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Infection by Coronavirus JHM of Rat Neurons and Oligodendrocyte-Type-2 Astrocyte Lineage Cells during Distinct Developmental Stages

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Primary telencephalic cultures derived from neonatal Wistar Furth rats were able to support the growth of coronavirus JHM if a viable neuronal population was maintained. This occurred under serum-free defined, but not serum-supplemented, growth conditions. The importance of neurons in establishing infections in mixed cultures was confirmed by immunocytochemical and electron microscopic studies. Glia, although more abundant than neurons in these cultures, were less frequently infected during the initial 48 h postinoculation. The two glial lineages present in mixed telencephalic cultures were separated into type-1 astrocytes and oligodendrocyte-type-2 astrocyte (O-2A) lineage cells and individually assessed for their ability to support virus growth. Infection could not be established in type-1 astrocytes regardless of the culture conditions employed, consistent with our previous study (S. Beushausen and S. Dales, Virology 141:89–101, 1985). In contrast, infections could be initiated in selected O-2A lineage cells grown in serum-free medium. Virus multiplication was however significantly reduced by preconditioning the medium with mixed telencephalic or enriched type-1 astrocyte cultures, suggesting that intercellular interactions mediated by soluble factor(s) can influence the infectious process in O-2A lineage cells. This presumption was supported by eliciting similar effects with basic fibroblast growth factor and platelet-derived growth factor, two central nervous system cytokines known to control O-2A differentiation. The presence of these cytokines, which synergistically block O-2A cells from differentiating into oligodendrocytes was correlated with specific and reversible resistance to JHM virus (JHMV) infection. These data, combined with our finding that accelerated terminal differentiation of the oligodendrocyte phenotype confers resistance to JHMV (Beushausen and Dales, Virology, 1985), suggest that the permissiveness of O-2A cells for JHMV is restricted to a discrete developmental stage.

Much attention has recently been focused on the pathogenesis of virus-induced demyelinating diseases in rodent model systems. Among the agents studied is the neurotropic murine coronavirus JHM, which is capable of inducing central nervous system (CNS) disease in susceptible mice and rats. A spectrum of pathological processes is observed after intracranial inoculation of JHM virus (JHMV) into preweanling Wistar Furth (WF) and Wistar Lewis rats, ranging from acute fulminant encephalitis to delayed onset and chronic demyelination (41, 42, 63). Previous studies have shown the nature of the disease process predominating in rat pups inoculated intracranially to be a function of several host and viral determinants including the following: strain of rat employed, postnatal age at time of inoculation, length of time elapsing between inoculation and development of clinical signs, immunologic status of the host (62–64, 72), and variable molecular phenotype of S, the virion's major spike glycoprotein (9, 12, 17, 39, 65, 69).

In inbred WF rats, intracranial challenge during the first week of life with an adequate dose of virus invariably causes acute encephalitis, whereas challenge thereafter, up to weaning, frequently results in chronic, demyelinating disease (62). Although rats normally become resistant to JHMV postweaning, if they are treated with immunosuppressant drugs or if they are genetically athymic, they can remain susceptible to infection into adulthood (64, 72). These findings implicate the role of the cellular immune response in resistance. However, the disease provoked by the inoculation of JHMV postweaning occurs predominantly in grey rather than white matter regions of the CNS (72). The pathology observed suggests that both immunologic and nonimmunologic developmental changes taking place within the rat during the first 3 weeks of postnatal life influence this age-dependent response.

Undoubtedly, the in vivo interactions occurring among host, virus, and environment implicated in controlling the outcome of infection are dynamic, complex, and thus onerous to access. These difficulties in carrying out fundamental studies directed at elucidating pathogenic mechanisms prompted our laboratory to make extensive use of experimentally amenable in vitro systems utilizing dissociated primary neural cultures. Employing such cultures, which were secondarily enriched for specific forebrain-derived macroglia, we previously demonstrated that rat cells of the lineage giving rise to oligodendrocytes can act as conditionally permissive targets for JHMV whereas type-1 astrocytes cannot (5). Such a specificity in glial cell tropism of JHMV is not found among murine cells (70). Additionally, several observations indicated that the state of differentiation of rat-derived oligodendrocytes may be an important means by which the host could control virus replication within white matter regions, thus influencing the development of demyelinating disease (5, 6).

Several laboratories are employing in vitro model systems for studying gliogenesis, with emphasis placed on determinants controlling the differentiation of the oligodendrocyte-type-2 astrocyte (O-2A) lineage, which is specialized for

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myelination within the CNS (16). Although the details are far from complete, the emerging picture suggests that cell-cell interactions play a prominent role in this process. In this regard, type-1 astrocytes have been shown to produce the A-chain of platelet-derived growth factor (PDGF) (49, 54), a mitogen for O-2A progenitors derived from rat optic nerve (43, 49) and perinatal cerebral hemispheres (4). In addition, most murine neurons situated throughout the embryonic and adult CNS express the A-chain of PDGF at high levels (71). PDGF, active as either A-A homodimers or A-B heterodimers, binds to surface receptors and induces O-2A progenitors to undergo several cycles of division before reaching an intrinisically determined nonmitotic state, thought to be a prelude to terminal differentiation into oligodendrocytes or type-2 astrocytes (29, 49; for a review, see reference 46). Recently, the combination of PDGF and basic fibroblast growth factor (bFGF) was shown to exert a synergistic influence on O-2A progenitor mitogenesis, resulting in the inhibition of differentiation into oligodendrocytes (7, 37); this apparently is due to the action of bFGF in upregulating the expression of PDGF receptors (37). bFGF, present during CNS development, has been shown to originate from various cultured CNS cell types, among them murine cerebellar astrocytes (23), a human astrocytoma cell line (57), and human fetal neurons (66). In addition, rat CNS neurons synthesize another 18-kDa heparin-binding growth factor, which is immunologically distinguishable from bFGF (53).

The present study demonstrates that culture conditions and cell-cell interactions, which control the survival, proliferation, and differentiation of cell populations in primary rat telencephalic cultures, also influence the establishment of JHMV infection.

MATERIALS AND METHODS

Continuous line of cells. L-2 mouse fibroblasts (55) were used for propagating virus stocks as well as for plaque assaying virus from inoculated neural cell cultures, as described previously (32).

Viruses. Murine hepatitis virus strain JHM originally obtained from the American Type Culture Collection (Rockville, Md.) and the Indiana strain of vesicular stomatitis virus (VSV) were propagated and titers of the viruses on murine L-2 fibroblasts were determined as previously described (5).

Mixed telencephalic cultures from neonatal rats. Neonatal WF rat pups were taken within 12 h of birth and in most cases within 6 h of birth for the preparation of primary dissociated telencephalic cultures. For the purposes of age assessment of cultures, this time interval was referred to as postnatal day 0 (P0). After the meninges were carefully dissected from the surface of the cerebral hemispheres, the telencephalon was placed into ice-cold Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 10 μg of gentamicin per ml ($DMEM_{10}$). The telencephala were then washed three times with cold $DMEM_{10}$, and any adherent meninges were carefully removed. The tissue was minced with scalpel blades into fine pieces and washed once with cold Ca^{2+}-and-Mg^{2+}-free phosphate-buffered saline (PBS), pH 7.2, before digestion with 1× trypsin–EDTA (Sigma) at 37°C for 15 to 20 min with intermittent swirling. The tissue was washed three times with cold $DMEM_{10}$ and triturated gently, using fire-polished Pasteur pipettes in the presence of 20 μg of DNase I (Sigma) in a volume of 5 to 7 ml. The resulting cell suspension was passed by gravity through 130- and 33-μm-pore-size nylon meshes (Nitex). The resulting filtrate was then layered over 5 ml of cold fetal bovine serum in a 15-ml conical centrifuge tube (Corning) and centrifuged at 200 × *g* for 8 min. The cell pellet was resuspended in $DMEM_{10}$, and viable cell count was determined on the basis of trypan blue exclusion. The cell suspension was seeded onto either 12-mm-diameter glass coverslips or 35-mm-diameter plastic petri dishes (Nunc, Roskilde, Denmark) pretreated with poly-L-lysine (Sigma) at 5 to 10 μg/cm^2. The seeding density ranged from 2.5×10^5 to 5.0×10^5 viable cells per cm^2. All cultures were grown in $DMEM_{10}$ for the first 3 days in vitro (DIV). At this time, some of the cultures were switched to a serum-free, chemically defined medium first described by Bottenstein (8). Briefly, this medium consisted of DMEM supplemented with 1.2 g of $NaHCO_3$ per liter, 15 mM HEPES (pH 7.3), 5 μg of bovine insulin (Sigma) per ml, 50 μg of human transferrin (Sigma) per ml, 30 nM sodium selenite (Sigma), 10 ng of *d*-biotin (Sigma) per ml, and modified to include 30 nM triiodothyronine (T3) (Sigma), and is hereafter referred to as O1/T3. The remainder of the cultures were maintained in $DMEM_{10}$. Thereafter, the medium, serum-containing or serum-free, was changed every 3 days.

Secondarily derived glial cultures enriched for either O-2A lineage cells or type-1 astrocytes. Enriched glial cultures of either type-1 astrocytes or O-2A cells were prepared by following the outline described by McCarthy and deVellis (36). Complete medium changes were made at 4, 6, and 8 DIV. At 8 to 10 DIV, the cultures, consisting predominantly of a bed layer of type-1 astrocytes and top-dwelling cells of the O-2A lineage at various stages of differentiation, were washed three times with warm Ca^{2+}-and-Mg^{2+}-free PBS, pH 7.2, followed by the addition of fresh $DMEM_{10}$. Cultures were placed on a rotary shaker for 2 h at 120 rpm to free dead cells. The supernatant was replaced with fresh $DMEM_{10}$ at 37°C, and the loosely attached, top-dwelling O-2A lineage cells were removed by sharply tapping the sides of the flask. The cell suspension was pelleted by centrifugation at 200 × *g* for 5 min, and the cells were resuspended and deposited in 24-well dishes (Nunc) at densities ranging from 1.25×10^5 to 3.0×10^5 cells per cm^2 in $DMEM_{10}$. Twenty-four hours later, the $DMEM_{10}$ was carefully removed and replaced with test medium for 48 to 72 h followed by inoculation with virus. In some experiments, process-bearing, O-2A lineage cells were shaken from mixed cultures at 5 DIV instead of 8 to 10 DIV. This was done to increase the percentage of cells that were still in the progenitor state. Where indicated, 10 ng of recombinant human bFGF and/or the A-B heterodimeric form of recombinant PDGF, both obtained from Upstate Biotechnology Inc., Lake Placid, N.Y. were added to medium O1/T3 at 36-h intervals.

