Theiler's murine encephalomyelitis virus 3D RNA polymerase: its expression in the CNS and the specific immune response generated in persistently infected mice

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Intracerebral inoculation of the neurotropic murine picornavirus, Theiler's murine encephalomyelitis virus (TMEV), results either in an acute encephalitis (GDVII strain) or in the establishment of a persistent infection with the development of demyelinating lesions (BeAn strain). In this article, the expression of the viral RNA polymerase was studied in the central nervous system of both acutely and persistently infected mice and in infected cells in tissue culture. Similar numbers of acutely infected glial cells (80–85%) expressed both viral polymerase and structural proteins in vitro while a much smaller proportion of persistently infected glial cells (0.6–0.9%) expressed these proteins. Following infection of mice with GDVII, many cells in the brain were found to express polymerase. However, in the spinal cord of mice persistently infected with BeAn, very few cells were found to express the polymerase while many more cells showed the presence of viral structural proteins. This suggests that a restriction in viral replication, possibly at the level of polymerase expression, may be a feature of the persistent infection. However, enough polymerase was expressed to maintain a polymerase-specific antibody response in a number of infected animals as late as 21 months post-infection. Mechanisms that may be involved in the establishment and maintainance of TMEV persistence are discussed with reference to these findings.

Introduction

Theiler's murine encephalomyelitis virus (TMEV) was first isolated in 1934 from a mouse suffering from spontaneous flaccid paralysis of the hind limbs (Theiler, 1934). The virus is a picornavirus, belonging to the genus Cardiovirus, and is a natural enteric pathogen of mice that infects the cells lining the gastrointestinal tract and only causes central nervous system (CNS) disease with very low frequency (0.02–0.05%) (Theiler & Gard, 1940). There are two groups of TMEVs, the Theiler's original (TO) strains which include the BeAn and DA isolates and the virulent strains GDVII and FA. CNS disease can be induced routinely by intracranial inoculation of TMEV. Inoculation with the virulent strains results in a rapid and fatal encephalomyelitis with severe necrosis of neurons in the hippocampus, cerebral cortex, basal ganglia, hypothalamus, substantia nigra, pons and spinal cord grey matter (Olitsky, 1945; Stroop et al., 1981; J. P. Simas, H. O'Shea, A. A. Nash & J. K. Fazakerley, unpublished results). In 3- to 6-week-old susceptible mice, the TO strains cause a characteristic biphasic disease. The acute phase is characterized by acute inflammatory lesions of the brain and spinal cord and lasts for the first month of infection. Survival and recovery from this phase is coincident with the appearance of high titres of circulating virus-specific IgG and neutralizing antibody (Welsh et al., 1987). Despite this antibody response, survivors of the acute phase do not clear the virus from the CNS and develop a chronic, persistent infection involving oligodendrocytes, astrocytes and macrophages (Aubert et al., 1987; Clatch et al., 1990) and may also develop inflammatory, demyelinating lesions (Daniels et al., 1952; Lipton, 1975). The demyelination is associated with strong virus-specific delayed-type hypersensitivity (DTH) responses which, in susceptible SJL/J mice, are directed at a dominant T cell epitope in the VP2 protein (Gerety et al., 1994). Demyelination is thought to be brought about via a non-specific bystander mechanism caused by the recruitment and activation of macrophages by these T cells in areas containing viral antigen. These then strip...
the myelin lamellae from neurons. There is also the possibility that direct viral lysis of infected cells may occur (Brahic et al., 1981; Blakemore et al., 1988) or that TMEV-specific cytotoxic T cells are able to lyse infected oligodendrocytes (Rodriguez & Sriram, 1988; Borrow et al., 1992).

TMEV persistence in the CNS is not well understood. A number of mechanisms have been proposed to be involved in virus persistence; these include the development of viral antigenic variants, antibody-mediated persistence or the presence of some virus- or cell-specific factors that affect the viral replicative cycle (Roos & Whitelaw, 1984; Cash et al., 1985, 1988; Tolskaya et al., 1992). There is little evidence to support involvement of the first two mechanisms in TMEV persistence in the CNS. However, Cash et al. (1985, 1988) suggested that a block in viral RNA replication may occur and from this hypothesis that lack of a cellular factor may be involved in this restriction. However, in persistently infected cells, little is known about the expression of the TMEV non-structural proteins which are involved in viral RNA replication and possibly in viral protein expression. One study has shown the expression of the 3C and 3D proteins of the TO strain DA in productively infected BHK cells (Ozden et al., 1988) but no studies have been carried out on cells persistently infected with TO virus strains either in vitro or in the CNS of infected mice.

