Isolation and Characterization of Subacute Sclerosing Panencephalitis Virus Nucleocapsids

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Nucleocapsids from subacute sclerosing panencephalitis (SSPE) virus-infected CV-1 cells were concentrated by differential centrifugation employing sucrose cushion techniques and further purified by centrifugation through a linear CsCl density gradient. The buoyant density of ^3H -uridine-labeled nucleocapsids in CsCl was found to be 1.31 g/cm^3 . Ultraviolet absorption spectra of the purified SSPE nucleocapsid showed an absorption maximum at 260 to 265 nm and a 280/260 ratio that corresponded to a nucleic acid content of approximately 4.3%. Negatively stained preparations of SSPE nucleocapsids were found to have a width of $18 \pm 1 \text{ nm}$, a periodicity of 5 to 6 nm, and a length between 1–1.4 μm , with the greatest number at 1.3 μm .

The presence of paramyxovirus-like nucleocapsids in inclusion bodies detected in thin sections of brain tissues has been one of the diagnostic signs of subacute sclerosing panencephalitis (SSPE) (4). These nucleocapsid structures have been observed in explanted cultures of SSPE brain tissue as well as in established cell lines co-cultivated or fused with infected brain cultures (1, 16, 17). Estimates of the size of the SSPE nucleocapsids observed in both the cytoplasm and nucleus of infected cells have been made (13, 24). Histochemical investigations revealed that SSPE virus-specific inclusion bodies stained positively for ribonucleic acid (RNA) and protein but negatively for deoxyribonucleic acid and lipid (21, 25), and immunological evidence has shown SSPE virus-infected cells to contain antigen detectable by fluorescent anti-measles serum (7, 20). Based on these reports, it has been generally believed that the synthesis of the nucleocapsids in cells is a direct consequence of the infection of the cell by a viral agent, probably a measles-like RNA virus.

Despite the seeming abundance of nucleocapsid structures in SSPE virus-infected cells, there have been no reports of their isolation. This paper reports the development of a procedure for concentrating and purifying the nucleocapsids from cell culture fluids and SSPE virus-infected cells. In addition, size measurements from negatively stained preparations of the purified samples are presented.

MATERIALS AND METHODS

Virus and cell cultures. The virus used in these experiments was the LEC strain of SSPE virus originally

isolated by fusing brain biopsy explant cultures, obtained from a SSPE patient, with CV-1 cells (1). This SSPE isolate has been passaged continuously in CV-1 cells, and all experiments used virus from the 24th to 26th passage. In the remainder of this paper, infectious SSPE fluids will be referred to as virus, or virus-containing, cell culture fluids.

CV-1 cells, obtained from American Type Culture Collection, were used between the 27th and 35th passage level and grown in T-75 tissue culture flasks at 37°C in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. A humid atmosphere of 5% CO_2 in air was maintained when cells were cultured in petri dishes.

Chemicals and isotopes. Actinomycin D (Sigma Chemical Co., St. Louis, Mo.) was dissolved in distilled water at a concentration of 100 $\mu\text{g/ml}$ and kept frozen in the dark. Uridine-5,6- H^3 (New England Nuclear Corp., Boston, Mass.; specific activity, 42.4 Ci/mmoles) was used at a concentration of 10 to 20 $\mu\text{Ci/ml}$ throughout these experiments to label the RNA of the SSPE nucleocapsid. ^{14}C -amino acids, (New England Nuclear Corp.) were used at a concentration of 0.03 $\mu\text{Ci/ml}$ to label cellular proteins.

Stock solutions of CsCl were made by dissolving appropriate amounts of CsCl in NT buffer (0.13 M NaCl, 0.05 M tris[hydroxymethyl]aminomethane [Tris]-hydrochloride, pH 7.4). Sucrose solutions were also prepared in NT buffer. Densities of both CsCl and sucrose solutions were determined from refractive index measurements.

Assay of infectious virus. Confluent monolayers of CV-1 cells in 60-mm plastic tissue culture dishes were infected with 0.2 ml of virus inoculum/dish at the appropriate dilution. After an adsorption period of 1 hr at 37°C, the inoculum was removed, and the cell monolayer was washed with sterile NT buffer and overlaid with MEM containing 2% fetal bovine serum and 0.5% agarose. The plates were incubated at 37°C for 2 to 3 days, an additional overlay of agarose contain-

ing 0.2% neutral red was added, and plaques were counted 12 to 16 hr later.

