The Effect of L3T4 T Cell Depletion on the Pathogenesis of Theiler's Murine Encephalomyelitis Virus Infection in CBA Mice

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SUMMARY

Theiler's murine encephalomyelitis virus (TMEV) gives rise to a biphasic disease of the central nervous system (CNS) following intracranial inoculation of susceptible strains of mice. The early phase, during the first month, resembles poliomyelitis and in the late phase the mice suffer from inflammatory demyelination reminiscent of multiple sclerosis. In order to investigate the role of helper T cells in the acute and chronic phases of the disease we depleted mice of their L3T4 T cells in vivo with rat monoclonal antibodies, prior to infection and prior to the onset of clinical signs of demyelination. Mice depleted of their helper cells failed to produce antibodies to TMEV and consequently were unable to clear virus from the CNS and died within the first month of infection. Depletion of T cells before the demyelinating phase of the disease resulted in a marked decrease in the incidence of disease from 77% of the immunocompetent animals with clinical signs of paralysis to 36%. Immunocompetent TMEV-infected mice also developed antibodies against myelin suggesting that autoimmune mechanisms may play a role in TMEV-induced demyelination.

INTRODUCTION

Theiler's murine encephalomyelitis virus (TMEV) belongs to the Picornaviridae and has many biological characteristics in common with poliomyelitis virus (Theiler, 1934). TMEV is a natural pathogen of mice which causes an asymptomatic enteric infection and occasionally paralysis. This virus can be subdivided into two groups: one includes encephalitic strains such as GDVII which when injected intracranially gives rise to a rapid and fatal encephalitis, and the other group contains Theiler's original strains (To) which produce a biphasic disease of the central nervous system (CNS) in susceptible strains of mice. During the early part of infection (up to 30 days) mice suffer from 'polio-like' symptoms possibly as a result of lytic infection of motor neurons in the spinal cord (Lipton, 1975). At later times, during the chronic phase, the disease resembles multiple sclerosis with inflammatory demyelinating lesions confined to the white matter. The virus is thought to persist in the glial cells of mice throughout their lifetime (Brahic & Stroop, 1981). In fact, the persistent infection of oligodendrocytes may be directly responsible for the demyelination seen in the chronic disease (Penney & Wolinsky, 1979). However, the demyelination may in part be immune-mediated since it can be prevented by immunosuppressive therapies (Lipton & Dal Canto, 1976; Roos et al., 1982) and by monoclonal antibodies (MAbs) against the Ia region products (Rodriguez et al., 1985). In this context, delayed type hypersensitivity (DTH) reactions have been shown to correlate with susceptibility to demyelination, thus implicating T cell immunity in this process (Clatch et al., 1986).

The detailed immunology of this virus-host relationship is poorly understood. In particular, the nature of the T cell response to the virus is confined to studies on DTH, little or no information is available on T cells involved in protective immunity or directly in the pathological responses seen in the CNS.

In order to study the T cell response in this infectious disease, we have used the powerful system described by Cobbold et al. (1984) to deplete T cell subsets in vivo using rat MAbs. In this
paper, we investigate the course of infection in mice depleted of L3T4 positive T cells, and pose two questions. What role do these cells play in the immune response to this virus and do they contribute to the demyelinating disease in the same way as the autoimmune T cells in experimental allergic encephalomyelitis?

**METHODS**

**Animals.** Four- to five-week-old female CBA mice (Department of Pathology, Cambridge University, Cambridge, U.K.) were thymectomized by standard procedures under hypnorn-valium anaesthesia and used one week later.

**Virus.** The BeAn 8386 strain of TMEV (a gift from Dr H. L. Lipton, Department of Neurology, University of Chicago, Ill., U.S.A.) was grown in BHK 21 cells and the culture supernatant containing infectious virus was divided into samples and stored at -70 °C before use.

**Virus assays.** Brains and spinal cords were removed from infected mice and placed in Glasgow modified Eagles's MEM (GMEM) containing penicillin, streptomycin, fungizone, 200 mM-glutamine and 20 mM-HEPES buffer. The samples were then homogenized with a Polytron P1035 apparatus (Northern Media Supply Ltd., North Humberside, U.K.). The homogenized tissues were clarified by centrifugation at 2000 r.p.m. for 10 min and then stored at -70 °C. Supernatants were serially diluted and assayed for infectivity on BHK cells (Rueckert & Pallansch, 1981).

Serial dilutions of serum from infected mice were assayed for neutralizing activity as previously described (Rabinowitz & Lipton, 1976).