In addition, little is known about the immune responses raised against the non-structural proteins of picornaviruses following infection. The presence of antibodies specific for the 3D RNA polymerase has only been described previously in the diagnosis of foot-and-mouth disease virus (FMDV)-infected cattle (Cowan & Graves, 1966; Kleid et al., 1981; Bergmann et al., 1993). This paper describes the characterization of reagents that can detect TMEV 3D RNA polymerase expression after the establishment of persistent infections in vitro and in vivo. It also shows that an antibody response directed against the 3D RNA polymerase is present in susceptible mice up to 21 months post-infection (p.i.)

Methods

**Cells and viruses.** The BeAn and GDVII strains of TMEV were originally provided by Prof. H. L. Lipton (Mount Sinai Medical Centre, New York, USA). Virus was grown in BHK-21 cells cultured in the Glasgow modification of Eagle’s medium containing 10% newborn calf serum and 10% tryptose phosphate broth at 37 °C. Culture supernatants containing infectious virus were passaged three times and were aliquoted and stored at −70 °C. An immortalized glial cell line (MGCI) was generated from a primary mixed glial cell culture prepared as described by McCarthy & DeVellis (1980) with multiple passaging to enrich for astrocytes. These cells were then immortalized by transfection with a construct expressing temperature-sensitive simian virus 40 large T antigen (Jat & Sharp, 1989) and were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 1 mm-sodium pyruvate at 34 °C. After eight passages, subconfluent cells were infected with BeAn at an m.o.i. of 0.1. Extensive CPE was observed from 3 days p.i. with approximately 80–90% cell death. To establish a persistently infected cell culture, surviving cells (PGC) were passaged and maintained in tissue culture for several weeks during which time 10^5–10^6 p.f.u. of virus was detectable in the cell medium.

**DNA constructs.** The BeAn infectious cDNA clone, pKLT7BA (Law, 1990) was developed in the Department of Pathology, Cambridge University from the virus isolate supplied by Prof. H. L. Lipton. The viral sequence is flanked by a T7 promoter and a NotI cloning site in a pUC118 plasmid background (Vieira & Messing, 1987; Fig. 1a). The DNA fragments indicated in Fig. 1(a) were excised, made blunt-ended and ligated to NotI-cleaved shuttle vectors pJH1, pJ14 or pJ35 (Fig. 1b). The BamHI cassettes containing the TMEV fragments were then cloned into the BamHI site of the expression vector, pET3a (Studier et al., 1990; Fig. 1c) to create T7 gene 10–TMEV fusion protein constructs. The intermediate cloning step allowed the insertion, between the gene 10 and TMEV sequences, of a tetrapeptide sequence Ile–Glu–Gly–Arg, that is specifically recognized and cleaved by blood coagulation factor X, (Magnusson et al., 1975). The pJ4 BamHI cassette also contains an extra 4 nucleotides upstream of the TMEV sequence that maintains the reading frame of the 3D polymerase on subsequent expression from the pET3a construct and the pJ15 cassette contains an extra 5 nucleotides. The final constructs expressed the following regions of BeAn: pET–VP4/VP2 (VP4 13–100 and VP2 1–117); pET–VP2/VP3 (VP2 44–236 and VP3 1–154); pET–VP3/2B (VP3 155–232, VP1, 2A and 2B 1–12); pET–3D–pol (3C 208–217 and 3D). Two constructs were also assembled for in vitro transcription of the GDVII 3D polymerase gene: ca7 (Fig. 1d) and by21. These constructs are in a pUC118 background and contain a DNA fragment extending from the PsI site at 6697 to the 3’ end of the viral genome (amino acids 35–461).

**Expression and purification of the pET–TMEV fusion proteins.** The expression protocol for the pET–TMEV fusion proteins was as described previously (Studier et al., 1990). The insoluble proteins were then purified by detergent precipitation (Nagai & Thogersen, 1987), SDS–PAGE and electroelution.

**Preparation of antibody to the TMEV fusion proteins.** Rabbits (Dutch or Dutch/New Zealand White cross, Department of Pathology Animal Unit, University of Cambridge, UK) were immunized with 500 μg of gel-purified fusion protein emulsified in 2 volumes of Freund’s complete adjuvant and were given three boost inoculations with 200 μg of protein in Freund’s incomplete adjuvant at monthly intervals. The rabbits were bleed 2 weeks after each immunization and the polyclonal serum was clarified by centrifugation.