Infection of cell cultures. CV-1 cell monolayers were infected with 0.02 plaque-forming units (PFU) of the virus per cell, unless otherwise stated, and refed with fresh media after 1 hr of adsorption at 37°C. When isotopically labeled nucleocapsid preparations were prepared, actinomycin D (0.2 µg/ml) was added 24 hr postinfection, followed by the addition of ³H-uridine (10 µCi/ml) 4 to 6 hr after the addition of the drug.

Determination of radioactivity and optical density. Samples were absorbed into Whatman no. 3 filter paper pretreated with 5% trichloroacetic acid (12). The radioactive papers were washed three times with cold 5% trichloroacetic acid followed by two washes with cold ether. The filter papers were then dried, placed in scintillation vials containing 10 ml of toluene-based scintillation fluid, and counted in a scintillation spectrometer.

The optical density of samples was measured in a Gilford spectrophotometer, and all values were corrected for light scattering by assuming that the optical density at 320 nm represented only light scattering and that scattering increases inversely with the fourth power of the wave length (10).

Electron microscopy. Samples were suspended in a small amount of culture media, spread on carbon-coated grids, and negatively stained with 2% potassium phosphotungstate which was neutralized to pH 7.0 with KOH. Specimens were examined in a Hitachi HS-8 electron microscope at 50 kv. The microscope was calibrated by carbon grating replica (E. F. Fullam Inc., New York).

RESULTS

Growth and stability of the virus. The LEC strain of SSPE virus (1) used in these studies had an initial infectivity titer of 3×10^3 PFU/ml. Its pattern of cytopathic change was similar to that caused by measles virus (9, 22) and consisted of formation of syncytia, vacuolization, and the ultimate destruction of the areas of syncytia. During the course of these experiments, it was observed that harvesting the virus from the infected cells and the culture fluid just before total lysis of the cell monolayer increased the titer of the stock virus pool to 2×10^5 PFU/ml. Figure 1 shows time course of virus synthesis in CV-1 cells. In this experiment an input multiplicity of infection of 0.1 was used, and cell-associated virus was first detected at 18 hr. Electron microscopy examination of thin sections of the infected cultures showed nucleocapsids in the syncytia-forming cells. A maximum cell-associated viral infectivity was reached at 36 hr postinfection, at which time the entire cell monolayer was a mass of syncytia. The CV-1 cell sheet rapidly degenerated and lysed after 36 hr, and the cell-associated infectivity declined over 100-fold during the next 24 hr. Infectious virus was not released into the media until the cell sheet was almost completely lysed, suggesting that the infectivity associated

with the culture fluids was a result of virus released from lysed cells. If a lower input multiplicity of infectious virus was used (i.e., 0.02), the period of viral production was prolonged 24 to 48 hr, although syncytia were still first observed at 24 hr postinfection and cytopathic effects were first observed at 30 hr.

The addition of 0.1 µg of actinomycin D/ml, either 12 hr prior or 12 hr after infection, did not alter the final yield of infectious virus (extracellular and cell-associated virus, *see below*) even though RNA synthesis, measured by ³H-uridine incorporation, was reduced by over 90% (Table 1).

FIG. 1. Time course of synthesis of infective SSPE virus. Monolayers of CV-1 cells were infected with SSPE virus at a multiplicity of infection of 0.1. At designated times the medium was removed and virus was quantitated by plaque titration (○-----○). The cells were suspended in an equal volume of medium, homogenized in a tight-fitting Dounce homogenizer, clarified, and titered (○—○).

TABLE 1. Effect of actinomycin D on viral production

Sample	Preincubation with actinomycin D ^a	Actinomycin D during infection	PFU ^b /ml × 10 ⁻³
1	+	+	1.60
2	—	+	1.45
3	—	—	1.53

^a Actinomycin D, 0.1 µg/ml, was preincubated for 12 hr prior to infection or added immediately after infection.

^b Plaque-forming units.

Virus infectivity was highly sensitive to heat (Table 2), since the infectivity decreased 100-fold after the virus was held at 37 C for 4 hr. The virus was more stable at lower temperatures and could be stored at -70 C for many months without loss of infectivity.

