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Differences in Cell-to-Cell Spread of Pathogenic and Apathogenic Rabies Virus In Vivo and In Vitro

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Pathogenic parental rabies virus and apathogenic variant virus were shown to differ in their ability to infect neurons in vivo and neuroblastoma cells in vitro. After intracerebral inoculation, the distribution of infected neurons in the brain was similar for both viruses, but the rate of spread throughout the brain, the number of infected neurons, and the degree of cellular necrosis were much lower in the case of apathogenic virus. After adsorption to mouse neuroblastoma cells, apathogenic virus was less rapidly internalized than pathogenic virus, and cell-to-cell spread of apathogenic variant virus was completely prevented by the addition of rabies virus-neutralizing antibody, whereas the spread of pathogenic virus was not affected.

The replication of rabies virus in vivo is almost entirely restricted to nerve tissue, and neurotropism is the main feature of rabies infection. After intramuscular inoculation, rabies virus replicates first in the nearby striated muscle cells, then enters the sensory nerve on unmyelinated neural endings in motor nerve endplates or neuromuscular or neurotendinal spindles, and spreads along peripheral nerves up to the spinal cord into the brain (10). Unlike many other virus infections, in which neutralizing antibody limits virus spread, rabies virus infection spreads from cell to cell in tissue culture despite a continuous overlay of antirabies serum (16), and it has been postulated that this escape from neutralization may reflect the mechanisms of spread during natural infection (16). The basic mechanism(s) involved in the pathogenicity of fixed rabies viruses and factors that determine manifestation of the disease after infection are not well understood but most likely involve multiple factors, including the immunological competence of the host.

Recent investigations with apathogenic rabies variant viruses indicate that the glycoprotein of rabies viruses is a major determinant of virus pathogenicity (3, 4). The antigenic variant (RV194-2) selected for its resistance to neutralizing monoclonal antibody 194-2 exhibited a modified pathogenicity in adult mice (4), a change which corresponds to a single amino acid substitution of the glycoprotein molecule at amino acid position 333 (Arg → Ile or Gln) (4). This amino acid substitution at position 333 was also identified in the apathogenic fixed rabies virus strains Flury HEP and Kelev (W. H. Wunner and C. L. Smith, unpublished data), which also resisted neutralization by monoclonal antibody 194-2. Thus, the amino acid substitution at position 333 appears to be a molecular marker of the apathogenic phenotype. The major biological difference in the in vivo behavior of pathogenic and apathogenic rabies viruses is the extent of virus spread in the central nervous system, the apathogenic viruses being significantly less neuroinvasive.

In the present study, we compared the pathogenic parental rabies virus and the biochemically and biologically well characterized apathogenic RV194-2 variant virus for their ability to infect neuronal cells in vivo and to spread from cell to cell in a tissue culture system that accurately reflects the in vivo behavior of pathogenic and apathogenic fixed rabies virus.

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MATERIALS AND METHODS

Cells. Baby hamster kidney cell line BHK-21 (14) and C 1300 clone NA mouse neuroblastoma (NA) cells (9) were propagated in Eagle minimal essential medium supplemented with 10% fetal calf serum.

Viruses. Clone-purified challenge virus standard CVS-11 (6) and ERA (1) parental stocks were produced in BHK-21 cell monolayers infected at a multiplicity of infection (MOI) of 0.1 PFU per cell and incubated at 33°C for 4 days in the presence of Eagle minimal essential medium supplemented with 0.2% bovine serum albumin.

Variant strains expressing an antigenically altered glycoprotein were selected from CVS-11 and ERA virus stocks after treatment with monoclonal antibodies and cloning of neutralization-resistant mutants as described previously (18). The neuropathogenicity of variants for adult mice was evaluated based on survival after intracerebral inoculation of 5- to 6-week-old female ICR mice. The nonpathogenic variants of CVS-11 and ERA viruses were selected with monoclonal antibody 194-2 (4).

Virus infectivity titration. Monolayers of hamster CER cells were infected with 0.1 ml of virus at serial fivefold dilutions and incubated for 60 min to allow virus adsorption, overlaid with nutrient agarose medium (17), and incubated at 35°C for 4 days. Plaques were counted after removal of agarose and staining with crystal violet solution.