**Infection and sampling of mice.** CBA, BALB/c, SJL/J and PL/J mice were obtained from the Department of Pathology Animal Unit (University of Cambridge, UK) and Olac (Bicester, UK). CBA mice (3–4 weeks old) were anaesthetized and infected intracerebrally (i.c.) with 10^4 p.f.u. of GDVII virus in 20 μl of PBS. Mice infected i.c. with BeAn virus were between 3 and 6 weeks old and received between 10^4 and 10^5 p.f.u. of virus in 20 μl of PBS, depending on the experiment. In general, mice were sacrificed after acquiring clinical signs of disease. However, owing to the reorganization of the Pathology Department animal facilities, a number of long-term, asymptomatic BeAn-infected mice (13/17) were also euthanized and their sera tested for TMEV antigen reactivity (Fig. 6). Symptoms of acute encephalitis included ruffled fur, unsteady gait, breathing difficulties and flaccid paralysis of the hind limbs. Characteristics of the later, chronic disease included hunched posture, weight loss, spastic paralysis of the tail and paralysis of the limbs: more usually observed in the hind limbs.
Fig. 1. The basic plasmids used in this study. The pKLT7BA cDNA clone of the BeAn virus is based on the pUC118 plasmid. The transcription vector contains a T7 promoter at the 5' terminus and a NotI cloning site at the 3' terminus. The four DNA restriction enzyme fragments shown in (a) were excised from the full-length clone, made blunt-ended and were inserted into a purpose-built StuI-cleaved shuttle vector (b). These were constructed by ligating self-annealed oligonucleotides #922 5' GATCCATCGAGGGTAGGCCTACCCTCGATG 3', ICDJ4 5' GATCCATCGAGGGTAGGAAGGCCTTCCTACCCTCGATG 3' or ICDJ5 5' GATCCATCGAGGGTAGGATAGGCCTACCCTCGATG 3' to BamHI-cleaved pUC118 (b). The vector (pIJ1, pIJ4 or pIJ5) was chosen depending on the reading frame of the TMEV fragment. The Bsu36I-BamHI and HindIII-EcoRI fragments were cloned into pIJ1, the EcoRI fragment into pIJ5 and the PvuII-NotI fragment into pIJ4. The BamHI cassette containing the TMEV fragment was then ligated to BamHI-cleaved pET3xa expression vector (c) (Studier et al., 1990). Subsequent expression of this construct results in a fusion of the viral protein sequence to the first 260 amino acids of the gene 10 protein of bacteriophage T7, the two sequences being separated by the Ile-Glu-Gly-Arg tetrapeptide sequence recognized by blood coagulation factor Xα. (d) Construct ca7 for in vitro transcription of the GDVII polymerase. Construct by21 was identical except for the lack of a XhoI site. The hatched area indicates the 3' untranslated region. AmpR, ampicillin resistance gene; ori, T7 intergenic region; φ10, bacteriophage φ10 T7 promoter sequence; Φφ, transcription terminator sequence.
Preparation of radiolabelled infected cell lysate. Confluent BHK cells were infected with BeAn virus at m.o.i. of 3. [35S]Methionine was added (1 μl for every 5 × 10⁶ cells) and the cells left in methionine-free medium overnight. All cells and cellular debris were collected in 100 μl RIPA buffer (50 mM-Tris-Cl pH 7.2, 150 mM-NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100) per 5 × 10⁶ cells, vortexed vigorously three times and the insoluble matter removed by centrifugation. The supernatant (cell lysate) was collected and stored at -70 °C.

In vitro transcription and translation. Plasmids were linearized with either SphI (by21) or XhoI (ca7) under standard conditions and the linear template was recovered by a single extraction with phenol followed by ethanol precipitation in the presence of 3–5 m-sodium acetate. Any interfering impurities were removed by Sephadex G-50 chromatography. Linearized template (1–2 μg) was transcribed and purified as described previously, but in the absence of cap (Brierley et al., 1989). RNA was recovered by ethanol precipitation, dissolved in water and checked for integrity by electrophoresis on 15% agarose gels containing 0.1% SDS. Dilutions of purified RNA were translated in rabbit reticulocyte lysates as described previously (Brierley et al., 1989).

Radioimmunoprecipitation assay. For the specific immunoprecipitation of the products of an in vitro transcription or radiolabelled viral proteins from a viral cell lysate, 4 μl of the translation product or 10 μl of cell lysate was made up to 20 μl with RIPA buffer and incubated with 2–5 μl of antisera. Protein–antibody complexes were precipitated using Protein A–Sepharose beads (Sigma Biochemical) and standard techniques. Recovered protein samples were then electrophoresed through 15% SDS-polyacrylamide gels.

Western blot analysis. For the detection of TMEV-specific antibodies, a Western blot assay was used. Fifty μg of the pET–TMEV gel-purified fusion proteins were mixed with the same amount of gel-purified T7 gene 10 protein. The protein samples were separated by SDS–PAGE on a 15% gel without wells and transferred to nitrocellulose paper. After transfer, the filter was cut up into 7 mm wide strips, each of which was incubated with a different mouse serum at 1/200 dilution. Blocking and incubation steps were carried out in a solution of 5% milk powder (Marvel), 2% fetal calf serum in PBS at 37°C. Filter washing was performed using 1% Nonidet P-40 and 1% FCS in PBS. The filters were then labelled by the ECL method according to the manufacturer’s instructions (Amersham International) and bound antibody visualized by exposure to X-ray film.

