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Lymphocyte recognition elements on the VP1 protein of Theiler's virus

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SUMMARY

Theiler's virus is a murine picornavirus that persists in the central nervous system in susceptible mouse strains, and gives rise to immune mediated demyelinating disease. Antiviral CD4 T cells are necessary to protect from overwhelming virus replication in the acute phase of the disease, and are thought to act by stimulating the antibody response. The present study used overlapping synthetic peptides to map the location of epitopes recognized by CD4 T cells. One T-cell epitope was identified between amino acids 33–47 of VP1, which was recognized by virus-reactive T cells. 'Cryptic' epitopes were also present within VP1 at postitions 153–167, 166–180, 225–239 and 233–247. A linear B-cell epitope was identified in the C-terminal region 225–276. Immunization of CBA mice with inactivated virus, but not peptides containing VP1 B- or T-cell epitopes, reduced the virus titre in the CNS in the acute phase of the disease.

INTRODUCTION

Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus belonging to the cardiovirus genus, which normally causes an asymptomatic enteric infection in its natural host, the mouse. After intracerebral inoculation of 'Theiler's original' virus strains into certain inbred mice, a biphasic disease ensues consisting of an acute paralytic disease followed by chronic inflammatory demyelinating pathology.[1] Demyelination is observed from about 8 weeks post-infection in CBA mice, and lesions are histologically similar to those observed in CNS tissue from multiple sclerosis (MS) patients.[2] Thus TMEV infection serves as a useful animal model to study the processes leading to demyelination. The CBA strain is a strain of intermediate susceptibility to demyelination, in contrast to the polarized forms of disease seen in fully resistant (e.g. C57BL/6) or fully susceptible (e.g. SJL/J) strains.

The processes in Theiler's virus-induced demyelination are complex, involving the cytopathic effects of the virus and the pathological action of the immune response. Immunosuppression results in a decrease in demyelination if administered after the acute phase of the disease.[3–6] Comparisons between susceptible and resistant mice indicated that the antiviral DTH response, mediated by CD4 T cells, was responsible for the immunopathology.[7,8] Removal of these cells before the chronic phase of the disease resulted in a lower incidence of demyelinating disease.[9]

In contrast, a lack of CD4 T cells during acute infection results in overwhelming virus replication in the CNS leading to death.[9] Treatment of these animals with hyperimmune anti-TMEV serum prevents death, suggesting CD4 T cells are required to provide immunological help for the antibody response during the acute phase.[10] Alternative methods of CD4 T-cell antiviral activity may also be important, as passively transferred antibody does not completely substitute for an intact immune system.[10]

Given the importance of CD4 T lymphocytes in the acute phase infection, the aim of the current study was to investigate the specificity of virus-reactive T cells in order to dissect further the molecular relationship between the virus and the immune system. Overlapping synthetic peptides were constructed that contained the sequence of the VP1 and VP2 capsid proteins of the BeAn strain of TMEV. These two proteins are recognized by polyclonal anti-TMEV antibodies,[11–13] and it was considered likely that they also contained T-cell epitopes, as T- and B-cell epitopes sometimes coincide on the capsid proteins of other picornaviruses.[14,15] The same peptides were also used to define the B-cell epitopes on these proteins.

Several 'cryptic' T-cell epitopes were identified on the VP1 protein, these are epitopes that are not presented after processing of intact TMEV, but which are immunogenic when inoculated into animals in peptide form.

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Abbreviations: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; GMEM, Glasgow's minimum essential medium; TMEV, Theiler's murine encephalomyelitis virus.

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MATERIALS AND METHODS

Animals

Female CBA mice were obtained from Olac (Bicester, UK). Unless stated otherwise mice were injected with peptides or virus at 6–10 weeks of age.

Virus, synthetic peptides and fusion proteins

The BeAn 8386 and GDVII strains of TMEV were obtained from Professor H. L. Lipton (Mount Sinai Hospital, New York, NY). For use in proliferation assays virus-containing supernatant was heat inactivated at 56° for 1 hr.

A panel of synthetic peptides encompassing the complete amino acid sequence of VP1 and VP2 (BeAn strain[16]) was synthesized in collaboration with Professor Nigel Groome (Brookes University, Oxford, UK) using an Amimed multiple peptide synthesizer. Each peptide was 15 amino acids long and overlapped the peptide either side by seven amino acids.

Peptides were dissolved at a concentration of 1 mg/ml in RPMI 1640 medium supplemented with 2 mM glutamine and 0·08% sodium bicarbonate and stored at 4°. In proliferation assays peptides were diluted in supplemented RPMI to give final concentrations of 50 μg/ml, 10 μg/ml and 5 μg/ml.