Secondary cultures enriched for type-1 astrocytes were prepared from the mixed glial cultures as described by McCarthy and deVellis (36). After any residual cells of the O-2A lineage were released by means of vigorous shaking, the remaining astrocyte bed layer was passaged by dissociation from the plasticware by using 1× trypsin–EDTA. Astrocytes were seeded onto poly-L-lysine-coated 35-mm-diameter petri dishes and grown in either $DMEM_{10}$ or the chemically defined medium O1/T3.

Conditioned medium production. The chemically defined medium O1/T3 was conditioned for 72 h by exposure to either mixed telencephalic cultures (P3 to P12) or subconfluent, enriched type-1 astrocyte cultures. After residual serum was washed from cultures, 25 ml of O1/T3 was added to each 150-cm^2 culture vessel and then collected after a

period of 72 h. The medium was centrifuged at 2,000 rpm for 10 min at 0°C in a Sorvall RT 6000 tabletop centrifuge to remove any cellular debris and then divided into aliquots and immediately stored at −70°C.

Inoculation of neural cultures and assaying infectivity. Mixed primary telencephalic cultures grown in 35-mm-diameter petri dishes were inoculated with JHMV in a volume of 350 μl at multiplicities of infection (MOIs) of ~1. Mixed telencephalic cultures grown on 12-mm-diameter glass coverslips were inoculated at MOIs of ~5 in 90 μl. The virus was adsorbed for 1 h at 37°C in a humidified atmosphere with 5% CO_2, unbound inoculum was removed, and cultures were washed twice with warm PBS before reconstitution with medium. Secondary cultures of type-1 astrocytes were grown in 35-mm-diameter petri dishes and inoculated with JHMV at MOIs from 1 to 50. Adsorption and washing of inoculum was performed as described for mixed telencephalic cultures. Secondary O-2A lineage cultures were grown in 24-well dishes and inoculated as described above with JHMV at MOIs of ~1 in 100 μl. The medium in all neural cultures was changed daily during the initial 48 h postinoculation and every second day thereafter.

Immunostaining of neural cultures. Monoclonal antibody (MAb) A2B5 (American Type Culture Collection), which recognizes the ganglioside G_{T3} (13) present on O-2A progenitor cells (50), type-2 astrocytes (47), and neurons (14), was used in ascitic fluid at a 1:4 dilution. MAb O4 (59), which recognizes surface sulfolipids on cells that are transitional intermediates between O-2A progenitors and oligodendrocytes (58), was used in tissue culture supernatant at a 1:2 dilution. Rabbit antiserum to galactosyl cerebroside (GC) and rabbit antiserum to guinea pig myelin basic protein (MBP), employed previously (5), were used at dilutions of 1:10 and 1:30, respectively. MAbs against the 160-kDa neurofilament polypeptide (Amersham) in tissue culture supernatant were diluted 1:5, rabbit antibodies against rat neuron-specific enolase (NSE) (Polysciences, Inc., Warrington, Pa.) were diluted 1:900, MAbs against JHMV nucleocapsid (N) (a generous gift from M. J. Buchmeier, Scripps Clinic and Research Foundation) in tissue culture supernatant were diluted 1:8, and rabbit antibodies against bovine glial fibrillary acidic protein (GFAP) (Dakopatts, Glostrup, Denmark) were diluted 1:40. Primary antibodies were diluted in PBS (pH 7.2) containing 3% (wt/vol) bovine serum albumin. The fluorochrome-conjugated secondary antibodies were used in PBS at the following dilutions: goat anti-mouse immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) (Sigma), 1:30; goat anti-mouse IgM (μ chain specific)-FITC (Sigma), 1:30; goat anti-rabbit (heavy and light chains)-rhodamine isothiocyanate, 1:40 (Cappel Laboratories); and goat anti-rabbit (heavy and light chains)-Texas Red (TR) (Jackson Immunoresearch Laboratories), 1:50. Primary and secondary antibodies were applied at room temperature in a humidified atmosphere for 25 to 35 min. Staining for surface antigens was done on living cells. For labelling both surface and internal antigens, cells on coverslips were fixed with 4% paraformaldehyde in PBS for 5 min at room temperature, after completion of surface labelling reactions. Cells were then permeabilized in acid ethanol (5% glacial acetic acid–95% ethanol [vol/vol]) for 10 min at −20°C, washed in PBS, immunolabelled, and then postfixed in 4% paraformaldehyde in PBS.

Electron microscopy of infected telencephalic cultures. JHMV-infected and uninfected mixed telencephalic cultures in 35-mm-diameter petri dishes were prepared for electron microscopy as previously described (11). Briefly, cultures were washed with 0.1 M sodium phosphate, pH 7.0, and then fixed in situ for 5 min with 1% glutaraldehyde in 0.1 M sodium phosphate (vol/vol). Following fixation and three washes with 0.1 M sodium phosphate, cells were postfixed with 1% OsO_4 in 0.1 M sodium phosphate (vol/vol), dehydrated by using a series of graded ethanol solutions, and embedded in epoxy resin. Sections were cut both horizontally and vertically to the plane of the attachment surface. After the sections were stained with uranyl acetate and then with lead citrate, specimens were viewed in a Philips EM 300 electron microscope.

Immune-mediated lysis of selected cell populations in infected mixed cultures. One day postinoculation with JHMV, quadruplicate 35-mm-diameter plates containing mixed telencephalic cultures were incubated with either MAb A2B5 diluted 1:4 in $DMEM_{10}$ or rabbit antibodies against GC diluted 1:10 in $DMEM_{10}$ as described by Beushausen and Dales (5). After incubation for 60 min at 20°C, unbound antibody was removed by two washes of $DMEM_{10}$. Half of the antibody-reacted cultures were exposed at 37°C for 30 min to rabbit Lo Tox complement (Cedarlane, Hornby, Ontario, Canada) diluted 1:14 in $DMEM_{10}$. Unfixed complement was removed by washing twice with 1 ml of $DMEM_{10}$ before the addition of medium.

RESULTS

Primary mixed telencephalic cultures. (i) Growth characteristics in serum-containing and serum-free defined culture environments. Cultures derived from neonatal rat telencephala were grown with 10% fetal bovine serum supplementation during the initial 3 DIV. Within 24 h of establishing the cultures, neurons began sprouting neurites, and by 3 DIV, cell aggregates and a complex network of processes were well established. At this time, a proportion of the cultures were maintained in $DMEM_{10}$, while the remainder were switched to the serum-free, chemically defined culture medium O1/T3. Cultures grown under the two conditions remained morphologically indistinguishable up to 6 DIV. At this point in time, aggregates of cells in $DMEM_{10}$ began to detach from the substrate coincident with the undergrowth of flat astroglia. This cell behavior was not observed with cultures grown in O1/T3. By 7 to 8 DIV, cultures in $DMEM_{10}$ took on the typical stratified appearance originally described by McCarthy and deVellis (36) in which phase-lucent, flat, epithelioid astrocytes made up the bed layer on which phase-dense, process-bearing cells of the O-2A lineage were superimposed. In sharp contrast, cultures placed in O1/T3 medium beginning at 3 DIV retained aggregates of rounded cells separated from each other by zones permeated with a network of numerous processes. Figure 1 illustrates the microscopic differences observed between the two culture conditions at 9 DIV.

Phenotyping of cultures was carried out between 5 and 21 DIV by using monospecific and monoclonal antibodies. At 5 DIV and P5, neurons identified by the presence of neurofilaments and NSE, were present in both O1/T3 and $DMEM_{10}$ cultures. These same markers revealed that neurons were nearly totally lost from $DMEM_{10}$ cultures by 8 DIV but were still numerous in cultures grown in O1/T3 until at least 21 DIV and P21 (Fig. 2). The loss of neurons from cultures maintained in $DMEM_{10}$ appeared to coincide with the proliferation of epithelioid astrocytes, similar to the results reported by Gilad et al. (21) in neonatal rat cerebellar cultures. Electron microscopic examination of O1/T3 cultures sampled at 15 DIV and P15 revealed the presence of

