Immunofluorescence and Cytochemical Studies of Visna Virus in Cell Culture

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Received for publication 3 July 1967

Sequential morphological changes occurring in sheep choroid plexus cells infected with visna virus were studied by direct immunofluorescence, acridine orange, and hematoxylin and eosin staining methods. Specific immunofluorescence was first detected in the perinuclear cytoplasm of solitary cells 24 hr after infection. As the infection progressed, viral antigen appeared in an increasing number of cells, and rounded globular cells with long slender processes harboring intense fluorescence were seen. Nuclear fluorescence was not observed in infected monolayers. Polykaryocytes formed within 6 hr after inoculation due to the direct cell-fusing effect of the virus inoculum did not show specific fluorescence. Viral antigen was found, however, in the cytoplasm of multinucleated giant cells in cover slips harvested after new infective virus had been released, and later in the course of infection circular fluorescent inclusions were seen in the cytoplasm of polykaryocytes. Comparable eosinophilic inclusions were observed in hematoxylin and eosin preparations, and acridine orange staining of infected monolayers demonstrated similar inclusions which fluoresced with the color characteristic of single-stranded nucleic acid and were susceptible to digestion with ribonuclease. Visna virus appears to be a ribonucleic acid virus which replicates in the cytoplasm.

Visna virus is a yet unclassified virus which causes a slow, progressive, and fatal disease of the nervous system of sheep (31, 32). Propagation in cell cultures derived from the sheep choroid plexus (33) has resulted in characterization of a number of the biological, physical, and chemical properties of the virus (16, 38-40).

Although electron microscopic studies indicate that virus particles are formed by budding on the cytoplasmic membrane of host cells, the fine structure of the nuclei and cytoplasm of infected cells looks much like that of uninfected control cells (36), and the cellular site of virus synthesis has not yet been established.

The present communication describes experiments performed to determine the site of visna virus replication in sheep choroid plexus cells by use of immunofluorescence and cytochemical techniques. The results suggest that visna virus is a ribonucleic acid (RNA) virus which is synthesized in the cytoplasm of infected cells.

MATERIALS AND METHODS

Cell cultures. Sheep choroid plexus (SCP) cells were prepared by trypsin dispersion of choroid plexuses removed from the brains of exsanguinated domestic Hampshire or Suffolk sheep as previously described

(16). Cells were grown in reinforced Eagle's medium (1) containing 10% fetal bovine serum in 250-ml plastic flasks and incubated at 37 C. Cell lines prepared in this manner consist of elongated fibroblastic cells which survive 10 to 12 serial passages.

Virus. Visna virus K485 was obtained from H. Thormar and P. A. Pálsson, Institute for Experimental Pathology, University of Iceland, and was carried through eight serial passages in SCP cells. Eighth passage virus containing $3.4 \times 10^{6.0}$ TCID₅₀/ml was used in the experiments. Concentrated visna virus containing $10^{8.0}$ TCID₅₀/ml was prepared by clarification at $1,500 \times g$ for 10 min, followed by centrifugation at $78,000 \times g$ for 6 hr and suspension of the pelleted material in one-fiftieth of its original volume in reinforced Eagle's medium plus 0.5% bovine plasma albumin (Fraction V, Armour Pharmaceutical Co., Kankakee, Ill.); this concentrated preparation was used in an experiment to produce rapid cell fusion. All virus stocks were stored at -70 C until they were used.

Visna virus antiserum. Serum 4992 from a sheep infected with visna virus was kindly supplied by H. Thormar. This serum has a specific neutralization titer of 1:1,024 and was used in the preparation of fluorescein-labeled antiserum.

Infection of cell cultures. Experiments were performed by infecting confluent SCP monolayers grown in 60-mm plastic tissue culture dishes containing two 18-mm square glass cover slips. Replicate dishes were

washed twice with 5.0 ml of phosphate-buffered saline (PBS), pH 7.2 (9), and inoculated with 0.5 ml of unconcentrated or concentrated virus. After adsorption for 3 hr at 37 C, the inoculum was removed and the cell sheet was washed with PBS. Maintenance medium (reinforced Eagle's medium and 2% inactivated lamb serum) was added, and the cultures were incubated at 37 C in a humidified atmosphere of 5% carbon dioxide. At intervals after infection, the medium was harvested, and bovine plasma albumin (BSA) was added to a concentration of 0.5%. The harvested medium was then frozen and stored at -70 C until it was assayed for infective virus. Cover slips were removed and fixed for cytological studies. Uninfected cultures inoculated with 0.5 ml of reinforced Eagle's medium and 0.5% BSA were handled in the same manner and served as controls.