Radioimmunoassay. Serial twofold dilutions of inactivated virus (50 μg per well) in sodium carbonate-bicarbonate buffer (pH 9.5) were air dried on soft plastic 96-well plates (Dynatech Laboratories, Inc.) and incubated for 1 h with phosphate-buffered saline (PBS) containing 10% agamma horse serum. Monoclonal antibody 509-6, specific for rabies virus glycoprotein, and antibody 502-2, specific for rabies nucleocapsid antigen, were added as ascites (25 μl) at a 1:1000 dilution to a series of wells to which antigen was adsorbed. Plates were incubated for 1 h at 37°C and washed three times with PBS. ^{125}I-labeled goat anti-mouse immunoglobulin (25 μl; specific activity, 0.5 μCi/μg) containing 30,000 cpm was added to each well. Plates were incubated at 37°C for 1 h and washed three times with PBS. The bottoms

of the wells were then cut with an incandescent wire, and radioactivity in the wells was measured in a gamma counter. The highest dilution of antigen giving 10% of the maximum counts per minute over the background was considered the endpoint of titration.

Immunohistopathology. Six-week-old ICR female mice were injected in the frontal lobe of the cerebrum with 25 μl of CVS-11 or CVS RV194-2 virus (10^4 PFU) with a 27-gauge needle. At the indicated times, four animals were anesthetized and perfused with periodate-lysine-paraformaldehyde fixative (8). Brains were removed, kept in the same fixative for 24 h, paraffin embedded, and sectioned by standard procedures. A peroxidase-anti-peroxidase technique (15) was used to demonstrate rabies virus antigen. Briefly, deparaffinized brain tissue sections were treated for 24 h with rabbit anti-rabies nucleocapsid serum diluted 1:200. This antibody detected CVS-11 and CVS RV194-2 antigens with similar sensitivity. Sections were then incubated for 30 min with goat anti-rabbit immunoglobulin G (Cappel Laboratories) diluted 1:100 and for 30 min with peroxidase-anti-peroxidase complex diluted 1:600 (Cappel Laboratories). Slides were then washed with 0.5 M Tris hydrochloride (pH 7.6) and stained with diaminobenzidine tetrachloride (type II; Sigma Chemical Co.) as described previously (15) and counterstained with Mayer's hematoxylin.

Immunofluorescent antibody staining. Viral antigen in infected cells was detected by the direct immunofluorescent antibody staining technique. Cells were washed with PBS, fixed for 30 min in cold acetone, and air dried. Cells were then incubated with fluorescein isothiocyanate-labeled rabbit polyclonal antinucleocapsid antibody for 30 min, washed in PBS and distilled water, and examined under UV illumination. The anti-rabies nucleocapsid antibody used was equally efficient in detecting CVS-11 and CVS RV194-2 viruses. The percentage of infected cells was determined by counting at least 1,000 cells.

Isolation of virus from infected mouse brain. At 2, 3, and 4 days postinfection (p.i.), three animals infected with RV194-2 virus were sacrificed, and the brains were removed and homogenized. The virus was then isolated on BHK-21 cell cultures as previously described (19). The antigen makeup of this virus has been determined (4).

RESULTS

Immunohistochemical and histological studies. Pathogenic parent CVS virus and apathogenic variant virus were examined for their ability to infect neurons in vivo. Brain tissue from mice infected intracerebrally with pathogenic or apathogenic virus was examined histologically at 3, 5, and 7 days p.i. The immunoperoxidase staining technique was used to demonstrate the presence of rabies antigens at the cellular level (Fig. 1). Frozen sections of infected mouse brains stained by direct immunofluorescence with anti-rabies nucleocapsid serum or anti-rabies glycoprotein serum also gave similar results (data not shown).

