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Investigation of the role of delayed-type-hypersensitivity responses to myelin in the pathogenesis of Theiler's virus-induced demyelinating disease

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SUMMARY

The contribution of autoimmune responses to the pathogenesis of Theiler's virus-induced demyelinating disease was investigated. Delayed-type hypersensitivity responses to myelin were examined in both symptomatic and asymptomatic mice at different times post-infection, in order to determine whether autoreactivity correlates with the development of demyelination. The results indicate that although autoimmune responses probably do not play a major role in the initiation of demyelination at early times post-infection, autoreactivity to myelin antigens dose eventually develop in symptomatic animals, perhaps through the mechanism of epitope spreading. Autoimmunity to myelin components is therefore an additional factor that may contribute to lesion progression in chronically diseased animals.

INTRODUCTION

Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus which induces a biphasic disease of the central nervous system (CNS) following intracranial inoculation of susceptible strains of mice.[1] An acute, polio-like disease with grey matter pathology occurs during the first month post-infection, and is followed in surviving animals by a chronic demyelinating disease which represents a valuable model for multiple sclerosis (MS) in man (reviewed in ref. 2). Primary demyelinating lesions associated with inflammatory cell infiltrates are found in the spinal cord, and are accompanied by clinical signs of spastic paralysis.

The pathogenesis of Theiler's virus-induced demyelinating disease (TVID), is still not completely understood: by analogy with other virally induced demyelinating diseases of the CNS, and hypotheses that have been proposed for the aetiology of MS, direct viral damage and/or immunopathology may be involved. TMEV typically infects oligodendrocytes *in vitro*[3] and can produce demyelination directly *in vivo*, e.g. in nude mice where virus replication proceeds unchecked in the absence of T-cell responses.[4,5] Genetically susceptible immunocompetent strains of mice fail to clear virus from the CNS and infected oligodendrocytes, astrocytes and microglia/macrophages can be detected throughout the chronic disease;[6,7] however, virus replication appears to be restricted,[8,9] and there is evidence to suggest that much of the demyelination in these animals is immunopathologically mediated. Morphological studies indicate a close correlation between inflammatory cell infiltrates and areas of demyelination,[10] and the lesions have a distribution and appearance similar to those observed in experimental autoimmune encephalomyelitis (EAE),[7] where T cell-mediated damage is known to be of pathogenic importance. Immunosuppression has been shown to reduce incidence/severity of the chronic disease phase, and the fact that anti-Ia[11,12] and anti-CD4 antibodies[13] also reduce demyelination, suggests that class II-restricted $CD4^+$ T cells are involved in disease pathogenesis. However, immunogenetic experiments indicate that at least one of the genes important in determining disease susceptibility/resistance maps to major histocompatibility complex (MHC) class I,[14–16] suggesting that a class I-restricted ($CD8^+$ T cell-mediated) response also plays a role in TMEV infection. *In vivo* T cell depletion experiments, work with β_2-microglobulin knock-out mice (which lack functional $CD8^+$ T cells) and adoptive transfer experiments have implicated $CD8^+$ T cells in viral clearance from the CNS.[17–21] $CD8^+$ T cells may also modulate the demyelinating phase of the disease,[17,19,21] perhaps by the production of cytokines, e.g. interferon-γ (IFN-γ), for which both protective and pathogenic roles have been suggested in TMEV infection.[23–25]

Immunopathological responses within the CNS may be directed against virus-encoded determinants and/or autoepitopes. Initial evidence for the involvement of virus-specific responses in disease pathogenesis came from the correlation

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observed by Clatch *et al.*,[26] between the ability of different inbred mouse strains to mount an antiviral delayed-type hypersensitivity (DTH) response after intracranial infection with TMEV and their susceptibility to demyelinating disease. More recently, it has been shown that priming of DTH responses to TMEV and adoptive transfer of $CD4^+$ virus-specific T cells into TMEV-infected recipient mice both increase the incidence and accelerate the onset of clinical disease[27] and it has been demonstrated that induction of peripheral tolerance to viral antigens will inhibit the development of demyelinating disease.[28] Virus-specific $CD4^+$ T cells thus clearly play an important role in the pathogenesis of TVID. However, it is also possible that as in other animal models of demyelinating disease such as EAE or that produced in mice/rats infected with the JHM strain of coronavirus mouse hepatitis virus (MHV),[29] autoimmune responses directed at myelin components may also be induced and make some contribution to the demyelinating disease. The work described here addresses the question of whether or not this is the case in TVID. Autoimmune T-cell responses are examined over time post-infection in two strains of mice of differing disease susceptibility, in order to reveal whether autoimmune reactivity correlates with disease development, and hence what role autoimmunity may have in disease induction and/or progression.