A fusion protein containing the complete sequence of VP1 was used in some experiments. This protein encompassed the 2A region plus the flanking C terminal 77 amino acids of VP3 and the N terminal 12 amino acids of 2B, linked to 260 amino acids of gene 10 of bacteriophage T7.

Virus infectivity assays

Tissues to be assayed for virus content were homogenized in 0·5–1 ml HEPES-buffered Glasgow's modified Eagles medium (GMEM). Samples were stored at −70° and clarified prior to use.

Virus-infectivity assays followed the protocol previously described.[17] Briefly, serial dilutions of the test samples were added to monolayers of BHK-21 cells and absorption allowed to occur at room temperature. Monolayers were then overlayed with 0·6% agar (DIFCO Laboratories Ltd, Molesey, Surrey, UK), followed by 1 ml of plaque assay medium (GMEM plus 0·5% bovine serum albumin, 0·075 mg/ml diethylaminoethyl dextran and 40 mM magnesium chloride). The monlayers were incubated at 37° for 3 days, then fixed with 4% formal saline and stained with 0·01% toluine blue.

Virus neutralization assays

Serum samples obtained from infected or immunized mice were assayed for neutralizing antibody as previously described.[18] Briefly, twofold serial dilutions of sera were prepared, and incubated with 100 PFU of TMEV for 1 hr at room temperature. The residual virus infectivity was then assayed as described above. Virus plus medium alone served as a negative control. The dilution of serum that neutralized approximately 50% of infectious virus was calculated, and the reciprocal of this number defined as the neutralization titre of the sample.

Immunization regimes

To elicit a response to antigen, mice were injected subcutaneously with an emulsion of antigen in complete Freund's adjuvant. 8 days later the draining lymph nodes and/or spleens were removed and a suspension of lymphocytes obtained. In some experiments mice were boosted with a second dose of antigen in incomplete Freund's adjuvant (IFA). Spleens, lymph nodes and serum were removed 7–8 days later.

The immunizing dose of peptide and fusion protein was 30–100 μg per mouse. Mice immunized with virus received 10^6–10^7 PFU of heat-inactivated BeAn.

Lymphocyte proliferation assays

Single-cell suspensions from lymph nodes or spleens were suspended in supplemented RPMI medium, and washed in this medium prior to use in the assays. In the case of spleen preparations, red blood cells were lysed by incubating the cell pellet with 0·17 M NH_4Cl in 0·017 M Tris buffer for 5 min at room temperature.

Proliferation assays were performed in U-bottomed 96-well plates. 100 μl of cell suspension containing 5×10^5 cells was aliquoted into each well, followed by 100 μl of antigen dissolved in RPMI supplemented as described above. Unless otherwise stated the assay medium contained a final concentration of 2% horse serum. Assay plates were incubated at 37° for 72 hr. 8 hr before the end of the incubation, 50 μl of supplemented RPMI containing 0·5 μCi of tritrated thymidine ([^{3}H]TdR) (Amersham International PLC, Little Chalfont, Bucks, UK) was added to each well. The plates were subsequently harvested using a Skatron cell harvester (Lier, Norway) and the incorporated radioactivity measured with a 1205 Betaplate liquid scintillation counter (LKB).

Where antibodies to major histocompatibility complex (MHC) antigens were included in lymphocyte cultures, the appropriate dilution of antibody was added at the start of the 72-hr culture period. Anti-class I antibody (MCA 172, Serotec, Oxford, UK) recognized H-2K^k and H-2D^k. Anti-class II antibody (MCA 24, Serotec, Oxford, UK) recognized H-2-I-A^k and H-2-I-A^s.

Peptide enzyme-linked immunosorbent assay (ELISA)

Peptide-specific antibodies were detected using an ELISA technique modified from that previously described.[19] Briefly, 5 μg of peptide was added to each well on 96-well plates, then incubated with a 1/100 dilution of serum from infected CBA mice 2 months after intracranial infection with 10^4 PFU BeAn. Horseradish peroxidase conjugated sheep anti-mouse IgG was used at a dilution of 1/1000 to detect bound antibody. Finally the substrate 5-aminosalicilic acid was added and the optical density measured with an ELISA plate reader.

