reactivities to individual capsid proteins at different time points by Western blot analysis (Fig. 4). Twenty to forty days after i.c. infection, antibodies reactive to individual TMEV capsid proteins could be detected. In SJL mice, only reactivity to VP1 is readily detected, and only reactivities to VP2 and/or VP4 (VP0 = VP4 + VP2) are observed in either C57BL/6 or BALB/c mice. A particularly high level of antibody response to A1Cb is likely to reflect strong reactivity to VP1 protein in SJL mice, compared with high levels of antibodies to VP2 epitopes in C57BL/6 and BALB/c mice. As predicted on the basis of an immunodot assay (Fig. 1), sera from mice s.c.

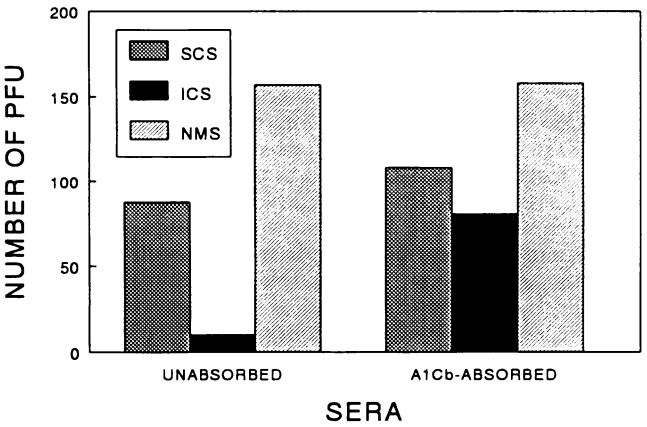

FIG. 5. Determination of abilities of sera from mice i.c. infected with live virus and mice s.c. immunized with UV-inactivated virus to neutralize virus infection in vitro. Virus infectivity was determined by assessing the number of PFU on BHK-21 monolayers after incubating virus in the presence of pooled sera (1:100) from normal, i.c. infected or s.c. immunized SJL mice. The antibody titers of sera from both virus-infected and immunized mice were identical to the results of assays directed toward TMEV. Aliquots of these sera were passed through identical immunoabsorbent columns conjugated with A1Cb-BSA, and their neutralizing activities were measured after adjusting the final volumes to the equivalent of a 1:100 dilution of the original serum pool.

immunized with TMEV recognized VP1 and VP2 capsid proteins in all three mouse strains tested. Interestingly, only antisera from SJL mice recognized the VP3 capsid protein, despite the reactivity to the A3A epitope detected in other mouse strains by immunodot assay. This discrepancy may reflect the differences in antibody recognition between sera from SJL mice and sera from C57BL/6 or BALB/c mice of the A3A epitope presented by the VP3 protein and by peptide conjugate. Alternatively, the titer of antibody to A3A could be lower for C57BL/6 mice than for SJL mice.

In order to verify that the above-described differential recognition of viral proteins with pooled sera is not a reflection of any individual variations within the mouse strain, sera from five individual mice per strain i.c. infected with TMEV for 70 days were tested (data not shown). All five individual C57BL/6 mice produced a detectable level of antibody recognizing only VP2. In contrast, four of five SJL mice produced antibodies predominantly against a VP1 epitope(s). Further testing of these sera by an immunodot assay (data not shown) indicated that the majority of anti-VP1 antibodies recognize the A1Cb epitope as shown with the pooled sera (Fig. 1). These results indicate that experimental results with pooled sera reflect the antibody response of the majority of mice of each strain.

Virus-neutralizing activities of sera from SJL mice infected or immunized with TMEV. Because the antibodies specific for the A1Cb epitope exhibited efficient viral neutralization in vitro (39) and because SJL mice infected with TMEV demonstrate the highest level of antibodies to A1Cb (Fig. 1), we examined the neutralizing activity of pooled sera from SJL mice i.c. infected with virus and compared it with that of pooled sera from SJL mice s.c. immunized (Fig. 5). The antibody titers of the pooled sera from i.c. infected mice and from s.c. immunized mice to whole TMEV were identical (2^9). However, sera from SJL mice infected with TMEV exhibited far stronger neutralizing activity than sera from SJL mice s.c. immunized with UV-inactivated virus. Thus, neutralizing antibodies may not be effective in protecting mice from developing the disease.