Concentration of nucleocapsids. SSPE-infected CV-1 cultures were harvested 72 hr after infection in the following manner. Virus-containing cell culture fluid and NT buffer used to rinse the infected cells were pooled and centrifuged in a Sorvall SS-34 rotor at $8000 \times g$ for 15 min to remove cellular debris. The supernatant fluid was then centrifuged at 5 C in a Spinco 19 rotor for 2 hr at $50,000 \times g$, and the resulting pellet was resuspended in NT buffer. Infected cells were scraped from the bottles with a rubber policeman, washed three times with NT buffer, and lysed in 5 to 10 ml of cold distilled water for 20 min. Cellular debris and subcellular particles were removed by centrifugation, and the lysate of the infected cells was combined with the resuspended pellet obtained from the culture media and wash fractions. The combined suspension was centrifuged through 30% sucrose onto a 65% sucrose cushion in a Spinco model SW41 rotor at $150,000 \times g$ for 1.5 hr. This procedure resulted in the further concentration of particulate material and, in addition, freed the suspension of relatively small-molecular-weight material. The particulate material collected at the 30 to 65% sucrose interface was examined by electron microscopy using the negative staining technique and was found to contain large quantity of both intact and partially fragmented nucleocapsid material as well as cell membranes and other cellular material. Equivalent preparations of noninfected control cells showed only membranes and some cellular debris. The sucrose interface fraction was used as starting material for additional purification.

Purification and characterization of the nucleocapsids. A concentrated ^3H -uridine-labeled nu-

cleocapsid suspension (0.5 ml) from the 30 to 65% sucrose interface was dialyzed against NT buffer after the addition of 1% bovine serum albumin (BSA), layered on a preformed CsCl density gradient (1.25 to 1.35 g/cm³), and centrifuged to equilibrium ($200,000 \times g$ for 20 hr in a Spinco SW50.1 rotor). The resulting profile (Fig. 2a) was constructed by measuring the incorporation of ^3H -uridine into trichloroacetic acid-insoluble material and the optical density at 260 nm of each fraction. A peak of optical density and radioactivity coincided at a density position of 1.31 g/cm³, and the presence of partially purified SSPE nucleocapsids in this fraction was confirmed by electron microscopy.

The lighter material (1.20 to 1.25 g/cm³) was found to be predominantly composed of cellular material by electron microscopy. Without adding BSA to stabilize the nucleocapsid preparation, the peak of radioactivity at 1.31 g/cm³ decreased about fivefold, and the nucleocapsid continued to degrade rapidly even when stored at 4 C. A control experiment (also plotted in Fig. 2a) in which uninfected cells labeled with ^3H -uridine and prepared in parallel with an infected preparation showed less than 1% of total acid-insoluble ^3H -uridine radioactivity in the density region, 1.31 g/cm³, although considerable radioactivity was present at the region of lighter density. The partially purified SSPE nucleocapsids could be rebanded after overnight dialysis against NT buffer if 1% BSA was added to the sample before dialysis. BSA stabilized the nucleocapsids during the dialysis and subsequent centrifugation in CsCl gradient. When BSA was omitted, very little nucleocapsid was recovered. Figure 2b shows the CsCl density gradient profile of the partially purified nucleocapsids after the second equilibrium centrifugation. Again the peak of optical density corresponded to the peak of ^3H -uridine-incorporated trichloroacetic acid-insoluble material, both centering at a density position of 1.31 g/cm³. The recovery of the nucleocapsid material after the second equilibrium centrifugation, measured by acid-insoluble ^3H -uridine incorporation, was about 25% of the applied sample.

To evaluate the amount of cellular debris contaminating the nucleocapsid fractions during various steps of the purification procedure, the following experiment was devised. An SSPE virus-infected culture was labeled with ^3H -uridine, whereas an uninfected culture was labeled with ^{14}C -amino acids. The crude cell lysates of the two cultures were mixed, and the extent of nucleocapsid purification was determined by following the removal of ^{14}C -amino acid-labeled cellular proteins at each purification step. Table 3 shows

TABLE 2. Effect of temperature on viral infectivity

Temp (C)	Incubation time (hr)	PFU/ml ^a
37	0	2.6×10^5
	4	5.0×10^3
	24	$< 10^{-1}$
4	4	8.0×10^4
	24	4.3×10^4
-20	24	1.6×10^5
-70	24	2.1×10^5

^a Virus from stock virus pool, harvested in culture medium, was incubated at specific temperatures and titered.

FIG. 2. Equilibrium centrifugation in a CsCl gradient of ^3H -uridine-labeled SSPE nucleocapsids. Samples were centrifuged for 20 hr at $200,000 \times g$ in a Spinco SW50.1 rotor, fractionated, and assayed for acid-insoluble radioactivity and absorbancy at 260 nm. Symbols: ---, density; ●, acid-insoluble ^3H -uridine incorporation of SSPE virus-infected preparation; ■, acid-insoluble ^3H -uridine incorporation of uninfected control cells; ○, optical density at 260 nm of infected preparation. (a) Dialyzed nucleocapsid fraction from a 30 to 65% sucrose interface. (b) Second equilibrium centrifugation of the 1.31 g/cm^3 peak fractions after dialysis.

