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Human Fetal Schwann Cells Support JC Virus Multiplication

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The human papovavirus JC virus (JCV), the etiologic agent of progressive multifocal leukoencephalopathy, displays a narrow host range for growth, preferentially infecting oligodendrocytes, the myelin-producing cells of the central nervous system. In tissue culture, human fetal brain cells have been used for JCV propagation because of their ability to support JCV virion production. In this study, we present evidence that a human fetal cell type derived from the peripheral nervous system can be productively infected with JCV. Schwann cells, the cell type responsible for myelination in the peripheral nervous system, support the expression of JCV T antigen and JCV DNA replication. However, viral proteins and DNA replication were not detected either in dorsal root ganglion neurons or fibroblasts. These results extend the host range of JCV to include another cell of the glial lineage whose function is myelin formation.

The human papovavirus JC virus (JCV) causes a demyelinating disease in the brains of immunocompromised individuals termed progressive multifocal leukoencephalopathy (PML) (17, 20). The site of JCV multiplication in the brain is the macroglial cell, particularly the oligodendrocyte, the myelin-producing cell of the central nervous system. JCV growth in tissue culture closely reflects this cell tropism, since JCV grows most efficiently in cells from human fetal brain (16). The cellular composition of human fetal brain derived from 7- to 16-week fetuses has been shown to be composed mainly of large, flat type I astrocytes and bipolar undifferentiated precursor cells (4, 16). Both cell types have been reported to support the growth of JCV (9). In this study, we designed experiments to test the infectivity of JCV in human fetal Schwann cells, which are the myelin-forming cells of the peripheral nervous system. In order to conduct these experiments, we established a culture system in which each neural component of the peripheral nervous system was individually investigated for its susceptibility to JCV.

Establishment of human fetal cultures. Sciatic nerve ganglia or dorsal root ganglia (DRG) were surgically removed from 9- to 18-week fetuses and collected in sterile culture media. Dissociation and cultivation of Schwann cells were performed according to a method previously described (2). Briefly, DRG were treated with 1% collagenase (Sigma Co., St. Louis, Mo.) for 1 h prior to the surgical removal of the enveloping connective tissue sheath and trituration through a 22-gauge needle. Dissociated cells were seeded on rat tail type 1 collagen in Dulbecco modified Eagle medium containing 10% fetal calf serum and 50 ng of nerve growth factor (NGF) per ml (gift from G. Gurroff). After 48 to 72 h in culture, cells were treated for 24 to 48 h with 10 μg of cytosyl fluoroarabinoside per ml. Cells were then washed and transferred to antimitotic-free medium. Schwann cells were also obtained from these DRG cultures by the removal of NGF from the culture medium, which caused neuronal death. The remaining cells enriched in Schwann cells were further subcultured.

To ensure cell specificity in infection experiments, we took additional care to determine the purity of the cell cultures. Cell types were identified by using antibodies to specific cell markers. Human fetal Schwann cells, which express NGF receptors on their surfaces (2), were identified by using monoclonal antibody (MAb) ME20.4, a monoclonal immunoglobulin G (diluted to 2.5 μg) (gift from M. Bothwell). Approximately 90% of the cells in the cultures were bipolar cells and reacted with the anti-NGF receptor antibody; the remainder (10%) were fibroblasts. These results contrast with the composition of mixed DRG cultures used in the cultivation of human immunodeficiency virus infection (21), in which Schwann cells constituted only 20 to 25%. DRG were identified by using polyclonal antineurofilament antibody (diluted 1:100) (Chemicon Inc., El Segundo, Calif.) and morphologically by the presence of neurite outgrowths in the presence of NGF. To compare the DRG with the central nervous system cells, fetal brain cultures were established as previously described (2, 9). By using a polyclonal antibody raised against glial fibrillary acidic protein, a marker for astrocytes (Chemicon Inc.), about 90% of the cells from brain cultures were identified as astrocytes.