In order to examine whether the high neutralizing activity seen in sera from mice i.c. infected with virus reflects the predominant antibody response to A1Cb, antibodies reactive to this epitope were eliminated after absorption using an A1Cb-conjugated affinity column. No reduction of TMEV neutralizing activity was seen with a similar column conjugated with an unrelated peptide (data not shown). This specific elimination of anti-A1Cb antibody from sera of virus-infected mice drastically reduced the neutralizing activity compared with the neutralizing activity after the same treatment of sera from mice immunized with the virus (Fig. 5). This result strongly suggests that the major population of neutralizing antibodies in sera from virus-infected mice is reactive with A1Cb.

Neutralization of in vitro viral plaque formation by antibodies specific for individual epitopes. In order to determine whether antibodies to the individual linear antibody epitopes are able to neutralize in vitro viral infection, monospecific antibodies to the linear epitopes were prepared in C57BL/6 mice by s.c. immunization with synthetic peptides conjugated to keyhole limpet hemocyanin. The resulting antibodies specifically recognized the respective TMEV viral proteins when assessed by Western blot analysis (Fig. 6A). These antibodies were subsequently tested for their abilities to inhibit viral infection in vitro (Fig. 6B). The results of viral plaque assay on BHK-21 monolayers indicate that antisera specific for A1Cb, A2A, and A2B can neutralize viral plaque formation (Fig. 6B). Antibodies specific for A3A showed only a marginal inhibition, and antibodies specific for the other epitopes (A1A and A1Ba) did not display such an activity. In order to determine a relative level of neutralization by anti-A1Cb antibody compared with levels of neutralization by antibodies to other epitopes, titers of these antisera against the corresponding peptide conjugates were normalized and then tested for their neutralizing capabilities (Fig. 6C). The results strongly suggest that A1Cb is the most potent neutralizing epitope among the linear antibody epitopes.

On the basis of a space-filling model (Fig. 7) of the recent X-ray crystallographic structure of TMEV BeAn (33), the linear antibody epitopes are generally external and are located at the extruding areas of the individual capsid proteins, although the degrees of exposure vary somewhat. Interestingly, the neutralizing epitopes appear to be most exposed and are located near the putative cellular receptor binding site (18, 33). Since the A1Cb epitope appears to be the closest to the receptor binding site and exhibits the strongest neutralizing activity, antibodies to these epitopes may interfere with the binding of virus via cellular receptors. A nonneutralizing A1A epitope is located near the potential receptor binding site directly beneath the A1Cb epitope. The lack of viral neutralization by antibodies reactive to this epitope may reflect the poor accessibility of the epitope.

DISCUSSION

It has previously been shown that antibodies with relatively restricted heterogeneity are found in the spinal fluids of virus-infected mice (47) as well as multiple sclerosis patients (34). In addition, B cells producing antibodies to VP1 and VP2 proteins are also found in the demyelinating lesions (4). However, the role of humoral antibodies in the demyelination process is not yet clear. Mice with B cell deficiency induced by neonatal treatment with anti-μ antibodies exhibited an exacerbated disease course, suggesting that antibodies specific for