Assay of infective virus. Confluent SCP monolayers in 60-mm plastic petri dishes, four plates per dilution, were washed twice with PBS and inoculated with serial 10-fold dilutions of virus in reinforced Eagle's medium with 0.5% BSA. After a 3-hr adsorption period at 37 C, 5.0 ml of maintenance medium was added, and the cultures were incubated at 37 C in a humidified atmosphere of 5% carbon dioxide. Cultures were examined after 14 days for cytopathic changes, and 50% infectivity end points were calculated by the method of Reed and Muench (29).

Staining procedures. Immunofluorescence studies were performed by the direct staining method of Coons and co-workers (7, 8). The globulin fraction of visna virus antiserum 4992 was precipitated with sodium sulfate and conjugated with fluorescein isothiocyanate (35). Fluorescein-labeled serum was absorbed with rat and mouse liver powder before use and retained a neutralizing titer of 1:200 against 20,000 TCID₅₀ of visna virus. Cover slips to be stained by the immunofluorescence method were fixed in acetone, washed with PBS, and stained for 30 min at 25 C with a 1:4 dilution of fluorescein-labeled visna virus antiserum. Cover slips were then washed with PBS and mounted on microscope slides in buffered glycerin.

Hematoxylin and eosin staining was done on cover slips fixed in Zenker's fluid for 60 min by the method of Enders and Peebles (11).

Acridine orange staining was performed on cover slips fixed in Carnoy's fixative, stained with 0.05%

acridine orange (Chroma-Gesellschaft, Stuttgart, Germany) in acetate buffer (pH 5.4), and mounted in glycerin and acetate buffer (12).

Digestion with nucleases was performed by incubating Carnoy-fixed cover slips with either 0.05% five times crystallized ribonuclease in acetate buffer (pH 5.4) containing 0.003 M MgCl₂ or 0.01% once crystallized deoxyribonuclease in 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.3) containing 0.003 M MgCl₂ at 37 C for 1 hr. Nucleases were obtained from Sigma Chemical Co., St. Louis, Mo. Control cover slips were incubated with buffer alone.

Fluorescent-antibody- and acridine orange-stained preparations were examined by use of a Reichert Fluorex microscope with a BG12 exciter filter and an OG4 barrier filter. Photographs were taken on Anscochrome D-200 or Kodachrome X film.

RESULTS

Growth of visna virus in SCP cells. The amount of visna virus sequentially released in SCP cultures infected at a multiplicity of 4 TCID₅₀ per cell is shown in Table 1.

Newly released virus was first detected in medium harvested 24 hr after infection. An exponential increase then occurred during the next 24 hr, and peak titers were found in medium harvested at 96 and 120 hr after infection. This pattern of viral multiplication is in general agreement with previously reported studies on the growth of visna virus in SCP cell cultures (16, 37).

Morphological changes in visna virus-infected SCP cells. Fluorescent-antibody staining of visna-infected cell monolayers was performed on cover slips harvested at 6, 24, 31, 43, 72, 96, and 120 hr after infection. The results of these studies are summarized in Table 1.

Specific fluorescence was first detected in isolated cells 24 hr after infection. Fluorescent antibody was found to be localized in the cytoplasm of scattered fibroblastic cells constituting less than 1% of the cell population of the monolayer. Such fluorescence was often noted to be more intense about the nucleus of the cell, and

TABLE 1. Immunofluorescence of visna virus-infected sheep choroid plexus cells

Time after infection (hr) ^a	Infective virus (TCID ₅₀ /ml)	Intensity of specific fluorescence			Inclusions	Approximate percentage of cells stained
		Fibroblasts	Polykaryocytes	Rounded cells		
24	1.0 × 10 ³	+	0	0	0	<1
31	6.3 × 10 ³	+	0	0	0	10
48	3.5 × 10 ³	++	+	+	0	15
72	6.3 × 10 ³	++	+	+	0	30
96	2.2 × 10 ⁷	+++	++	++	+	70
120	4.3 × 10 ⁷	+++	+++	+++	+++	70

^a Time after inoculation of sheep choroid plexus monolayers with visna virus at a multiplicity of 4 TCID₅₀ per cell and incubation at 37 C.

