Detection of Polyoma Virus DNA in PML-Brain Tissue by 
(in situ) Hybridization

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(Accepted 3 July 1978)

SUMMARY

The human papova (JC) virus was extracted from brain of a patient with pro-
gressive multifocal leukoencephalopathy. A single band of virus was obtained at a
density of 1.345 g/ml CsCl. JC virus DNA was purified and a highly specific cRNA 
was generated in vitro. In situ hybridization with JC virus cRNA and autoradi-
ography on sections of the same brain revealed silver grains over oligodendrocytes,
astrocytes and possible vascular endothelial cells, indicating the presence of JC
virus DNA in these different cell classes.

INTRODUCTION

Progressive multifocal leukoencephalopathy (PML) is a subacute demyelinating disease 
related to the chance infection of the human brain with polyoma viruses. It is a rare disorder 
that usually occurs as a complication of a grave underlying disease. Neuropathologically, 
there are widely disseminated foci of demyelination, forming plaque-like lesions. The 
oligodendrocytes, the cells which maintain the myelin sheaths, appear to be lytically infected. 
They are depleted within demyelinated foci and packed with virus particles at the periphery 
of the lesions. Astrocytes, the other major glial cells of the white matter, also appear to be affected. 
In the areas of oligodendrocyte destruction, the astrocytes only rarely contain virus 
particles but are distorted with bizarre nuclear forms and mitoses resembling malignant cells 
(Aström et al. 1958; Narayan, 1976). The hypothesis that oligodendrocytes are permissively 
and lytically infected and that astrocytes are non-permissively infected acquiring some 
characteristics of transformed cells is supported by experiments in cell cultures. Shein (1967) 
infected cultures of human foetal glial cells with simian virus 40 (SV40) and demonstrated 
lysis of the spongioblasts -- thought to be precursors of the oligodendrocytes -- and trans-
formation of foetal human astrocytes.

Moreover, two patients with PML exhibited co-existent gliomas (Richardson, 1961; 
Castaigne et al. 1974). Although tumour (T) antigen of polyoma viruses has not been ob-
served in frozen sections of the brain, cells grown from the brains of patients with PML have 
been positive for T antigen and these were presumed to represent populations of astrocytes 
(Narayan, 1976; F. Cathala, unpublished data). The present study attempts to test this 
hypothesis by using in situ hybridization with cRNA of JC virus in a case of progressive 
multifocal leukoencephalopathy.

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METHODS

Brain material. Brain material was obtained from a 56-year-old man with PML. Details about the clinical course and histological findings have been previously published (Weiner et al. 1973). For this study, multiple frozen coronal sections of the brain were kindly supplied by Dr W. Tourtelotte, Department of Neurology, Wadsworth VA Hospital, UCLA, Los Angeles, California.

Purification of virus. JC virus was purified directly from human brain: 60 g of brain were ground in a frozen mortar with a small amount of Hanks' balanced salt solution (BSS), suspended in 10% BSS and extracted twice with Freon as previously described (Penny et al. 1972). The virus suspension was pelleted through a 30% sucrose cushion in a Beckman centrifuge, SW27 rotor at 27,000 rev/min, 3.5 h, 4 °C. The pellets were homogenized in PBS in a Dounce homogenizer and solid CsCl was added to a final density of 1.32 g/ml. The virus was banded to equilibrium in a Beckman SW50.1 rotor at 36,000 rev/min, 36 h, 20 °C.

SV40 was propagated in Vero cells which were grown in Eagle's MEM containing 5% foetal calf serum. Confluent monolayers of cells were infected with SV40 at an input multiplicity of 1 p.f.u./cell. After adsorption of the inoculum for 1 h, medium containing 2% foetal calf serum was added and the cells were incubated at 37 °C. Virus was harvested out of the medium when most of the cells had detached from the glass surface. Purification was done as described for JC virus.