JCV infection and transfection of enriched cultures. Cells were seeded in poly-D-lysine-coated 24-well trays, each well containing an 18-mm-diameter round coverslip, or in 35-mm-diameter petri dishes. Infections were initiated with 100 to 200 hemagglutination (HA) units of the Mad-1 (prototype) strain of JCV. As early as 6 days following the initial infection, the JCV T protein was detected in Schwann cell cultures. Two antibodies from independent sources reacting to JCV T protein were utilized: the murine MAb PAb416 (diluted 1:10) directed against the amino-terminal end of the simian virus 40 (SV40) T protein (Oncogene Sciences, Inc., Manhasset, N.Y.) and a tumor-bearing-hamster anti-SV40 serum (diluted 1:20) (8). MAbs to SV40 T antigen cross-reacting to JCV T antigen are not common; however, PAb416 has been shown to specifically label both SV40 and JCV T antigens (10, 18). For immunofluorescence staining, primary antibodies were incubated at room temperature alone for 30 min and then for 30 min with goat anti-mouse and anti-hamster rhodamine-conjugated antibodies (diluted 1:40), respectively, for PAb416 and anti-SV40 serum. All fluorescence microscopy was performed with a Zeiss Axiphot epifluorescent microscope.

Polyomaviruses like JCV multiply in the nuclei of permissive cells, where productive infection is often associated with a variety of nuclear changes (13). In our study, immu-

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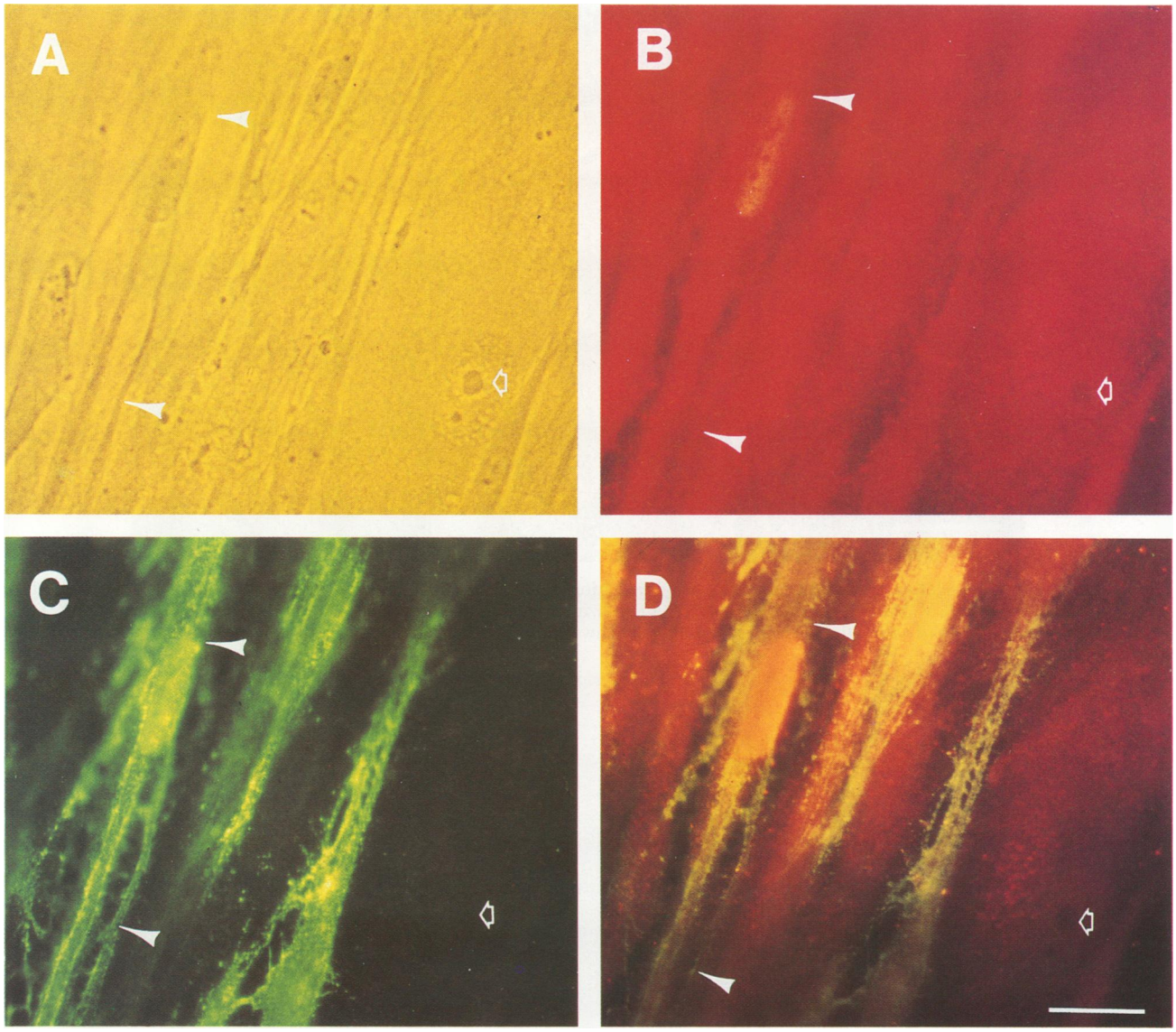