At 3 days p.i. with apathogenic virus, viral antigen was identified in neurons caudal to the injection site in the neocortex and the hippocampus of both hemispheres. However, in the cerebellum, which does not project directly to the neocortex, there was no evidence of immunoreactive virus (Fig. 1A). In contrast, immunoperoxidase-stained cerebellum sections of mouse brain infected with pathogenic virus revealed strong evidence of viral antigen in the white matter (Fig. 1D, see area marked by star).

At 5 days p.i. in the apathogenic virus-infected tissue, viral antigen was detected in the cerebellum, mostly confined to axons in the white matter (Fig. 1B), although an occasional neuron in the cerebellar cortex also contained viral antigen. The intensity of staining and the number of neurons with immunoreactive viral protein in the neocortex and the hippocampus also increased compared with those found after 3 days (data not shown). Similarly, affected neurons were also recognized in the thalamus and the hypothalamus. At this time, in the pathogenic virus-infected cerebellum, infection already progressed to a larger number of Purkinje cells (Fig. 1E). Also, in contrast to the pathogenic virus infection, a definite leptomeningitis was observed in mouse brains with apathogenic virus (data not shown). The infiltrate was composed of mononuclear cells, and similar infiltrates were observed around blood vessels as they entered the brain parenchyma. This could not be demonstrated throughout the infection with pathogenic virus.

By 7 days p.i. in the apathogenic virus-infected mouse brain, immunoreactive viral antigen was detected throughout the brain, including the brain stem. Cerebellar Purkinje neurons containing immunoreactive viral antigen were readily identified but were not numerous at this time (fewer than 20 per section) (Fig. 1C). Most neurons, however, including those revealing viral antigen, appeared healthy. In the pathogenic virus infection, neurons were much more intensely stained, and many appeared to be undergoing necrosis. In addition, the neurophil became vesicular or rarified, consistent with intracerebral edema and suggesting that mortality resulted from brain swelling.

These results of comparative studies indicate that both apathogenic and pathogenic viruses spread within the brain in similar pathways; however, the spread of apathogenic virus was slower, as evidenced by the amount of viral antigen detected at different times examined. This difference in the rate of spread of pathogenic versus apathogenic virus was a consistent observation in all four mice examined at each of the different time points. Virus isolated 3 and 4 days p.i. from brains of apathogenic CVS RV194-2 virus-infected mice retained the antigenic phenotype of RV194-2 virus, i.e., resistance to neutralization by monoclonal antibody 194-2. No virus could be recovered from CVS RV194-2 virus-infected mouse brains 2 days p.i. Therefore, the limited spread is not due to a phenotypic reversion that has been observed after virus passage in newborn mouse brain (4).

In vitro studies with pathogenic and apathogenic rabies viruses. The cell-to-cell spread of virus was studied with either NA cells (C 1300 clone NA) (Fig. 2B) or BHK-21 cells (Fig. 2A). Monolayers of NA or BHK-21 cells were infected at an MOI of 0.1 PFU per cell with either pathogenic or apathogenic variant virus. At 1 h p.i., antirabies serum, at a final dilution of 1:100 and corresponding to a neutralization index of 7.6, was added to the infected cells, and at 24 h p.i., less than 10% of BHK and NA cells infected with pathogenic and apathogenic virus, respectively, contained rabies-specific antigen, as demonstrated by fluorescent antibody staining. In BHK-21 cells infected with either pathogenic or apathogenic variant CVS virus, the infection spread in the presence of neutralizing antibody to uninfected neighboring cells until 100% of the cells were infected at 96 h p.i. (Fig. 2A). In NA cells infected with pathogenic CVS virus (Fig. 2B), the kinetics of virus spread in the presence of neutralizing antibody was similar to that in BHK-21 cells. However, in NA cells infected with the apathogenic variant virus derived from CVS (Fig. 2B) or ERA virus (data not shown), no spread of infection was observed in the presence of virus-neutralizing antibody.

