Examination of Eight Cases of Multiple Sclerosis and 56 Neurological and Non-neurological Controls for Genomic Sequences of Measles Virus, Canine Distemper Virus, Simian Virus 5 and Rubella Virus

By S. L. COSBY, 1,2 S. MCQUAID, 1 M. J. TAYLOR, 2 M. BAILEY, 1 B. K. RIMA, 2 S. J. MARTIN 2 AND I. V. ALLEN 1

The Queen's University of Belfast, 1Multiple Sclerosis Laboratory, Institute of Pathology, Royal Victoria Hospital, Belfast BT12 6BL and 2NICGENE, School of Biology and Biochemistry, 97 Lisburn Road, Belfast BT9 7BL, U.K.

(Accepted 10 April 1989)

SUMMARY

In situ hybridization studies have been carried out on brain samples from eight cases of multiple sclerosis (MS) and 56 non-neurological and neurological controls, using single-stranded 35S-labelled RNA probes prepared against genomic RNA sequences of measles virus, canine distemper virus, rubella virus and simian virus 5. Foci of hybridization were found using probes against the measles virus nucleocapsid protein (N), phosphoprotein and fusion protein gene sequences in two of the MS cases, and also in one control, a case of disseminated cytomegalovirus infection with spinal cord necrosis. This result was confirmed using biotinylated probes prepared against the measles virus N genomic sequence. No hybridization was found in any of the MS or control cases using any of the other viral genome-specific probes.

INTRODUCTION

Direct evidence for a viral aetiology for multiple sclerosis (MS) is still lacking, despite a large amount of circumstantial evidence from a variety of disciplines (reviewed by Cook & Dowling, 1980; McFarlin & McFarland, 1982; ter Meulen & Stephenson, 1983; Johnson, 1985). Epidemiological and serological studies have suggested the involvement of a number of different enveloped RNA viruses in MS, including measles virus (MV), canine distemper virus (CDV), simian virus 5 (SV5) and rubella virus (Haire et al., 1973; Norrbey et al., 1974; Haire, 1977; Meurman et al., 1977; Cook et al., 1978; Goswami et al., 1987). Measles virus is still a prime candidate on the basis of evidence from serological studies of MS patients and controls (Adams & Imagawa, 1962; Haire et al., 1973; Norrbey et al., 1974; Haire, 1977; Meurman et al., 1977; Cook et al., 1978; Goswami et al., 1987). Measles virus is still a prime candidate on the basis of evidence from serological studies of MS patients and controls (Adams & Imagawa, 1962; Haire et al., 1973; Norrbey et al., 1974; Haire, 1977; Meurman et al., 1977; Cook et al., 1978; Goswami et al., 1987). Measles virus is still a prime candidate on the basis of evidence from serological studies of MS patients and controls (Adams & Imagawa, 1962; Haire et al., 1973; Norrbey et al., 1974; Haire, 1977; Meurman et al., 1977; Cook et al., 1978; Goswami et al., 1987). Measles virus is still a prime candidate on the basis of evidence from serological studies of MS patients and controls (Adams & Imagawa, 1962; Haire et al., 1973; Norrbey et al., 1974; Haire, 1977; Meurman et al., 1977; Cook et al., 1978; Goswami et al., 1987).
The evidence for and against the presence of MV sequences in both MS and non-MS control brains is therefore conflicting. In view of these inconsistencies and the suggested involvement of other RNA viruses in the disease, we have used 35S-labelled ssRNA probes to MV, CDV, SV5 and rubella virus to examine brain tissue (by ISH) from eight cases of classical MS in which inflammation in the central nervous system (CNS) was marked, two cases of subacute sclerosing panencephalitis (SSPE) and 56 neurological and non-neurological controls. Biotinylated RNA probes have also been used in specific cases to confirm results obtained with radioactively labelled probes.