Immunohistochemistry. For use in immunostaining procedures, BHK cells and glial cell lines were grown on microscope slides that had previously been coated with 0.01% poly-L-lysine or 2% Bovod (British BioCell International) and sterilized. For preparation of tissue samples, animals were perfused with PLP fixative (2% paraformaldehyde, 75 mM-lysine HCl, 30 mM-Na₂HPO₄, 17 mM-NaH₂PO₄, 3.6% sodium periodate). The tissues were then cryoprotected in increasing concentrations of sucrose in PBS (12%, 16%, 18%) before flash freezing in dry ice-cooled isopentane. Frozen sections (5–10 μm) were cut and were stored dry at -70 °C. Standard peroxidase staining techniques were used for both tissue culture cells and histological sections. Briefly, sections or tissue culture cells were treated with 0.3% hydrogen peroxide in methanol to inactivate any endogenous peroxidases. The samples were then rehydrated through graded ethanol, permeabilized by treatment with 0.1% Triton X-100 and blocked with 5% normal goat serum in PBS. The samples were incubated at room temperature with the primary antibody and 1% normal goat serum for 1 h. After washing, the samples were again blocked before incubation for 45 min at room temperature with the secondary antibody, a biotinylated goat anti-rabbit IgG serum (Vector Laboratories). A Vectastains ABC kit (Vector Laboratories) was used to amplify the signal which was visualized using diaminobenzidine as substrate according to the manufacturer’s instructions. Samples were lightly counterstained with haematoxylin.

In situ hybridization. Riboprobes were transcribed from a recombinant pGen3 vector containing the BeAn Sal–XhoI restriction fragment (nucleotides 1729–4733). Probes were labelled with [35S]ATP using a Gemini riboprobe labelling kit (Promega), hydrolysed to a mean size of 200 bases and in situ hybridization performed as described previously (Fazakerley et al., 1993).

Results

Antiserum raised against the pET-3D-pol fusion protein has specific binding activity to the TMEV 3D RNA polymerase

The specificity of the rabbit antiserum raised against the bacterial polymerase fusion protein was tested in a number of in vitro systems (Fig. 2). Cell lysates were prepared from unlabelled BeAn-infected and mock-infected BHK cells and equivalent amounts were separated by SDS–PAGE on a 15% gel. After transfer to nitrocellulose, a protein of approximately 55 kDa was detected using the polymerase-specific antiserum in the BeAn-infected, but not the mock-infected cell lysate (Fig. 2 a). This corresponds well with the predicted size of the protein (Pevear et al., 1987; Law & Brown, 1990). The specificity of the serum was further investigated by immunoprecipitation of radiolabelled viral proteins. Firstly, constructs coding for the majority of the GDVII 3D polymerase (ca7 and by21; see Fig. 1 d) were linearized and transcribed in vitro. Dilutions of the transcripts were translated in a rabbit reticulocyte lysate system in the presence of [35S]methionine and the radiolabelled proteins were immunoprecipitated with the polymerase serum (Fig. 2 c). The size of the major product (46 kDa) corresponded well with the size predicted for this truncated protein from the amino acid sequence. Secondly, immunoprecipitations were carried out from a radiolabelled BeAn-infected BHK cell lysate using the polymerase serum and rabbit sera raised against the pET-VP3-2B bacterial fusion protein and heat-inactivated BeAn virus (Fig. 2 b). The sera specific for viral capsid proteins precipitated whole virions or protomers whereas the polymerase serum precipitated one protein of 54 kDa. No specific proteins were precipitated from a mock-infected, radiolabelled BHK cell lysate and the pET-VP3-2B serum did not precipitate the 2A protein or its precursors.

3D RNA polymerase expression in infected fibroblast cells in vitro

BHK cells were grown on microscope slides and infected as detailed in the Methods. The cells were fixed in 1% paraformaldehyde 18 h p.i. and immuno-
Theiler's virus polymerase expression

