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Characterization of a Glial Cell Line Persistently Infected with Borna Disease Virus (BDV): Influence of Neurotrophic Factors on BDV Protein and RNA Expression

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Borna disease virus (BDV) infects cells of the nervous system in a wide range of species. Previous work suggests that there are differences in BDV replication in neuronal cells and glial cells. Many neurons are lysed by the immunopathologic response to BDV; lysis of dentate gyrus neurons in the absence of encephalitis is seen in rats inoculated with BDV as neonates. In contrast, persistently BDV-infected astrocytes increase over the course of BDV infection. Therefore, we compared BDV replication in neuronal (SK-N-SH and SK-N-SHEP) and astrocytic (C6) cell lines. While SK-N-SH cells produced more infectious virions per cell, the C6 cells contained more BDV proteins and RNA. BDV sequences in the supernatants of both cell types were identified, despite low titers of infectious virus, suggesting the release of incomplete virions into the medium. C6 cells secreted a factor or factors into the medium that enhanced the production of BDV proteins and RNA in other cell lines. In addition, nerve growth factor treatment produced the same enhancement. Thus, BDV replication in certain neural cells in vitro may be linked to the production of cell-specific factors which affect viral replication.

Borna disease virus (BDV) replicates in the nervous system of most species inoculated experimentally, from birds to primates (5, 6, 28). BDV was determined to be a single-strand RNA virus only recently (18, 26, 42, 49, 50, 54). Further molecular characterization has demonstrated this agent to be a negative-strand RNA virus (5). Although some sequence homology exists with paramyxoviruses and rhabdoviruses, BDV remains unclassified (29).

After intracranial inoculation, BDV initially replicates in neurons of the limbic system, e.g., CA3/CA4 region of the hippocampus (6, 9). As disease progresses, BDV-infected neurons die, both from direct viral lysis and from immunopathological responses, while BDV-infected astrocytes appear to increase (8–10). Neural cell-specific variability in survival after viral infection has been demonstrated for other viruses, such as herpes simplex virus (HSV) (25, 45). In the latter case, however, astrocytes are lytically infected while neurons are relatively resistant to lysis in vitro.

Determination of the host cell characteristics responsible for susceptibility or resistance to BDV infection would provide valuable information regarding the mechanism of viral pathogenesis for BDV and other neuroviruses (1, 31). Susceptibility of primary neural cell cultures and permanent cell lines to BDV infection in vitro and relative efficiency of BDV production of viral antigens and infectious virus have been reported (14, 15, 20, 24, 30, 37). However, the results of many of the studies are difficult to compare because of differences in methods of virus quantitation. In addition, when these earlier studies were performed, cDNA clones specific for BDV were not available, and thus, BDV RNA production could not be determined.

Nonneural tissues and cells can also be infected with BDV, but only under special conditions. By using tissue culture assays or polymerase chain reaction (PCR), infectious virus and/or BDV-specific nucleic acids have been recovered from skin, bone marrow, spleen, liver, or salivary glands from rats inoculated either as neonates or as adults after receiving immunosuppressive doses of cyclosporin A and from adult rats with chronic Borna disease (greater than 2 months postinoculation) (23, 46). However, the interpretation of these results is difficult, since whole-organ homogenates may be contaminated by BDV in neural processes innervating the tissues.

Neural cell susceptibility to BDV infection might be due to the presence of BDV-specific receptors on the cell surface; however, there has not yet been a concrete demonstration of BDV-specific receptors on any cell. An alternate explanation for tissue-specific infection with BDV is that BDV-susceptible cells may produce factors that enhance BDV protein and RNA production in these cells. For example, a preferred site for BDV replication, the hippocampus, is rich in a variety of neurotrophic factors (33). In the rat, the hippocampus is the brain region richest in nerve growth factor (NGF) (36), suggesting a link between replication of BDV and the presence of this or other neurotrophic factors. NGF affects the replication cycle of other viruses and is associated with latent HSV infection; the absence or removal of NGF is associated with recrudescence of productive HSV replication (11, 57).

In order to identify cell-specific variations in BDV replication in vitro, we examined the susceptibility of several neural cell lines to BDV. We used a BDV permissive cell line, the rat astrocytoma C6, to identify some cell characteristics that influence the ability of BDV to replicate in this cell

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line (17). To this end, we compared the production of BDV proteins and RNA in C6 cells with that in a kidney cell line, MDCK, and in the neuronal cell lines SK-N-SH and SK-N-SHEP.