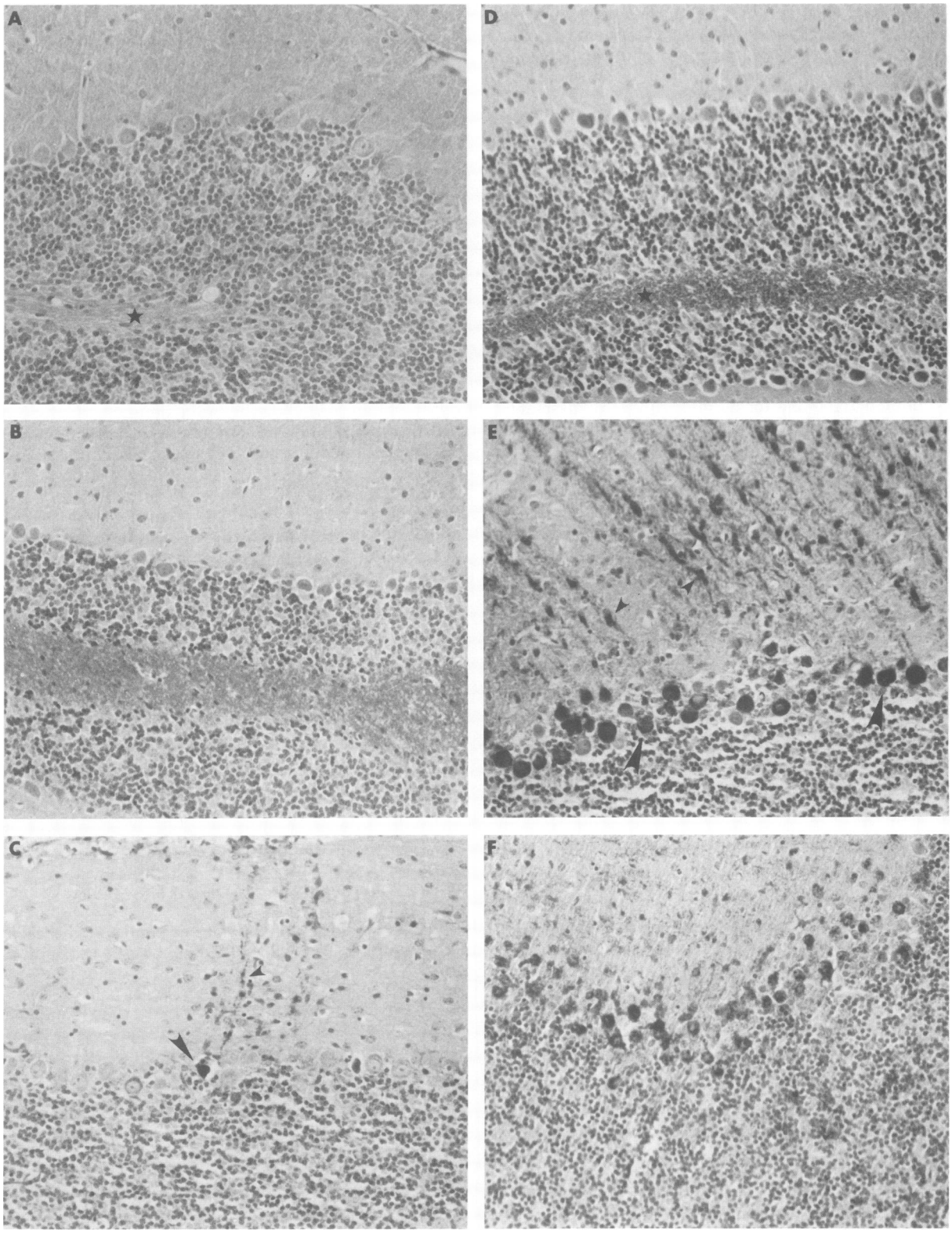

FIG. 1. Photomicrographs of apathogenic CVS194-2 (A through C) and pathogenic CVS-11 (D through F) virus-infected mouse cerebellum sections. The mice were sacrificed on day 3 (A and D), 5 (B and E), or 7 (C and F) after a neocortical (frontal lobe) injection of either virus. Viral nucleocapsid antigen was detected as described in Materials and Methods. The immunostained patterns of the cerebellum exemplify the differences in the behavior of these viruses. At 3 days, no evidence of spread of the apathogenic virus to the cerebellum was seen (see star), while immunoreactive pathogenic virus was present mainly in the white matter (D). At 5 and 7 days, Purkinje neurons (large arrows indicate perikarya; small arrows indicate dendrites) were the predominant class of cerebellar neurons in which immunoreactive pathogenic (E and F) or apathogenic (B and C) virus was found. The difference between the two viruses in the cerebellum and elsewhere was primarily quantitative; many more Purkinje cells contained immunoreactive pathogenic virus at early survival times compared with the apathogenic virus. All sections were lightly counterstained with hematoxylin. Magnification, ×184.