FIG. 1. Detection of viral T protein by immunofluorescence in Schwann cells infected with JCV. Schwann cells from sciatic nerves of a 13-week fetus were infected with 200 HA units of the Mad-1 strain of JCV. Following a 10-day incubation period, cultures were acetone-methanol fixed and immunolabeled. Closed arrowheads indicate Schwann cells; open arrowheads indicate fibroblasts. (A) Phase-contrast micrograph of the culture; (B) detection of JCV T protein with a tumor-bearing-hamster serum raised against a cross-reacting SV40 T protein; (C) same field as in panels A and B, but stained with monoclonal immunoglobulin G anti-human NGF receptor (MAb ME20.4), expressed exclusively on Schwann cells in these cultures; (D) double exposure of fluorescence micrographs in panels A and B. Bar = 25 μm.

noreactivity was localized exclusively to the nuclei of morphologically bipolar cells (Fig. 1B, closed arrowheads), which correspond to Schwann cells. The nuclei of infected cells were generally oblong or oval, although occasionally a few were enlarged two to three times their normal sizes and were hyperchromatic. However, in contrast to astrocyte cultures used as positive controls, infected Schwann cell cultures did not demonstrate polycaryotic nuclei. Fibroblasts in the Schwann cell cultures were flat and did not express the intranuclear JCV T antigen (Fig. 1B, open arrowhead). Expression of the early JCV mRNA for large T protein synthesis is transcriptionally regulated and depends upon the recognition of JCV enhancer and/or promotor by a tissue-specific factor(s) synthesized in the host (5). Schwann cells appear to contain similar cell-specific factors for JCV.

To confirm the infectivity of JCV to Schwann cells, double-labeling experiments using antibodies directed to viral and cellular proteins were undertaken. MAb ME20.4, which was raised against human NGF receptor, and the hamster antiserum which recognizes the JCV T antigen were mixed to the appropriate final concentrations and detected with fluorescent conjugated serum. Results are shown in Fig. 1D. Bipolar Schwann cells expressing surface NGF receptors also expressed the JCV T protein. Approximately 30% of the cells in the infected Schwann cell cultures were doubly labeled (Fig. 1D, closed arrowheads). Fibroblasts in the