MATERIALS AND METHODS

Virus stock. BDV stock was prepared from homogenized BDV-infected rat brain tissue as described earlier (6) and had a viral titer of 10^6 50% tissue culture infectious doses ($TCID_{50}$) per ml when assayed on primary fetal rabbit glial cells (FRB).

Cell lines and conditioned medium. The cell lines used were the following. FRB is a primary fetal rabbit brain cell grown from embryonic day 19 brain tissue as described previously (6). SK-N-SH (American Type Culture Collection [ATCC]) and SK-N-SHEP (Joan Schwartz, National Institute of Neurological Disorders and Stroke, Bethesda, Md.) were derived from a human neuroblastoma tumor. Cloned from SK-N-SH, SK-N-SHEP has epithelioid qualities and is considered less neuronally differentiated (39). C6 cells (ATCC), derived from a rat glioma, have astrocyte-like characteristics (55). MDCK cells (ATCC) are dog kidney cells which replicate BDV; MBV cells are persistently BDV-infected MDCK cells (S. Herzog and R. Rott, Giessen, Germany). The PC-12 cell line (ATCC) is from a rat pheochromocytoma and differentiates into a neuron-like cell in the presence of NGF (22, 32).

C6-conditioned medium (C6-CM) was harvested 4 days after placing fresh medium (minimal essential medium [GIBCO, Gaithersburg, Md.] with 2% fetal calf serum [GIBCO]) on the C6 cells. The C6-CM was tested for NGF content on mouse dorsal root ganglia neurons by using a modification of a technique reported earlier (21). The sensitivity of the assay was $\geq$0.5 pg/ml.

BDV-susceptible cells were treated either with NGF (Bioproducts for Science, Indianapolis, Ind.) or with C6-CM to determine whether these substances would affect production of BDV proteins or RNA. Pretreatment of the cells with neurotrophic factors was performed to evaluate changes in the ability of viruses to bind to, enter, and infect the cell. Cell monolayers were pretreated either with 50 ng of NGF per ml in growth medium or with a 1:1 dilution of C6-CM in fresh medium every other day for 5 days prior to infection (D$-$5 to D$-$1). After removing all pretreatments, BDV (10^5 $TCID_{50}$) was added to the cell monolayers overnight (D0) and then replaced with growth medium. Since BDV binds to and enters cells within 4 h and BDV proteins appear within 24 h after infection (20), a postinfection treatment with the neurotrophic factors would effect viral replication in the already-infected cells. Some cell monolayers were post-treated with NGF or C6-CM every other day, as described above (D+1 to D+9). To some samples, polyclonal goat anti-NGF was added (E. M. Johnson, Jr., Washington University, St. Louis, Mo.).

On D+8 to D+11, the monolayers were resuspended and split into two aliquots. One aliquot was cytocentrifuged onto glass slides, fixed in cold acetone, and stained in an indirect immunocytochemistry assay (Vectastain ABC; Vector Laboratories, Burlingame, Calif.) for BDV antigens by using polyclonal rabbit anti-BDV, as described earlier (6). BDV antigens were visualized after addition of either AEC or diaminobenzidine (Vector Laboratories). The cells were counterstained with hematoxylin. The percentage of cells expressing BDV proteins was determined by counting the infected (brown) and uninfected (blue) cells in at least 10 high-power fields.

A duplicate aliquot of cells was tested for BDV RNA content by RNA enzyme immunoassay (RNA-EIA) as described below.

Infectious virus in supernatants and cell lysates. Fresh medium was placed on nearly confluent flasks of persistently infected cells: C6BV, MBV, SK-N-SH-BV, and SK-N-SHEP-BV. The cell supernatant was collected 48 h later. The infected cell monolayer was then suspended in 5 mM EDTA (GIBCO), and the cells were counted. The cells were placed on ice and disrupted by two 15-s ultrasonic bursts; lysis of the cells was confirmed by phase-contrast microscope examination. Both supernatant and cell samples were centrifuged (400 $\times$ *g* for 15 min) to remove cell debris, and the clarified samples were serially diluted in half-log increments and inoculated on a BDV-sensitive cell line, either FRB or C6. Indirect immunofluorescence assays detected BDV antigens by using polyclonal rabbit anti-BDV and a fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin (Vector Laboratories).