Fig. 2. Characterization of rabbit antisera. (a) Western blot detection of BeAn 3D RNA polymerase in infected BHK cell lysates. Unlabelled cell lysate (25 µl) was prepared from BeAn-infected (+) or mock-infected (−) BHK cells. The samples were separated by SDS-PAGE on a 15% gel, transferred to nitrocellulose, blocked and then incubated with a 1/1000 dilution of the polymerase-specific serum. Blocking and incubation steps were as detailed in Methods. The bound antibody was detected using 125I-labelled Protein A and visualized by autoradiography. Unpurified bacterial fusion protein pET-3D-pol (10 µg) was also transferred (pET-3D-pol) and a shorter exposure shows binding to the fusion protein product. The arrow marks the 55 kDa TMEV 3D RNA polymerase. (b) Radioimmunoprecipitation of viral proteins from BeAn-infected (+) and mock-infected (−) [35S]methionine-labelled cell lysates (details in Methods). Three rabbit sera were used, raised against the following antigens: heat-inactivated BeAn virus; pET-VP3-2B fusion protein and pET-3D-pol fusion protein. The viral proteins precipitated from the infected cell lysate are indicated. (c) Radioimmunoprecipitation of in vitro translations of GDVII 3D RNA polymerase constructs ca7 and by21. The translation products were labelled with [35S]methionine, separated on a 15% polyacrylamide gel and visualized by autoradiography. Water indicates a negative control where no transcript was added to the translation reaction. In (a) and (b) M indicates 14C-labelled molecular mass standards.

cytochemical staining was carried out using the polymerase-specific and capsid-specific primary rabbit antisera and rabbit Ig-specific biotin-conjugated secondary goat antibody. Very little non-specific staining was seen on mock-infected BHK cells with either serum (Fig. 3a and d). However, large amounts of reaction product were seen in the cytoplasm of infected cells stained for polymerase (Fig. 3b). In many cells, the antigen was localized in foci which were much more intensely stained than the surrounding cytoplasm (Fig. 3c). Cells were stained with differing intensities ranging from apparently uninfected cells, to cells which had lost the natural BHK cell morphology and had a rounded appearance, associated with intense staining for polymerase. An identical range of staining patterns was seen when either the capsid-specific serum was used (Fig. 3e) or serum raised against heat-inactivated virus (not shown) and between 60 and 65% of surviving cells were positively stained for TMEV antigens (Table 1). TMEV RNA distribution in infected cells can also be focal (Brahic et al., 1981). This suggests that TMEV RNA, structural and non-structural proteins may all be associated with virus replication complexes as is seen in poliovirus-infected cells (Yin, 1977; Bienz et al., 1992). A previous report on the DA strain 3C protease and 3D polymerase protein distribution in infected BHK cell cytospins has shown cytoplasmic staining but with the antigen appearing to surround the nucleus (Ozden et al., 1988). That this was not seen here, suggests that this phenomenon may have been due to intracellular disruption caused by trypsin treatment and centrifugation of the cells.

3D RNA polymerase expression in acutely and persistently infected glial cell cultures

In the CNS of persistently infected mice, one of the cell types in which TMEV can be detected is the astrocyte. This cell type has also been demonstrated to present TMEV antigens to T cells in vitro (Borrow & Nash,
Fig. 3. BHK-21 cells were grown on microscope slides and infected (b, c and e) or mock-infected (a and d) with BeAn virus. After 18 h, the cells were immunoperoxidase stained for the presence of polymerase (a–c) or capsid antigens (d and e). 1/500 dilutions of the specific rabbit sera raised against pET-3D-pol and pET-VP3-2B were used as the primary antibody. Cells were counterstained with haematoxylin. In all micrographs the bar marker represents 50 μm except in (e) and the inset of (c) where it represents 20 μm and 100 μm respectively.

1992). It was therefore of interest to determine whether 3D polymerase expression following productive and persistent infection of this cell type was different to that seen in BHK cells. The MGC7 glial cell clone was grown and infected or mock-infected with BeAn virus on microscope slides as described for BHK cells, except for the use of glial cell culture conditions. The persistently infected PGC cells were cultured for 2 days on microscope slides before fixation and immunostaining. Polymerase and capsid antigens were detected in the MGC7 cells with a similar varied distribution to that described above for BHK cells, with strong cytoplasmic staining, often focal, in 82–86% of surviving cells (Fig. 4 a and b; Table 1). The PGC cells showed cytoplasmic staining for polymerase and capsid antigens only in a subpopulation of cells (Table 1) and in most cells this staining was evenly distributed (Fig. 4d). A subpopulation of cells was also positive for viral RNA by
Table 1. Proportion or number of cells in which TMEV antigen was detectable in vitro and in vivo