MATERIALS AND METHODS

Mice

Two inbred strains of mice were used in this study: SJL mice, which are highly susceptible to TVID, and CBA mice, which are of intermediate susceptibility. The latter mouse strain was used in addition to the former because its lower disease incidence provided the opportunity to compare clinically healthy with diseased animals even at late times post-infection. Female CBA mice (Department of Pathology, Cambridge University, Cambridge, UK) and SJL mice (obtained from Olac, Bicester, UK) were infected when 4–5 weeks old.

Virus

The BeAn 8386 strain of TMEV was a gift from Dr H. L. Lipton (Northwestern University, Evanston, IL). Virus was grown in BHK-21 cells, and the culture supernatant containing infectious virus was aliquoted and stored at $-70°$ before use. The viral titre was determined by plaque assay on BHK-21 cells.[30]

Infection of mice and assessment of clinical signs of demyelination

Mice were anaesthetized with ether and injected intracranially (i.c.) into the right cerebral hemisphere with 10^4 plaque forming units (PFU) of BeAn in a 20-μl volume; control animals were injected i.c. with 20 μl of phosphate-buffered saline (PBS).

After infection (day 0) all animals were examined twice weekly for the development of clinical signs indicative of demyelinating disease. Clinical signs were scored on a scale from 0 to 6, where 0 indicates a healthy animal and 1–6 represent gradually increasing severity of signs as follows: 1, ruffled fur and/or hunched posture; 2, ruffled fur and hunched posture plus unsteady gait; 3, very unsteady gait, weak grasp response when placed on wire grid, and sometimes slight hind limb monoparesis; 4, severe hind limb weakness and/or paralysis, weight loss (especially CBA mice); 5, paraparesis of hind limbs (forelimbs sometimes involved), severe weight loss, frequently diarrhoea; 6, moribund/dead.

Histology

Mice were anesthetized with ether and perfused via the left ventricle with 4% glutaraldehyde in phosphate buffer pH 7·2. The fixed spinal cords were removed and cut into four or five blocks which were post-fixed with 1% osmium tetroxide, dehydrated with graded ethanol and embedded in TAAB resin (Taab Laboratory Equipment, Aldermaston, UK). Full face coronal sections were cut at 1 μm from one end of each block and stained with alkaline toluidine blue. Histological assessments were made without prior knowledge of experimental protocol or disease classification.

Preparation of myelin

Myelin was prepared from the brains of adult SJL mice by sucrose density gradient fractionation according to the method of Bradbury *et al.*[31] The protein content was determined by the Folin method.[32]

Myelin-specific DTH testing

DTH testing was performed on groups of 5–10 TMEV-infected mice and sham-injected age-matched controls at different times post-infection. Mice to be DTH tested were selected at random from the available animals at that time-point with a clinical score >3 (SJL mice) or either $\geq$ or <3 as appropriate (CBA mice). Each animal was only DTH tested once during the course of the experiment. Ear pinna thickness was measured with a Mitutoyu engineer's micrometer and then the left ear was injected intradermally in the dorsal surface with 10 μg mouse myelin in 20 μl PBS, and the right ear was simultaneously injected with PBS only. Twenty-four hours after challenge the increase in thickness of each ear over prechallenge measurements was determined and the results were expressed as the myelin-specific increase (i.e. increase in myelin-injected ear minus increase in PBS-injected ear) in units $cm \times 10^{-3}$. Ear swelling reactions showed typical DTH kinetics, i.e. the maximal swelling occurred at 24–48 hr. An analysis of variance was used to determine the statistical significance of DTH test results.