METHODS

Clinical material. Brains from eight cases of classical MS, two cases of SSPE, one case of disseminated cytomegalovirus (CMV) infection, with spinal cord necrosis, one case of cerebral thrombosis and four non-neurological controls were obtained 1 to 12 h post-mortem (the majority under 6 h). Blocks were either dissected from fresh brain and snap-frozen or obtained from sectioned formalin-fixed brains. Biopsy material from four cases of cerebral abscess were also snap-frozen. A detailed account of the pathology and clinical history of the MS and control cases will be reported elsewhere (Allen et al., unpublished). Snap-frozen samples (three to four blocks per brain) from 23 cases of schizophrenia and 23 non-neurological controls were obtained from the MRC Brain Bank (Addenbrooke's Hospital, Hills Road, Cambridge, U.K.).

Virus and cell culture. The origin and methods of propagation of the human 2 strain of MV and the Onderstepoort strain of CDV have been previously described (Rima & Martin, 1979; Campbell et al., 1980; Cosby et al., 1981). SV5 was supplied by Dr R. E. Randall (University of St Andrews, U.K.) and propagated in a manner similar to that described for MV and CDV. Rubella virus was supplied by Dr P. V. Shirodaria (Department of Microbiology and Immunobiology, Queen's University of Belfast, U.K.). Cells were infected with undiluted inoculum, which was removed after 1 h and replaced with Eagle's MEM containing 20% kaolin-adsorbed serum (prepared as described by Schmidt & Lennette, 1970). Vero cells, obtained from Flow Laboratories were grown in Eagle's MEM (Glasmov modification) supplemented with 5% newborn calf serum and infected with virus. A primary human astrocytoma cell culture, derived from a biopsy of an astrocytoma (supplied by Dr D. McCormick, Neuropathology Laboratory, Queen's University of Belfast, U.K.) was grown in RPMI 1640 medium (Gibco) supplemented with 10% foetal calf serum and infected with virus, for the ISH analyses.

Probes. The measles N gene clone was supplied by Professor S. Rozenblatt (University of Tel Aviv, Israel) and has been previously described (Rozenblatt et al., 1985). The MV phosphoprotein (P) and fusion protein (F) gene clones and the CDV N gene clone were constructed as previously described (Hull et al., 1984; Russell et al., 1985; Richardson et al., 1986). Cloned rubella virus DNA (150 bp insert of rubella virus genome) was supplied by Dr R. F. Pettersson (University of Helsinki, Finland) and a clone of the SV5 N gene by Dr R. A. Lamb (Northwestern University, Evanston, Ill., U.S.A.). Specific regions of each viral sequence were subcloned into gemini in vitro transcription vectors (Promega Biotec) and RNA probes produced to genomic sense N (186 bp), P (500 bp) and F (647 bp) gene sequences of MV, an N gene sequence (570 bp) of CDV, an N gene sequence (260 bp) of SV5 and a transcript (non-specific 1050 bp genomic fragment) which hybridized to both genomic and subgenomic rubella virus RNA. Transcripts of the vector sequences were used as control probes. Inserts were labelled by in vitro transcription (Melton et al., 1984) with either 16 μM (50 μCi) uridine 5’-[α-35S]triphosphate, triethylammonium salt (SP6 grade, 1250 Ci/mmol; Amersham) or 400 μM-biotin-11-UTP (Bethesda Research Laboratories). The radioactively labelled probes had a specific activity of 2 × 106 d.p.m./μg.

Northern blot analysis. RNA extraction and Northern blot analysis were carried out as described by Russell et al. (1985).