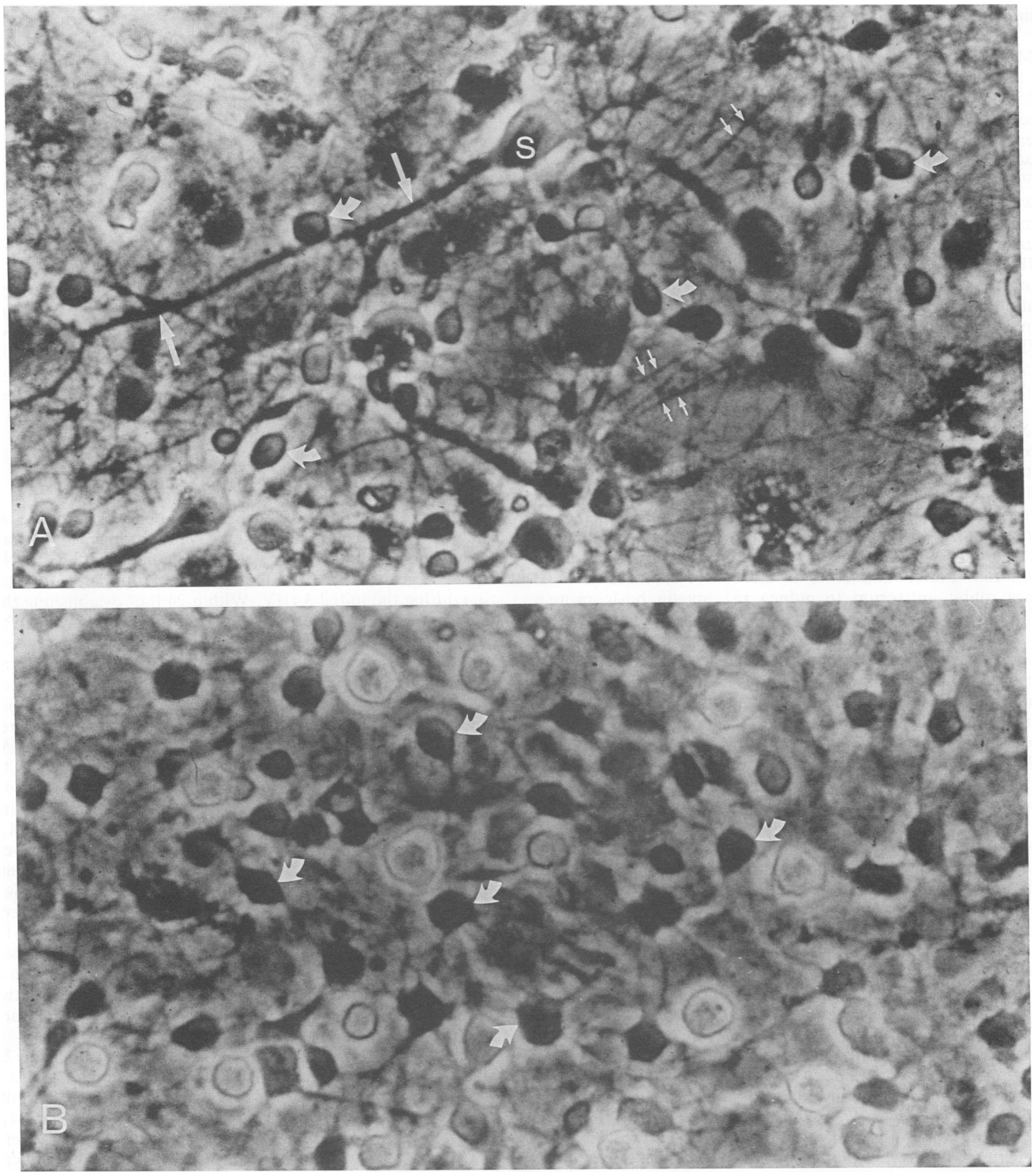

FIG. 1. Appearance at 9 DIV and P9 of dissociated mixed telencephalic cultures derived at P0 from WF rats. (A) Typical culture placed in O1/T3 at 3 DIV. S marks the soma of a large cell extending a prominent process (indicated by large straight arrows). Small straight arrows point to numerous, smaller processes, and curved arrows point to smaller cells typical of the O-2A lineage. (B) Typical culture in $DMEM_{10}$. Process-bearing O-2A lineage cells (curved arrows), dwell above a phase-lucent bed layer of type-1 astrocytes. Magnification, ca. ×1,500.

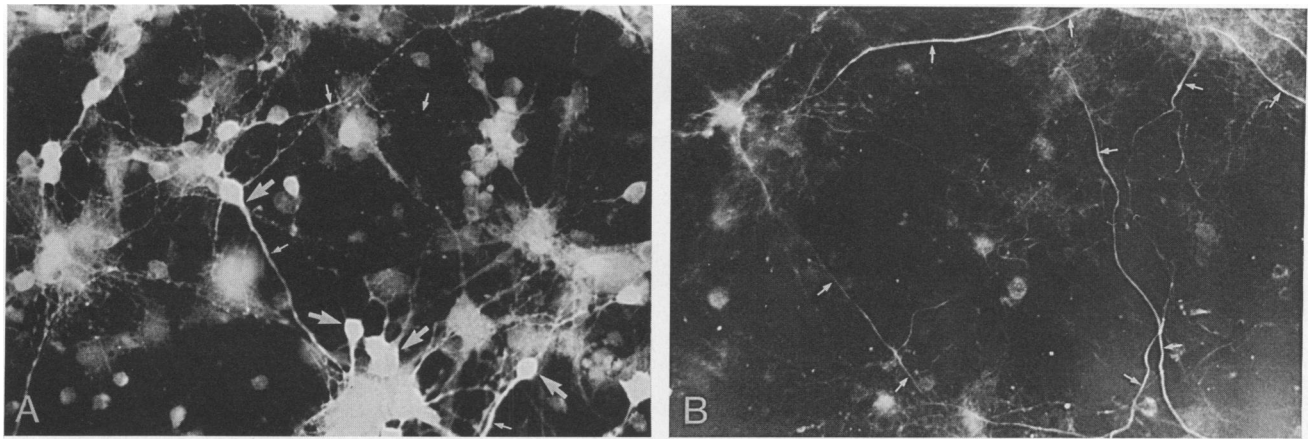

FIG. 2. Detection of neurons at 10 DIV and P10 in dissociated mixed telencephalic cultures grown in O1/T3. (A) Neuronal cell bodies (large arrows) and processes (small arrows) visualized after reaction with rabbit anti-NSE and goat anti-rabbit IgG (TR conjugated). (B) Neurites coursing over the culture surface (arrows) are revealed with MAb against 160-kDa neurofilament protein and goat anti-mouse IgG (FITC conjugated). Magnification, ca. ×700.

cells possessing the fine structure typical of neurons and a profuse network of neurites containing numerous microtubules (Fig. 3A to C). $GFAP^+$ astrocytes were abundant under both growth conditions. However, astrocytes possessed stellate morphology in O1/T3 and polygonal morphology in $DMEM_{10}$. In both $DMEM_{10}$ and O1/T3 media, an abundance of bi- and tripolar $A2B5^+$ O-2A progenitor cells was observed at 5 DIV and P5, along with fewer GC^+ oligodendrocytes, in agreement with previous reports (4, 18, 24). There was a progressive, time-related differentiation of O-2A lineage cells, detected by the loss of A2B5 immunoreactivity coupled with increased anti-GC and anti-MBP immunoreactivity. This shift in antigenic phenotype was coupled with morphologic differentiation identified by the change from bipolar or simple multipolar forms, which predominated at 4 to 5 DIV, to more-complex branching patterns evident by 10 DIV (data not shown), consistent with previous reports (4, 18, 24). Morphologic differentiation of oligodendrocytes progressed to the extent where myelination of axons in selected areas of the culture was observed as determined by immunocytochemical (data not shown) and electron microscopic methods (Fig. 3B and C), as already described for primary telencephalic cultures by others (40).

These observations illustrate the dynamic nature of this culture system and indicate that the milieu of the culture medium influenced profoundly the survival of specific neural cell types, particularly neurons. Survival of neurons was in turn associated with differences in the histotypic organization that these cultures adopted, the development of which was likely contingent upon intercellular interactions and cellular differentiation.

(ii) Infection with JHMV. To determine whether the differences observed in cultures grown in $DMEM_{10}$ and O1/T3 media influenced the ability to infect with JHMV, a series of mixed telencephalic cultures, grown in one or the other medium were inoculated at 10 DIV and P10 at a MOI of 1. The data in Fig. 4 illustrate unequivocally that the culture milieu strongly influenced both the magnitude and longevity of virus production. In some experiments, cultures in O1/T3 that were inoculated at P10 remained productively infected for at least 6 weeks, yielding titers in the order of 1,000 PFU/ml (data not shown).

Connection between the acceleration of oligodendrocyte differentiation and exposure to the cyclic AMP (cAMP) analog O2′-dibutyryladenosine 3′:5′-cyclic monophosphate (dbcAMP) (5, 38, 51) or to activators of adenylate cyclase (6, 52) prompted us to assess the effects of pretreatment with dbcAMP. Our previous work had shown that if cultures secondarily enriched for O-2A lineage cells were treated with compounds affecting adenylate cyclase metabolism before inoculation, subsequent establishment of JHMV infection was inhibited (5, 6). The consequence of pretreatment of mixed telencephalic cultures grown in O1/T3 was of interest to us, since these cultures contained O-2A lineage cells and neurons, both targets of JHMV in vivo (61). Treatment of O1/T3 cultures with 2.5 to 5.0 mM dbcAMP 72 h before and 48 h after inoculation with JHMV at P10 resulted in no adverse effects on virus production; to the contrary, dbcAMP frequently enhanced virus growth, which was most notable during the initial 5-day period postinoculation. A similar dbcAMP-related enhancement, albeit very minor, was also observed occassionally in $DMEM_{10}$ cultures (Fig. 4). The enhancement was attributed to cAMP per se, because equimolar concentrations of sodium butyrate actually inhibited virus growth, resulting from the necrosis of cells of non-type-1 astrocytic phenotype (data not shown).

The addition of T3, implicated in the process of postnatal oligodendroglial differentiation and myelination (2; for a review, see reference 44), to cultures grown in serum-free medium was also without effect in either the establishment of infection or long-term production of virus (Fig. 4).

To ascertain whether the age of the culture influenced virus production, cultures grown in O1/T3 were inoculated with JHMV at a MOI of 1 at 4 DIV and P4, 6 DIV and P6, 10 DIV and P10, 15 DIV and P15, and 21 DIV and P21. The influence of continuous treatment with 2.5 mM dbcAMP 48 h before and 48 h after inoculation was also assessed at P6, P10, P15, and P21. The results, summarized in Table 1, demonstrated first of all that an infection was most difficult to establish in very young P4 cultures, which contained a high frequency of bipotential O-2A progenitor cells and neurons elaborating an extensive network of neurites. Second, the highest virus yields were obtained from cultures inoculated at P10, suggesting that the number and/or suscep-