RESULTS

Development of demyelinating disease in TMEV-infected SJL mice

SJL mice are highly susceptible to the development of chronic demyelinating disease following infection with TMEV. This is illustrated by the results of the experiment shown in Fig. 1, where a group of 50 SJL mice were infected i.c. with TMEV and their clinical signs were monitored over time. The BeAn 8386 strain of TMEV used here was tissue culture-adapted, and produced only mild (if any) signs of TMEV-induced early acute grey matter disease, but over the first 6 months post-inoculation, almost all (46/50) of the SJL mice infected with this virus developed a chronic disease characterized by clinical signs of gradually increasing severity, from hind limb weakness, gait abnormalities and hunching, to paralysis and in some animals, death. These clinical signs were scored on a scale

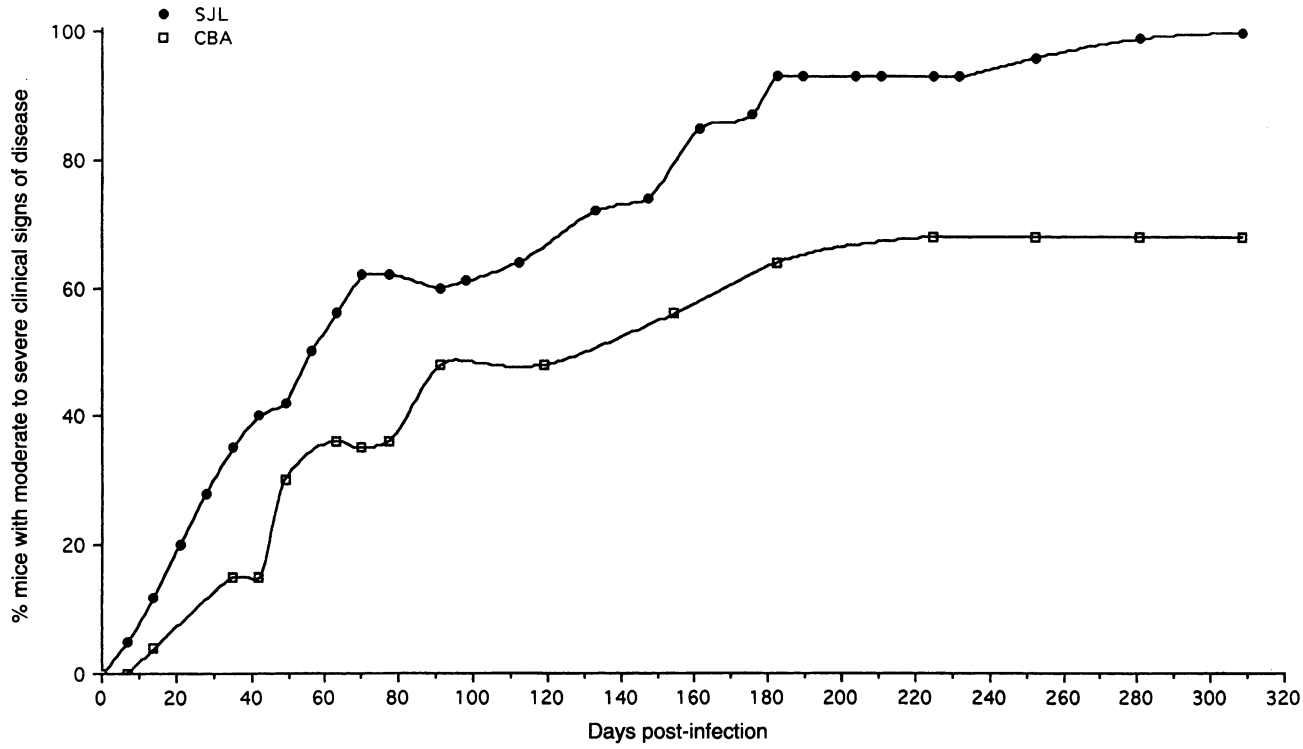

Figure 1. Time–course of development of clinical signs of disease in SJL and CBA mice infected with TMEV. Two groups of 50 SJL and CBA mice were infected i.c. with 10^4 PFU BeAn strain of TMEV on day 0, and mice were examined over time thereafter for clinical signs of disease. The results shown are the % of animals exhibiting moderate to severe clinical signs of disease (a score of ≥ 3 on a scale from 0–6; see the Materials and Methods for details) at the indicated times after infection.

from 0 (healthy) to 6 (moribund) (see the Materials and Methods for details). Histological analysis of the CNS of SJL mice with different clinical scores revealed that inflammatory demyelinating lesions (Fig. 2a) were consistently found in the brain and spinal cord of animals with a clinical score of three or more (i.e. exhibiting moderate to severe signs of demyelinating disease).