ISH. Formalin-fixed (paraffin-embedded) sections (4 μm) or frozen sections (7 to 9 μm) were cut onto glass slides (organosilane-treated; Tourtellotte et al., 1987). Tissue culture cells were centrifuged (Shandon cytocentrifuge) onto treated slides. Frozen tissue sections and cells were fixed for 20 min in 3:1 (v/v) ethanol-acetic acid. The ISH method was carried out as described by Haase et al. (1984a) omitting the 2 × SSC prehybridization step at 70°C. When an RNase step was required, it was carried out after the proteinase K (Sigma) treatment. Tissue was treated with RNase A (Sigma) at a concentration of 100 μg/ml in 2 × SSC, followed by extensive washing in 2 × SSC and dehybridization through graded alcohols. Tissue sections or cell deposits were covered with 100 μl of hybridization buffer, containing either 5 × 106 c.p.m. (35S-labelled probe) or 200 ng (biotinylated probe) and slides were placed in a humidified atmosphere. Sections were either hybridized at room temperature for 48 to 72 h (35S-labelled probes) or at 37°C overnight (biotinylated probes). In subsequent experiments hybridizations with 35S-labelled and biotin-labelled probes were carried out under both sets of conditions and no appreciable difference was found in the signal obtained. After hybridization, washes were carried out first in washing buffer (0.6% NaCl, 10 mM-Tris-HCl pH 7.0, 1 mM-EDTA) at room temperature for 5 min, then in 45% (v/v), biotin-labelled probes) or 50% (v/v) (35S-labelled probes) formamide in washing buffer.
for 30 min at room temperature, and finally under a range of stringency conditions as specified in the text.
Radioactively labelled slides were dipped in either Kodak NB2 emulsion or Ilford K5 emulsion. Exposure time
was from 3 to 7 days. Sections hybridized with biotin-labelled probes were treated as described by Allan et al.
(1989).

RESULTS

Detection of viral sequences in tissue culture cells

Infected human brain tissue was available for only MV (SSPE tissue). Therefore a human
astrocytoma cell line was chosen for infection with the various viruses under test for ISH so that
positive controls could be carried out in a human neural cell system. Hybridization was carried
out with both $^{35}$S-labelled and biotin-labelled probes. The final post-hybridization wash in each
case was carried out in 0.1 x SSC at 50 °C for 30 min. Hybridization was detected in cells
infected with each virus by both autoradiographic and non-radioactive methods. Hybridization
is shown in SV5-infected astrocytoma cells, treated with the SV5 N gene biotin-labelled probe (Fig. 1 a). Infected cells treated with the gemini control probe (Fig. 1 b) and mock-infected cells
treated with the SV5 probe (Fig. 1 c) gave no hybridization.

Examination of MS, SSPE and control cases

All brain sections were initially pretreated with relatively high proteinase K concentrations
(1 µg/ml for 5 min for frozen tissue and 20 µg/ml for 30 min for paraffin-embedded tissue at
37 °C) and examined by ISH, using $^{35}$S-labelled probes to the MV N gene sequence, other viral
gene sequences in the study and vector control sequences. Sections were washed initially at
relatively low stringency (2 x SSC at room temperature for 30 min).

Hybridization occurred extensively with the MV N gene probe in a number of different areas
in the two SSPE brains. Several labelled neurons (Fig. 2a) and inflammatory cells (in a
perivascular infiltrate; Fig. 2b) are shown in a section from the right temporal lobe of one case of
SSPE. However not all perivascular infiltrates contained inflammatory cells showing positive
hybridization. The CDV N gene probe was used to treat an adjacent section (Fig. 2c); this gave
no hybridization which underlines the specificity of the reaction for MV.

Fig. 1. Human astrocytoma cells infected with SV5 (a, b) or mock-infected (c), hybridized with SV5
biotinylated probe (a, c) or with gemini control probe (b). Bar marker represents 40 µm. Labelled cells in
(a) are shown by arrows.
Focal areas of hybridization were also observed with the MV N genomic probe in two of the MS cases (cases 1 and 2) and the case of disseminated CMV infection with spinal cord necrosis. No hybridization was observed with any probes in any of the other neurological or non-neurological control cases. Higher levels of non-specific background were seen in the four cases of cerebral abscess, possibly due to the agonal state of the tissue. This was greatly reduced when a final wash in 0.1 × SSC at 40 °C was carried out on adjacent sections.