**Preparation of MAbs directed against T cell determinants.** Rat MAbs to several mouse T cell antigens were prepared by Dr H. Waldmann and Dr S. Cobbold (Department of Pathology, Cambridge University) using the Y3/Ag 1.2.3. myeloma fusion partner (Galfre et al., 1979). The hybridomas were amplified in the ascitic fluid of Pristane-primed (LOU × DA) F1 rats (Cobbold et al., 1984). Partial purification by 50% ammonium sulphate was followed by exhaustive dialysis against phosphate-buffered saline (PBS). The antibody was stored at -20 °C at a concentration of 10 mg/ml. The specificities of the MAbs used were as follows: YTS 169.4, anti-Lyt 2 expressed on cytotoxic and suppressor T cells; YTS 191.1, anti-L3T4 expressed on helper T cells; YTS 154.7, anti-Thy 1 expressed on all T cells and YTS 121.5, anti-Lyt 1 expressed on most T cells.

**Determination of TMEV antibodies.** GDVII TMEV partially purified on a density gradient (a gift from Dr J. Harvey, CRC, Northwick Park, London, U.K.) at a concentration of approximately 2 × 10^11 p.f.u./ml was diluted 1/2000 in 0.05 M-sodium carbonate buffer pH 9.6 and 100 µl samples (approximately 10^7 p.f.u./well) were added to wells of microtitre plates (Dynatech Laboratories, Alexandria, Va., U.S.A.) for use in an ELISA as previously described (Groome et al., 1985). The specificity of the assay system was assessed with hyperimmune anti-ovalbumin sera and normal mouse sera (both gave OD < 0.15 at a dilution of 1/50). The endpoint in both assay systems was defined as the dilution of antisera to give an OD of 2 × S.D. above the background (i.e. second antibody alone).

**Preparation of myelin and detection of antibodies to myelin.** Myelin was prepared from SJL/J mouse brains according to the method of Bradbury et al. (1984) by sucrose density gradient fractionation. The myelin was then incorporated in an ELISA as previously described (Groome et al., 1985). The specificity of the assay system was assessed with hyperimmune anti-ovalbumin sera and normal mouse sera (both gave OD < 0.15 at a dilution of 1/50). The endpoint in both assay systems was defined as the dilution of antisera to give an OD of 2 × s.d. above the background (i.e. second antibody alone).

**Histological examination of spinal cords.** Mice were anaesthetized with ether and perfused through the heart with 0.1 M phosphate buffer (pH 7.4) containing 2% paraformaldehyde and 2.5% glutaraldehyde. The spinal cords were dissected out and post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer. The tissues were dehydrated through a graded series of ethanol and embedded in Spurr's resin. Sections were cut on a Porter-Blum MT1 Ultramicrotome, stained with uranyl acetate and lead citrate and examined at 60 kV in a Hitachi H7500 transmission electron microscope.

**Assessment of clinical symptoms of demyelination.** Mice were weighed twice weekly and examined for clinical evidence of demyelination without prior knowledge of the experimental protocol. The mice were judged to show evidence of demyelination if there was a weak grasp response when placed on a wire grid, absence of the righting reflex and evidence of incoordination and/or incontinence.
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, U.K.). Full face coronal sections were cut at 1 μm from one end of each block and stained with alkali Toluidine Blue. Thin sections were cut from areas containing lesions for examination by electron microscopy. Histological assessments were made without prior knowledge of experimental protocol or disease classification.

RESULTS

The effect of T cell depletion on the survival of TMEV-infected mice

Clinical signs were manifest between 9 and 14 days post-infection in depleted and immunocompetent mice. During the first month of TMEV infection approximately 12% of immunocompetent CBA mice died with early ‘polio-like’ symptoms i.e. total hind limb paralysis. A further 25% of intact mice died during the following 2 months with paralysis and signs of demyelination (Fig. 1). Removal of L3T4-positive T cells (helpers and inducers) resulted in approximately 80%o mortality by 3 weeks post-infection and 100% by day 50 post-infection. The mice were suffering from encephalitic symptoms: hunched posture, hind limb paralysis, ruffled fur and marked decrease in body temperature. There were no deaths in a control group of mice injected intracranially with PBS.

The replication of TMEV in the CNS of untreated and L3T4-depleted mice

Three experimental animals per group were killed on days 1, 3 and 7 post-infection and then at weekly intervals for 3 months in the case of immunocompetent mice. The brains and spinal cords were homogenized and the virus content assayed by infectivity on BHK cells.