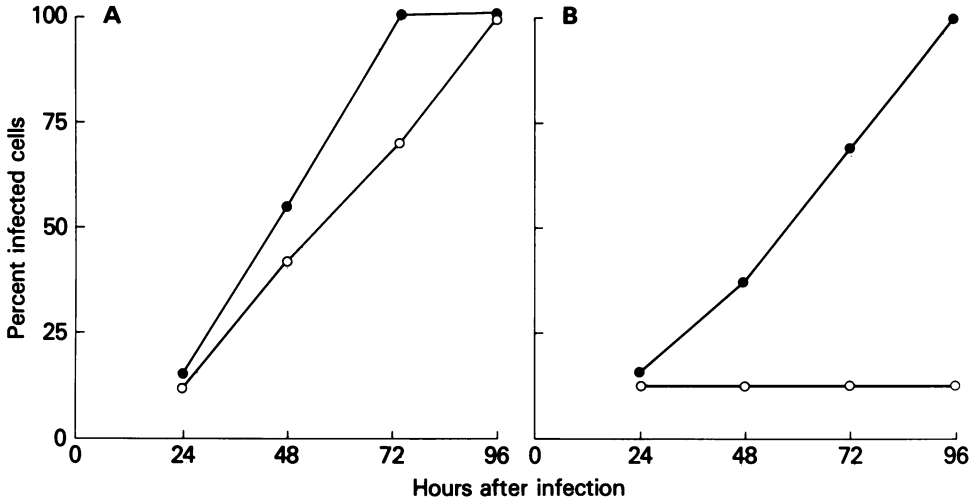

FIG. 2. Cell-to-cell spread of pathogenic CVS-11 virus and apathogenic CVS RV194-2 variant virus in BHK cells (A) and mouse NA cells (B). Monolayers of BHK cells or mouse NA cells were infected at an MOI of 0.1 PFU per cell with either pathogenic CVS-11 virus (●) or apathogenic CVS RV194-2 virus (○). After the addition of antirabies serum at 1 h p.i., infected cells were examined at 24, 48, 72, and 56 h p.i. for the presence of rabies antigen by fluorescent antibody staining technique.

We then tested the susceptibility of BHK-21 and NA cells to infection by pathogenic and apathogenic variant virus. The titers for both viruses, as determined by plaque assay in CER monolayers, were identical ($10^{7.6}$ PFU/ml). Cells were infected with twofold dilutions of virus, and at 24 h p.i. the percentage of infected cells was determined by the fluorescent antibody technique. At each dilution of pathogenic and apathogenic virus, a similar number of BHK cells was infected (Fig. 3A). However, eightfold more apathogenic variant virus than pathogenic virus was needed to infect a comparable number of NA cells (Fig. 3B).