BDV protein detection. (i) Western immunoblot analysis. After a washing to remove medium, monolayers of C6, C6BV, MDCK, and MBV cells were lysed in 1% Nonidet P-40, 150 mM NaCl, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) on ice. We determined the protein concentration of each lysate by using the bichinchoninic acid system (Pierce, Rockford, Ill.) and adjusted each sample to equivalent total protein concentration. The lysate was mixed with an equal volume of sample buffer with 2-β-mercaptoethanol (Sigma) and boiled. The proteins were separated by electrophoresis in a 10 to 20% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel (Jule Inc., New Haven, Conn.) and transferred to nitrocellulose. The nonspecific antibody-binding sites were blocked with a solution of 10% horse serum (GIBCO) and 0.05% Tween 20 (Sigma) in phosphate-buffered saline (pH 7.4). Polyclonal mouse anti-BDV or polyclonal rabbit anti-BDV antibody was applied. After washing, peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin (Vector Laboratories) was added. Specific binding of anti-BDV antibodies was detected by TMB membrane peroxidase substrate solution (KPL, Gaithersburg, Md.).

(ii) Immunoprecipitation. Infected and uninfected cell monolayers were rinsed and incubated in methionine-free minimal essential medium (GIBCO) for 4 h at 37°C. One millicurie of [^{35}S]methionine (Amersham, Arlington Heights, Ill.) was added to the flasks and incubated overnight. The cells were rinsed and lysed as described above. Aliquots of cell lysates were normalized for radioactive signal and mixed with polyclonal rabbit anti-BDV. Normal rabbit serum served as a control. The antibody-antigen complexes were captured on staphylococcal protein A-coated Sepharose beads (Pharmacia, Piscataway, N.J.). After being washed and boiled, the immunoprecipitated proteins were separated by SDS–12% polyacrylamide gel electrophoresis (PAGE). The gel was dried and exposed to X-ray film overnight.

BDV RNA determination. (i) Northern (RNA) blot analysis. Total RNA from BDV-infected rat brain tissue and MBV, C6, and C6BV cells was extracted by using guanidinium isothiocyanate, size fractionated on denaturing gels, transferred to nylon membranes, and hybridized with a ^{32}P-labelled BDV cDNA pAF4. Clone pAF4 represents the open reading frame for the 24-kDa protein of BDV (26). The specific activity of the probe was 5×10^8 cpm/μg of DNA. Ten micrograms of total RNA was loaded in each lane.

(ii) **RT/PCR-EIA.** Samples were collected from supernatants and cells as described above, in such a manner as to avoid cross-contamination of samples. The RNA was extracted and reverse transcribed with random hexanucleotides and 200 U of mouse mammary leukemia virus reverse transcriptase (RT) (Boehringer Mannheim, Indianapolis, Ind.). The cDNA was amplified in a 100-μl reaction volume for 25 cycles, with 1.25 U of Amplitaq DNA polymerase (Cetus-Perkin Elmer, Emeryville, Calif.). The following primers were derived from the BDV cDNA clone pAB5: 5′-GATGACGATCCTATCCAACC-3′ and 5′-GTCACGGCG CGATATCTTTC-3′ (18, 26, 29). Clone pAB5 represents sequence from the 38/40-kDa BDV protein. The PCR product was 271 bp in length.

Probe was prepared by first amplifying pAB5 sequences in a PCR by using the following nested set of primers, also from pAB5: 5′-GTAATGAGCAACAATGGCTG-3′ and 5′-TTAA TACGACTCACTATAGGGGCCCAGCCTTGTGTTTCTA T-3′ (T7 promoter sequences underlined). The PCR products were used as a template in a transcription reaction by using T7 RNA polymerase and biotinylated UTP (Enzo Diagnostics Inc., New York, N.Y.). After DNase I treatment (Promega, Madison, Wis.), the sample was incubated at 37°C to digest the DNA template and the primers. The DNase was heat inactivated at 94°C. The labelled RNA was purified on a NAP-5 chromatography column with Sephadex G-25 (Pharmacia LKB, Piscataway, N.J.) by using a 0.5% SDS buffer. The probe was stored in aliquots at −70°C.