Analysis of myelin-specific DTH responses in SJL mice undergoing TMEV-induced demyelinating disease

To determine whether autoimmune T-cell responses may be contributing to the pathogenesis of the chronic demyelinating disease exhibited by BeAn-infected SJL mice, at both early times (4 and 8 weeks) and late times (36 and 48 weeks) post-infection, groups of 7–10 animals exhibiting moderate to severe clinical signs of disease were tested for DTH reactivity to myelin. The use of whole myelin rather than individual myelin components for DTH testing allowed simultaneous detection of responses against a mixture of potential oligodendrocyte autoantigens. The results obtained are shown in Fig. 3a. Mice in the early stages of the disease (tested 4 and 8 weeks post-infection) did not exhibit significant myelin-specific DTH reactivity; but by contrast chronically diseased animals (tested at 36 and 48 weeks post-infection) did show DTH reactivity to myelin. Analysis of variance of the myelin-specific increases in ear thickness revealed that the DTH response mounted by diseased animals 36–48 weeks post-infection was statistically significant ($P<0{\cdot}05$). This response did not reflect the spontaneous development of myelin-autoreactivity in SJL mice with age, since the myelin-specific response made by age-matched uninfected control mice at each time point did not differ appreciably from that mounted by uninfected SJL mice at time 0.

CBA mice are less susceptible to the development of demyelinating disease following infection with TMEV

A group of 50 CBA mice infected with the BeAn strain of TMEV were also scored for the development of clinical signs of disease over time post-infection as described above for SJL mice (Fig. 1). Whereas almost all of the TMEV-infected SJL mice had developed clinical signs of disease by 4–5 months post-infection (Fig. 1) only 68% of similarly-infected CBA mice were found to have done so even by 1 year post-infection (Fig. 1). In the CBA mouse strain, therefore, both apparently healthy and symptomatic animals could easily be identified even at late times post-infection. Histological analysis of the CNS of CBA mice exhibiting clinical signs of different severity revealed that as with SJL mice, demyelinating lesions could consistently be found in the brain and spinal cord of animals with a clinical score of three or more (i.e. with moderate to severe symptoms of disease) (Fig. 2b). Animals with lower clinical scores failed to show pronounced histological abnormalities. The lesions in CBA mice (Fig. 2b) were less extensive than those in SJL mice (Fig. 2a). SJL mice had more pronounced cellular infiltrates with inflammatory cells in areas where no demyelination was evident. Axonal degeneration was also a feature of the demyelinating lesions in SJL mice. By contrast in the CNS of CBA mice, the inflammatory infiltrates

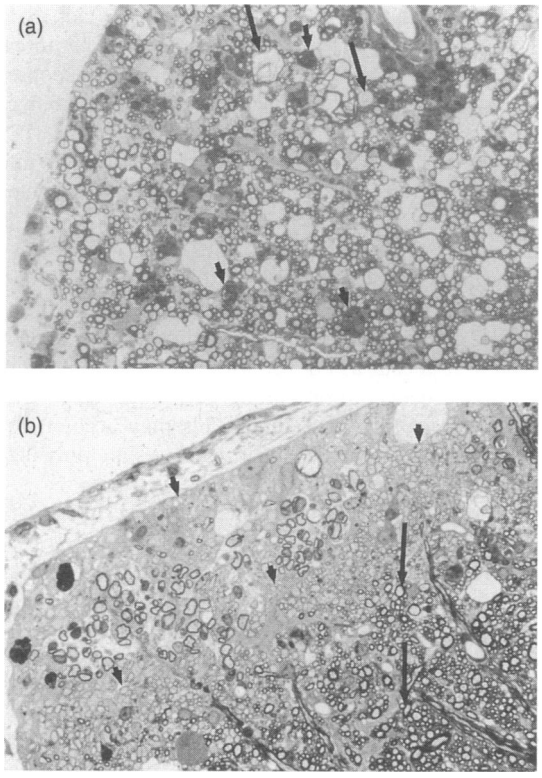

Figure 2. (a) Alkaline toluidine blue staining of a coronal section of the spinal cord lesion in a BeAn-infected SJL mouse 12 weeks post-infection. Demyelinating axons (arrows) and numerous myelin-debris-filled macrophages are evident (arrow heads). Magnification ×450. (b) Alkaline toluidine-blue staining of a coronal section of the spinal cord lesion in a BeAn-infected CBA mouse with a clinical score of ≥3 at 12 weeks post-infection. Areas of normal myelin (arrow) and demyelination (arrowhead) are evident. Magnification ×450.

were much more closely localized to the areas of demyelination and there was no evidence of axonal degeneration.