RESULTS

Epitope recognition in the TMEV-specific proliferative T-lymphocyte response

CBA mice were immunized with heat-inactivated BeAn in complete Freunds adjuvant and 8 days later draining lymph nodes were removed. Lymphocytes from these nodes were cultured with various peptides covering the sequence of VP1 and VP2. Figure 1 shows the proliferative response to VP1 and VP2 peptides. Three concentrations of peptide were used, only the highest is shown. Most peptides gave rise to responses greater than the background (medium alone); however, only

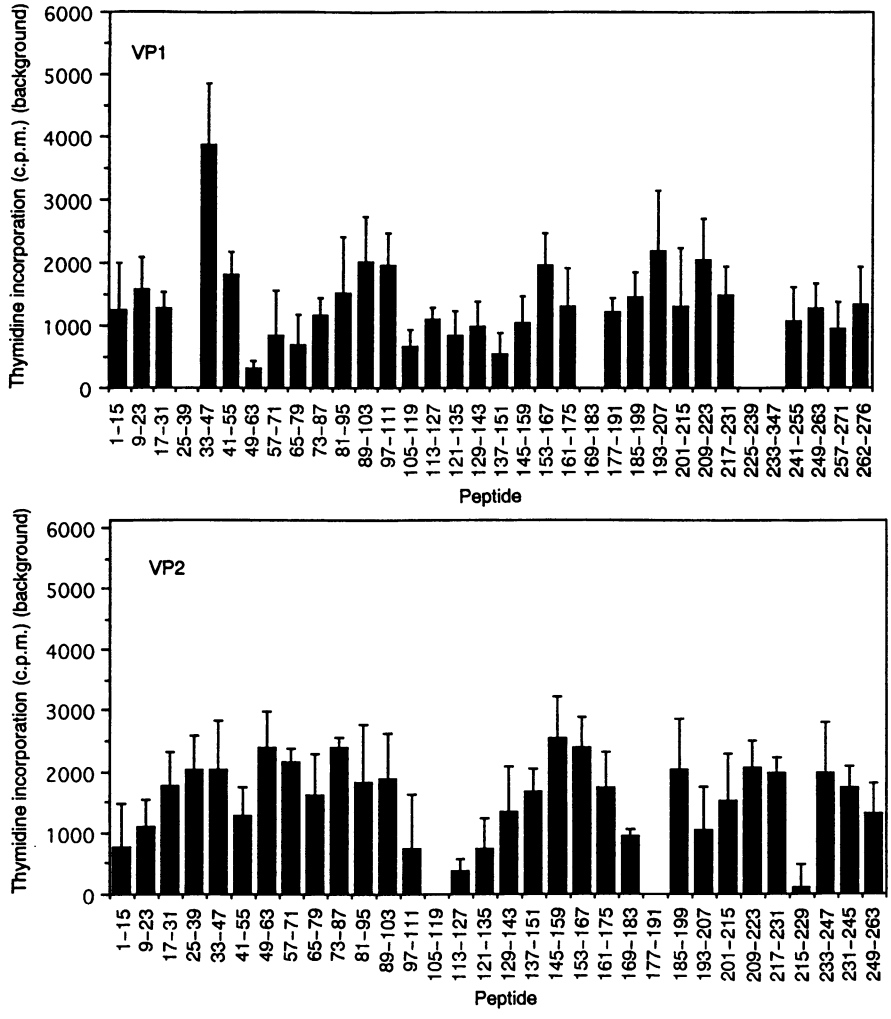

Figure 1. Responses of TMEV-specific lymphocytes to peptides representing the sequence of VP1 and VP2. CBA mice were primed with heat-inactivated BeAn and the draining lymph nodes removed 8 days later. Lymphocytes were cultured with peptides at concentrations of 5, 10 and 50 μg/ml. Graphs show responses to 50 μg/ml peptide. Each bar represents the mean of quadruplicate cultures, error bars show one standard deviation. Background responses (medium alone) have been subtracted and were in the range 630–930 CPM. Response to heat-inactivated BeAn was 13 788 c.p.m.

one peptide, VP1 33–47, induced a response that was significantly greater ($P < 0{\cdot}01$ by Student's t-test) than the average peptide-induced proliferation.

T-cell recognition of epitopes on VP1 following immunization with VP1 fusion protein

CBA mice were immunized and boosted with a bacterial fusion protein containing the complete sequence of VP1. Lymphocytes from draining lymph nodes and spleens were then cultured with the panel of VP1 peptides to determine epitope specificity. As shown in Fig. 2, two peptides, 33–47 and 153–167, elicited significant responses ($P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively).