Purification of virus DNA. Virus DNA was extracted according to Tai et al. (1972). Sodium dodecylsulphate (SDS) was added to the virus suspension to give a final concentration of 1%. The solution was incubated at 37 °C for 10 min, solid CsCl was added up to 1 M and then kept for 15 min at 0 °C. Precipitating SDS was removed by low speed centrifugation at 0 °C. Ethidium bromide (200 µg/ml) was added and the density adjusted to 1.55 g/ml. After equilibrium centrifugation in a Beckman SW50.1 rotor at 35,000 rev/min for 50 h at 20 °C, one single band could be visualized by u.v. light in a position corresponding to the component I DNA band. The band was collected and the ethidium bromide removed by extractions with isoamyl alcohol followed by extractions with ether. The DNA was dialysed against 0.01 x 1.5 mM-sodium chloride - 0.15 mM-sodium citrate (0.01 x SSC). The amount of DNA was calculated from absorbance.

Transcription of virus DNA with E. coli RNA polymerase. Three µg of component I DNA were transcribed (zur Hausen et al. 1972). The reaction mixture contained, in a total volume of 0.3 ml, 20 µM of each of the four ribonucleoside-triphosphates (3H-UTP, sp. act. 23 Ci/mmol; 3H-CTP, sp. act. 22.6 Ci/mmol, obtained from Amersham, England; GTP and ATP were unlabelled). After incubation for 90 min at 37 °C, 0.3 ml of polymerase buffer (Burgess, 1969) containing 50 µg/ml DNase was added and further incubated for 30 min at 37 °C. After extraction with the same volume of saturated phenol, pH 7.4, the aqueous phase was separated by low speed centrifugation and chromatographed on a Sephadex G-50 column (Pharmacia, Sweden), 10.4% of the total radioactivity appeared in the first peak. These fractions were pooled and stored at -70 °C. Sedimentation analysis of this material revealed a heterogeneous banding profile between 12 and 6S. After boiling for 10 min the material was sedimented as one peak at about 6S.

DNA-cRNA hybridization. For preparation of cellular DNA, organic material was cut in small pieces, ground with sterilized sand and prepared as a 10% solution in 0.1 M-tris, pH 7.4. This material, as well as tissue culture cells, was lysed with 2% sarcosyl (Serva, Germany), digested with 1 mg pronase/ml for 30 min at 37 °C and subsequently extracted with phenol, chloroform-4% isoamyl alcohol and ether. The nucleic acids were incubated with RNase
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(50 µg/ml for 30 min at 37 °C), re-extracted and dialysed extensively against 0.01 × SSC. For hybridization according to zur Hausen et al. (1972), 50 µg purified DNA was heat-denatured by boiling for 15 min followed by rapid cooling in ice-water. Varying amounts of JC virus DNA were mixed with calf thymus DNA to an amount of 50 µg DNA/filter. The probes were brought up to 2 × SSC and slowly filtered through 0.45 µm nitrocellulose filters. The filters were incubated for 4 h at 80 °C. After heat denaturation, 5 × 10^4 ct/min cRNA (in 2.5 × SSC, 50% formamide, 0.05% SDS) were added on each DNA-loaded filter which was gently shaken over 5 days at 43 °C. The filters were washed with 2 × SSC, treated with RNase (50 µg/ml for 30 min at room temperature), washed again and monitored for radioactivity.

For in situ hybridization, multiple areas of frozen brain tissue from the same patient were examined. The areas selected for intensive study represented areas of cerebral white matter with demyelinated foci. Sections were cut at 15 µm in a cryostat. Serial sections were prepared for in situ hybridization and conventional histological staining with haematoxylin eosin (H & E) or luxol-fast blue, stained directly after fixing in formaldehyde, and a specific astrocyte stain (FAN; Miquel et al. 1968). Frozen sections of brain from patients dying of multiple sclerosis and Huntington’s chorea as well as biopsy sections of a glioblastoma were prepared as controls. For hybridization, frozen sections were fixed in methanol–acetic acid (3:1) and treated with 0.07 N-KOH for 3.5 min. After washing in 2 × SSC they were annealed under a coverslip with 50,000 ct/min of JC cRNA for 5 days at 43 °C, treated with RNase and exposed under Ilford G5 emulsion for two weeks to produce autoradiographs. After development, slides were stained with Giemsa solution (Wolf et al. 1973).