Analysis of myelin-specific DTH responses in TMEV-infected CBA mice

At different time-points following infection with TMEV, groups of five CBA mice which were either exhibiting moderate–severe clinical signs of disease or that appeared healthy/mildly symptomatic were tested for DTH reactivity to myelin (Fig. 3b). The results obtained in the groups of CBA mice with a clinical score ≥3 paralleled those obtained in the SJL mouse strain (where all animals tested had clinical scores in this range): i.e. significant myelin-specific DTH responses were not observed in mice tested at early times post-infection (which had only recently developed clinical scores indicative of demyelinating disease), but highly significant ($P<0.001$) responses were observed in chronically-diseased animals (nine mice tested 36–48 weeks following infection). By contrast, CBA mice which remained relatively asymptomatic over the entire course of the infection did not exhibit myelin-specific DTH reactivity even when tested at very late times (five mice tested at 36–48 weeks) post-infection. This demonstrates that myelin-specific DTH responses are developed only in animals undergoing demyelinating disease.

DISCUSSION

We have examined T-cell responses to myelin in TMEV-infected mice of different strains at various times post-infection to determine whether correlations exist between the development of autoreactivity and demyelination that might indicate a causative link. Minimal DTH responses following peripheral challenge with myelin were observed in the first few months post-infection. However, when animals were tested at very late times post-infection, it was found that symptomatic but not asymptomatic animals of both strains did then possess significant myelin-specific DTH reactivity. We have also obtained similar results in CBA and SJL mice infected with the DA strain of Theiler's virus (data not shown). It thus appears that autoimmune responses to myelin are not of importance in the initiation of early demyelinating disease in TMEV-infected mice. However, they are induced at very late times post-infection, although only in animals in which there has already been damage to myelin within the CNS. It is possible that DTH to myelin could contribute to lesion progression during chronic disease.

Early experiments to examine whether autoimmune responses are induced in TMEV-infected mice involved unsuccessful attempts adoptively to transfer demyelinating disease from TMEV-infected animals to naive syngeneic recipients with spleen cell preparations[33] (our unpublished observations). In this paper, we directly tested cell-mediated autoimmune responses in TMEV-infected mice. In agreement with results of other groups who also tested DTH responses[34,35] and spleen cell proliferative responses[33,36] to myelin and myelin components over the first 2–3 months following infection of mice with TMEV, we were unable to detect autoimmune T-cell responses in the periphery at early times post-infection. It is always possible that myelin-specific responses develop within the CNS of TMEV-infected mice at these times which are not detected peripherally. Musette *et al.*,[35] observed that there was a selective expansion of T cells with receptors composed of particular Vβ–Jβ combinations in the spinal cord but not the spleen of TMEV-infected SJL/J mice, suggesting that a local antigen-driven response was occurring, although they did not address the specificity of this response. However, even if autoimmune responses are initiated in the CNS over the first few months post-infection, they do not appear to make a significant contribution to the demyelinating disease process at this time, as the induction of tolerance to spinal cord homogenate did not influence the course of demyelinating disease over the first 60 days post-infection.[36]

Autoreactive T-cell responses have not previously been examined in the later stages of the chronic demyelinating disease induced by TMEV in mice. However, some evidence that autoimmunity may develop in TMEV-infected mice at late times post-infection was reported by Cash *et al.*,[37] who studied the specificity of immunoglobulin secreted by B cells isolated from the CNS of SJL/J mice infected 3–7 months previously with TMEV-DA, and found antibodies which reacted with two non-viral white matter components which were present only in infected animals. In addition, serum autoantibodies to myelin have been detected in BeAn-infected CBA mice.[13] In the present study, we tested autoreactive T-cell responses and found that DTH responses to myelin were readily detected in the periphery of diseased but not asympto-