The EIA was performed as described previously (13). The PCR products were hybridized to the biotinylated RNA probe at 78°C for 60 min, and the hybridized material was captured on a plate coated with goat anti-biotin antibodies. After the plates were washed, the Fab′ fragment of a monoclonal antibody, coupled to β-galactosidase, and directed against DNA-RNA hybrids, was added. The reaction was developed by adding 4-methylumbelliferyl-D-galactoside (Sigma), and the amount of fluorescence generated after an hour was measured in a Dynatech Microfluor microtiter plate fluorometer.

RNA hybridization capture EIA (RNA-EIA). Cytoplasmic RNA was extracted by the Nonidet P-40 lysis procedure, as described earlier, designed to avoid denaturing agents that could destroy the function of antibodies used in later steps (12). RNA was hybridized in solution to a biotinylated, double-stranded cDNA probe synthesized from pAB5. The hybridized mixture was added to anti-biotin antibody-coated plates, and an EIA was performed to detect DNA-RNA hybrids. The relative change in BDV RNA was calculated on the basis of a standard curve performed for each assay as described earlier (12).

RESULTS

BDV infection of cell lines. The infection of neural and nonneural cell lines by direct inoculation with BDV was examined by using neuron-derived (SK-N-SH and SK-N-SHEP) and astrocyte-derived (C6) cell lines. All three cell lines were infected by direct application of BDV into the cell culture medium. BDV replicated in all cell lines noncytopathically, and BDV antigens were detected immunocytochemically both in the nuclei and in the cytoplasm (data not shown). Persistently infected cell lines (SK-N-SH-BV, SK-N-SHEP-BV, and C6BV) were developed by serial passage of the infected cells, resulting in cell lines in which >90% of cells were infected with BDV.

TABLE 1. BDV replication in neuronal and astrocytic cell lines

Cell type and cell line	Intracellular BDV/cell ($TCID_{50}$)[a]	Supernatant	
		BDV/ml ($TCID_{50}$)[a]	PCR (FU^b) per 100 μl
Neuronal			
SK-N-SHEP-BV	0.0004	120 ± 40	ND[c]
SK-N-SH-BV	0.05	60 ± 40	1,922 ± 6
SK-N-SH			8 ± 1
Astrocyte			
C6BV	0.0008	7 ± 13	1,548 ± 29
C6			7 ± 1
Kidney			
MDCK-BV (MBV)	0.02	360 ± 280	1,800 ± 22
MDCK			6 ± 1
Rat brain			
Infected	10^{6d}	NA[e]	2,445 ± 45
Uninfected			2 ± 1

[a] Reflects an average of two or three experiments.
[b] FU, fluorescent units in RT/PCR-EIA.
[c] ND, not determined.
[d] Per gram rather than per cell.
[e] NA, not applicable.

Release of infectious BDV into the supernatant of these persistently infected cell lines was then assessed (Table 1). Since BDV caused a nonlytic infection in these cell lines, cell-free infectious virus in the medium was released from intact cells. Most cell lines produced between 1 and 2 log units of virus per ml of cell supernatant. The C6BV cells released the least infectious virus into the medium. Using RT-PCR, we also examined the supernatants from infected cells for the presence of BDV-specific nucleic acid sequences (Table 1). The signal per milliliter of cell supernatant approximated that of a gram of BDV-infected rat brain.

Intracellular, infectious BDV was assessed on a per cell basis in lysates from all three cell lines (Table 1). The SK-N-SH-BV cell lysates contained 0.05 infectious virions per cell, while the C6BV and SK-N-SHEP-BV produced 100-fold less virus per infected cell.

Comparison of C6BV and MBV cell lines. In order to examine cell-specific differences in BDV replication, we tested a neural (C6BV) and a nonneural (MBV) cell line for BDV protein and RNA expression. Both cell lines produced infectious BDV, and >90% of the cells were infected. The MBV cell line, derived from MDCK cells, was developed by cocultivation with BDV-infected FRB cells (24), since MDCK cells appeared resistant to infection by BDV after direct inoculation with virus. In our experiments, 2 to 3% of MDCK cells became infected after direct inoculation with BDV (data not shown). In contrast, the C6BV cell line was developed in our laboratory by direct inoculation with BDV. Thus, a major difference between these cell lines was illustrated by ease of infection after direct inoculation with BDV.

We examined the expression of BDV proteins in the MBV and C6BV cells by Western blot analysis. Equal amounts of protein from lysates of confluent monolayers were loaded onto and separated by SDS-PAGE, blotted onto nitrocellulose paper, and stained with polyclonal mouse anti-BDV antibody (data not shown) or polyclonal anti-rabbit antibody (Fig. 1). By using either serum, both C6BV and MBV contained proteins consistent with the three major BDV proteins, p38/40, p24, and p14.5.