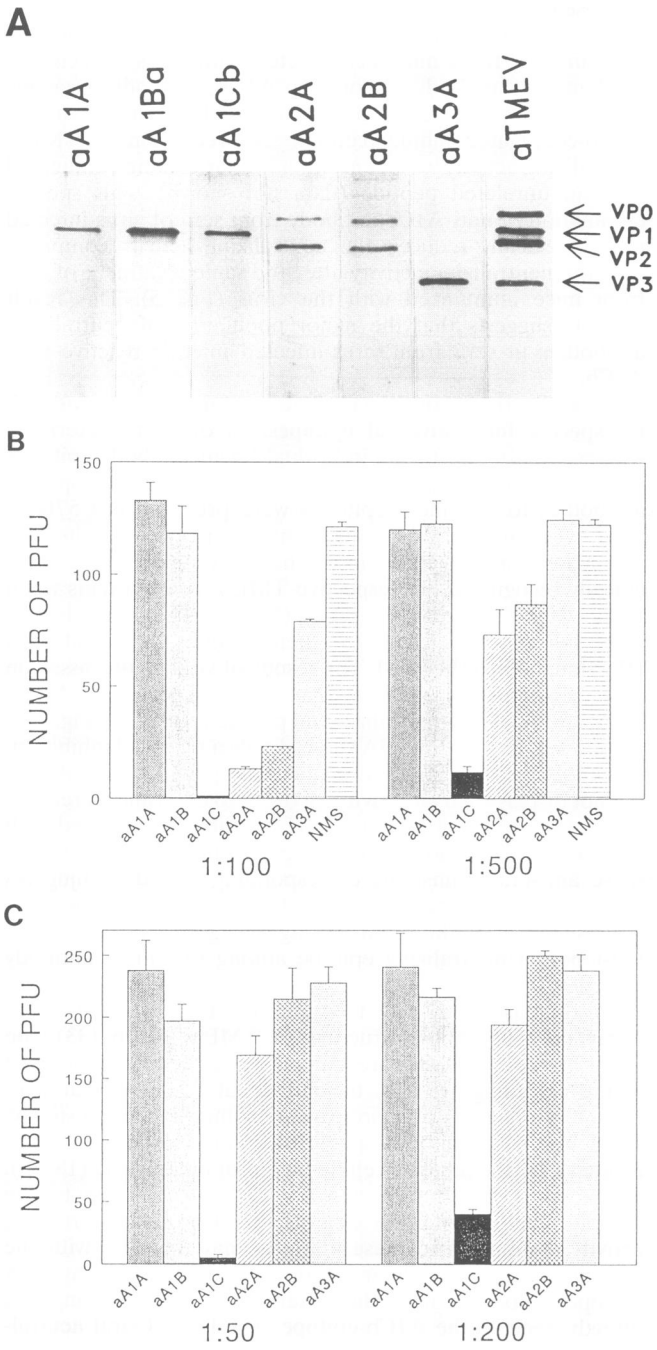

FIG. 6. In vitro neutralizing activities of monospecific antibodies to individual linear antibody epitopes. (A) Antibodies prepared against the individual peptide-keyhole limpet hemocyanin conjugates specifically react with the respective viral capsid proteins containing the epitopes. Western blot analysis was used to determine the reactivities of epitope-specific antibodies against virus-infected BHK-21 culture supernatants as described above. (B) Determination of the abilities of epitope-specific antibodies for in vitro viral neutralization. Two different dilutions (1:100 and 1:500) of antibodies were used to examine the effect on viral neutralization as described above. (C) Comparison of relative neutralizing activities of the epitope-specific antibodies. The individual antibodies were adjusted to yield similar ELISA titers against the respective peptides, and then their abilities for viral neutralization were examined.

TMEV may be involved in the protection of the host (46). The ability to generate virus-specific IgM antibodies may account for resistance in certain mouse strains, as shown by protection by passive transfer of such antibodies from resistant C57BL/6 to susceptible SJL mice (49). Interestingly, a recent report suggests that SJL mice may produce anti-TMEV antibodies with a higher IgG2a/IgG1 ratio than that of C57BL/6 mice when the BeAn viral strain is used (43). Therefore, differences in Ig class or subclass may influence the progression of the disease and/or protection of the host from the disease.

In this paper, we verify and further define the predominant antibody epitopes reflecting linear regions of viral capsid proteins by using synthetic peptides with 15 or fewer amino acid residues (Table 1). The number and regions defined by synthetic peptides are in good agreement with our previous results obtained by using recombinant λgt11 clones expressing overlapping TMEV capsid regions (8, 24). Interestingly, most of the linear antibody epitopes appear to be externally exposed and clustered along the side of the junction where two icosahedral units meet (Fig. 7). Of the six predominant linear antibody epitopes, three are involved in viral neutralization (Fig. 6), and these neutralizing epitopes are located near the putative cellular receptor site (18, 33). These observations are parallel to those for antibody responses to other picornaviruses (2, 5, 17, 19, 55, 58). However, comparison of antibodies specific for individual epitopes from virus-infected mice is rather difficult, due to the relatively low level of antibody titers. Similarly, it is difficult to compare the relative titers of antibodies against conformational and linear epitopes, because of the potential conformational changes under the antibody assay conditions. Consequently, our experimental results for assessing antibodies are not fully quantitative. However, we have clearly demonstrated the relative differences in the levels of antibodies against the individual linear antibody epitopes in three different mouse strains either infected or immunized with TMEV.