ISH was repeated on adjacent sections using the MV N gene probe and vector control probes in the two positive MS cases, the disseminated CMV case and the two positive cases of SSPE, using a final stringency wash of 0.1 × SSC at 40 °C for 30 min. Hybridization still occurred in all cases. In addition, when further sections were available these were hybridized with either 35S-labelled P- or F-encoding MV gene sequences or both (and the relevant vector sequences). In all cases foci of hybridization remained positive in several adjacent sections with the N, P or F gene probes. Further adjacent sections in these positive areas showed hybridization (depending on the availability of remaining tissue) using biotinylated probes to the MV N gene and control.
vector sequences. In order to allow a more accurate histological interpretation of the results, less severe proteinase K treatment (1 μg/ml for 1 min) or in some cases no enzyme treatment was carried out on these sections. Again the original focal areas remained positive. The detection method was also carried out on adjacent sections which had been covered in hybridization buffer but not with the probe. Non-specific deposition of the enzyme product did not occur. Pretreatment of adjacent sections with RNase prior to hybridization completely prevented hybridization in all cases.

**Anatomical location of hybridization with MV probes in the two MS cases and in the one disseminated CMV case**

A total of 30 (28 frozen, two paraffin-embedded) blocks were examined in MS case 1 of which six (frozen) showed areas hybridizing with MV probes. One was in the frontal lobe (grey and white matter), another in the temporal lobe (grey and white matter), two in the occipital lobe (grey and white matter) and two in the regions of periventricular plaques. All sections contained plaques, and foci of positive hybridization were generally in the white matter in the periplaque region, where active demyelination was still occurring. Fig. 3(a) shows an area of plaque and periplaque in the frontal lobe (case 1) where approximately 50% of the cells are labelled with the MV N probe. At higher magnification (Fig. 3(b)) both macrophages and astrocytes are shown to be labelled and both cytoplasmic and nuclear hybridization is observed in different cells. No hybridization occurred with the gemini control probe in an adjacent section (Fig. 3(c)). Fig. 4(a) shows a region of periventricular plaque formation (transverse through a blood vessel), from case 1, where some inflammatory cells in the perivascular infiltrate are 35S-labelled in a similar way to those in the SSPE brain. In addition several cell types, including macrophages, are labelled in the surrounding area. No hybridization occurred when an adjacent section was pretreated with RNase before hybridization with the MV N probe (Fig. 4(b)) or in an adjacent section treated with the gemini control probe (Fig. 4(c)).
DISCUSSION

We have examined brain samples from eight cases of classical MS and 56 neurological and non-neurological controls for MV, CDV, rubella virus and SV5 RNA sequences. Foci of hybridization with MV genomic probes were found in two of the MS cases, and in a case of disseminated CMV infection (with spinal cord and brain lesions) but not in any of the other control cases. Hybridization was not found in any of the cases in the study with any of the other viral probes. The specific hybridization with MV probes only (in some cases demonstrated with
Viral genomic sequences in human brain

probes against two or more genomic sequences) and the reproducibility of results in adjacent sections at different times and with different hybridization detection systems indicates strongly that MV genomic RNA is present in these cases. However sequences of three other RNA viruses that have been previously implicated in MS (Meurman et al., 1977; Cook et al., 1978; Goswami et al., 1987) were not detected in any of the 64 brains examined in this study.