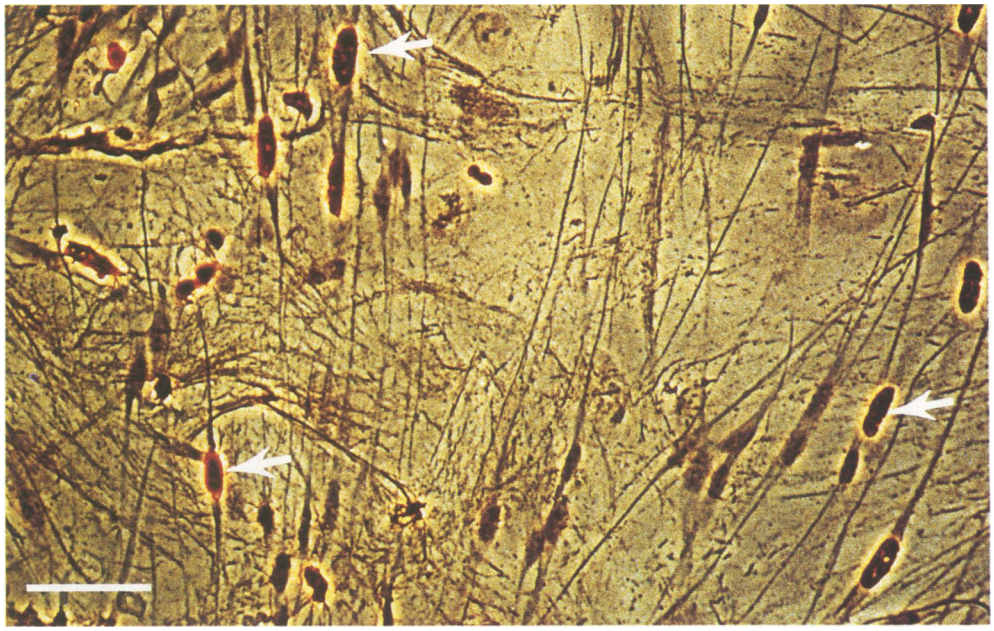

FIG. 2. JCV DNA replication by in situ DNA-DNA hybridization of Schwann cells infected with JCV. Cells were cultured 10 days following infection with JCV Mad-1. In situ DNA-DNA hybridization was performed with a biotin-labeled DNA probe. A positive hybridization signal is localized within the nuclei of JCV-infected Schwann cells, as indicated by the brown diaminobenzidine reaction product (arrows). Bar = 50 μm.

cultures were negative for both NGF and JCV T antigen, which suggested that fibroblasts are not permissive to JCV infection (Fig. 1D, open arrowhead). In PML brain tissue, neurons are not infected by JCV (20, 22). We have established cultures of human fetal DRG neurons which can be used as a model to test the infectivity of neurotropic viruses. In this study, we made multiple attempts to infect human fetal DRG neurons with JCV but failed to demonstrate viral infectivity. In addition, the presence of infected Schwann cells in DRG cultures did not alter the neuron's nonpermissiveness to JCV infection.

JCV DNA replication in Schwann cells. In situ DNA-DNA hybridization experiments were carried out to establish the proportion of cells replicating JCV DNA (1, 3). Also, newly replicated DNA was extracted from Schwann cell cultures and examined by restriction endonuclease digestion and Southern blot transfer. Replication of JCV DNA was examined in infected Schwann cell cultures 5 days to 2 weeks following the initial infection. The method utilized a JCV DNA biotin-labeled probe as previously described (1, 9). A strong hybridization signal indicated by a brown precipitate was localized to the nuclei of the bipolar Schwann cells (Fig. 2, arrows). As in the JCV T protein experiments, the nuclei of infected cells were generally enlarged compared with the nuclei of their uninfected counterparts. Approximately 10 to 15% of the Schwann cells hybridized to the JCV biotinylated probe. In contrast, hybridization was not detected in control cultures. In addition, the few fibroblasts contained in the Schwann cell cultures remained negative. In DRG cultures which contained neurons and small numbers of Schwann cells, the only cell type infected by JCV virus was the Schwann cell. JCV DNA was prepared from infected Schwann cells by Hirt extraction (7) and detected by Southern transfer and hybridization to a ^{32}P-labeled JCV probe. Results of DNA analysis are shown in Fig. 3. Restriction