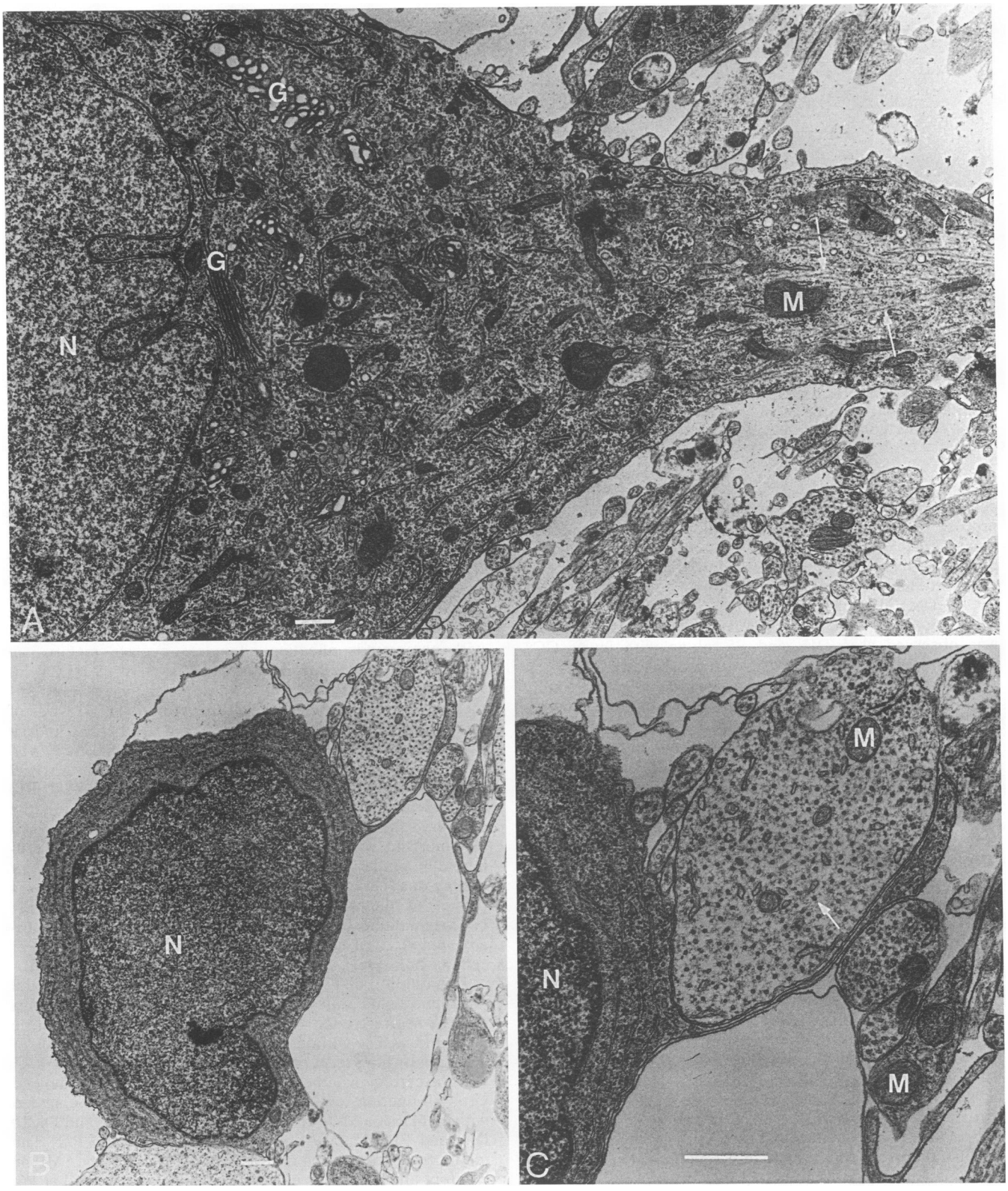

FIG. 3. Electron microscopy of selected cells in an uninfected telencephalic culture from neonatal WF rats preserved in situ at P15. Following explantation, the cultures were grown in $DMEM_{10}$ for 3 days and then maintained in O1/T3 for 12 days. (A) Portion of a cell with neuronal morphology contains a central nucleus (N) with homogeneous karyoplasm, prominent Golgi membranes (G), numerous mitochondria (M), and bundles of microtubules (small arrows) inside the process. Numerous smaller processes in cross-section containing microtubules are also evident at the bottom right. (B) A smaller, dense cell, superimposed on the neuronal cell layer, possesses the fine structure of an oligodendrocyte. (C) A portion of the cell as in panel B illustrates at a higher resolution a process from the adherent cell which partially surrounds the prominent neurite filled with microtubules (arrow). Bars = 0.5 μm.

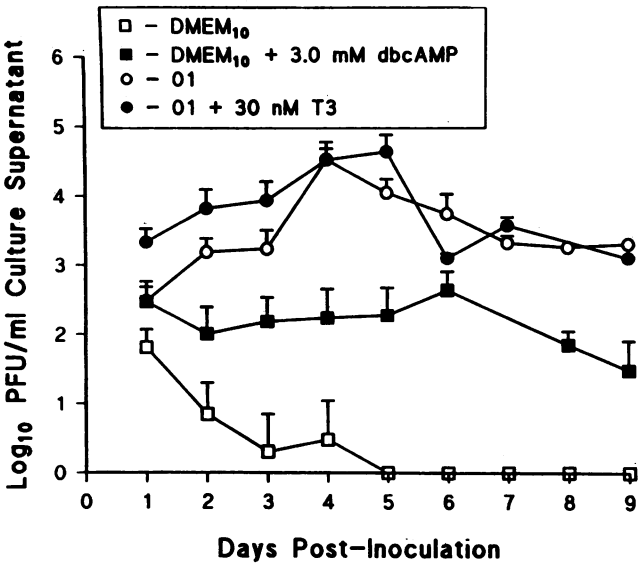

FIG. 4. JHMV replication in primary dissociated mixed telencephalic cultures derived from WF rats at P0. Cultures were grown continuously in $DMEM_{10}$ or were switched at 3 DIV to serum-free O1 medium with or without T3 supplementation. $DMEM_{10}$ cultures receiving dbcAMP were treated 48 h before and 48 h after inoculation with JHMV. All cultures were inoculated at 10 DIV and P10 with a MOI of 1. The data are the mean titers ± standard deviations from four identical experiments.

tibility of target cells is optimal at this stage. Third, the enhancing effect of dbcAMP was most pronounced when inoculation occurred at P10 to P21. These differences on rates of JHMV replication led us to attempt further elucidation of the cell type(s) supporting the infection.

(iii) Identification of virus-infected cells within mixed telencephalic cultures. Mixed telencephalic cultures established on glass coverslips were inoculated at P10 and monitored at 24-h intervals for virus released into the supernatant. As anticipated, cultures maintained continuously in $DMEM_{10}$ were devoid of neurons, determined as described above, but contained numerous type-1 astrocytes and O-2A lineage cells and failed to produce infectious particles or viral N antigen at 1, 2, and 5 days postinoculation. In contrast, cultures maintained in O1/T3 commencing at 3 DIV became efficient virus producers 24 h postinoculation, expressing N antigen in both isolated individual cells and clusters of 4 to 5 cells. By 48 h postinoculation, the infected-cell foci became larger. This was accompanied by the intensification of N antigen staining within the cytoplasm and spread along elongated processes radiating from the cell body as discrete puncta (Fig. 5). By simultaneous immunolabelling, it was demonstrated that these cells were NSE^+, GC^-, and $GFAP^-$, establishing their identity as forebrain neurons. It should be mentioned that colabelling for NSE and N was evident only during the initial 24 h postinoculation, then became weaker, and ultimately disappeared. The simultaneous presence of N^+ $GFAP^+$ and N^+ GC^+ cells, which together made up less than one-fourth of all those scored as N^+ within the initial 48 h revealed that cells of astrocyte and oligodendrocyte phenotypes had also been infected. By 5 days postinoculation, the formation of syncytia, which were sometimes quite extensive, obliterated morphological details of the infected polykaryocytes. Nevertheless, prominent foci in the cell mass colabelled for N and GFAP. Inoculations carried out at P6 and P17 resulted in similar labelling patterns.

As with the above data from immunocytology and plaque assays, we did not find any evidence of JHMV infection in $DMEM_{10}$ or $DMEM_{10}$ plus dbcAMP cultures by electron microscopy, but we observed cells containing coronavirus particles and inclusions of viral material in O1/T3 and O1/T3 plus dbcAMP cultures (Fig. 6). There was close correspondence in neuronal morphology of infected cells observed by light and electron microscopy with respect to the typical pyramidally shaped cell bodies extending long processes, presumably neurites, which were replete with microtubles (Fig. 6). The prominent cytoplasmic inclusions consisted of aggregates of N previously identified by us in other host cells (35). In favorable orientations, the N component was regularly distributed along the microtubule arrays, extending from the soma into processes (Fig. 6A and C). Particles with the morphology of coronavirions were readily found either

TABLE 1. JHMV replication in primary mixed telencephalic cultures inoculated at different ages postnatally[a]

Age at challenge	Treatment	Titer[b] at the indicated day postinoculation:			
		1	2	3	4
P4	None (control)	ND[c]	0.4 ± 0.4	0.2 ± 0.07	5.6 ± 1.1
P6	None (control)	4.6 ± 0.6	24.0 ± 19.8	54.0 ± 13.4	340.0 ± 35.4
	dbcAMP	3.9 ± 0.5	7.4 ± 4.7	22.1 ± 21.1	197.5 ± 123.7
P10	None (control)	30.2 ± 11.7	116.8 ± 29.3	245.0 ± 99.0	250.0 ± 21.2
	dbcAMP	10.8 ± 3.2	52.0 ± 32.5	142.5 ± 60.1	272.5 ± 60.1
P15	None (control)	ND	4.1 ± 0.6	32.0 ± 0.7	10.0 ± 0
	dbcAMP	ND	53.8 ± 41.5	248.8 ± 136.8	109.0 ± 36.8
P21	None (control)	8.2 ± 0.7	11.0 ± 0.5	13.8 ± 6.7	18.0 ± 4.2
	dbcAMP	8.4 ± 1.0	48.8 ± 15.9	137.2 ± 19.4	217.5 ± 95.4

[a] Primary, mixed, dissociated telencephalic cultures were established using newborn WF rat pups. Duplicate cultures from each treatment were inoculated with JHMV at a MOI of 1 at the ages indicated. Cultures in the control group were grown in O1/T3 medium beginning at 3 DIV. dbcAMP-treated cultures were exposed to 2.5 mM of this agent 48 h prior to inoculation with virus and continued for 48 h postinoculation. Fresh dbcAMP was added daily during this period.

[b] All titers are the means of duplicate cultures and are expressed as 10^2 PFU/ml of culture supernatant.

[c] ND, not determined.