Materials. All chemicals were obtained from Merck, Germany; enzymes were purchased from Serva, Germany. E. coli RNA polymerase was kindly provided by Dr H. zur Hausen, Institute of Hygiene and Microbiology, Freiburg, Germany.

RESULTS

JC virus extracted directly from human brain and purified in a CsCl gradient by centrifugation to equilibrium revealed only one single band at a density of 1.345 g/ml CsCl (Fig. 1a). This has a slightly greater density than SV40 purified under the same conditions (Fig. 1b). JC virus failed to show a second peak or shoulder of less dense virus as is routinely found with other polyoma viruses grown in tissue cultures (Fig. 1c).

JC virus DNA was purified by lysing the particles with SDS and centrifuging to equilibrium in a CsCl-ethidium bromide gradient. One single band at a density of component I DNA could be visualized under u.v. light. The single peak of dense virus obtained in CsCl as well as the large amount of component I DNA extracted from virus particles suggested that most of the particles in PML brain material are complete. Calculations from absorbance of the banded DNA showed that 6 µg of virus DNA had been obtained from 60 g of brain, indicating the presence of over 2 × 10^10 virus particles per g of brain.

To test the specificity of hybridizations with JC virus cRNA, increasing amounts of JC virus DNA were mixed with 50 µg of calf thymus DNA and then annealed with a constant input of JC cRNA. The result is shown in Fig. 2. JC virus DNA bound the corresponding cRNA in a linear reaction from 0.3 up to 20 ng. DNA extracted from control brain material as well as from HeLa and Vero tissue culture cells hybridized with a background value of 0.08% (40 ct/min) indicating the specificity of JC cRNA for JC virus DNA.

Histological staining of multiple sections of PML brain material selected for this study showed many demyelinated lesions. Along the margin of these lesions (Fig. 3a) a marked
Fig. 1. (a) CsCl gradient of JC virus. The virus was extracted directly from the brain with Freon, pelleted through a 30% sucrose cushion and banded to equilibrium in CsCl. (b) CsCl gradient of SV40 virus. The virus was grown in Vero cells and purified from the medium. Gradients were collected from the bottom and monitored for refractive indices and absorbance at 260 nm.

Fig. 2. Reconstruction test with JC virus cRNA. Various amounts of JC virus DNA were mixed with 50 μg calf thymus DNA fixed on filters and hybridized with 3 × 10⁶ ct/min JC virus cRNA.

Microglial cell reaction, in addition to signs of inflammation and many enlarged distorted cells representing altered oligodendrocytes, were detectable (Fig. 3b). Inclusion bodies could not be identified. Sections taken deeper into the lesions contained giant astrocytes, fat laden macrophages, and occasionally blood vessels with perivascular, mononuclear inflammatory cells. The FAN staining confirmed that most of the cells in the centre of the lesions were giant astrocytes.

Serial sections adjacent to those for histological staining were hybridized in situ with JC complementary RNA. Whereas the control brain sections showed no clustering of label or accumulation of grains over nuclei, labelling of nuclei was clearly present in sections of PML brain. Along the periphery of several lesions studied, many enlarged nuclei of oligodendroglia cells were detected which showed heavy label with silver grains indicating the presence of JC DNA (Fig. 3c, d). In the totally demyelinated core of the lesions, giant bizarre nuclei surrounded by a stainable cytoplasm with processes were easily identified as astrocytes. Some of these nuclei were clearly labelled (Fig. 4a, b). However, comparison with the adjacent FAN stained sections revealed that only a minority of the astrocytes were labelled (Fig. 4c).