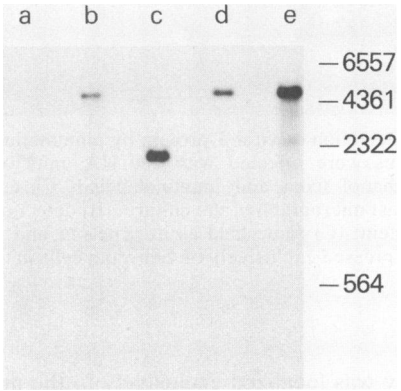

FIG. 3. Southern blot transfer of Hirt-extracted DNA from JCV-infected Schwann cells. Viral DNA was extracted from Schwann cells by the Hirt method, treated with restriction endonucleases, and electrophoresed in a 1% agarose gel at 125 mV for 3 h. DNA was transferred to a nytran filter and hybridized to ^{32}P-labeled Mad-1 DNA (specific activity, 8.1×10^8 dpm/μg). The filter was washed with 0.1 M SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate at 65°C for 30 min. Lanes a and b, DNA extracted from infected Schwann cells and treated with *Eco*RI-*Bam*HI and *Eco*RI, respectively; lanes c and d, the corresponding controls from Mad-1 JCV DNA fragments; lane e, *Eco*RI fragment of linear JCV DNA used as a control for the probe. Lambda *Hin*dIII marker positions are indicated to the right of lane e.

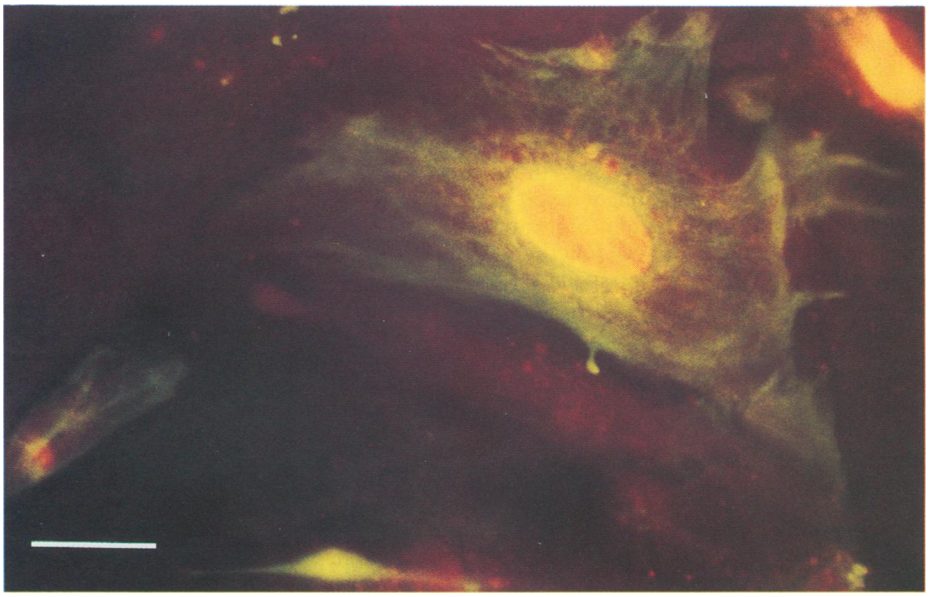

FIG. 4. Astrocytes infected with JCV passed from Schwann cell extract. A lysate of sodium deoxycholate-treated Schwann cells infected with JCV (13 days) was used as an inoculum in the infection of human fetal astrocytes. Following a 7-day incubation, cells were sequentially acetone-methanol fixed and immunolabeled with glial fibrillary acidic protein, incubated with a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody and with a tumor-bearing-hamster antiserum raised against a cross-reacting SV40 T protein, and incubated with rhodamine-conjugated anti-hamster antibody. Bar = 25 μm.

endonuclease fragments of DNA extracted from Schwann cells 10 days after infection (Fig. 3, lanes a and b) are similar in size to DNA fragments of the control, the Mad-1 strain of JCV (Fig. 3, lanes c and d). The restriction DNA fragments of the viral DNA replicated in the Schwann cells were similar to those of the JCV DNA from the Mad-1 virus (12).