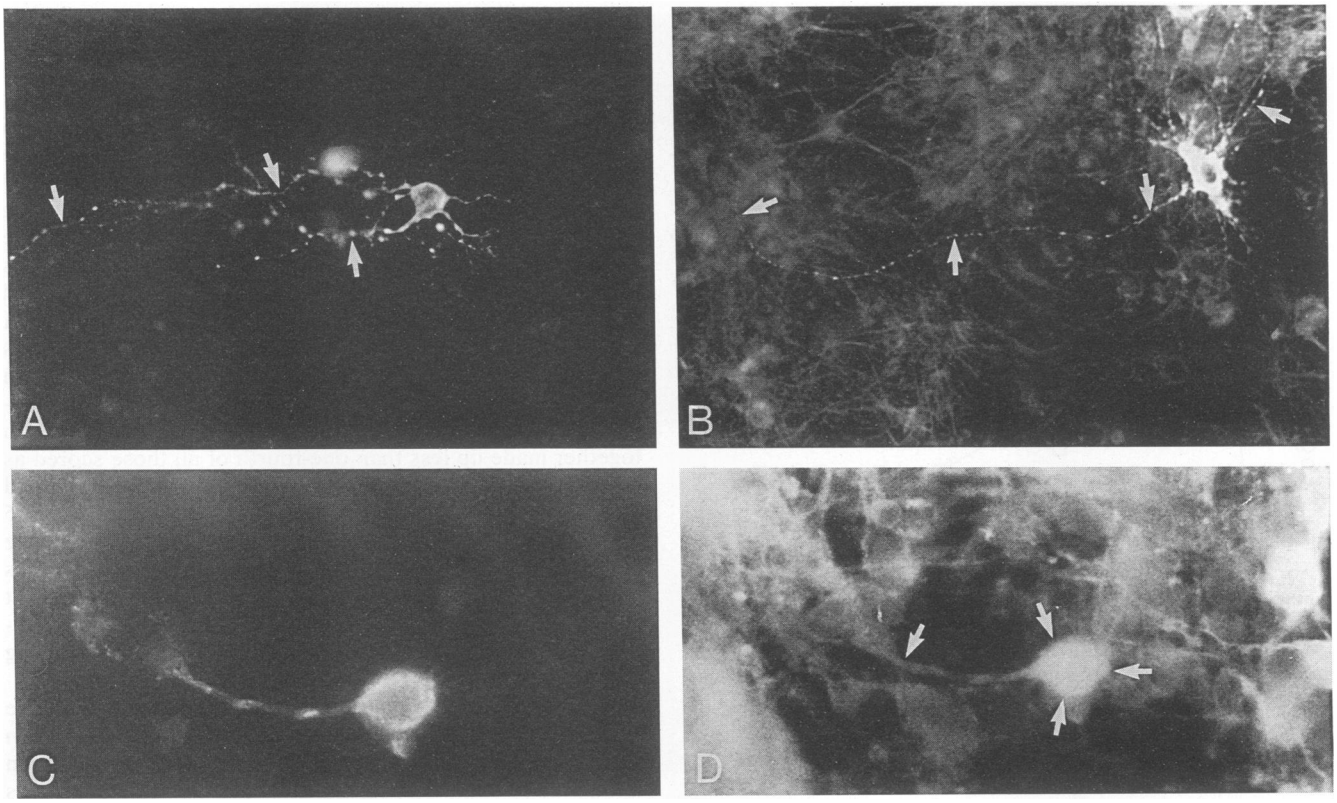

FIG. 5. Identification of JHMV-infected cells in primary mixed telencephalic cultures propagated in O1/T3 medium. (A) Following inoculation at P6, the culture was labelled at 3 days postinoculation by reaction with MAbs against JHMV N and goat anti-mouse IgG (FITC conjugated). Note the presence of N immunoreactivity in the soma and along the cell processes (arrows). Magnification, ca. ×920. (B) Another cell with comparable morphology and distribution of N, sampled 2 days postinoculation in a culture inoculated at P10. Note that intense fluorescence of RITC-tagged GFAP of astrocytes is not completely suppressed by the FITC filter. (C and D) Culture inoculated at P6 sampled and labelled 1 day postinoculation. Magnification, ca. ×920. In panel C, the N^+ cell is identified as in panel A; in panel D, the same cell as in panel C (arrows) was colabelled with rabbit anti-NSE and goat anti-rabbit IgG (TR conjugated). Magnification, ca. ×1,400.

as budding or free forms within endoplasmic reticulum (Fig. 6B) or at the surface.

To further identify the cell type(s) supporting replication of JHMV, we employed complement-mediated immune cytolysis. Binding of polyclonal antibodies against GC was used for eliminating cells of the oligodendrocyte phenotype, and MAb A2B5 was used to select neuronal (14), O-2A progenitor (50), and type-2 astrocytic cells (47). Immune lysis was carried out 24 h after inoculation at P10, and the amounts of virus subsequently produced were determined. As evident from the data in Table 2, cultures exposed to MAb A2B5 plus complement produced virus an order of magnitude less than those left untreated or treated individually with A2B5 or complement. Application of antibodies against GC plus complement reduced the virus titer marginally and at later times than observed with MAb A2B5 plus complement. This was taken as suggestive evidence that $A2B5^+$ rather than GC^+ cells were the primary producers of JHMV, consistent with immunocytological and electron microscopic observations which implicated the neurons. However, some ambiguity remained as to the $A2B5^+$ cells affected, since MAb A2B5 can also recognize O-2A progenitors and type-2 astrocytes. It, therefore, became imperative to ascertain the role of glial cells in JHMV replication.

Secondary glial cultures. (i) JHMV replication in cultures enriched for type-1 astrocytes or O-2A lineage cells. Previous work in our laboratory identified the tropism of JHMV for rat cells within the O-2A lineage and the lack of tropism for type-1 astrocytes. This is to be contrasted with the unambiguous tropism of a closely related coronavirus murine hepatitis virus type 3 for type-1 astrocytes (5). To account for the inefficient infection of O-2A lineage cells situated in mixed cultures grown in O1/T3 medium and the absence of virus growth by cultures in $DMEM_{10}$, we invoked epigenetic effects due to components in the culture milieu. This idea was tested by infecting enriched type-1 astrocyte or O-2A lineage cultures when these were grown under the conditions used for propagating mixed telencephalic cultures.

Type-1 astrocytes were flat and epithelioid in form when grown in the presence of serum supplement, but they possessed a stellate outline with numerous processes when grown in O1/T3. Process formation became even more accentuated upon the addition of dbcAMP, consistent with previous reports (1, 20). However, regardless of the culture conditions employed, type-1 astrocytes did not support JHMV replication, consistent with our published results (5, 6). This drew our attention to an apparent anomaly concerning JHMV expression in $GFAP^+$ cells, documented above.

Secondary cultures of O-2A lineage cells (90 to 95% purity) were established at densities ranging from 1.25×10^5 to 2.5×10^5 cells per cm^2. Following 24 h in $DMEM_{10}$, the cultures were grown for a further 48 or 72 h in O1/T3, O1/T3

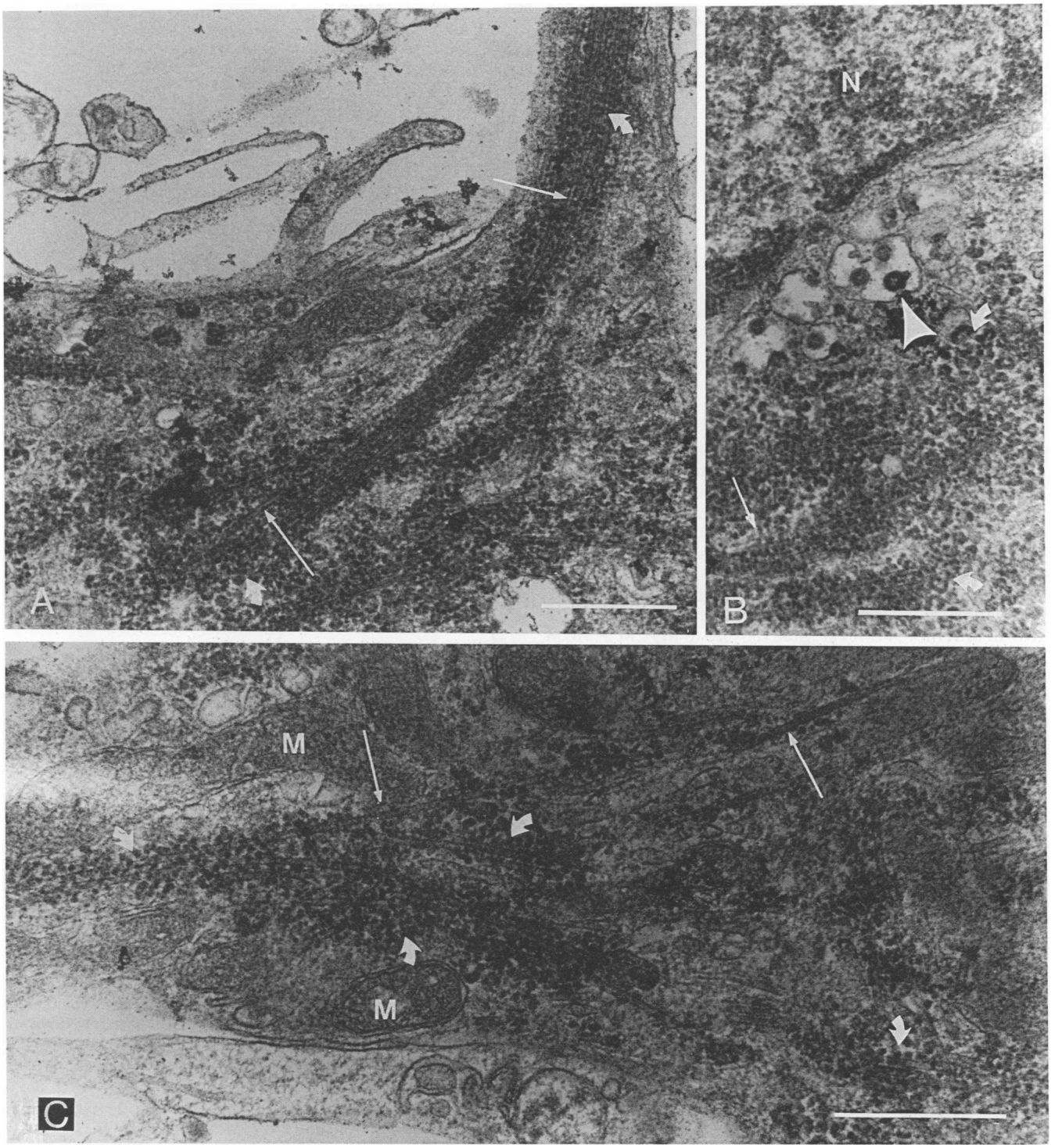

FIG. 6. Electron microscopy of selected examples of portions of neuronal type cells from a telencephalic explant culture infected and sampled as in Fig. 3. (A) Portion of a cell with a prominent neurite and numerous dense cytoplasmic inclusions. (B and C) Higher-resolution images reveal a close association between microtubules (small arrows) and dense, granular nucleocapsid material of a coronavirus (curved arrows), corresponding in structure to that described previously (35). Individual JHM virions (large arrowhead) are evident inside cytoplasmic vacuoles near the nucleus (B). N, nucleus, M, mitochondria. Bars = 0.5 μm.