FIG. 1a to 1d. Immunofluorescent staining of sheep choroid plexus cells infected with visna virus. Cells were inoculated at a multiplicity of 4 TCID_{50} per cell and at intervals were fixed and stained by the direct immunofluorescence technique. (1a) 72 hr after infection, specific fluorescent staining in the cytoplasm of a multinucleated giant cell, $\times 125$; (1b) 96 hr after infection, fluorescent antibody localized to the cytoplasm of fibroblasts and in several round globular cells with elongated processes, $\times 50$; (1c) 120 hr after infection, specific fluorescence in the cytoplasm of fibroblast and multinucleated giant cell, $\times 125$; (1d) 120 hr after infection, aggregates of fluorescent antibody appearing as inclusions in the cytoplasm of a multinucleated giant cell, $\times 125$.

FIG. 2. Circular eosinophilic inclusion in polykaryocyte 120 hr after inoculation with visna virus. Hematoxylin and eosin stain. $\times 100$.

FIG. 3. Intracytoplasmic inclusions in polykaryocytes stained with acridine orange 120 hr after inoculation with visna virus. Inclusions demonstrate the red fluorescence associated with single-stranded RNA. $\times 100$.

was fairly homogeneous in character. Polykaryocytes were observed at 6 and 24 hr after infection, and were probably the result of the direct fusing effect of the visna virus inoculum (16). Such multinucleated cells did not show fluorescence.

The findings 31 hr after infection were similar to those at 24 hr, but at 31 hr viral antigen was seen in the cytoplasm of a larger number of fibroblastic cells.

After 48 hr, approximately 15% of the cells

in the monolayer showed intracytoplasmic fluorescence. Polykaryocyte formation appeared more prominent, and a few such multinucleated cells showed intracytoplasmic fluorescence. In addition, there appeared for the first time rounded globular cells with two or more long slender processes; both the cell bodies and processes of such cells exhibited an intense bright apple-green fluorescence. Such globular spindle or spiderlike cells were also found in cover slips harvested later than 48 hr and may well represent visna-infected SCP cells in the terminal phases of degeneration.

At 72 hr after infection, a sizeable number of cells appeared to have become detached from the glass surface, and approximately 30% of the remaining attached cells contained cytoplasmic fluorescence. Such fluorescence was observed in fibroblastic cells, polykaryocytes (Fig. 1a), and globular cells.

After 96 and 120 hr, when peak titers of infective virus were found in the medium, both the intensity of immunofluorescent staining and the number of cells containing viral antigen were maximal. Viral antigen remained localized in the cytoplasm (Figs. 1b, c, and d), and approximately 70% of the cells showed fluorescent staining. Specific fluorescence was again observed in fibroblastic cells, in polykaryocytes (Fig. 1c), and in rounded shrunken, cells with elongated processes (Fig. 1b). In addition, dense round aggregates of intensely fluorescent material were observed within the cytoplasm of many of the multinucleated giant cells in the culture (Fig. 1d).

Specific fluorescent staining was never observed in the nucleus of infected SCP cells.

Uninfected SCP cells did not stain with fluorescein-labeled visna virus antiserum, and infected SCP cells were not stained by the application of fluorescein-labeled anti-human γ -globulin sheep serum. Specific fluorescent staining of infected SCP cells by labeled visna virus antiserum was blocked by prior treatment with unlabeled specific antiserum.

Hematoxylin and eosin staining revealed changes comparable to those observed in cover slips stained by the immunofluorescence method. Rounded cells with long fine processes similar to those showing intense staining with fluorescent visna virus antiserum were first observed in cover slips harvested 48 hr after infection; they were deeply basophilic.

Approximately 30% of polykaryocytes 120 hr after infection contained eosinophilic cytoplasmic inclusions (Fig. 2). These inclusions were observed only in multinucleated giant cells and clearly corresponded to the aggregates of specific fluorescence seen in polykaryocytes stained by

the fluorescent-antibody technique; they were often surrounded by a crescent of nuclei (Fig 2).

Acridine orange stains viruses that contain double-stranded RNA or deoxyribonucleic acid (DNA) orthochromatically green and single-stranded RNA and DNA metachromatically red under the staining conditions employed in the present experiments (13, 14, 22). SCP monolayers harvested 120 hr after infection with visna virus and stained with acridine orange showed numerous intracytoplasmic inclusions which corresponded in size, shape, and distribution to those observed in multinucleated giant cells after staining with either fluorescein-labeled antibody or hematoxylin and eosin. As illustrated in Fig. 3, such inclusions exhibited the orange-red color that is associated with single-stranded nucleic acid.