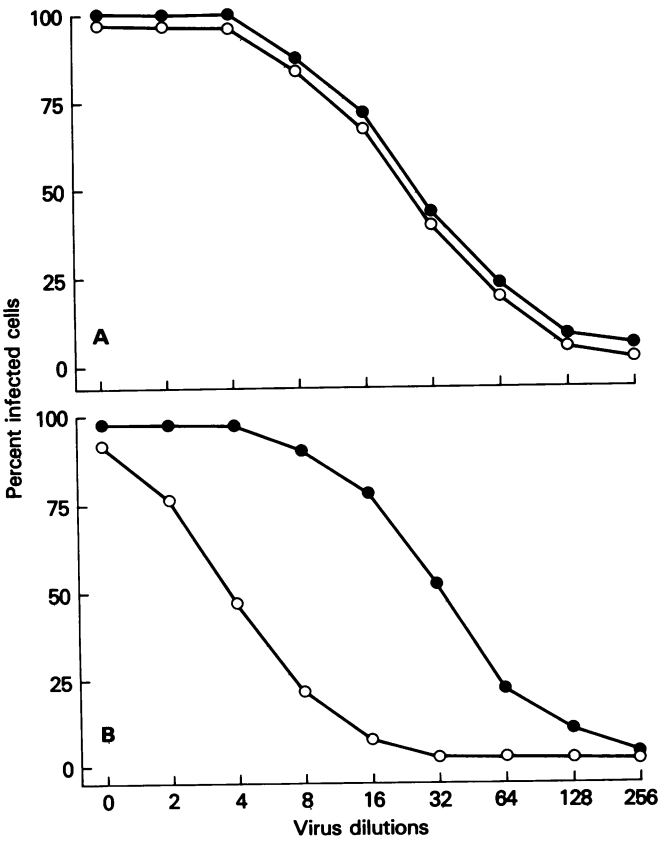

FIG. 3. Susceptibility of BHK-21 (A) and NA (B) cells to infection by pathogenic CVS-11 or apathogenic CVS RV194-2 virus. Cells were infected with twofold dilutions of pathogenic (●) or apathogenic (○) virus. The titer for both undiluted viruses was $10^{7.6}$ PFU/ml. At 24 h p.i., the percentage of infected cells was determined by the fluorescent antibody technique.

Figures 4 and 5 illustrate the kinetics of infectious virus production and the kinetics of viral protein synthesis, respectively, in NA cells. Cells were infected at an MOI of 0.1, PFU per cell, and at 1 h p.i., the cells were treated with antirabies serum for 1 h to neutralize nonadsorbed virus. In NA cells, 100 times more pathogenic virus than apathogenic virus was produced by 12 h p.i. (Fig. 4). At 36 h p.i., the yields of pathogenic and apathogenic viruses were equal. To assess viral protein synthesis, the amount of nucleocapsid protein and glycoprotein present in cell lysates was determined by radioimmunoassay with monoclonal antibodies specific for N or G protein. Similar to the production of virus, the synthesis of G and N protein at 12 h p.i. was significantly lower in NA cells infected with apathogenic virus than in NA cells infected with pathogenic virus (Fig. 5A and B).

Previous studies (12, 20) revealed no differences in competitive inhibition by the parent virus and RV 194-2 apathogenic virus for binding of radiolabeled virus to BHK-21 or NA cells. Therefore, we investigated whether differences in the kinetics of virus internalization might account for the different rates of infecting NA cells with pathogenic and apathogenic virus. Monolayers of NA cells were incubated with 10 PFU of virus per cell for various times, and noninternalized virus was neutralized by the addition of antirabies serum. The cells were then examined at 18 h p.i. for the presence of viral antigen by fluorescent antibody staining. The number of infected cells was similar

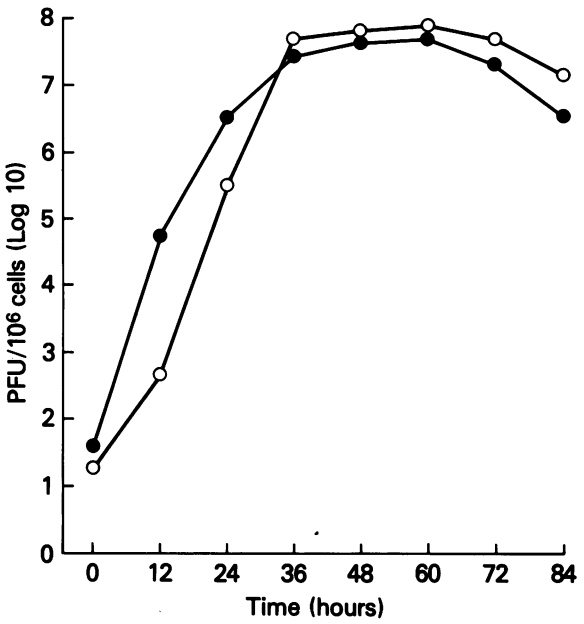

FIG. 4. Kinetics of production of infectious virus in NA cells. NA cells were infected at an MOI of 0.1 PFU per cell with pathogenic CVS-11 (●) or apathogenic CVS RV194-2 (○) virus. At 1 h p.i., cells were treated with antirabies serum for 1 h to neutralize noninternalized virus. Antiserum was removed, cells were incubated for the indicated times at 37°C, and the amount of virus released into the tissue culture medium was determined by plaque assay.