Antigenic properties of VP1 peptide 33–47

In order to test the antigenicity of peptide VP1 33–47, a group of CBA mice was immunized with peptide 33–47. Draining lymph node cells were then cultured with peptide VP1 33–47, BeAn or peptide VP1 89–103, which acted as a negative control. The results are shown in Table 1A, lymphocytes primed with VP1 33–47 mounted a significant proliferative response to homologous peptide and BeAn ($P < 0{\cdot}001$ in both cases), but not VP1 89–103. Conversely, lymph node cells from animals immunized with peptide VP1 137–151 recognized neither the homologous peptide nor VP1 33–47. The BeAn preparation used in these studies had a mild mitogenic effect on lymphocytes, consequently stimulation indices of 4 or more were considered a significant response to virus. Thus the response of peptide VP1 137–151-primed cells to virus was not considered significant.

To determine the MHC restriction of the T-cell epitope in VP1 33–47, peptide-reactive cells were cultured with peptide plus anti-MHC class I or class II antibodies. Lymph node cells from mice immunized with VP1 33–47 were cultured with 50 μg/ml peptide 33–47 in the presence of monoclonal anti-H-2K^k antibody (MCA 172, Serotec), monoclonal anti-H-2I-A^k antibody (MCA 46, Serotec) or no antibody. Anti-MHC class II antibody completely inhibited the proliferative response

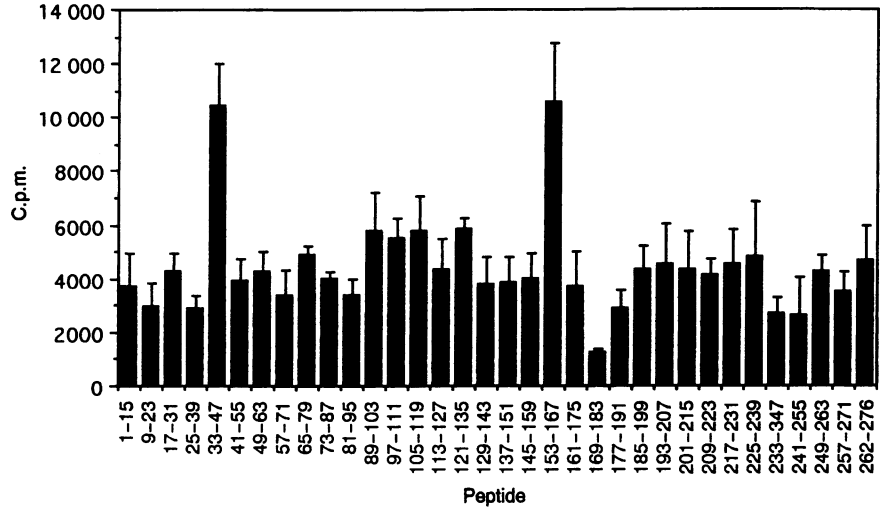

Figure 2. Responses of fusion protein-specific lymphocytes to peptides representing the sequence of VP1. CBA mice were primed with a fusion protein containing the complete sequence of VP1. Draining lymph nodes were removed 8 days later and the lymphocytes cultured with peptides at concentrations of 5, 10 and 5 μg/ml. The graph shows responses to 50 μg/ml peptide. Each bar represents the mean of quadruplicate cultures, error bars show one standard deviation. Response to heat-inactivated BeAn: 18 842 c.p.m., to medium alone: 5 728 c.p.m.

against peptide VP1 33–47, at concentrations as low as 5 μg/ml (data not shown). Anti-H-2K^k antibody had no effect on peptide-specific proliferation.

'Cryptic' T-cell epitopes within VP1

To test whether any subdominant or 'cryptic' epitopes were present within VP1, CBA mice were immunized with a mixture of peptides in CFA and screened for T-cell responses to individual peptides. Two peptide cocktails were used, one containing VP1 peptides 76–92, 86–100, 91–105, 96–110, 101–115 and 153–167 (cocktail 1), the other contained VP1 peptides 141–155, 146–160, 156–170, 161–175, 166–180 and 80–95 (cocktail 2). The results are shown in Fig. 3. Mice primed with cocktail 1 mounted significant T-cell responses against peptides VP1 153–167 ($P < 0.001$) and VP1 166–180 ($P < 0.001$) and BeAn ($P < 0.001$). Mice primed with cocktail 2 also gave significant responses to VP1 153–167 ($P < 0.05$) and VP1 166–180 ($P < 0.001$) and BeAn ($P < 0.001$).

B-cell epitopes within VP1

An ELISA system was used to determine which peptides contained antibody binding sites. Peptides bound onto microtitre plates do not assume the same conformation as corresponding regions on the intact virion, so this assay could only detect linear B-cell epitopes. Polyclonal serum from infected CBA mice bound to several VP1 peptides (Fig. 4), the strongest binding was to peptides from the C terminal 52 amino acids (225–276). Peptides 25–39, 81–95 and 121–135 were also strongly recognized by the polyclonal serum.