To examine the infected Schwann cell cultures for progeny virions, cell lysates were tested for hemagglutination of human type O erythrocytes (16). Schwann cell cultures from a 14-week fetus were infected with an inoculum of 4 HA units of JCV Mad-1 strain. Ten days after the initial infection, the HA titer was 32 HA units. Titers indicated low-level production of JCV infectious particles by Schwann cells; however, these levels in Schwann cell lysates were sufficient to initiate a secondary JCV infection in astrocytes. Successful passage of the infection was indicated by the simultaneous detection of astrocytes and JCV T protein by double-labeling immunofluorescence with antibodies raised against glial fibrillary acidic protein and SV40 T antigen (Fig. 4). These results show that a productive infection takes place in Schwann cells and that complete mature viral particles formed in Schwann cells can be used to infect astrocytes.

To compare the efficiency of the JCV regulatory region in human fetal Schwann cells with that of astrocytes, we utilized the pM1-CAT plasmid, which contains regulatory sequences of JCV upstream of the chloramphenicol acetyltransferase (CAT) gene (12). The pSV2CAT plasmid, which contains the SV40 enhancer-promoter sequences, and the pSV0CAT plasmid, which does not contain either the promoter or the enhancer, were used as positive and negative controls, respectively (6). CAT activity was measured 48 h after transfection (15), and the results are shown in Table 1. The extracts prepared from human fetal astrocytes transfected with pM1-CAT produced an 8.4-fold-higher CAT activity (430 pmol h^{-1} mg^{-1}) than did extracts prepared from Schwann cells (51 pmol h^{-1} mg^{-1}). Transfection efficiency could account for the differences between the two cell types, since the levels of CAT activity obtained with plasmids containing SV40 promoter-enhancer regions were 7.8-fold greater in astrocytes than in Schwann cells.

The host range of JCV in the nervous system has been shown to be limited to glial cells in brain tissue (17, 20) and human fetal brain-derived cells in culture (5, 9, 11, 16). The data reported here indicate that Schwann cells, a glial cell type derived from the human peripheral nervous system support JCV gene expression leading to virus multiplication. Fibroblasts and neurons did not produce detectable levels of JCV T antigen or viral DNA. The fact that neurons did not show signs of JCV infection is consistent with previous reports on PML tissues (22), except for one account of positive in situ hybridization labeling of cortical neurons (14). The amount of virus produced by Schwann cells was lower than that routinely obtained with brain-derived cells (9, 16). This observation may be due to factors that affect virus entry or transfection efficiency. Other possible reasons for low virus productivity in Schwann cells could involve regulation at the molecular level of recognition of the JCV regulatory sequences. More importantly, in this report, we

TABLE 1. Transient expression of CAT reporter gene in cultures of human fetal glial cells[a]

Plasmid	CAT activity (pmol h^{-1} mg^{-1}) in:	
	Schwann cells	Astrocytes
pM1-CAT	51	430
pSV2CAT	833	6,499
pSV0CAT	31	141

[a] Preparation of Schwann cell and astrocyte cultures is described in the text. Cultures were transfected with 10 μg of DNA. pSV2CAT and pSV0CAT were used as positive and negative controls, respectively.

present evidence for JCV infectivity of a glial cell type not described as a JCV host before. These data are consistent with the observation that JCV preferentially infects cells of glial origin, which could be due to the fact that expression of the early region of JCV is dependent on the presence of regulatory elements in glial cells (5, 19). Other cell types (19), possibly fibroblasts and neurons, either lack these elements or express elements that instead might exert a down regulation of the JCV genome. In addition, Schwann cells, like oligodendrocytes, are a cell type whose function is the synthesis and maintenance of myelin. Permissiveness of myelin-producing cells to JCV may be an indication of unknown intrinsic cellular properties associated with the phenotypic expression of myelin. Nevertheless, results presented herein warrant the extension of this study to pathological tissues from PML patients.

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