<table>
<thead>
<tr>
<th>Sample</th>
<th>Antigen load</th>
<th>Cell type</th>
<th>pET-VP3-2B</th>
<th>Heat-inactivated BeAn</th>
<th>pET-3D-pol</th>
<th>Viral RNA</th>
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<tr>
<td>Tissue culture cells positive for viral antigen (%)*</td>
<td></td>
<td>BHK</td>
<td>60.0</td>
<td>61.1</td>
<td>64.9</td>
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<td></td>
<td></td>
<td>MGC</td>
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<td>83.4</td>
<td>82.0</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td>PGC</td>
<td>0.6</td>
<td>0.9</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>BeAn-infected spinal cord tissue (chronic)†</td>
<td>Low</td>
<td>High</td>
<td>37.4</td>
<td>26.0</td>
<td>3.8</td>
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<tr>
<td></td>
<td>Mean</td>
<td>Low</td>
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<td>1.7</td>
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<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>12.0</td>
<td>8.9</td>
<td>1.3</td>
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<tr>
<td>BeAn-infected brain tissue (acute)§</td>
<td>Antigen load</td>
<td></td>
<td>61.4</td>
<td>54.0</td>
<td>56.0</td>
<td></td>
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<tr>
<td>GDVII-infected brain tissue (acute)§</td>
<td></td>
<td></td>
<td>ND</td>
<td>&gt; 200/field</td>
<td>&gt; 200/field</td>
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</tr>
</tbody>
</table>

* Approximately 2000 tissue culture cells (BHK, MGC7 and PGC) were counted after immunoperoxidase staining with the rabbit TMEV antigen-specific antisera indicated and 1150 PGC cells were examined for the presence of BeAn RNA by in situ hybridization.
† The average number of TMEV antigen-positive cells present per section in cryostat sections of spinal cord taken from BeAn-infected CBA mice killed 70 days p.i. A total of 207 spinal cord sections were screened using the antisera indicated. The sections could be divided into two groups on the basis of a high number of positive cells (< 10) or a low number (> 10). The mean value is also shown.
‡ The average number of TMEV antigen-positive cells present per section in cryostat sections of brain taken from CBA mice acutely infected with BeAn, killed 10-18 days p.i. A total of 15 sections were screened.
§ The minimum number of TMEV antigen-positive cells present per × 16 field of view in cryostat sections of brain taken from CBA mice acutely infected with GDVII. A total of 31 sections were screened.

in situ hybridization (Fig. 4c; Table 1). Occasional cells had a speckled appearance in the cytoplasm, with numerous small foci staining for viral polymerase (Fig. 4e and 4f) although this pattern was seen less frequently for capsid proteins. Dense, focal staining as seen in acutely infected cells was very rare, but some cells were found with a rounded morphology (Fig. 4g). Similarly small numbers of cells were positive for polymerase, capsid and viral genome (0.6–1 %; Table 1), observations that would be consistent with the hypothesis that the persistently infected culture is a ‘carrier culture’, with only a small number of cells becoming infected (Walker, 1964).

3D RNA polymerase expression in the mouse CNS

The TMEV 3D polymerase and structural proteins could be detected in similar numbers of infected cells in vitro. To determine whether differential expression of TMEV proteins might play a role in TMEV persistence in vivo, we investigated the distribution of TMEV antigens in the mouse CNS following both acute (BeAn and GDVII) and persistent (BeAn) infections. Cryostat sections were prepared from infected CBA and control BALB/c mouse CNS tissue as detailed in Methods and Fig. 5. Mice infected with 10⁴ p.f.u. of GDVII were sampled at 4 days p.i. when they became moribund. The sections were then immunoperoxidase stained for the presence of 3D RNA polymerase. A number of regions of the GDVII-infected mouse brains (cortex, basal ganglia and hippocampus) contained cells that expressed this antigen (Table 1). These regions also contained cells expressing viral capsid proteins (Table 1) and containing viral genome (data not shown). Each field of view in an infected area had at least 200 TMEV-positive cells. Cells were also found positive for polymerase and capsid antigens in sections of brains from BeAn-infected CBA mice that developed symptoms of acute encephalitis 10–18 days p.i. The number of antigen-positive cells was much lower than following GDVII infection, but both capsid and polymerase antigens appeared to be present in similar numbers of cells (Table 1). Cryostat sections (10 μm) were also cut from the spinal cords of CBA mice 70 days p.i. with 10⁴ p.f.u. of BeAn. These mice had developed clinical signs of disease including hunched posture and gait abnormalities 3–4 days previously. Brain material was not examined as no virus can be isolated from the brain of CBA mice later than 21 days p.i. with BeAn virus (Borrow et al., 1993). Sections were stained for the presence of viral polymerase and a number of positive cells were seen in the white matter (Fig. 5b; Table 1). Serial sections were also cut to show the fidelity of the polymerase antisera on cryostat sections and the adjacent sections were stained using sera specific for viral capsid proteins (Fig. 5c–h). The serial sections show a small focus of infection in the white matter of the spinal cord. A glial cell is stained with the pET-VP3-2B serum (Fig. 5c and f) and the cell body of this cell or a neighbouring cell contains viral polymerase (Fig. 5d and g). The following section shows cellular debris or processes stained with the serum raised against heat-inactivated BeAn virus (Fig. 5e and h). Numerous cells were seen stained with the sera raised against the pET-VP3-2B fusion protein and heat-inactivated BeAn
in other sections of spinal cord white matter, but 90\% fewer cells were seen stained for viral polymerase irrespective of whether a high or low number of TMEV-positive cells were present (Table 1; Fig. 5b). The specificity of all sera was good with very low background binding activity in the CNS of uninfected animals.
Antibody responses to TMEV 3D RNA polymerase are detectable in infected mice at times up to 21 months p.i.