Viral replication occurred in the brain (Fig. 2) and spinal cord (Fig. 3) of immunocompetent mice during the first month after infection, reaching a peak at day 14. A dramatic reduction of the virus titre in the CNS occurred by day 28 which coincided with the development of high titres of neutralizing IgG antibody specific for TMEV (Fig. 4). In the untreated group there were no animals displaying clinical symptoms at day 28. However, at day 42, three immunocompetent mice developed paralysis and virus was isolated at relatively high titres from their brains and spinal cords. All these animals were showing symptoms of paralysis. In the late phase of the disease (56 to 77 days post-infection) virus was isolated from the CNS of six animals, of which two (taken at day 70 and 77) had clinical evidence of demyelination.

In the L3T4-depleted mice, viral titres also peaked at day 14 but unlike the undepleted group, virus was not cleared from the CNS at day 28 (mean virus titre in the brain 5·4 log_{10} p.f.u. and in the spinal cord 5·2 log_{10} p.f.u.). The peak viral titre coincided with the development of clinical symptoms in both groups of mice.

Induction of virus-specific antibody in intact and depleted mice

Sera from the groups of mice killed for the determination of the viral content of the CNS were assayed for the presence of class-specific antibody against TMEV. Sera from the L3T4-depleted mice were assayed in the ELISA using a second antibody against mouse IgM, IgG, and IgA (GAM, Serotec).

TMEV-specific IgM antibodies were detected early in the infection in intact mice, at day 3 the mean antibody titre was \( \log_{2} 8.9 \pm 1.4 \). The IgG antibody was first detected at day 14, peaking at day 28 (mean titre \( \log_{2} 12.9 \pm 0.6 \)). Neutralizing antibodies (> 1/100) were also detectable at day 28 post-infection (data not shown).

L3T4-depleted mice did not produce any detectable serum antibodies against TMEV. Sera from these animals gave titres of \( \log_{2} 6-6 \) with the GAM reagent in comparison to intact animals which had titres of \( \log_{2} 9-6 \) at day 3 and day 14, using this reagent.

Detection of antibodies to mouse myelin in infected mice

Antibody responses to mouse myelin developed in untreated CBA mice by day 28 after infection (mean antibody titre \( \log_{2} 8.1 \pm 1.4 \)) and reached a peak between day 42 to 56-post-infection (mean antibody titre \( \log_{2} 8-6 \)). Depletion of L3T4-positive T cells prevents all antibody production and so no antimyelin antibodies were detected (data not shown).
The effect of T cell depletion on the chronic demyelinating phase of the disease

In order to investigate the role of T cells in the pathology of TMEV-induced demyelination, groups of 11 mice were treated with anti-L3T4 or a mixture of the four MAbs (see Methods) 65 and 72 days post-infection. Thereafter, the mice were observed twice weekly for the development of clinical signs of demyelination and sampled for antibody response to myelin and TMEV and for presence of virus in the spinal cord between 119 and 240 days post-infection. The results of the clinical observations are shown in Fig. 5. The most notable effect of the removal of T cells was the reduction in the incidence of mice developing severe symptoms of demyelination. At day 155 post-infection 75% (6/8) of intact mice were suffering from signs of disease compared to 12% (1/8) in L3T4-deficient mice and 22% (2/9) of the total T cell-depleted mice. At day 212 post-infection, when histological examination of the cords was carried out, 62% (5/8) of the intact mice had severe symptoms whereas all of the L3T4- and total T cell-depleted mice were normal. During the course of these experiments, 77% (10/13) of the intact mice showed severe
**Fig. 3.** The mean virus content of the spinal cord of (a) intact mice and (b) L3T4-depleted mice as determined by plaque assay on BHK cells. The results are expressed as $\log_{10}$ p.f.u./g of tissue. The results obtained with individual animals are represented by a single dot and the curve was drawn through the geometric mean of the group sampled at the time indicated by the y-axis.

**Fig. 4.** Antibody levels to TMEV and myelin in the sera of mice used in experiments reported in Fig. 2 and 3. (a) The mean IgG (solid lines) and IgM (dotted lines) antibody titre against TMEV as determined by ELISA, in intact mice. The results are expressed as the mean (± standard deviation) of a group of at least three mice at a given time post-infection as indicated on the y-axis. The − signs indicate the absence of neutralizing anti-TMEV antibody, the number of + signs indicates the number of animals with neutralizing antibody titres $>1/100$. (b) The autoantibody titre against mouse myelin as determined by ELISA. The results are expressed as the mean (± standard deviation) of a group of three or more mice at a given time post-infection as indicated on the y-axis.
Table 1. Incidence and onset of clinical disease in mice depleted of T cell subsets in the chronic phase of TMEV infection