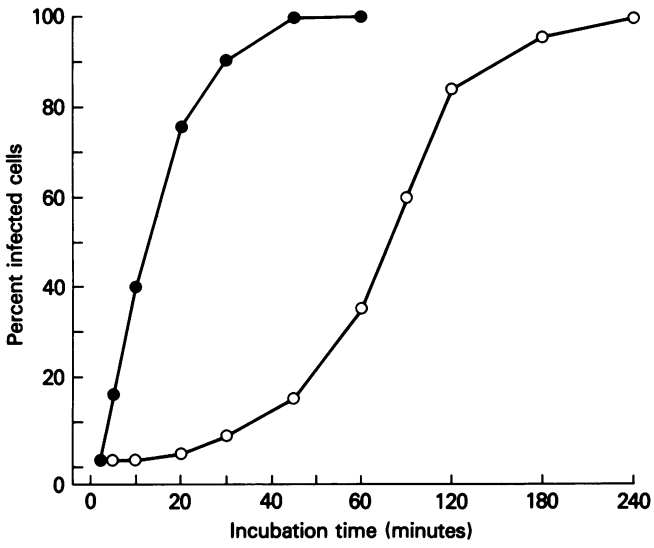

FIG. 6. Kinetics of internalization of pathogenic CVS-11 (●) and apathogenic CVS RV194-2 (○) virus. Monolayers of NA cells were incubated with 10 PFU per cell of pathogenic or apathogenic virus for various time periods. At the indicated times, antirabies serum was added to the culture medium to neutralize noninternalized virus. At 18 h p.i. the cells were examined for the presence of viral antigen by fluorescent antibody technique.

at 12 and 18 h p.i.; however, the amount of viral antigen per cell was greater at 18 h. When rabies virus-neutralizing antibody was added at 30 min p.i., viral antigen could be demonstrated in 90% of NA cells infected with pathogenic virus but only in 10% of NA cells infected with apathogenic virus. When neutralizing antibody was added at 60 min p.i., 30% of apathogenic virus-infected NA cells contained viral antigen, and 90% of the cells were infected when antibody was added at 180 min p.i. (Fig. 6).

DISCUSSION

The immunohistological data demonstrate qualitative similarities in the infection of the central nervous system tissue after intracerebral inoculation of mice with the pathogenic