'Cryptic' T-cell epitopes within the antibody binding region of VP1

It was of interest to determine whether subdominant T-cell epitopes were present in the peptides that were recognized by polyclonal serum.

CBA mice were inoculated with a cocktail of peptides 225–239, 233–247, 241–255 and 262–276 in CFA. The response of

Table 1. Peptide-specific proliferative responses from immunized mice. CBA mice were immunized with peptide(s) in CFA and draining lymph node cells removed 8 days later. Results show mean of quadruplicate cultures ± SD. Each peptide was used at three concentrations, the table gives responses to the highest concentration, 50 μg/ml. Heat-inactivated virus (BeAn strain) was used at 3.5×10^7 PFU/ml

Immunizing peptide	Test peptide	[^{3}H]TdR incorporation (CPM)	
VP1 33–47	Virus	8911 ± 733	$P < 0.001$
VP1 33–47	VP1 33–47	10878 ± 520	$P < 0.001$
VP1 33–47	VP1 89–103	1617 ± 519	NS
VP1 33–47	Medium	729 ± 321	
VP1 137–151	Virus	1036 ± 252	NS†
VP1 137–151	VP1 33–47	360 ± 47	NS
VP1 137–151	VP1 137–151	389 ± 103	NS
VP1 137–151	Medium	480 ± 155	
B-Cell-epitope pool*	Virus	1661 ± 128	NS†
B-Cell-epitope pool*	VP1 225–239	16091 ± 3740	$P < 0.001$
B-Cell-epitope pool*	VP1 233–247	4123 ± 1192	$P < 0.001$
B-Cell-epitope pool*	VP1 241–255	319 ± 85	NS
B-Cell-epitope pool*	VP1 262–276	2520 ± 589	$P < 0.001$
B-Cell-epitope pool*	Medium	828 ± 366	

* B-cell-epitope pool consisted of VP1 peptides 225–239, 233–247, 241–255, 262–276.

† Virus antigen had a small mitogenic effect on lymphocytes, therefore only stimulation indices of above 4 were considered significant.