Our results contrast with previous reports where either no MV RNA was detected in any MS or control brains tested (Stevens et al., 1980; Dowling et al., 1986) or viral RNA was reported to be present in both MS and normal controls (Haase et al., 1984b). These differences may relate to the specificity and the sensitivity of the probes used, the extent of sampling carried out and the initial pathological selection (of cases showing a marked degree of inflammation) and assessment of the tissue. Given the very focal nature of the hybridization in the brains studied it is possible that localized regions containing viral RNA could have been overlooked in the previous studies by Stevens et al. (1980) and Dowling et al. (1986). No indication was given of the extent of sampling of different areas in each brain or of the neuropathological evaluation of inflammatory and other changes. We have found that extensive sampling was necessary to detect the positive foci in the cases examined. Although hybridization was found generally in periplaque regions or areas showing inflammation, many blocks from the same brains exhibiting these pathological changes were completely negative. Therefore taking a small number of samples from plaque and normal white matter may not be sufficient. It is possible that our failure to detect MV sequences in some but not all of the six MS cases examined in this study may be due to the limited number of tissue blocks available.

The sensitivity of the probe and the technique employed are also of major significance in the detection of viral RNA. All the previous studies have used 3H-labelled DNA probes, either reverse-transcribed from purified genomic RNA (Stevens et al., 1980; Haase et al., 1981) or nick-translated cloned DNA (Dowling et al., 1986). In particular the solution hybridization method carried out by Stevens et al. (1980) was of relatively low sensitivity. They calculated that 14·1 genome equivalents per diploid brain cell would be necessary for the detection of viral RNA. Dowling et al. (1986) estimated that this was approximately 30-fold less sensitive than their own dot blot assay. It is therefore not surprising, particularly in the region where viral RNA is limited to restricted foci, that examination of RNA from gram quantities of homogenized brain tissue failed to result in hybridization.

The sensitivity of detection (0·021 genome equivalents per cell) calculated by Dowling et al. (1986) is based on their estimate of 21 genome equivalents of MV sequences per cell of SSPE brain. Although we find variation in the levels of MV RNA in different samples of SSPE brain (due to both the number of cells apparently infected and the number of copies per cell), by using 32P-labelled RNA probes in Northern blot analysis we estimated the detection of between several hundred and several thousand copies of the viral genome per cell (unpublished data). This is in agreement with a previous study by Cattaneo et al. (1987). On this basis the sensitivity of detection of the assay by Dowling et al. (1986) would be 10-fold to 100-fold lower than calculated. Our own comparative studies by both dot blot and Northern blot analysis of the relative sensitivity of nick-translated and ssRNA probes show the former to be at least 100-fold lower in their level of detection (unpublished data). This is also relevant to the relative sensitivity of detection of viral sequences in our own and in the ISH study by Dowling et al. (1986).

Although these workers suggest that their failure to detect MV RNA may have been due to the use of probes derived from internal gene sequences (P, M and H) we consider this unlikely as we were able in selected cases to detect RNA using probes to P and F as well as N genomic sequences.

The results obtained by Haase et al. (1981) and Haase et al. (1984b) suggest that MV RNA is found in a large number of both MS and control brains. However, several factors must be considered. As previously indicated by Dowling et al. (1986) the 50S RNA used as the template for reverse transcription may have contained contaminating Vero cell RNA. It is therefore possible that expression of host cell sequences was detected in many of the brains examined. We have found it of particular importance to remove GC tails and internal GC-rich sequences from in vitro transcripts which can often give rise to hybridization to ribosomal sequences (McClure &
Perrault, 1985, 1986). Although we also report the detection of MV sequences in a case other than MS, demyelination is also present in the CNS of this case of disseminated CMV infection. Also MV RNA sequences were only found in a specific periplaque region, which may indicate the involvement of MV in the demyelinating process. However, because CMV-like inclusions are also present in the brain, the role of each virus in relation to the neuropathological changes is unclear. The underlying cause of the disseminated CMV infection (possibly linked to severe immunosuppression) is now under further investigation and should help to elucidate the course of events in the disease process.