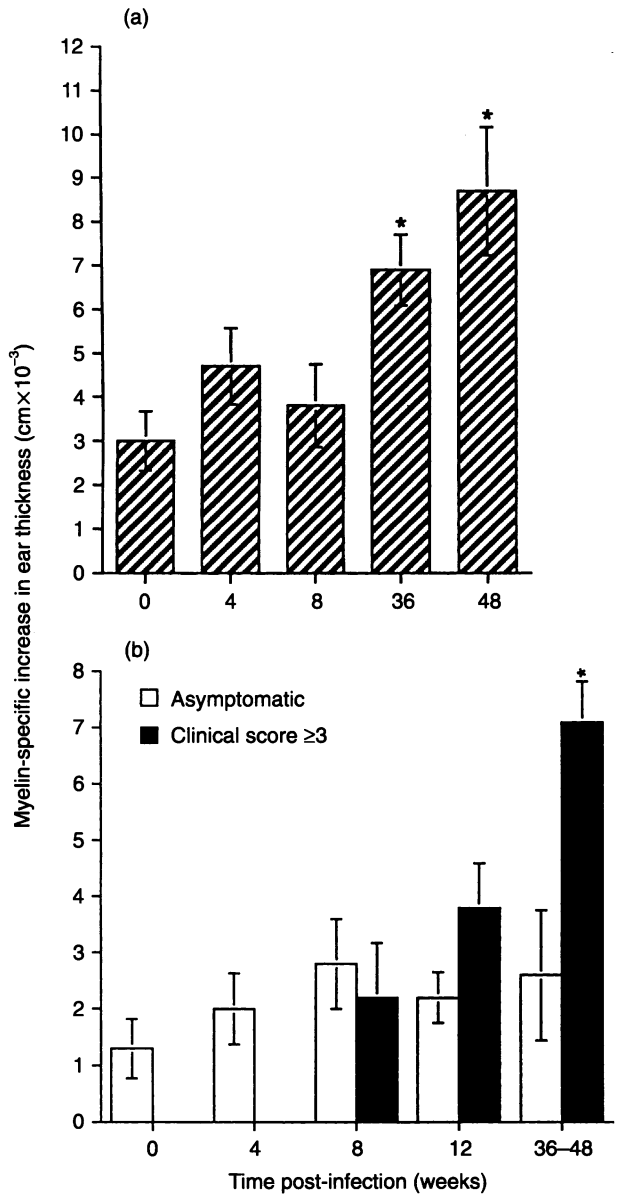

Figure 3. (a) Myelin-specific DTH reactivity of SJL mice at different times following infection with TMEV. Groups of 7–10 SJL mice and age-matched sham-infected controls were tested just prior to infection with TMEV, and at the indicated times following infection, for DTH reactivity to myelin as described in the Materials and Methods. All infected animals used in this experiment had a clinical score of ≥ 3 (i.e. exhibited moderate–severe clinical signs of disease indicative of CNS demyelinating disease). Data for the mice tested prior to infection and all of the sham-infected age-matched control mice at subsequent time-points is recorded as the 0 time-point; data at subsequent time-points represents the results obtained with infected mice at the indicated time post-inoculation. The results shown are the mean myelin-specific increase in ear thickness of each group of animals; the bars indicate one standard error above and below the mean. *indicates a DTH response to myelin shown to be significantly greater than the control response ($P<0.05$). (b) Myelin-specific DTH reactivity of CBA mice at different times following infection with TMEV. At the indicated times post-infection, groups of five CBA mice which were asymptomatic or exhibiting mild disease signs (open bars); or groups of five mice with a clinical score of ≥ 3 i.e. exhibited moderate–severe clinical signs of disease indicative of CNS demyelinating disease (black bars) (nine symptomatic mice were tested at 36–48 weeks post-infection) were tested for DTH reactivity to myelin. *indicates a DTH response to myelin shown to be significantly greater than the control response ($P<0.001$). Details as for (a).

matic animals at late times post-infection. The fact that only mice undergoing demyelinating disease developed autoreactive DTH responses suggests that the autoimmune responses did not represent cross-reactivity with viral antigens, but rather were related to the demyelinating disease process.