As 3D polymerase expression was detectable in the CNS of persistently infected mice, we decided to screen the sera from a selection of infected mice for the presence of polymerase-specific antibodies at various times p.i. Infected mice were bled from the tail vein at various times p.i. and serum clarified by centrifugation after clot formation. Samples were from PLJ mice at 112 days p.i., SJL/J mice at 181 days p.i., PLJ mice at 209 days p.i. and CBA mice at 638 days p.i. (Fig. 6). All mice were asymptomatic at the time of sampling apart from two PLJ mice (112 days p.i.) and two PLJ mice (209 days...
Fig. 6. Detection of antibodies specific for the structural proteins of TMEV in persistently-infected susceptible mice. A mixture of T7 gene 10 fusion proteins encompassing the regions shown were mixed along with the T7 gene 10 protein, separated by SDS–PAGE, transferred to nitrocellulose which was cut into 7 mm strips. Each strip was incubated with individual infected mouse sera and visualized by ECL and autoradiography (see Methods). The number of mouse sera giving a positive signal with each antigen is summarized. The CBA mouse immunized with pET-3D-pol consistently gave a positive signal to all T7 gene 10 proteins whereas the pooled normal CBA sera were always negative.

p.i.). These mice had symptoms of waddling gait and/or rear limb paralysis. The individual mouse sera were screened for the presence of TMEV antibodies by Western blot as detailed in the Methods. Polymerase-specific antibodies were detected in 8/30 sera tested (Fig. 6), and a representative blot is shown in Fig. 7. None of the mouse sera bound to the T7 gene 10 protein, indicating that the specificity was to the viral protein sequence and not the T7 fusion sequence. Pooled sera from four uninfected CBA mice did not react with either protein and serum from a CBA mouse immunized with pET-3D-pol reacted with both proteins. There was no correlation between clinical symptoms of disease seen in the four symptomatic PLJ mice and the presence of polymerase antibodies (data not shown), although the proportion of mice possessing this antibody specificity appeared to decrease with time after infection (5/7 PLJ mice at 112 days p.i. compared to 2/13 CBA mice at 638 days p.i.; Fig. 6). All mice that had detectable levels of polymerase-specific antibody in their serum also showed reactivity by Western blot to one or more viral capsid proteins (Fig. 6).
Discussion

The mechanisms involved in the establishment and maintenance of a persistent CNS infection by the TO strains of TMEV have not yet been elucidated. Once an infection has been established, the virus is able to persist in the CNS of some susceptible mice for many months or years as determined by virus isolation, in situ hybridization for viral RNA or PCR for viral RNA (Lipton, 1975; J. P. Simas, S. Amor, A. A. Nash & J. K. Fazakerley, unpublished results). The question arises of whether the persisting virus is actively replicating or whether merely virus RNA or virions are persisting.

In this paper, we have characterized a rabbit polyclonal serum that has specific reactivity with the TMEV 3D RNA polymerase. Immunocytochemistry with this serum has shown that in productively infected BHK-21 and MGC7 cells large amounts of polymerase are expressed with a similar cytoplasmic distribution to the viral structural proteins. The numbers of cells stained and the intensity of the staining were also similar for both cell types (Table 1). However, after the establishment of a persistent infection of an immortalized glial cell culture, heavy focal staining was rarely seen and only 0.6–1% of cells were positive for viral antigen. This suggests that a carrier culture, as described by Walker (1964), had been established. It is likely that the majority of glial cells in the infected culture were unable to support BeAn replication while the minority of cells susceptible to infection were able to maintain the titre of $10^4$–$10^6$ p.f.u. seen in the tissue culture supernatant. The significance of the speckled TMEV-specific staining patterns seen in PGC cells compared to the antigen distribution in MGC7 cells is not clear. Viral release in both infected MGC7 and PGC cells is presumed to be lytic, even if some restriction in viral replication were to be occurring in the PGC cells.