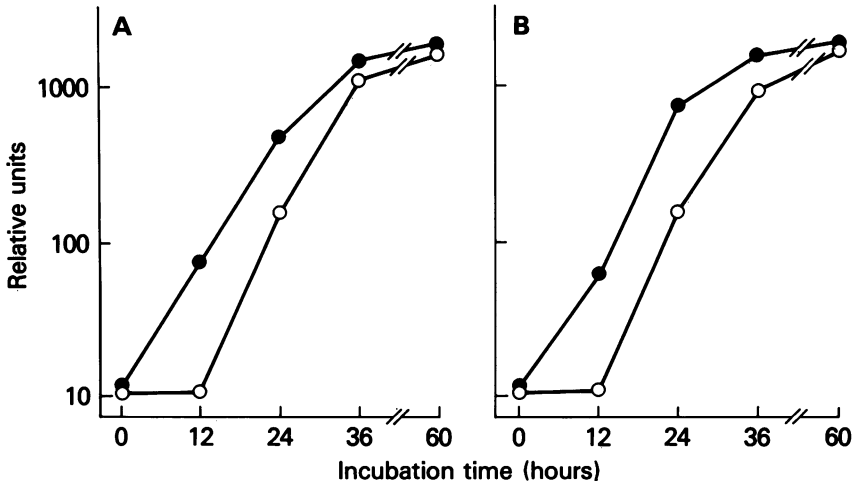

FIG. 5. Synthesis of glycoprotein (A) and nucleocapsid protein (B) in NA cells infected with pathogenic CVS-11 (●) or apathogenic CVS RV194-2 (○) virus. NA cells were infected at an MOI of 0.1 PFU per cell and, 1 h p.i., treated with antirabies serum for 1 h to neutralize noninternalized virus. Antiserum-containing tissue culture medium was replaced by fresh medium, and cells were incubated for the indicated times at 37°C and lysed with 1% Nonidet P-40. The amount of glycoprotein or nucleocapsid protein in the lysate was determined by radioimmunoassay.

CVS parent or apathogenic variant (RV194-2) virus. Differences in mouse brain infected with these viruses were mainly quantitative. The pathogenic virus spread more rapidly from the neocortex to the cerebellum and infected more neurons than did the apathogenic virus. It is possible that neural mechanisms controlling virus transport (10) determine the rates of dissemination of rabies virus and that such mechanisms are affected by the specific amino acid at position 333 of the viral glycoprotein molecule. This may be significant in determining lethality of the virus.

Our results on the spread of apathogenic virus in the central nervous system after direct inoculation into the brain are similar to those described by Kučera et al. (7), who demonstrated a reduced ability of apathogenic virus to spread to the central nervous system after inoculation of virus into the anterior chamber of the eye.

The observation that neuronal cells in vivo exhibit differences in susceptibility to pathogenic and apathogenic virus can be correlated with the behavior of these viruses in cultured NA cells, which retain several characteristics of neurons (2, 11, 13). Whereas no differences between pathogenic and apathogenic rabies virus infection were observed in BHK-21 cells, NA cells were much more susceptible to pathogenic than to apathogenic virus. Only 15% of NA cells were infected with apathogenic virus when antirabies serum was added 45 min after virus adsorption (Fig. 6). Antirabies serum added at the same time to NA cells infected with pathogenic virus failed to prevent complete infection of the cells. Since competition binding experiments did not reveal any differences in the attachment of pathogenic and apathogenic virus to NA cells (20), it seems likely that the pathogenic virus is more rapidly internalized than apathogenic virus.

Most interesting are the observed differences in cell-to-cell spread between pathogenic and apathogenic viruses which occur in NA cells in the presence of antirabies serum, although these experiments do not reveal the particular mechanisms involved. Possible reasons for these observed differences include: (i) altered fusion function of the glycoprotein due to the point mutation at residue 333, although no differences were observed in pH-dependent fusion of cells infected with apathogenic versus pathogenic rabies virus (unpublished observations); (ii) site-specific proteolytic cleavage involving arginine at position 333 in the pathogenic virus glycoprotein which may induce virus activation similar to that found in parainfluenza virus (5), although no evidence for any cleavage mechanisms has been found to support this hypothesis; and (iii) differences in affinity for putative receptors in viral attachment to cells in culture. Previously described binding studies involving competition for virus attachment to saturable cellular receptor sites have suggested that these two viruses compete for the same specific binding site (20). In the present studies, the number of virus particles of apathogenic virus required to infect an equal number of NA cells was 10 times greater than the number of particles of pathogenic virus required, suggesting that more of the apathogenic virus particles, which were apparently capable of binding to cell surface molecules, were incapable of entering the cell. It is possible that either the apathogenic virus interacts with the cell surface receptor in a manner that is different from that of pathogenic virus, resulting in a less efficient receptor function, or the two virus types attach to different species of cell surface receptors, one of which is less efficient for internalization of virus. The utilization of different receptor molecules on the NA cell membrane surface by pathogenic and apathogenic rabies virus would explain the differences in their infection rates and spreading patterns both in vivo and in vitro. We are currently isolating receptor molecules from NA cells and brain tissue which have binding specificity for pathogenic or apathogenic rabies virus or both. These experiments should reveal whether differences in virulence between pathogenic and apathogenic rabies virus are determined by the nature of the receptor responsible for virus-host cell binding in vivo or in vitro.

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