The metachromatic staining of the intracytoplasmic inclusions was completely abolished by prior digestion with ribonuclease. Incubation with deoxyribonuclease eliminated the green fluorescence of the nuclear chromatin, but failed to remove the orange-red fluorescence of the cytoplasmic inclusions.

Immunofluorescence studies of rapid virus-induced cell fusion. Exposure of SCP monolayers to visna virus at high multiplicity results in rapid cell fusion that involves the entire monolayer by 5 to 6 hr; large syncytia are formed which disintegrate well before new virus is released (16).

SCP monolayers were inoculated with concentrated visna virus at a multiplicity of 30 TCID₅₀ per cell; 6 hr later the cover slips were harvested, fixed, and stained by the direct immunofluorescent technique. Although extensive cell fusion had occurred, specific fluorescence was not seen in any of the multinucleated cells in the monolayer, indicating that visna virus-induced cell fusion can occur in the absence of detectable intracellular viral antigen.

DISCUSSION

In its morphology and some of its physical, chemical, and biological properties, visna virus shows similarities to the avian leukosis and murine leukemia viruses (39, 40). Cells infected with avian leukosis or mouse leukemia viruses show cytoplasmic localization of viral antigen when they are studied by the immunofluorescence technique under conditions like those that existed in the present study (2, 19, 24-27, 42, 43). The observation that visna virus antigen develops in the cytoplasm of infected cells points to yet another common feature between visna virus and the avian leukosis and murine leukemia viruses.

In a previous study of the sequential changes occurring in visna-infected cells with acridine orange staining (41), Thormar reported that the cytoplasm of infected cells developed an intense red fluorescence and that this fluorescence did not appear after treatment with ribonuclease. Increased orthochromatic greenish-yellow fluorescence was not detected in the cytoplasm of infected cells, nor were fluorescent cytoplasmic inclusions described. The present study demonstrates that inclusions of viral antigen observed late in visna virus infection show the orange-red fluorescence characteristic of single-stranded nucleic acid stained with acridine orange. The metachromatic fluorescence of these inclusions was removed by treatment with ribonuclease, but appeared unaffected by deoxyribonuclease digestion. Evidence now available from cytochemical studies indicates that visna virus contains single-stranded nucleic acid which is most probably RNA.

Polykaryocyte formation produced by the inoculation of SCP monolayers at high virus-cell multiplicities appears to be due to a direct effect of the virus particle on the cell membrane that is independent of virus replication (16). This concept gains support from the finding that rapid cell fusion occurs without the appearance of intracellular viral antigen.

The multiplicity of infection used in the present investigation on the site of visna virus synthesis was carefully chosen to provide an inoculum that would infect as many cells as possible and yet avoid extensive fusion and degeneration before new virus could be released (16). Nonetheless, surprisingly few cells contained viral antigen 24 hr after infection, and therefore it is probable that multiple cycles of infection occurred during the period of study. The reason for the relative inefficiency of infection as demonstrated by immunofluorescence is not clear; it may be related to restrictive limitations in virus penetration, uncoating, or other early steps in the viral replicative cycle.

Cytoplasmic inclusions similar to those noted in the present study have been observed in polykaryocytes produced by infection of cell cultures with several different syncytium-forming viruses (3, 4, 15, 17, 21, 23, 28, 30), and evidence has been presented which suggests that polykaryocytes contain little or no infective virus (5, 34), possibly because of alterations in the cell membrane or other cellular constituents which accompany the fusion process. Cytoplasmic inclusions occurring in polykaryocytes produced by the simian myxovirus SV5 (6) and in cells infected with the syncytium-forming type 2 parainfluenza virus (18, 20) or mumps virus (10) have been shown to be

composed of fibrils of viral ribonucleoprotein. The cytoplasmic inclusions found in polykaryocytes late in visna virus infection may well represent accumulations of viral nucleoprotein which the polykaryocyte cannot incorporate into mature infective virus particles.

ACKNOWLEDGMENTS

We thank Karen K. Funk for excellent technical assistance and Beatrice C. Seegal and John B. Zabriske for many helpful suggestions during the conduct of the experiments.

This investigation was supported by Public Health Service grant NB-06989 from the National Institute of Neurological Diseases and Blindness, grants AI-05474 and AI-05600 from the National Institute of Allergy and Infectious Disease, grant HE-03929 from the National Heart Institute, and by a gift from the Miles Hodson Vernon Foundation, Inc. D. H. Harter was recipient of Career Research Development Award 1K3NB34,900 from the National Institute of Neurological Diseases and Blindness.

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