It has been proposed that a block in viral RNA replication may occur in the majority of persistently infected cells in vivo, resulting in a reduced number of viral genome copies which in turn would lead to low expression of viral proteins (Cash et al., 1985, 1988; Cash & Brahic, 1986). This evidence involved quantification of the number of genome copies present in individual infected cells in the CNS. It was noted that a small proportion of viral genome positive cells contained a higher than average copy number and that only in these cells could viral proteins be detected by immunocytochemistry. The authors proposed that a cellular factor or factor(s) may be absent from the majority of infected cells and that only in a minority of cells would the presence of this factor result in viral replication and the expression of viral capsid proteins to a detectable level.

Immunohistochemical investigation of TMEV 3D polymerase expression in the CNS of infected mice has shown that similar numbers of cells are positive for this antigen as for structural proteins during acute infection with both the BeAn and GDVII strains of the virus. However, in persistently infected mice, polymerase expression can only be detected in a very small number of cells in areas of the spinal cord where many more cells contain detectable levels of capsid proteins. This indicates that during acute disease in the brain, when the
infection is predominantly neuronal (Lipton, 1975; Stroop et al., 1981; J. P. Simas, H. O'Shea, A. A. Nash & J. K. Fazakerley, unpublished results), there is no problem with accessibility of the 3D polymerase antisera to its antigen. Also, the differential staining seen for the 3D polymerase antigen in the persistently infected spinal cord is not likely to be due to differential affinities of the different antisera used. This is supported by the fact that the sera raised against pET-VP3-2B and pET-3D-pol both show similar reactivity at a number of dilutions in infected BHK cells, MGC7 cells, PGC cells as well as in the GDVII-infected mouse CNS (unpublished observations). It is therefore more likely that the quantities of antigen present are the limiting factors in detection. It should be noted that although spinal cord tissues were not examined during the acute infections, the viral tropism at this time is also predominantly neuronal.

Possible interpretations of this observation of reduced 3D polymerase expression in the spinal cord include: (i) there is some restriction in expression of the 3D polymerase protein compared to capsid proteins in the majority of infected cells and (ii) the 3D RNA polymerase protein is less stable than the capsid proteins and therefore either has a shorter half life in vivo, becomes inactivated by some factor or becomes degraded during immunohistochemical procedures.

In picornaviruses, the viral proteins are expressed as one single large precursor polypeptide from the positive sense genomic RNA. It is most likely, therefore, that any downregulation of the non-structural proteins would occur by premature termination of translation or alternatively by a post-translational mechanism. For example, following translation, stable, assembled virions could be able to persist in infected cells, perhaps in association with smooth membranes (Lorch et al., 1981; Frankel et al., 1986), whereas non-assembled structural proteins and non-structural proteins could be degraded. Indeed, by electron microscopy, viral particles have been observed in the cytoplasm of, and in association with, oligodendrocytes in the CNS (Blakemore et al., 1988). It is also possible that some cellular factor specifically inactivates the polymerase and so prevents viral RNA replication. If a cellular factor is involved, what induces it? TMEV infection of astrocytes in vitro is known to alter the expression of cellular genes, for example MHC and cytokine genes (Borrow & Nash, 1992; Rubio & Capa, 1993). It is also known that the expression of cellular genes following viral infection can lead to the restriction of viral replication in other RNA viruses; for example MxA protein restricts measles and influenza virus replication (Pavlovic et al., 1992; Schnorr et al., 1993). Some similar process may be occurring after TMEV infection.

As shown above, expression of the TMEV 3D polymerase during the chronic phase of TMEV infection of susceptible mice does occur. This protein is able to induce an antibody response in mice soon after infection, and this polymerase-specific antibody response can still be detected in some mice up to 21 months p.i. This is in spite of the fact that even at 70 days p.i., expression of the polymerase in the mouse CNS appears to be greatly restricted in comparison to the viral capsid proteins. It is possible to raise T cell responses against intracellularly expressed viral antigens (reviewed by Long & Jacobson, 1989), but it is much less likely that antibody responses could be primed against antigens that are not released from the cells. If the TMEV polymerase-specific antibody response is not of a very long duration, its detection could be used as a marker for recent virus expression in the animal and could indicate that a lytic infection of cells within the CNS was occurring at very late timepoints p.i.

It is unlikely that an immune response against the polymerase would have a role in the progression of TMEV-induced disease or in the protection of the host from its effects as preliminary experiments have indicated that immunization of CBA mice with the pET-3D-pol fusion protein prior to infection i.c. with BeAn does not protect mice against disease, whereas immunization with heat-inactivated virus does (data not shown).

It will be most interesting to determine whether the presence of detectable polymerase protein is linked to areas of demyelination and inflammation in the CNS and the presence of increased amounts of viral RNA. It has not yet been determined whether viral replication is required to induce an episode of inflammatory demyelination or whether the mere presence of persisting viral antigens is sufficient. It is also possible that the changed microenvironment within an area of inflammation could lead to the induction of viral gene expression and the escape from restriction.

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References


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