TABLE 2. Effects of immune lysis of GC^+ $A2B5^+$ cell populations on the replication of JHMV in mixed telencephalic cultures[a]

Treatment of culture	Titer[b] at the indicated day postinoculation:		
	2	3	4
O1/T3	6.8	9.1	12.6
O1/T3 + 2.5 mM dbcAMP	3.7	40.2	151.5
O1/T3 + complement	6.9	17.8	23.5
O1/T3 + A2B5	9.3	16.4	27.5
O1/T3 + anti-GC	16.5	13.5	36.2
O1/T3 + anti-GC + complement	7.4	10.0	17.0
O1/T3 + A2B5 + complement	1.7	2.4	2.9

[a] Mixed telencephalic cultures grown in 35-mm-diameter petri dishes were inoculated with JHMV at 10 DIV and P10 with a MOI of 1. Cultures receiving dbcAMP treatment were given the agent on a daily basis 48 h before and 48 h after viral inoculation. Antibody and complement treatments were initiated 24 h postinoculation as described in Materials and Methods.

[b] All titers are the means of duplicate cultures and are expressed as 10^2 PFU/ml of culture supernatant.

mixed 1:1 with O1/T3 conditioned by exposure for 72 h to mixed telencephalic cultures (MTCM), or O1/T3 mixed 1:1 with O1/T3 conditioned by 72-h exposure to type-1 astrocytes (ACM) and then inoculated with JHMV at a MOI of 1. In a number of experiments, O-2A lineage cells were also seeded directly onto a type-1 astrocyte bed layer and the cocultures were grown for 48 to 72 h in O1/T3 before inoculation. O-2A cultures grown in O1/T3 supported the growth of JHMV; however, preexposure to MTCM or ACM inhibited JHMV replication, with virus yields that were only 1/10 to 1/100 of those from control cultures. Inhibition was even more pronounced with O-2A lineage cells directly cocultured with type-1 astrocytes (Fig. 7). Since cell viability, assessed by trypan blue exclusion, was not altered under the above conditions, the differences in virus production could not be attributed to the loss of viable target cells. Enriched O-2A lineage cultures were mitotically more active in MTCM or ACM than controls in O1/T3, consistent with previous reports (19, 24, 28, 54). In addition, cells tended to maintain bipolar to simple multipolar forms in conditioned media but developed more complex forms typical of oligodendrocytes in unconditioned O1/T3. The suppressive effect on JHMV growth was evident in ACM diluted 1:10 before the effect disappeared, indicating that the activity was present at limiting concentrations. The suppression was specific to JHMV, because VSV replicated equally well in O-2A cells grown in conditioned or unconditioned O1/T3 (data not shown). Inhibition was also related specifically to cells of the O-2A lineage, because culture of L cells in O1/T3, MTCM, or $DMEM_{10}$ for 72 hours before and after inoculation with JHMV did not influence virus growth (Table 3). Evidently, the factor(s) in conditioned media inhibiting JHMV infections act(s) specifically on the O-2A cell type.

(ii) Modulation of JHMV replication in O-2A cells by the cytokines PDGF and bFGF. Inhibition of virus production by O-2A cultures shown in Fig. 7 suggested that the modulation involved diffusible cellular factor(s), possibly of heterotypic origin. Since suppression of JHMV growth in O-2A cells cultured in MTCM or ACM was correlated with enhanced mitotic activity and retention of simpler cell morphologies, we assessed the effect on virus replication of PDGF and

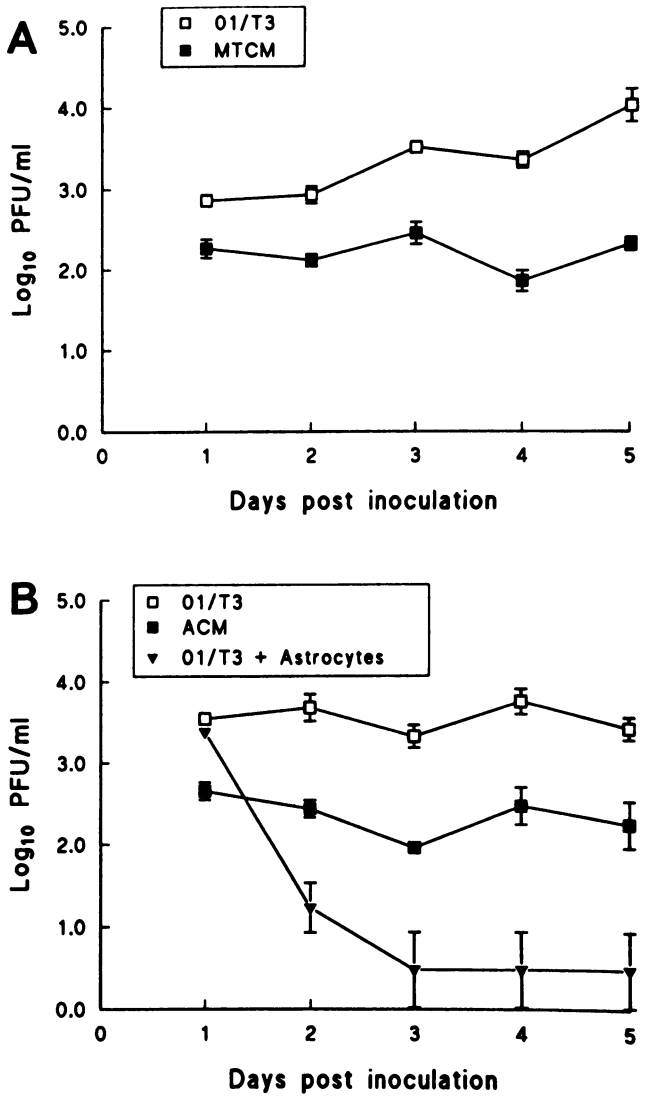

FIG. 7. Replication of JHMV in enriched O-2A lineage cultures derived from mixed telencephalic cultures at 9 DIV and P9. (A) Cultures plated at 1.25×10^5 cells per cm^2 were grown in O1/T3 or MTCM for 48 h prior to inoculating with JHMV at a MOI of 1. The data, which are mean titers ± standard deviations from triplicate cultures are representative of three independent experiments. (B) O-2A lineage cells, plated either directly onto the plasticware or onto a monolayer of type-1 astrocytes, were established at the same cell density as that for panel A. After growth in O1/T3 or ACM for 48 h, the cultures were inoculated with JHMV at a MOI of 1, with compensation being made for O-2A and type-1 astrocyte cocultures. Data are the means and standard deviations of titers from triplicate cultures and are representative of three independent experiments.

bFGF, two cytokines identified in effecting developmental control over cells of the O-2A lineage (7, 37, 49).

O-2A lineage cells isolated from mixed glial cultures at 9 DIV and P9 were grown for 72 h with PDGF (10 ng/ml), bFGF (10 ng/ml), or both factors, then were inoculated with JHMV at a MOI of 2. The virus titers produced in the presence and absence of cytokines are depicted in Fig. 8. Culturing O-2A lineage cells in the presence of bFGF, PDGF, and bFGF plus PDGF produced, respectively, 3.1-, 5.8-, and 5.5-fold increase in cell number over a 4-day period

TABLE 3. Effects of cell and virus specificities and culture conditions on virus growth

Cell/virus system	Culture conditions	Titer[a] (mean ± SD) at the indicated day postinoculation:			
		1	2	3	4
L cells[b]/JHMV (MOI of 0.1)	$DMEM_{10}$	21,000 ± 9,500			
	$DMEM_{10}$ + bFGF	23,000 ± 11,000			
	O1/T3	6,900 ± 2,700			
	O1/T3 + bFGF	6,700 ± 280			
	O1/T3 + PDGF + bFGF	7,000 ± 850			
	ACM	7,500 ± 1,200			
	MTCM	9,000 ± 1,400			
O-2A[c]/JHMV (MOI of 1)	O1/T3	ND[d]	10.7 ± 2.2	14.9 ± 8.9	30.8 ± 10.0
	O1/T3 + bFGF + PDGF	ND	0.2 ± 0.08	0 ± 0	0 ± 0
O-2A[c,e]/VSV (MOI of 1)	O/T3	326.7 ± 20.8	33.3 ± 13.9		
	O1/T3 + bFGF + PDGF	513.3 ± 110.6	306.7 ± 81.4		

[a] All titers are expressed as 10^2 PFU/ml of culture supernatant.
[b] L-cell cultures were completely lysed by 18 h postinoculation.
[c] Enriched O-2A progenitor cultures derived from 5 DIV and P5 mixed telencephalic cultures were established at a density of 1.25×10^5 cells per cm^2 and grown in O1/T3 supplemented with bFGF and PDGF (10 ng/ml each). At P9, half the cultures were switched to O1/T3 without cytokines and then inoculated at P13 with either JHMV or VSV.
[d] ND, not determined.
[e] O-2A cultures inoculated with VSV were completely lysed by 48 h postinoculation. Note that higher yields in cultures treated with O1/T3 plus bFGF plus PDGF correlated with higher cell densities.

relative to the number of cells grown on control O1/T3 medium. Therefore, the rates of virus replication were inversely correlated with the mitotic activity. bFGF displayed a potent inhibitory effect on its own which disappeared at concentrations below 10 pg/ml. Interestingly, the continual presence of bFGF has been reported to inhibit the differentiation of O-2A progenitors into oligodendrocytes (37). No such inhibitory influence of PDGF and bFGF on virus production was evident with L-cell cultures allowed to grow to confluency under identical conditions prior to inoculation, demonstrating that these cytokines affected the glia specifically (Table 3).