Autoimmune responses probably arise in TMEV-infected mice undergoing demyelinating disease via determinant spreading.[36] This phenomenon, which involves a change in T-cell specificity over time so that a broader array of epitopes start to be recognized, has been demonstrated in (SJL × B10.PL)F_1 mice suffering from EAE.[39] By analogy, in Theiler's virus-infected mice, the initial immune reaction within the CNS is mainly anti-viral, but as the myelin damage increases, later in the disease process, autoimmune responses may arise by intermolecular epitope spreading. This may account for the fact that the myelin-specific T-cell responses are only detected at later time-points.

Two pathological events which take place in the CNS of TMEV-infected mice that probably play key roles in the process of epitope spreading to autoantigens are damage to CNS myelin and the induction of MHC expression. Some oligodendrocyte destruction may be a direct result of the viral infection[4,5] but immune-mediated attack may be of greater importance. Histologically, there is evidence of myelin sheath degradation and uptake by macrophages in lesions in the CNS of TMEV-infected mice. Myelin antigens may be released to the periphery and autoimmune T cells primed there which then traffic back into the CNS and are retained following recognition of their specific antigen; or autoreactive T cells might potentially be induced within the CNS itself. In either case, MHC regulation within the CNS is critical to allow recognition of autoantigens to occur there.

Up-regulation of MHC class I, and also MHC class II antigens (in disease-susceptible mouse strains), has been demonstrated within the CNS of TMEV-infected mice.[21,40] Some viruses are able to achieve MHC induction by direct infection of CNS cells, e.g. the coronavirus MHV-JHM, which up-regulates MHC class II expression on infected astrocytes.[41] MHV-JHM induces a demyelinating CNS disease in Lewis rats. In this model diseased rats show T-cell reactivity to myelin basic protein[29] and the induction of MHC class II expression on glial cells and the presentation of degraded myelin is postulated to be a key factor in the autoimmune disease. Whilst the BeAn strain of TMEV is unable directly to up-regulate MHC class II expression on astrocytes in a similar way,[42] cytokines produced by T cells infiltrating the CNS to mediate control of virus replication at early times post-infection, e.g. IFN-γ, will induce MHC expression on CNS cells. Correlations have been observed between the ability of IFN-γ to induce MHC class II expression on astrocytes[42] and cerebrovascular endothelial cells[43] from different mouse strains and their susceptibility to TMEV-induced demyelinating disease. In these mouse strains, chronic MHC up-regulation on CNS cells in the context of autoantigen release will also set the stage for development of autoimmune responses.

The contribution that the autoimmune T-cell responses we have observed in mice undergoing TMEV-induced demyelinating disease makes to lesion spread and disease progression at late times post-infection remains unclear. There did not seem to be any correlation between clinical score and the strength of the DTH response to myelin autoantigens in individual SJL mice tested at 36–48 weeks (not shown), but as clinical scores from 3 to 5 did not correlate precisely with increasing extent of demyelination within the CNS, such a correlation would perhaps not have been expected. This question thus remains unresolved.

In summary, we report that in a CNS demyelinating disease triggered by a known viral infection, autoreactive T-cell responses ultimately develop in chronically diseased animals as a secondary phenomenon, the pathogenic significance of which has not been defined. This finding has important implications for hypotheses which have been proposed as to the aetiology of MS in man. MS is known to involve T cell-mediated damage to the CNS; but the process by which this is triggered and the specificity of T cells involved are currently unclear. T cell reactivity to myelin basic protein has been detected in the peripheral blood and/or cerebrospinal fluid of some MS patients,[44–47] although this is not always observed,[48] and such responses have been reported in some control subjects with other diseases that affect the CNS.[49] It has been suggested that these autoreactive T cells are central to disease production in MS. However, it is possible that as in TMEV-induced demyelination, they in fact represent only an epiphenomenon, being induced in some individuals only after damage to myelin has been produced by other mechanisms, which have yet to be identified.

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Note added in proof

Autoimmune responses to myelin peptides have also been demonstrated in SJL mice following infection with Theiler's virus by Dr Miller's group. Miller S. D., Vanderlugt C. L., Begolka W. S. *et al.* (1997) persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading. *Nat Med* **3,** 1133.