In addition, within the lesions, nuclei of some elongated cells resembling vascular endo-
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Fig. 3. (a) Small demyelinated lesion of a PML cryostat brain section from which adjacent sections were selected for histological staining and hybridization in situ (luxol-fast blue staining ×80). (b) Periphery of the lesion with several altered oligodendrocytes (luxol-fast blue staining ×525). (c) Autoradiograph (H & E staining) of the adjacent section hybridized in situ with JC cRNA showing the rim of the demyelinated lesion. Arrows indicate heavily labelled nuclei of oligodendroglia cells (×800). (d) Enlargement of a labelled nucleus of oligodendroglia origin (H & E staining ×950).
Fig. 4. Centre of a complete demyelinated lesion of a PML cryostat brain section. (a, b) Autoradiographs of a section hybridized in situ with JC cRNA showing specific labelled astrocytes (Giemsa staining ×1250, ×1600, respectively). (c) FAN staining of an adjacent section revealing several giant astrocytes (×400).

Theelial cells were marked by silver grains, suggesting the presence of JC virus DNA in cell types of the brain in which virus particles have not been identified (Fig. 5a, b).

DISCUSSION

Under laboratory conditions, it is very difficult to grow JC virus in primary human foetal cell cultures consisting of spongioblasts. In the present study, the difficulty in the preparation
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Fig. 5. Autoradiograph and Giemsa staining of a PML brain section hybridized in situ with JC cRNA. Labelled cells in the periphery of a lesion representing possible endothelial cells. Magnification (a) x1028; (b) x820.

of JC virus was circumvented by extracting virus directly from diseased human brain. A visible band of virus from 60 g of PML brain material was obtained, which confirms previous reports of the presence of large quantities of virus in such material (Penny et al. 1972). Extracted JC virus banded at a density of 1.345 g/ml CsCl indicating a slightly greater density than SV40 virus (Padgett et al. 1976). Moreover, the virus failed to show a second peak or shoulder of less dense virus as is routinely found with SV40 or BK virus grown in cell cultures.

The single peak of JC virus obtained in CsCl, as well as the relatively large amount of component I DNA extracted from virus particles, indicates that most, if not all, of the virus particles in this brain are complete and that probably no empty particles are present. However, whether the single peak of JC DNA contains defective DNA derived from defective particles could not be further investigated by restriction enzyme analysis since not enough brain material was available for virus extraction.
Complementary RNA, generated from JC virus DNA, specifically hybridized to cells in and around demyelinated lesions in PML brain corresponding to areas where virus particles can be found by electron microscopy. The majority of cells revealing a specific label were oligodendroglia cells, whereas only a small percentage of astrocytes showed a specific uptake of silver grains. The hypothesis that the bizarre astrocytes within the lesions are non-permissively infected or 'transformed' by JC virus was neither proved nor disproved by our results. The bizarre alterations in astrocytes, a hallmark of the histopathology of PML, might be explained by virus transformation or by a unique astrocytic reaction not dependent on direct infection of the astrocytic cells. Since only a minority of these cells reacted with cRNA, it is conceivable that they represent the astrocytes known to contain virus particles in this disease. The remaining unlabelled astrocytes may either be uninfected or may not contain enough JC DNA copies to be detected by in situ hybridization.

The possible reaction of JC cRNA with vascular endothelial cell nuclei in PML is of interest in view of a recent publication (Fareed et al. 1978) which reports that JC virus may replicate in human vascular endothelial cells in vitro. This observation may have important implications in the pathogenesis of this infection of man.

The application of the in situ hybridization technique in the detection of virus genomic information in brain sections of a human central nervous system disease has been shown to be a useful method. This reaction is highly specific and allows detection of virus infections in specific cell formations, but is limited in its sensitivity as compared to other hybridization techniques (Huang & Pagano, 1977).

It is conceivable that this approach could help to define virus-host relationships in other central nervous system infections provided that the appropriate biochemical probes are available.

This work was supported by the Deutsche Forschungsgemeinschaft Az. Me 270/17. R. T. J. was the senior U.S. Scientist recipient of the Alexander von Humboldt Stiftung. We gratefully acknowledge the helpful and critical discussions on the histopathology with Dr R. Meiermann, Department of Neuropathology, University of Göttingen.

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(Received 23 February 1978)