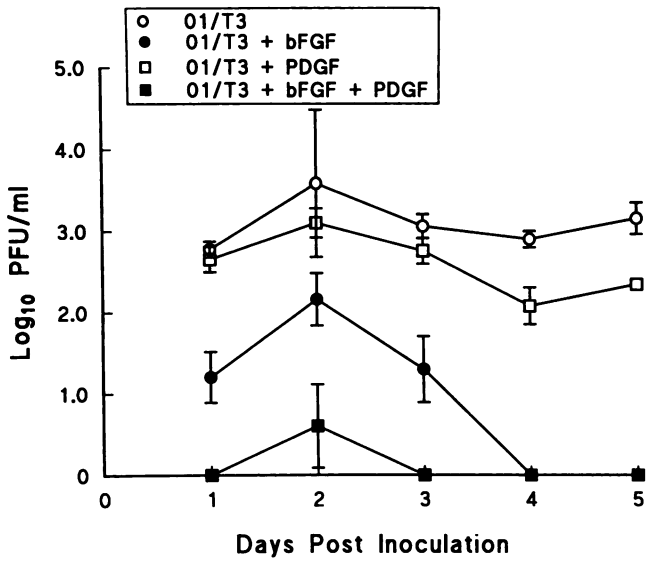

FIG. 8. Effects of bFGF and PDGF on the replication of JHMV in enriched O-2A lineage cultures. O-2A lineage cells derived from 9 DIV and P9 mixed telencephalic cultures were plated at 2.5×10^5 cells per cm^2 and grown for 48 h in O1/T3 alone or O1/T3 supplemented with bFGF (10 ng/ml), PDGF (10 ng/ml), or bFGF plus PDGF before inoculation with JHMV at a MOI of 2. Data are the means of titers ± standard deviations from triplicate cultures and are representative of three independent experiments.

In order to better define the relationship between the virus inhibitory state and O-2A cell differentiation mixed cultures at 5 DIV and P5 were used for isolating O-2A progenitor cells. At this stage, the majority of surface adherent cells were bipolar or had few processes and had a $A2B5^+$ GC^- phenotype (data not shown). After the released cells were plated at 1.25×10^5 cells per cm^2 on glass coverslips coated with poly-L-lysine and fibronectin (1 μg of fibronectin per cm^2), cultures were established in O1/T3 plus PDGF and bFGF (10 ng/ml each) (poly-L-lysine and fibronectin were used to enhance attachment and hence optimize survival of the O-2A cells after transfer from mixed cultures at a more juvenile stage, in accordance with the findings of Hunter and Bottenstein [25]). Figure 9 illustrates the appearance of a typical culture enriched for O-2A progenitors, 24 h after plating. Approximately 90% of the cells were bipolar or of a simple multipolar morphology and were $A2B5^+$ and $O4^-$. Only about 1% of the cells were GC^+, while the remainder were predominantly type-1 astrocytes. Some of these cultures enriched for progenitors were maintained continuously in the presence of PDGF and bFGF (replenished every 36 h), while others were switched at intervals to O1/T3 devoid of cytokines. Inoculation with JHMV, at a MOI of 1, was carried out at P6, P8, P9, and P13. Inoculation with VSV as the control virus was carried out at identical time points. During continuous presence of combined PDGF and bFGF, the O-2A progenitors continued to be mitotically active and possessed an $A2B5^+$ GC^- phenotype as previously reported (7, 37) and concomitantly resisted infection by JHMV, as judged by absence of viral antigen and released virus. By contrast, replication of VSV was unaffected (Table 3), demonstrating, as in the case of MTCM and ACM, the specificity of cytokines in suppression of JHMV. Upon withdrawal of the cytokines, only a small fraction of cells died. The surviving cells differentiated, as determined by shifts in expression of stage-specific phenotypic markers and acquisition of more-complex morphologies. Four days after the

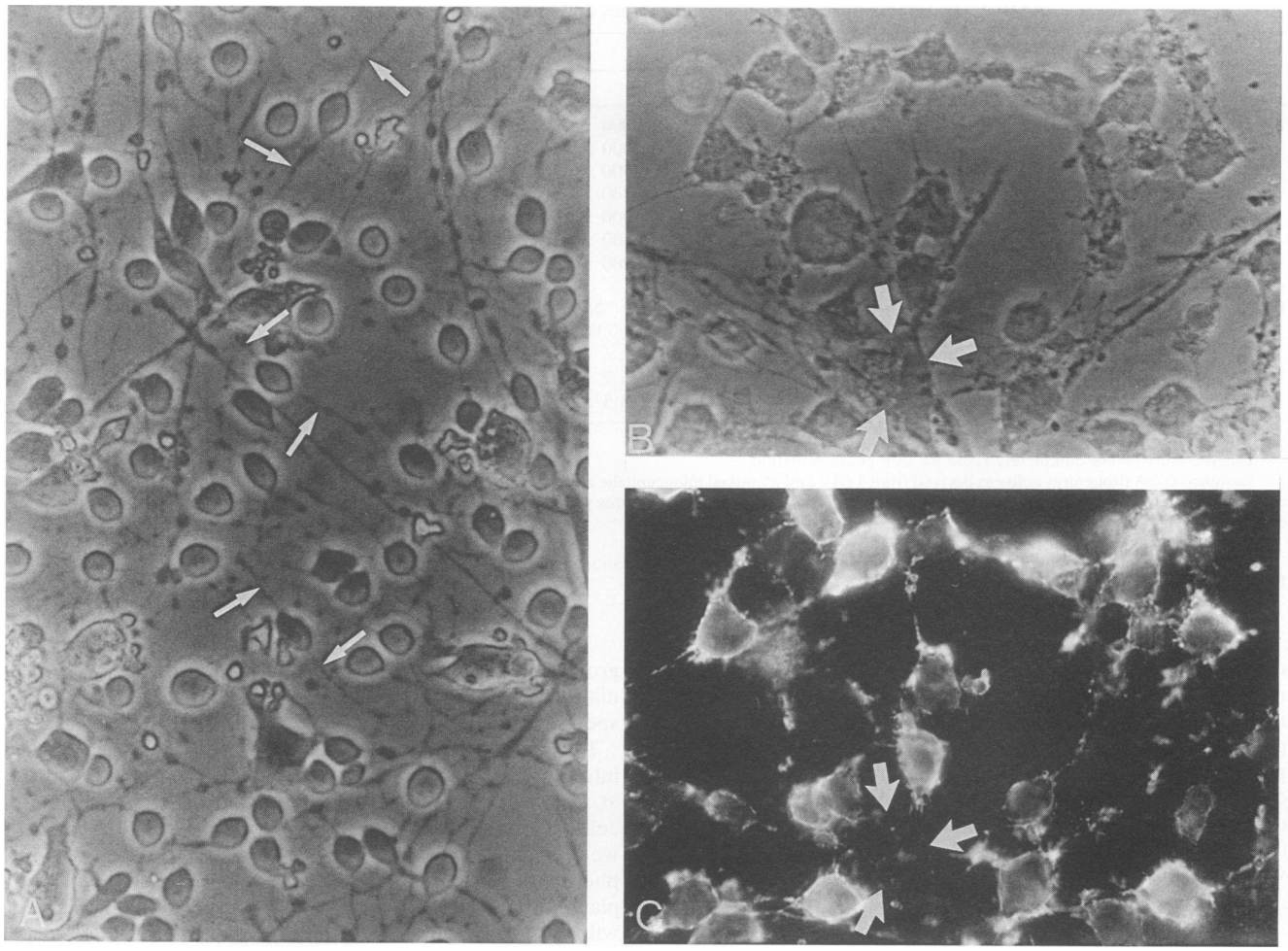

FIG. 9. Appearance of enriched O-2A progenitor cultures 24 h after plating (P6). (A) Phase-contrast image demonstrating cells with typical bipolar morphology (arrows) of O-2A progenitors. Magnification, ca. ×870. (B) Phase-contrast image of cells as in panel A at a higher magnification. Magnification, ca. ×1,200. (C) Same field as in panel B, demonstrating A2B5 surface immunoreactivity. Magnification, ca. ×1,200. Arrows in panels B and C point to an A2B5$^-$ cell.

removal of cytokines (P13), O-2A lineage cells were A2B5$^+$ O4$^+$ and weakly GC$^+$. By 7 days (P16), GC$^+$ and MBP$^+$ cells were present at 12.0 ± 4.1 and 15.4 ± 7.8 cells per ×500 microscopic field, respectively. By contrast, the continuous presence of bFGF and PDGF prevented the appearance of GC and MBP as previously reported (7, 37), but not of membrane sulfolipids recognized by MAb O4. Stellate GFAP$^+$ cells, some with A2B5 surface immunoreactivity, were also present in these cultures. This shift in phenotype was correlated with enhanced production of JHMV. This was most striking in cultures inoculated 4 days following the removal of cytokines (Table 3). These cultures produced 3,000-fold more virus than identically aged cultures continuously exposed to cytokines. Dual immunolabelling revealed that N antigen of JHMV was associated most frequently with GFAP$^+$ cells and less frequently with GC$^+$ cells (Fig. 10). Some of the JHMV N$^+$ cells were negative for both glial markers, as well as for the oligodendrocyte-specific MBP. Thus, repression of differentiation in O-2A progenitors was associated with a refractive state to JHMV infection, whereas progression toward more-differentiated states correlated with enhanced permissiveness.

Our present results when contrasted with those that demonstrated JHMV nonpermissiveness in oligodendrocytes in which differentiation was accelerated by cAMP analogs or by enhancement of adenyl cyclase metabolism (5, 6) suggest that among O-2A lineage cells, susceptibility to JHMV may be restricted to discrete developmental stages. These correlations may be of relevance to the age-dependent nature of the induction of demyelinating disease by JHMV observed in vivo with suckling rats (62, 63).