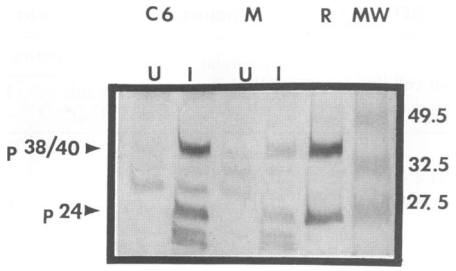

FIG. 1. Western blot demonstrating BDV proteins in infected C6 (C6) and MDCK (M) cells. Lane R, proteins from BDV-infected rat brain tissue for comparison; lane MW, molecular weight markers. Stained with polyclonal rabbit anti-BDV. U, uninfected; I, infected. Arrowheads illustrate BDV-specific proteins p38/40 and p24.

We compared BDV protein production in these two cell lines by in vitro labelling of proteins with [^{35}S]methionine, immunoprecipitation with polyclonal rabbit anti-BDV of aliquots of cell lysates that were normalized to the same radioactivity signal, and separation by SDS-PAGE. Our results documented that, over a 16-h period, the infected C6 cells (C6BV) contained more newly synthesized BDV-specific proteins than infected MDCK cells (MBV) (Fig. 2). Laser densitometry analysis of the bands revealed a 9.7-fold increase in p24 and a 4.9-fold increase in p38/40 in BDV protein signal in C6BV versus MBV.

The relative content of BDV RNA in the two cell lines was examined next. By using Northern blot analysis, 10 μg of total RNA from C6BV cells and MBV cells was compared after hybridization with a BDV-specific probe (Fig. 3). All four BDV RNA transcripts were seen in C6BV cells (8.5-kb genome and 3.5-, 2.1-, and 0.8-kb mRNA transcripts). There was increased hybridization of probe to the 3.5-kb RNA transcript in the C6BV cells compared with that in the infected rat brain. At the short exposure needed to delineate BDV RNA in C6BV cells, only the 0.8-kb transcript was seen in MBV cells. All four RNA species were demonstrated in the MBV cells with a longer exposure (data not shown).

Effects of C6-CM and NGF on BDV protein and RNA. After screening several different cell lines for susceptibility to BDV, we identified an astrocytoma cell line, C6, which expressed high levels of BDV proteins and RNA. Since C6 cells are known to secrete several neurotrophic factors into

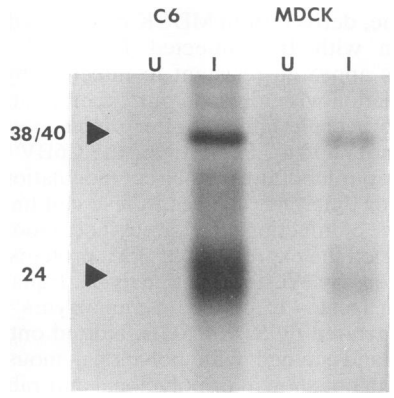

FIG. 2. Quantitative immunoprecipitation of radiolabelled BDV proteins from infected C6 and MDCK cells. Immunoprecipitated with rabbit anti-BDV. U, uninfected; I, infected.

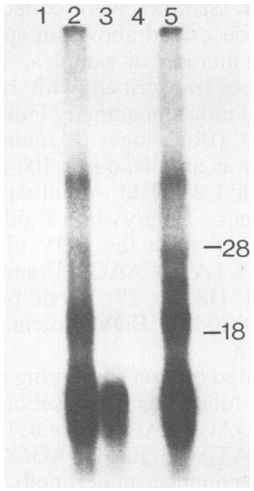

FIG. 3. Northern blot demonstrating BDV RNA in C6BV and MBV cells. Radiolabelled probe synthesized from BDV-specific cDNA clone pAF4. Lane 1, normal rat brain; lane 2, BDV-infected rat brain with 8.5-, 2.1-, and 0.8-kb BDV RNA transcripts; lane 3, MBV cells with 0.8-kb BDV RNA transcript; lane 4, uninfected C6 cells; lane 5, C6BV cells with the 8.5-, 3.5-, 2.1-, and 0.8-kb BDV RNA transcripts.

the tissue culture medium (34), we postulated that one or more of the factors produced by C6 cells might affect BDV replication and result in the enhanced production of virion components in these cells. We also tested a well-characterized neurotrophic factor, NGF, for effects on BDV replication, as NGF has been shown to alter the replication of other neuroviruses, such as HSV (11, 57).