<table>
<thead>
<tr>
<th>Cell depleted</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
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<tr>
<td>Mean day of onset</td>
<td></td>
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<tr>
<td>Animals with clinical signs (%)</td>
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signs of disease for longer than 1 week, whereas only 36% (4/11) of the L3T4-depleted group ever developed long term symptoms. Interestingly, 91% (10/11) of the total T cell-depleted group developed short episodes of clinical disease lasting between 1 and 7 days. Only 36% (4/11) of the total T cell-depleted group developed symptoms of longer duration. Mice depleted of T cell subsets showed a slight delay in the clinical signs of disease; the mean day of onset for both groups of depleted mice was between 107 and 109 days compared to 98 days for the intact mice (Table 1).

Infective virus was isolated only from the cords of mice showing clinical signs of demyelination at the time of sampling, either at 119, 214 or 240 days post-infection. The mice with normal clinical features had no detectable virus in their spinal cords (Table 2). Autoantibodies to myelin were detected in all the mice tested and although the numbers of animals tested for anti-myelin antibodies were small, there was a trend towards elevated autoantibody titres in immunocompetent mice with histological lesions (Table 2). In addition, untreated mice had elevated anti-myelin antibodies compared to the depleted groups. This observation may reflect the increased number of affected mice in the intact group. All the mice tested had high serum levels of anti-TMEV antibodies (Table 2). It should be stressed that depletion of L3T4 subsequent to antibody production does not affect the antibody response since memory B cells are now present.

Histological examination was performed on three to five mice per group at day 211 or 240 post-infection. Although the numbers of animals were small, there was a good correlation
Table 2. Comparison of viral titres in the CNS, antibody to TMEV and myelin in intact mice and T cell-depleted mice

<table>
<thead>
<tr>
<th>Presence of clinical signs of demyelination</th>
<th>Intact mice</th>
<th>L3T4 depleted</th>
<th>Total T cell depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean virus titre in spinal cord (log_{10} p.f.u./g)</td>
<td>4-6 &lt;2 5-0 &lt;2 4-0 &lt;2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean and range of antibody titre to TMEV (log_{2})</td>
<td>13-6 11-1 12-6* 12-9 11-6* 9-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean and range of antibody titre to myelin (log_{2})</td>
<td>7-6-11-6 10-6-11-6 8-6-9-6 8-6-10-6</td>
<td></td>
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* Results obtained from one animal.

between the results of the histological examination and the clinical signs shown by the animals. Active demyelination or repaired areas of demyelination were evident in spinal cords of mice that had suffered from clinical signs for a period of 1 week or longer.

DISCUSSION

The experiments reported here involve the use of CBA mice rather than the widely studied SJL/J mice, since the process of demyelination in the CBA strain is more similar to the human disease of multiple sclerosis. The lesions in the spinal cord of TMEV-infected CBA mice are characterized by large focal areas of primary demyelination in the lateral and ventral white matter. Infiltration of inflammatory cells is associated with the genesis of demyelination but unlike the lesions in the SJL/J mice the infiltration is localized in time and space to the areas of demyelination and there is no axonal degeneration (Dal Canto & Lipton, 1979; W. F. Blakemore et al., unpublished observations).

From these studies in CBA mice, four observations will be discussed. Firstly, the removal of L3T4 helper T cells prior to infection with TMEV results in the death of CBA mice within 1 month, due to an uncontrolled infection. Secondly, in the late stage of the infection in immunocompetent mice, virus was isolated only from the spinal cords of mice with clinical signs of demyelination. Thirdly, autoantibodies against mouse myelin were detected in the late disease (1 month post-infection). Lastly, the removal of L3T4 T cells or total T cells at day 65 and 72 prior to the onset of clinical signs of demyelination, reduced the incidence of disease by 50%.

Helper T cells are central to the recovery of mice infected with TMEV. L3T4-deficient mice are unable to control the infection and consequently elevated titres of the virus are observed in the cord and brain, 1 month post-infection, the time at which the virus is reduced to undetectable levels in control (untreated) mice. The most likely reason for the inability to clear virus from the CNS is the failure of depleted mice to produce TMEV-specific antibodies. Antibodies have been widely implicated in the recovery from infection with picornaviruses (Murphy & Glasgow, 1968; Rager-Zisman & Allison, 1973). However, the ability of antibodies to reduce viraemia, though clearly important, may not be the only mechanism operating to clear virus from a local site of infection. T cells may be more relevant here and preliminary data suggests that Lyt 2-positive T cells are active against TMEV-infected cells. An additional consequence of T cell depletion before infection with TMEV was the absence of viraemia in intracerebrally injected mice. Similar observations were made with nude BALB/c mice (Rosenthal et al., 1986).