The antibody titer of virus-infected SJL mice against the A1Cb epitope appears to be exceptionally high compared with titers of antibodies from C57BL/6 and BALB/c mice (Fig. 1 and 2). In contrast, these SJL mice produce relatively low levels of antibodies to A1A, A2A, and A3A epitopes, while high levels of antibodies are produced by similarly infected resistant C57BL/6 or BALB/c mice (Fig. 1 and 2). These preferential epitope recognition patterns by antibodies from virus-infected mice are consistent with our Western blot analysis (Fig. 4) demonstrating the differential viral protein reactivity, i.e., VP1 by SJL sera and VP2 by BALB/c and C57BL/6 sera. It is conceivable that the differences in the recognition patterns of antibodies from s.c. immunized and from i.c. infected mice may reflect viral structure altered by UV irradiation. However, the same immunization schedule with nonirradiated, intact virus resulted in an antibody recognition pattern similar to that of UV-inactivated virus (data not shown). This is consistent with the similar antibody responses to intact and to UV-inactivated rhinovirus and poliovirus, which are, however, different from the antibody response induced by heat-inactivated virus (20). Therefore, the predominance of antibody response appears to be influenced in our studies primarily by the route of immunization rather than by potential structural differences resulting from UV inactivation. A similar predominance is seen in the A1Cb recognition by antibodies from cerebrospinal fluids of virus-infected, clinically affected mice (Table 2). Antibodies recovered from the CNS may represent locally produced antibodies because no such CNS antibodies are detected from either hyperimmunized, healthy, susceptible SJL mice or virus-infected, resistant