C6-CM or NGF was applied to BDV-susceptible cell lines SK-N-SHEP and PC-12. PC-12 cells differentiate into neuron-like cells after exposure to NGF and C6-CM, while the SK-N-SHEP cells are less sensitive to NGF (2). The cells were treated with NGF or C6-CM either before or after infection with BDV in order to determine at what stage of viral infection the neurotropic factor exerted its effect. We quantitated changes in the percentage of cells expressing BDV proteins as well as changes in amount of BDV RNA per cell.

Postinfection treatment of SK-N-SHEP and PC-12 cells with either C6-CM or NGF resulted in a two- to fivefold increase in percentage of cells expressing BDV proteins (Fig. 4a and b). Both preinfection and postinfection treatment of PC-12 cells with NGF produced a similar increase in BDV protein-expressing cells, but pretreatment of SK-N-SHEP cells had no effect.

Anti-NGF blocked the effects of the NGF treatment on BDV protein expression in both cell lines. NGF was undetectable in C6-CM by bioassay (≤0.5 pg of NGF per ml [data not shown]). Anti-NGF blocked the effects of C6-CM on PC-12, but not SK-N-SHEP, cells. Immunoprecipitation of radiolabelled BDV proteins by polyclonal rabbit anti-BDV from NGF and NGF plus anti-NGF-treated SK-N-SHEP cells further substantiated that the enhancement of BDV protein expression was attributable to NGF (Fig. 5). Laser densitometry analysis of the p38 protein revealed that NGF treatment increased the BDV protein signal by 1.9-fold; NGF plus anti-NGF treatment resulted in a BDV protein signal that was 0.8-fold that of BDV-infected control.