Viral replication in the CNS occurred during the first month after intracranial inoculation of TMEV in immunocompetent CBA mice. The early IgM response to TMEV appeared to be ineffectual in the clearance of virus from the CNS but the development of neutralizing titres of IgG coincided with the removal of virus from the brain and spinal cord. In a similar virus-induced demyelinating disease resulting from the intracranial injection of JHM coronavirus, viral clearance from the CNS occurred by day 12 post-infection (Wege et al., 1983). In comparison, the clearance of TMEV was remarkably slow. The reason for this difference is
unclear but may be due to the importance of antibody in the clearance of picornavirus infections. Generally, the results reported in this investigation are similar to those originally reported by Lipton (1975) in SJL/J mice. However, in the late stages of the disease in CBA mice, relatively high titres of virus were isolated from the CNS of mice with clinical symptoms.

The presence of antibodies to myelin detected in the late disease, may result from damage to myelin as an epiphenomenon or they may play a pathogenic role in the demyelinating process. As yet it is not clear whether the antibodies are directed against myelin basic protein as previously reported (Rauch & Montgomery, 1986) or galactocerebroside as suggested by cross-reactivity studies with TMEV (R.S. Fujinami, personal communication). Autoantibodies to myelin, present in the late stage of TMEV infection, suggest that autoimmunity may be involved in the demyelinating process. However, there was no direct correlation between the titre of anti-myelin antibodies and the severity of clinical disease. Also the presence of antibodies to myelin was not indicative of demyelination as clinically and histologically normal animals had similar titres to those showing evidence of disease. However, autoantibodies often appear in patients' sera prior to the development of symptoms, e.g. anti-islet antibodies precede overt diabetes (Gorsuch et al., 1981). Also, during the period of chronic infection virus was isolated from the spinal cords of animals with evidence of clinical demyelination. This suggests a strong correlation between the presence of infective virus and the induction of demyelinating disease. Similar findings have been noted using in situ hybridization techniques (Chamorro et al., 1986).

In the chronic demyelinating stage of TMEV infection, mice depleted of T cell subsets had a delayed onset of clinical signs associated with demyelination compared to immunocompetent mice. Virus was consistently isolated from the spinal cords of mice with clinical signs of demyelination but not from infected normal mice. The incidence of severe disease and histological evidence of demyelination was reduced in all the depleted groups compared to control mice. At present, we have not detected any consistent difference in the histological appearance of lesions in any of the different groups of mice, but the number of animals with lesions examined in the treated group was small.

The pathogenic mechanisms involved in TMEV-induced demyelination have yet to be resolved. Some authors suggest that demyelination is due to direct cytolysis of oligodendrocytes (Penney & Wolinsky, 1979). Our observation of the correlation between the presence of virus and the clinical symptoms of demyelination would support such a postulation. However, viral 'reactivation' may also result in an influx of T cells into the CNS which could mediate demyelination by a bystander phenomenon (Rodriguez et al., 1983). Alternatively, DTH responses have been implicated in strains of mice that are susceptible to TMEV-induced demyelination (Clatch et al., 1986). It is suggested by the depletion experiments in vivo described here in the late phase of TMEV infection, that in the absence of T cells the incidence of clinical signs associated with demyelination is dramatically reduced. This supports early observations by Rabinowitz & Lipton (1976) who suppressed TMEV-induced demyelinating disease using anti-thymus sera and cyclophosphamide. The main question here is whether the T cells involved in the pathology are autoimmune. In demyelination induced by the JHM strain coronavirus, myelin-reactive T cells are present which can transfer the demyelinating disease to normal animals. At present we are examining this possibility in TMEV-infected mice. Myelin-reactive T or B cell-mediated autoimmunity may play a role in demyelination either by attacking virus-infected oligodendrocytes or through cross-reactive determinants that TMEV shares with myelin. A final mechanism for demyelination may involve the aberrant expression of Ia on astrocytes which triggers autoimmunity as seen in JHM coronavirus infections (Massa et al., 1986).

It is apparent from these results that T cells play a double role in the pathogenesis of TMEV infections. On the one hand they are essential for the recovery from a primary acute infection possibly as helper cells involved in the generation of anti-TMEV antibodies; on the other hand they appear to be involved in the pathology of demyelinating disease. Whether these cells are responding to reactivated virus or myelin (or both) is the subject of further investigations.

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REFERENCES


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