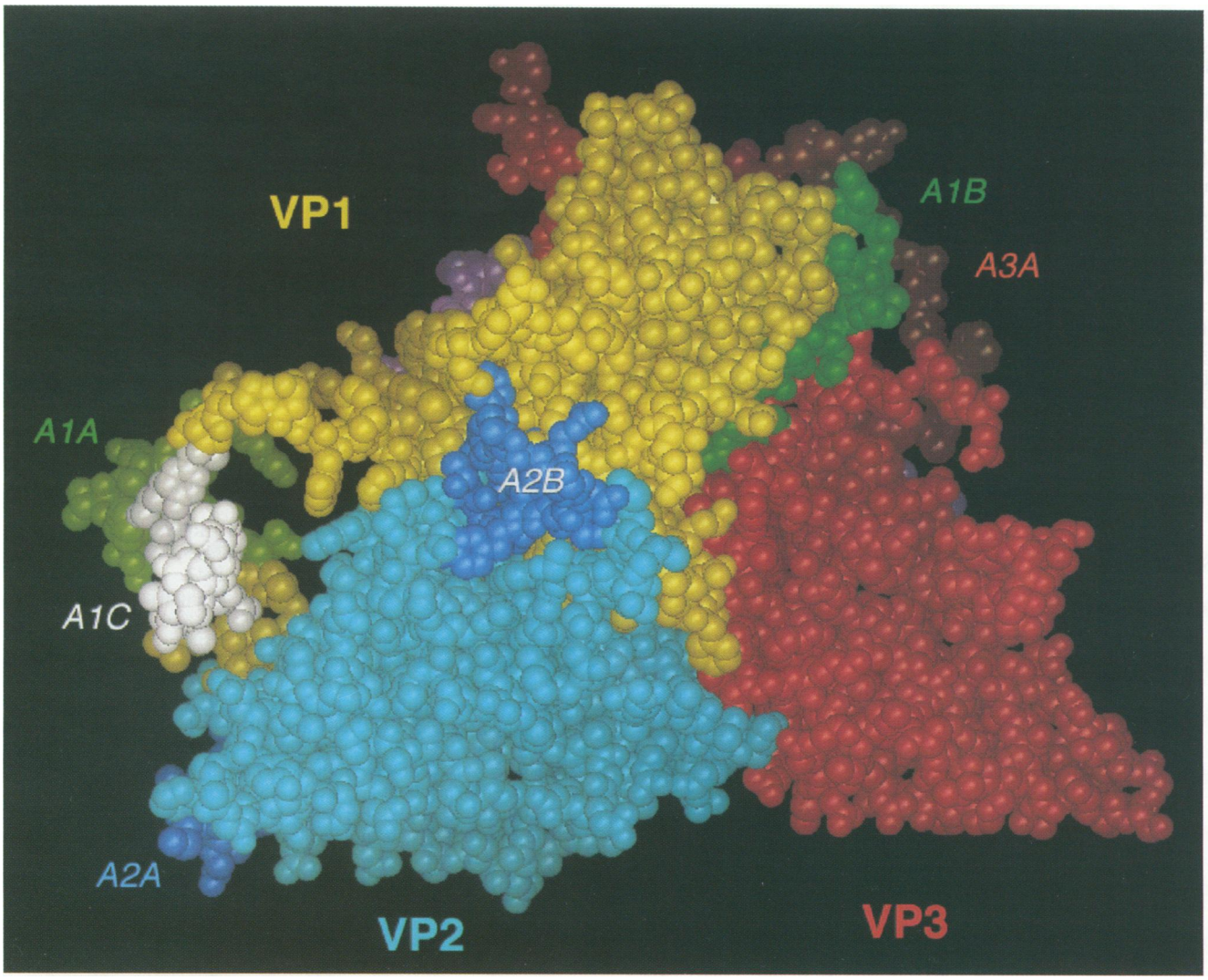

FIG. 7. Space-filling model of TMEV structural proteins based on the known X-ray crystallographic structure. One icosahedral unit of the capsid proteins is presented. The X-ray crystallographic data of TMEV BeAn strain (33) has been obtained from the protein data bank, Brookhaven National Laboratory, and the space-filling model was generated with InsightII software (Biosym Tech.) in a SiliconGraphics computer system. A1B represents the A1Ba epitope and A1C represents A1Cb. Other epitopes are the same as listed in Table 1. The purple polypeptide chain which is not labeled represents VP4.

BALB/c or C57BL/6 mice, whose serum antibody titers are greater than or equivalent to those of virus-infected SJL mice.

It is known that immunization with VP1 protein provides the best protection of the host from other picornavirus infections such as poliovirus and foot-and-mouth disease virus (2, 5, 17, 19, 55, 58). Since the VP1 capsid protein elicits strongly neutralizing antibodies against the viruses, the protection induced by immunization has been considered to be associated with the level of neutralizing antibodies. Similarly, it has been previously reported that TMEV mutants which escaped from neutralizing antibodies directed toward VP1 epitopes, including the A1Cb region, display altered low pathogenesis (48, 59). Antibodies to this epitope are known to exhibit a strong virus neutralization activity in vitro (39, 41). Therefore, the preferential reactivity to VP1 epitopes (and/or A1Cb) of sera and cerebrospinal fluids from SJL mice suffering from TMEV-induced demyelinating disease was unexpected. Hence, the high level of neutralizing antibodies to this epitope in virus-infected SJL mice appears not to protect the host from virus-induced demyelination. The relationship between the neutralizing activity of antibodies and their ability to protect the host from viral infection and/or virally induced disease is not always clear. In other virus systems, antibodies capable of neutralizing virus in vitro do not necessarily protect the host from viral infection while, in some cases, nonneutralizing antibodies are capable of protecting the host (3, 28, 57). It has been shown previously that a peptide containing this A1Cb epitope region can be generated by a trypsin cleavage of TMEV, and the resulting trypsin-cleaved virus is believed to be more pathogenic (39, 41). Therefore, it is possible that antibodies to this epitope may not be able to neutralize the virus in vivo.

The selective elevation of antibodies to the A1Cb epitope in demyelination-susceptible SJL mice may also enhance viral pathogenesis rather than protect the host. The presence of IgG2a antibodies to this epitope in virus-infected, but not

immunized, mice (Fig. 3) may affect the pathogenesis. The IgG2a subclass, which is favorably produced in the presence of Th1 cells involved in the inflammatory response (13, 37), is also known to be most effective in complement fixation (25) as well as antibody-dependent cell-mediated cytotoxicity (36). Therefore, it is conceivable that such antibodies may be involved in the exacerbation of inflammation or tissue destruction via antibody-dependent immune mechanisms (38). Antibodies to the other epitopes may also be more effective in eliminating chronic viral infection and may reduce viral antigen source involved in the stimulation of inflammatory T-cell populations (6). The experimental results (30, 35) indicating that viral persistence in the CNS is an important factor resulting in demyelinating disease support this possibility. Further investigations regarding the involvement of these viral epitopes in TMEV-induced demyelination may provide important insights into the pathogenic mechanisms of this virally induced model of human multiple sclerosis.

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