BDV mRNA expression in SK-N-SHEP cells was also

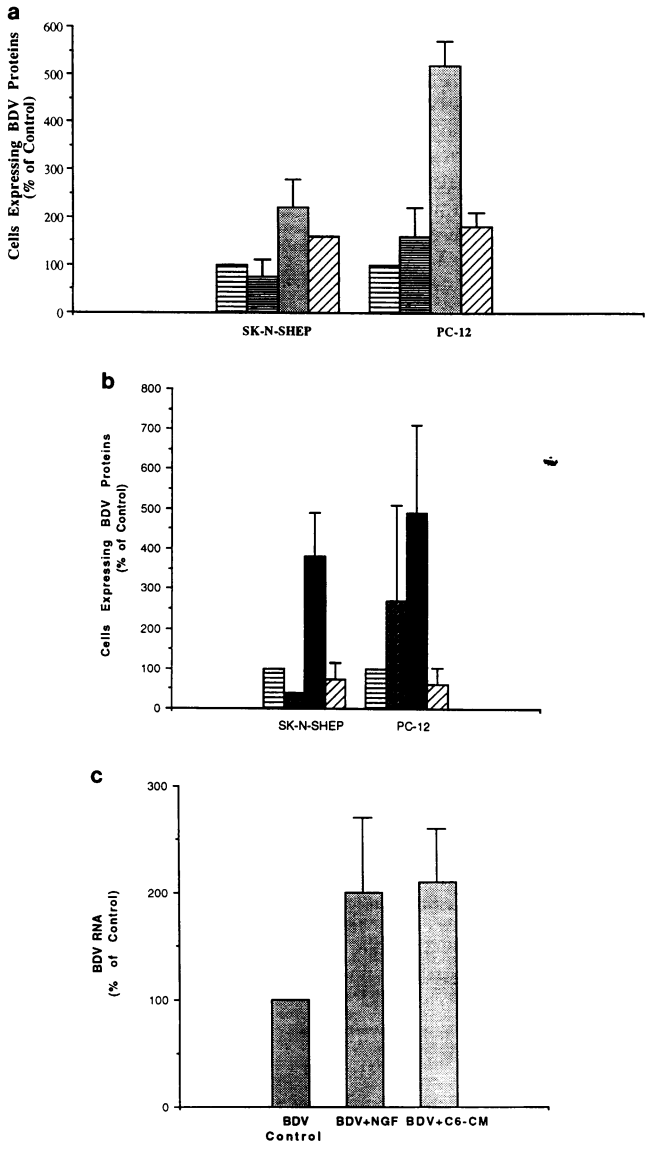

FIG. 4. (a and b) Neurotrophic factor treatment effects on BDV protein expression. By using immunocytochemical stains, the percentage of cells expressing BDV proteins was determined with and without C6-CM (a) or NGF (b) treatment of SK-N-SHEP and PC-12 cells. For each group of cells, the first column is infected without treatment, the second column is infected after pretreatment, the third column is infected before posttreatment, and the fourth column is infected before posttreatment with factor plus anti-NGF. (c) NGF or C6-CM effects on BDV mRNA in SK-N-SHEP cells. By using the RNA-EIA assay, the BDV mRNA in the cytoplasm was measured with and without neurotrophic factor treatment, after infection with BDV.

increased by C6-CM or NGF treatment after infection (Fig. 4c).

DISCUSSION

Cell-specific contributions to the tropism of viruses as well as the nature of virions produced have been described. For example, neuroblastoma cells have been shown to modify measles virus RNA during replication (38), and primary

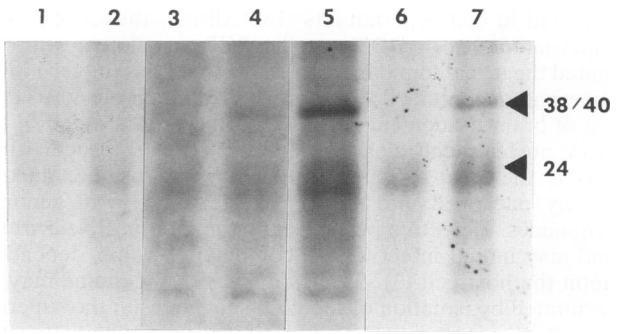

FIG. 5. Quantitative immunoprecipitation of radiolabelled BDV proteins from BDV-infected SK-N-SHEP cells with and without NGF posttreatment. All lanes were stained with polyclonal rabbit anti-BDV. Lane 1, normal cells, normal rabbit serum; lane 2, BDV-infected cells, normal rabbit serum; lane 3, BDV-infected cells, normal rabbit serum; lane 4, BDV-infected cells, rabbit anti-BDV; lane 5, BDV-infected cells treated with NGF, rabbit anti-BDV; lane 6, BDV-infected cells treated with NGF plus anti-NGF, normal serum; lane 7, BDV-infected cells treated with NGF plus anti-NGF, rabbit anti-BDV. Arrowheads illustrate BDV-specific proteins p38/40 (top) and p24 (bottom).

astroglial cell cultures have been shown to have defective replication cycles of measles virus (43). In vitro models of BDV replication in various cell lines and under the influence of defined and undefined growth factors permit evaluation of alterations in in vitro viral replication that then can be addressed in vivo. While findings in the in vitro model may or may not correlate with the in vivo setting, at the very least these in vitro findings can suggest future experimental directions for the in vivo model.

In vitro, BDV has a different pattern of replication in various neural cells. Both neuronal and astrocytic tissue culture cells were easily infected with BDV, as determined by BDV protein and RNA expression and infectious virus production. Of the neural cell lines tested, cells that were more neuronally differentiated (e.g., SK-N-SH) produced more infectious virus than the less neuronally differentiated cells (SK-N-SHEP).

Astrocytic cells were easily infected by direct inoculation of BDV and produced BDV proteins and RNA in abundance, yet these cells contained little infectious virus. The number of infectious virions produced per C6BV cell was similar to that reported previously (37). In chronic disease in vivo, astrocytes are the predominant BDV-infected neural cell; persistence of BDV in astrocytes in vivo could be associated with production of few complete virions and result in an accumulation of incompletely assembled viral components. For example, in chronic disease infectious BDV per gram of brain is 1 or 2 log units less than that found during the acute stage of infection, when BDV-infected neurons predominate (6, 8, 10).

Our neural cell lines produced no more infectious virus per cell than MBV cells, a kidney cell line. Kidney cells could be BDV-permissive cell in vivo as well, since BDV has been detected in the urine of rats infected as newborns (23, 44). Indeed, there have been recent demonstrations of NGF receptors on the developing kidney, which suggests that kidney cells may share similarities with cells of the nervous system (41).