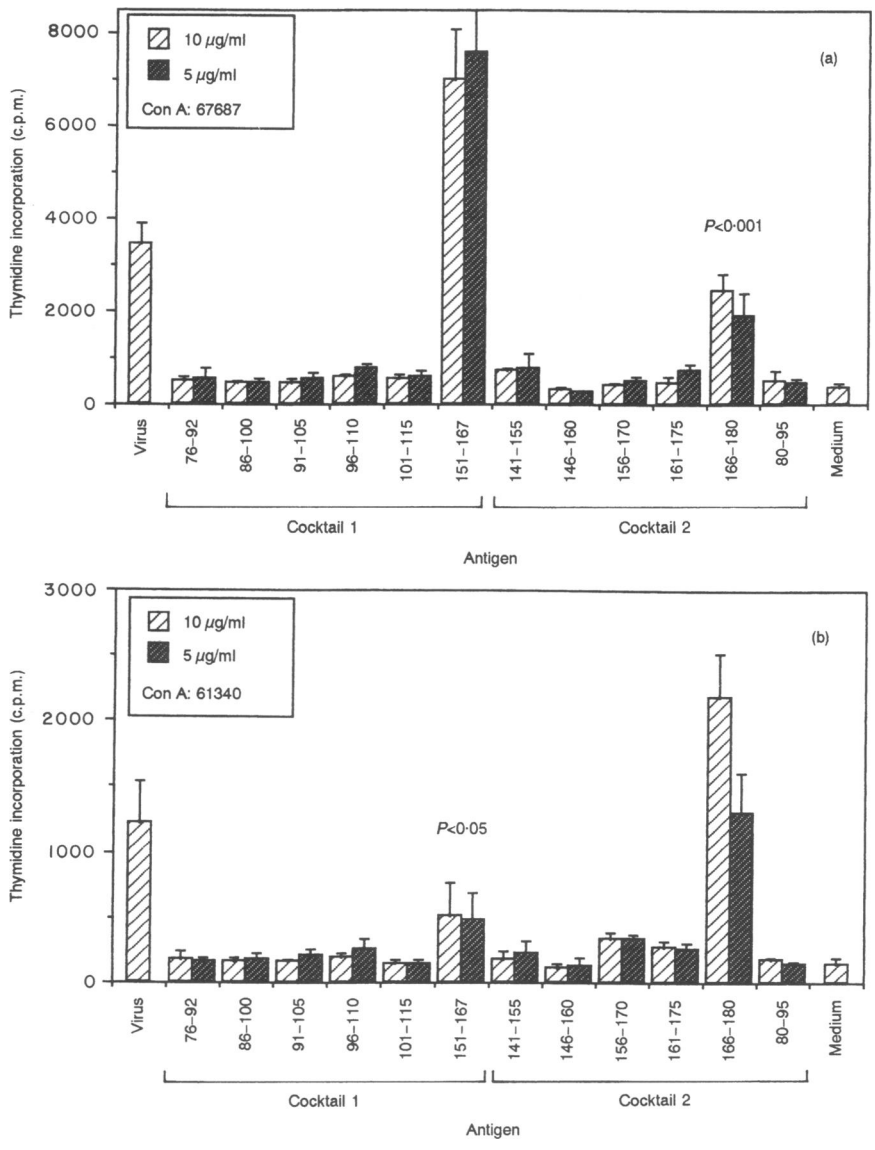

Figure 3. Effect of priming mice with cocktails of VP1 peptides. CBA mice were primed subcutaneously with cocktails of VP1 peptides in CFA. 8 days later draining lymph nodes were removed and lymphocytes from these used in proliferation assays. Graph (A): mice primed with cocktail 1; graph (B): mice primed with cocktail 2. Lymph node cells from 10 mice were pooled for each group. Data represents mean values from quadruplicate cultures, error bars show standard deviations. *P* values were calculated using a Student's *t*-test with Bessel's correction.

draining lymph node cells to individual peptides in the pool is shown in Table 1B. The proliferative response to VP1 225–239 was highly significant ($P < 0{\cdot}001$), and responses to VP1 233–247 and 262–276 were also significant (both $P < 0{\cdot}01$). Response to virus was not considered greater than the non-specific proliferation caused by the virus preparation, as the stimulation index was less than 4.

Effect of peptide immunization on viral titres in acute phase disease

In order to accelerate immune-mediated clearance of infectious virus, mice were injected with peptides to stimulate the CD4 T-lymphocyte subset alone (VP1 33–47) or the B- and T-lymphocyte subsets (C terminal VP1 peptides) prior to infection with BeAn. Other groups consisted of mice primed with heat-inactivated BeAn, VP1 fusion protein and immunization with the non-immunogenic peptide VP1 137–151 acted as a negative control.

At day 0 groups of 8 mice were immunized with the appropriate antigen in complete Freund's adjuvant (CFA). Mice were re-immunized with the same antigen in incomplete Freund's adjuvant on day 14. On day 21 all mice were infected with 10^5 PFU BeAn intracerebrally. Four mice from each group were killed at 7 and 14 days post-infection and brain and spinal cord removed for virus titration. Virus titre in the brain is shown in Fig. 5. The only group with a virus titre significantly lower than the control (peptide 137–151) was the

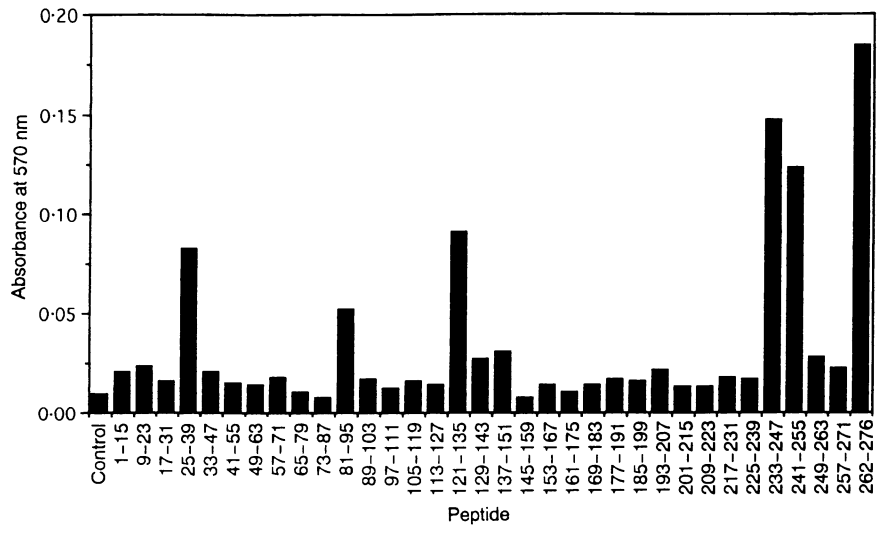

Figure 4. Binding of antibodies from BeAn-infected CBA mice to VP1 peptides. 96-well tissue culture wells were coated with 5 μg peptide, then incubated with serum from CBA mice at 4 weeks post-infection with BeAn. The plates were washed and a secondary HRP-conjugated anti-mouse IgG antibody used to detect antibody binding. The substrate was 5-aminosalicilic acid. An irrelevant peptide was used as a negative control for binding. Standard deviations were less than 10% of the readings.