DISCUSSION

This study describes an experimentally accessible, in vitro system potentially capable of providing further insight into the mechanisms by which JHMV induces demyelinating disease in susceptible rats. This system was particularly useful in identifying initial target cells within the CNS, as well as subsequent virus-host interactions that may explain the temporal shift in disease character observed as a function of age at inoculation. Our earlier studies (5, 6) recognized oligodendrocyte differentiation as an important determinant controlling JHMV multiplication. Since differentiation likely

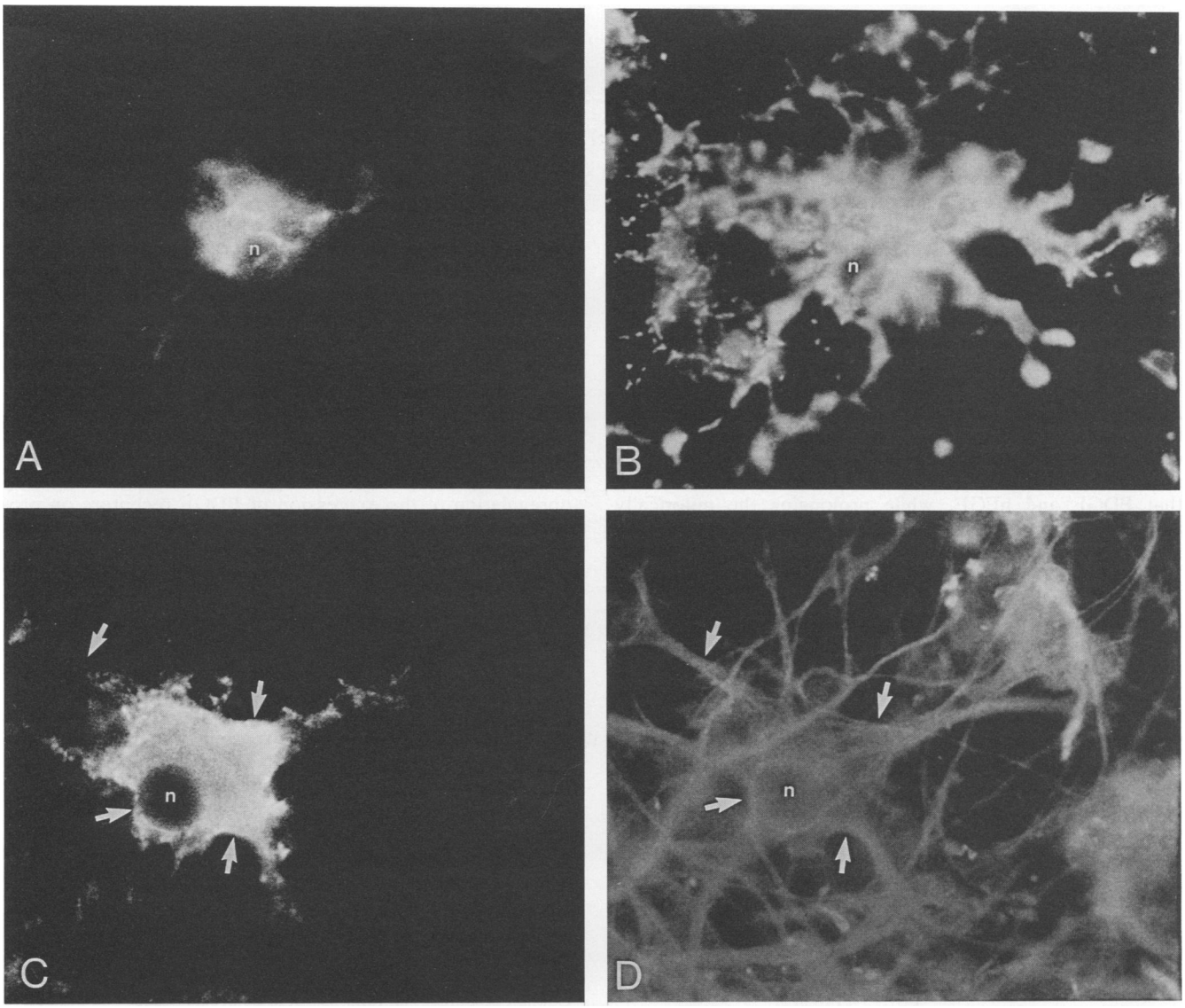

FIG. 10. JHMV-infected cells in enriched O-2A lineage cultures. These cells were isolated from mixed telencephalic cultures at 5 DIV and P5 as shown in Fig. 9. They were then grown in O1/T3 supplemented with bFGF plus PDGF until 9 DIV and P9, at which time they were switched to O1/T3 without cytokines and maintained for an additional 4 days before inoculation at P13 with JHMV. (A) N immunoreactivity in a cell 3 days postinoculation with JHMV. (B) The same cell as in panel A reacted with rabbit anti-GC followed by goat anti-rabbit Ig (TR conjugated). (C) N immunoreactive cell 3 days postinoculation with JHMV. (D) The same cell as in panel C demonstrating GFAP immunoreactivity by rabbit anti-GFAP followed by goat anti-rabbit Ig (TR conjugated). n are the nuclei in the same cells shown in panels A versus B and C versus D. Arrows in panels C and D point to a N^+ $GFAP^+$ cell. Magnification, ca. ×2,300.

involves an interplay between intrinsic cellular controls and extrinsic, epigenetic influences that induce or modulate a particular developmental program, the use of a defined culture medium aided in the systematic evaluation of developmental factors potentially involved in regulating viral permissiveness in O-2A lineage cells.

Unexpectedly, serum-free culture conditions which enhanced the survival of neurons in mixed telencephalic cultures were also associated with enhanced JHMV replication, illuminating the importance of neurons in the establishment of JHMV infections under organotypic situations. Immunocytochemical, electron microscopic, and immune-mediated cytolysis studies supported this contention. On the basis of cellular morphology and colocalization of viral N and neuron NSE antigens, it was evident that infection predominated in forebrain neurons within the first 24 to 48 h postinoculation. This was the case in cultures inoculated at P6, P10, or P17. The absence of NSE from infected neurons beyond 48 h may have been due to infection-induced leakage, increased turnover, and/or suppression of enzyme synthesis. The clear and precise association between JHMV nucleocapsid material and the microtubules within neurites, revealed by electron microscopy, corroborated immunocytological observations. Such an interaction, not previously reported to our knowledge, may have important implications for understanding how JHMV is disseminated and contributes to neuropathogenesis in the CNS.

The resistance to JHMV infection evident with mixed

telencephalic cultures grown continuously in $DMEM_{10}$ was unexpected, since these cultures contain an abundance of O-2A lineage cells shown previously to be permissive targets for this virus (5). Curiously, to establish infection in these cells, it was necessary to separate them from the others present in mixed glial cultures. Furthermore, in dissociated telencephalic cultures maintained in defined medium, less than 25% of all cells manifesting an infection during the initial 24 to 48 h were glia, suggesting that in vitro, a discrete albeit minor population of JHMV permissive O-2A lineage cells exists, among which virus susceptibility may somehow be connected with their state of differentiation. In this regard, MTCM and ACM which promoted mitogenesis and presumably blocked or delayed differentiation also caused a reduction of 10- to 100-fold in virus yields relative to those in unconditioned O1/T3 medium. These findings are consistent with a brief mention (67) of resistance to JHMV among $A2B5^+$ O-2A progenitors in primary dissociated cultures from Wistar rats. Our assumption that O-2A glia in mixed cultures are made nonpermissive by factors released into the medium by other cell types was supported by demonstrating that PDGF and bFGF, while prolonging the mitotically active, undifferentiated state, also conferred reversible resistance to JHMV.

Previous reports indicating that $GFAP^+$ cells in mixed brain cultures derived from perinatal Wistar Lewis rats were infected with JHMV (33, 34, 67) may appear to be in conflict with our original report (5), and present documentation of resistance by purified type-1 astrocytes. Observations from the present study can account for this apparent discrepancy if one recognizes that type-1 astrocytes and O-2A lineage cells, from which type-2 astrocytes are derived, arise from two distinct cell lineages within the CNS (48; for a review, see reference 46). Phenotypic plasticity, exemplified by the ability of cells of the O-2A lineage to coexpress oligodendrocytic and astrocytic markers has been documented in vivo and in vitro (22, 26, 27, 29, 68). Therefore, the viral antigen-positive $GFAP^+$ cells may either be those expressing a type-2 astrocytic phenotype at the time of infection or they may be O-2A bipotential cells susceptible to JHMV at an interval between the proliferative and terminally differentiated states. This may explain why both oligodendrocytes and cells with an astrocyte phenotype were observed manifesting JHMV infection in enriched O-2A cultures as well as in primary mixed telencephalic cultures employed here and by others (33, 34). Since the conditions we imposed on O-2A lineage cells were not conducive to acquisition of a stable type-2 astrocyte phenotype (30, 31), it is possible that the infected $GFAP^+$ cells were expressing GFAP only transiently or that the process of infection itself somehow induces GFAP expression in the manner of other in vitro environmental stimuli effecting the type-2 astrocyte phenotype (for a recent review, see reference 30).

Our previous demonstration that process-bearing O-2A lineage cultures become irreversibly nonpermissive for JHMV when pretreated with dbcAMP correlates with the accelerated terminal differentiation into oligodendrocytes (5, 6, 38, 51). This irreversible effect of dbcAMP should be contrasted with present data showing reversibility to an infectible state upon removal of PDGF and bFGF from the medium of progenitor-enriched cultures. Thus neither the mitotically active O-2A progenitor nor terminally differentiated oligodendrocyte stages are infectible, indicating that in this cell lineage, virus permissiveness occurs during a limited interim period.

Concerning JHMV-induced pathogenesis in the rat CNS, the following model is proposed. Present observations and previous in vivo studies (45, 60, 61) reveal that initial target cells are likely to be neurons, especially those in the hippocampus and cerebellum. Neurons may amplify the inoculum and possibly even act as a vehicle for dissemination to other target cells throughout the CNS. Demyelinating disease may ensue if O-2A lineage cells become infected at the susceptible stage of development, coinciding in vivo with the period when myelination is most active, during P10 to P21 (62). Beyond 3 weeks of postnatal life, completion of myelination in the rat coincides with the onset of resistance to the demyelinating form of disease, even when immunosuppressive therapy is used. Immunosuppression introduced at this stage accentuates an acute encephalitic disease due to fulminant neuronal infections (72). Within the first week of postnatal life, proliferating O-2A progenitor cells are migrating from subventricular zones to sites of future myelination (10). The O-2A progenitors migrate in response to PDGF (3, 43) produced by both type-1 astrocytes (49, 54) and neurons (56, 71). The synergistic effect of bFGF in extending the proliferative response due to PDGF is brought about by upregulation of the expression of PDGF receptors on progenitor cells (37). One may conclude from the present findings that the proliferative and migratory phase coincides with a state of resistance to JHMV. During the subsequent 2 weeks when mitogenesis of these migratory progenitors ceases, the differentiation phase related to myelination commences. During this period, bFGF mRNA levels (15) and p18 protein levels (53) have been reported to decrease in the rat brain, in concordance with accentuated oligodendrocyte differentiation and an age of perhaps the greatest susceptibility of O-2A lineage cells to productive infection with JHMV. These events in turn may have important consequences regarding the induction of demyelinating lesions by this agent.

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