Our negative findings in non-neurological and non-demyelinating neurological controls are in agreement with a recent ISH study (Moench et al., 1988) of post-mortem tissue from seven cases with acute MV encephalomyelitis. Moench et al. (1988), using a 35S-labelled MV N genomic ssRNA probe (similar in sensitivity to our own), failed to find MV RNA in the CNS of these cases whereas RNA was demonstrated extensively in other body tissues and in SSPE brain. These authors suggest that CNS invasion is not a common occurrence in MV infection and that acute MV encephalomyelitis may be brought about by an autoimmune phenomenon. A rapid clearance of virus from the CNS would serve as an alternative explanation. We cannot rule out the possibility in our own study that false negative results were obtained in control cases due to the limited material available (two to four blocks per brain). However, taking the rate of detection of MV sequences in the MS and demyelinating CMV cases (13 out of 79 blocks), the calculated probability of a control case being falsely negative is very low ($P < 0.0001$). Detection of MV RNA sequences in a case of systemic CMV infection suggests that virus may persist either in the CNS or at another site and may only be induced to levels detectable (by standard ISH techniques) by changes in the immune response. It is well documented (Brownell & Tomlinson, 1984) that children with leukaemia can develop measles inclusion body encephalitis. However in most cases recent infection with MV is known to have occurred.

Several important questions are raised by the results of our study. Only two of the MS cases gave positive hybridization for MV. This result could be explained in several ways. It could be due to sampling, because only a limited number of blocks were available in some of the cases. Secondly, it is possible that several viruses are capable of inducing MS in susceptible individuals. Thirdly, activation of virus in an MS brain may be a consequence of the disease process and not the primary cause.

Although the cases we examined for CDV, rubella virus and SV5 were all negative we cannot rule out the possibility that other viruses may be involved in some cases of MS. The recent association of human T cell lymphotropic virus type 1 with tropical spastic paraparesis (Jacobson et al., 1988) and the reported association of related viruses with MS (Koprowski et al., 1985; Reddy et al., 1989) suggests that such viruses may be candidates for long-term CNS infections. Another recent report describes the detection of herpes simplex virus type 2 antigen in three out of 31 MS cases (Martin et al., 1988). The detection of MV sequences in the case of disseminated CMV also indicates that it may be important to consider the role of multiple viral infections in the CNS. It will therefore be of interest to examine our MS cases for other neuropathological viruses not included in the present study.

It may be significant from the point of view of our own results that immunofluorescence titration studies carried out with the sera of MS patients in Belfast over a number of years (Haire et al., 1973; Haire, 1977) have shown that antibodies to MV are consistently higher than to other viruses tested. With relevance to our own study, MS case 1 had a known cerebrospinal fluid neutralizing antibody titre of 40. MV RNA may be detected (using the present techniques) if death occurs only during an active phase of the disease. The selective choice of 'inflammatory' cases may partly explain our positive results and other workers' negative findings. It is possible that MV enters the CNS at the time of childhood infection and remains in a persistent state with little RNA expression until an unknown triggering factor(s) induces increased replication and gives rise to a rapid inflammatory response. Alternatively virus may persist elsewhere in the body and enter the CNS possibly in infected lymphocytes or macrophages. Some of the same questions still remain concerning the pathogenesis of SSPE. The presence of MV RNA in inflammatory cells, particularly those in perivascular infiltrates, in MS and SSPE cases may be
of particular significance in the understanding of the pathogenesis and the relationship between these two diseases.

Examination of tissue has been carried out to date using probes directed against genomic RNA. This approach was chosen to ensure as far as possible the detection of viral RNA even if transcription were limited. We now wish to study mRNA expression in cases containing genomic sequences. Application of in situ hybridization can now be combined with polymerase chain reaction techniques to study very low levels of gene expression in both pathologically normal and abnormal regions of the CNS or other tissues. This combined molecular and pathological approach should further our understanding of where and how MV persists in the body and help to elucidate the exact role of the virus in MS and other demyelinating conditions.

This work was supported by the Multiple Sclerosis Society of Great Britain and Northern Ireland.

REFERENCES


(Received 20 December 1988)