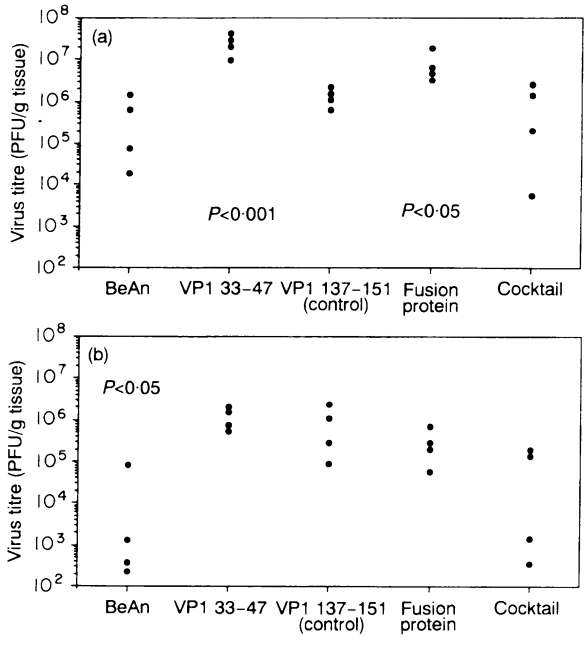

Figure 5. Effect of priming CBA mice with various antigens on the clearance of infectious TMEV from the brains of infected mice. 3–4-week-old CBA mice were immunized subcutaneously with the antigens shown above in CFA. Two weeks later the mice were boosted with the same antigen in IFA, and 7 days later the mice were infected intracerebrally with BeAn. Mice were killed 7 and 14 days post-infection and the brains titred for infectious virus content. Each point represents the brain titre from a single mouse. Fusion protein contained the complete sequence of VP1 plus part of the 2A sequence, 'cocktail' was a mixture of peptides containing putative B-cell epitopes (see text for details). Where there was a significant difference between control and experimental groups the P value is shown, calculated using a Student's t-test with Bessel's correction.

virus-immunized group ($P < 0{\cdot}001$), this was also true for the virus titres in the spinal cord (data not shown). Serum-neutralizing antibody titres from these mice are shown in Fig. 6, both the BeAn immunized group and the group immunized with the C terminal peptide pool gave rise to significant neutralizing titres. Thus, although the C terminal peptides could elicit a neutralizing antibody response, this was apparently not of sufficient titre to reduce the virus load detected in the CNS.

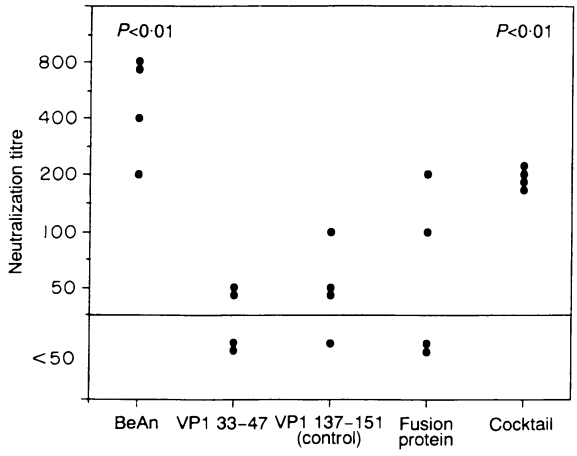

Figure 6. Antibody neutralization titres from mice immunized with various antigens. Blood samples were taken from all mice in the experiment described in Fig. 5. Serum was assayed for antibody neutralization titres 14 days after infection; in the graph shown each point represents the neutralization titres from individual mice. All points below the horizontal line represent titres below 50. Titres were defined as the reciprocal of the serum dilution which gave 50% neutralization of a standard inoculum of BeAn virus. Where there was a significant difference between control and experimental groups the P value is shown, calculated using a Student's t-test with Bessel's correction.

DISCUSSION

The results show that in CBA mice, a strain of intermediate susceptibility to TMEV-induced demyelination, T cells identify an epitope between amino acids 33–47 of VP1 following immunization with heat-inactivated BeAn. This epitope was I-A^k restricted, and when CBA mice were immunized with the VP1 33–47 peptide a response against inactivated virus could be detected, suggesting that processing of the virus resulted in presentation of this epitope.