Supernatants from BDV-infected cell lines have been reported to contain no infectious virus (14); in our experiments, however, low but reproducible levels of virus were

detected in cell supernatants. In addition, the infected-cell supernatant had a BDV-specific PCR signal that approximated the signal found in infected rat brain tissue, yet 1 ml of supernatant contained 4 log units less of infectious virus than 1 g of brain tissue. These findings suggest that much of the BDV nucleic acid released by the cell into the supernatant was in the form of replication-deficient virus, i.e., incompletely packaged into virions or lacking critical genomic sequences. Defective particles are common in RNA viruses and may inhibit infectivity of competent virions, depending upon the host cell (3). Studies of the BDV genome may be facilitated by isolation of the viral sequences in the supernatant fluid, since much of the difficulty in elucidating the genome of BDV stems from the inability to purify large amounts of cell-free virus.

Even when cells are >90% infected with BDV, either by cocultivation and single-cell cloning (MBV) or by direct inoculation (C6BV), there can be differences in the relative efficiency of production of BDV proteins and RNA. MBV cells replicated infectious virus but contained much less of the viral components than C6BV cells. Whether the increased expression of BDV components in the C6 cells represented increased production or decreased catabolism is not known. The C6BV cells produced relatively little infectious virus per cell, and the accumulation of BDV components in these cells may have resulted from defective maturation and decreased release of BDV virions. A similar phenomenon is seen with measles virus in subacute sclerosing panencephalitis (27, 35). Alternatively, this finding could be due to an accumulation of transcripts related to persistent infection, somewhat similar to the latency-associated transcripts seen in latent infections with HSV (19). As yet, there are no assigned functions to the BDV proteins and transcripts, although a sequence similarity between p38/40 and other viral polymerases has been found (29). Other investigators have found that HSV also causes a nonlytic, latent infection in C6 cells (16, 40).

NGF and C6-CM enhanced the levels of BDV mRNA and protein. It is possible that both NGF and C6-CM stimulated cell growth and production of cellular proteins and thus indirectly increased the expression of BDV components. NGF is inhibitory to the growth of adrenal cells (52, 53) but stimulates the production of many proteins. Even if NGF and C6-CM do not work directly on the viral replication cycle in vitro, these neurotrophic factors may exert an indirect effect on viral replication via a cellular RNA or protein synthetic effect. Although the data presented here were derived from in vitro experiments, it is of interest that BDV replication in vivo occurs preferentially in the hippocampus, the region of the rat brain with the highest NGF production.

RT-PCR detection of BDV sequences identifies even low-level BDV replication in cells. Before the advent of BDV cDNA clones, cells that were infected with BDV but produced few or no BDV proteins would not have been detected by immunohistochemical methods. Our findings of an increase in percentage of cells expressing BDV proteins after neurotrophic factor treatment suggested that cells can be infected with BDV yet not express BDV proteins in easily detected amounts. Alternatively, the neurotrophic factor treatments could have enhanced the spread of infection through virus released into the supernatant, although BDV is thought not to spread readily by this method in vitro (14, 15).

The use of anti-NGF was effective in blocking the effects of NGF on BDV replication but yielded conflicting results with C6-CM. Thus, it is unclear whether the C6-CM acted via NGF or another factor. It is possible, but unlikely, that the amount of NGF measured in the C6-CM could affect BDV replication, since even a 10-fold concentration of C6-CM did not produce physiologically active NGF in a bioassay. The active factor in C6-CM may have a structural resemblance to NGF, as is seen in the superfamily of known neurotropic factors: NGF, brain tissue-derived neurotrophic factor, and neurotropin-3 (51). Largely on the basis of bioassays of primary cell lines, other investigators have characterized several different neurotrophic factors in C6 cell supernatant (1, 34, 56).

SK-N-SHEP cells might have been expected to be resistant to the effects of NGF, since they are believed not to have NGF receptors (2, 39). It is possible, however, that BDV infection upregulated NGF receptors in these cells. This hypothesis is consistent with the finding that NGF exerted its effects on these cells only when given after BDV infection. The in vitro association between NGF, NGF receptors, and BDV infection is supported by the in vivo findings that NGF receptor expression on Schwann cells increases after axotomy (47, 48) and that BDV infection of Schwann cells is also increased after axonal damage (6). Damage to the central nervous system has also been shown to increase the NGF content of the hippocampus (53).

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