Several hidden or 'cryptic' epitopes were detected within VP1, peptides 153–167, 166–180 and 225–239 provoked strong proliferative responses after immunization with homologous peptide, and peptides 233–247 and 262–276 gave a somewhat weaker response. In the case of peptide pairs with partial overlap (153–167/166–180 and 225–239/233–247) there may be important residues in the overlap region, so that the responses were to the same epitope present in both peptides, alternatively two adjacent epitopes may be present. Immunization with a pool of peptides containing VP1 225–239, 233–247 and 262–276 resulted in responses to peptides but no significant response to BeAn; epitopes in these peptides may be regarded as 'absolute' cryptic determinants. Conversely, peptides 153–167 and 166–180 induced a response against BeAn in addition to the peptides themselves and can be regarded as 'latent' cryptic epitopes.[20]

Interestingly, immunization with the VP1-containing bacterial fusion protein resulted in responses against the cryptic epitope in peptide 153–167 in addition to peptide 33–47. This was presumably because of the differences in tertiary structure between the fusion protein and intact virions which resulted in differential protein processing.

T-cell epitopes on BeAn capsid proteins have been mapped in the susceptible SJL/J mouse strain.[21,22] Immunization or infection with BeAn stimulated both a proliferative and DTH response against amino acids 74–86 of the VP2 protein. In contrast, resistant C57BL/6 mice mounted proliferative and DTH responses to VP1, VP2 and VP3 after immunization with BeAn. Immunization of SJL/J mice with purified capsid proteins induced a response against the homologous protein for all three capsid proteins tested, indicating the presence of cryptic T-cell epitopes in VP1 and VP3 in addition to the immunodominant epitope in VP2. Whether the dominance of the VP2 epitope in susceptible mice relates to its susceptibility to demyelination or merely reflects the effect of a different H-2 haplotype is not known.

The precise antibody binding sites on TMEV have still to be resolved. Two DA strain monoclonal antibody escape mutants have been sequenced, bearing point mutations in VP1 residues 101[23,24] and 268.[25] Our results showed that peptides representing the C terminus of VP1 (225–276) of BeAn bind polyclonal neutralizing antibodies. The presence of a B-cell epitope near the C terminus of VP1 is supported by two other studies. Bacterial fusion proteins were utilized to demonstrate binding of polyclonal serum to proteins containing VP1 residues 13–27, 145–167 and 251–276, VP2 residues 2–14 and 165–179 and VP3 residues 24–43.[26,27] Later work showed that SJL mice recognize predominantly the 262–276 region of VP1,[28] whereas resistant strains recognized a broader range of epitopes. All these sequences are exposed on the surface of the virion, and represent linear B-cell epitopes, since the tertiary structure in fusion proteins is probably different from that in the intact virion. The C terminus of VP1 also contributes to an antigenic site on Mengovirus, a closely related cardiovirus.[19]

Three other peptides from VP1 were recognized by serum from infected animals. Peptide 81–95 is in loop 1, a prominent surface protrusion near the fivefold axis of the virion. Peptide 25–39 is located in the internal N terminus of VP1, a region that contains antibody binding sites in poliovirus type 1.[29] Antibody binding to a normally internal part of the protein is thought to occur through a reversible change in the capsid conformation. Peptide 121–135 is located in the β sheet core of VP1, and is therefore not exposed on the intact virion.

The TMEV epitope previously reported[21,22] is not close to any of these B-cell epitopes in the primary protein structure, although the epitope found in the current study (VP1 33–47) is close to one of the linear B-cell epitopes.[27] This is interesting in light of the previously mentioned association between some T- and B-cell epitopes on other picornaviruses.[30]

Immunization of CBA mice with inactivated BeAn prior to infection significantly reduced the titres of infectious virus in the CNS by 14 days post-infection. Pre-sensitization with peptide 33–47 or the peptide cocktail containing the putative B-cell epitope had no significant effect. Overall, the data supports the theory that neutralizing antibody is important in clearing infectious virus in the acute phase, as the treatment that caused a significant titre reduction also induced the highest neutralizing antibody titres. There appeared to be a 'watershed' titre of neutralizing antibody (1/200 dilution), above which there was a clear reduction in the infectious virus titre in the brain and spinal cord. A second plausable explanation is that the virus clearance and neutralizing antibody titre are both related to a third variable, for example a CD4 T-cell response stimulated by immunization with virus but not by the other antigens used.

In conclusion the data presented here reports a T-cell epitope in the VP1 protein of Theiler's virus. It also confirms the presence of a B-cell epitope in the C terminus of the molecule. Epitope-specific stimulation of the T-cell compartment did not have a significant effect on the rate of virus clearance from the CNS. Our data was consistent with the proposed role of neutralizing antibody as an important mechanism of reducing viral titres in the acute phase of the disease.

ACKNOWLEDGMENTS

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