TABLE 3. Purification of SSPE-specific nucleocapsids

Purification step	Volume (ml)	Total acid-insoluble counts/min $\times 10^{-6}$		Percent recovery	
		^3H	^{14}C	^3H	^{14}C
Crude extract (Nuclear-Spinco 19 pellet)	20	2.2	2.9	100	100
30 to 65% Sucrose interface	6	0.38	0.48	17.4	16.4
First equilibrium centrifugation in CsCl solution	3	0.165	0.15	7.5	5.1
Second equilibrium centrifugation in CsCl solution ^a	1	0.044	0.018	2.0	0.6

^a From an initial volume of 500 ml of cell extract and supernatant fluid, 0.55 mg of proteins/ml was recovered after the second CsCl equilibrium centrifugation.

that, after the second CsCl density centrifugation, 99.4% of the acid insoluble ^{14}C -labeled material had been removed from the sample. While the loss of ^3H -uridine-labeled viral nucleocapsid was

also substantial, CsCl equilibrium centrifugation removed more of the ^{14}C -labeled material than ^3H -labeled material. The ^3H -labeled material after the second CsCl centrifugation was judged to be almost pure nucleocapsids. Additional CsCl density gradient centrifugations did not result in further increase of the ^3H - ^{14}C ratio; on the contrary, further apparent loss of the nucleocapsids resulted.

An estimate of the nucleic acid content of nucleocapsid was obtained by measuring the ultraviolet absorption of the purified nucleocapsid material after the second CsCl density gradient centrifugation. The spectrum (Fig. 3) shown, after making the fourth-power light scatter corrections (see above), reveals a broad maximum at 260 to 265 nm and a minimum at 255 nm. The ratio of the optical density at 280 nm to that at 260, averaged from five spectra of different preparations, was 0.86, corresponding to a nucleic acid content of approximately $4.3 \pm 1.0\%$ (6).

Morphology of SSPE virus nucleocapsids. Material for morphological studies was obtained by disrupting infected cells by osmotic shock and pelleting the released material by centrifugation at $27,000 \times g$ for 30 min in an SS-34 rotor. The pellet was resuspended in a minimum volume of

FIG. 3. Ultraviolet absorption spectrum of purified SSPE virus nucleocapsids. The uncorrected absorption at 320 nm was 0.038 optical density unit.

media containing 0.4% BSA. Preparations obtained by these methods contained numerous strands of various lengths of tubular structures showing the typical herring-bone structure characteristic of paramyxovirus nucleocapsids (Fig. 4). These structures were never seen in preparations of uninfected cells and are considered to be viral nucleocapsids. They had a diameter of 18 ± 1 nm and a regular periodicity of 5 to 6 nm, depending on the extent of degradation and penetration of the staining solution and on the angle of view. Partial extension and uncoiling of the helical nucleocapsid structure was occasionally observed (Fig. 5). The helix appeared to be single-stranded, and an end-on view of the nucleocapsid revealed the existence of a central hole

with a diameter of 4 to 5 nm (Fig. 5).

The nucleocapsid material obtained at density 1.31 g/cm^3 after the CsCl centrifugation showed a large degree of clumping. When BSA was added prior to the dialysis and centrifugation steps, the degree of clumping was reduced.

Figure 6 is a histogram of the length distribution of 97 randomly selected nucleocapsid structures. As shown here, 65% fall in the range of 0.9 to $1.4 \mu\text{m}$, the largest number being at $1.3 \mu\text{m}$. The scarcity of pieces longer than $1.5 \mu\text{m}$ suggests that $1.3 \mu\text{m}$ may be the unit length of the SSPE nucleocapsid. The few nucleocapsids longer than $1.3 \mu\text{m}$ were probably formed by the end-to-end aggregation of shorter pieces, as has been suggested to explain similar observations of paramyxovirus nucleocapsids (6).

DISCUSSION

The growth properties of the LEC strain SSPE virus in CV-1 cells differ from the *in vitro* growth properties of measles virus in two respects. First, the SSPE virus-infected cells undergo cytopathic changes culminating in their destruction within 48 hr after infection, whereas measles virus-infected cells were able to maintain cell integrity for a much longer time (19, 22). Secondly, the titer of extracellular virus of SSPE-infected cultures never attains the high infections titer attained by measles virus infections (19, 22). Both facts suggest a difference in growth pattern between SSPE virus and measles virus in culture that should be examined further. The other growth properties, such as thermolability of the SSPE virus and the lack of effect of actinomycin D on virus production, are similar to that reported for measles virus (19).

These experiments demonstrate that nucleocapsids from SSPE virus-infected cells can be purified in quantities sufficient for biochemical and immunological studies. The labile nature of the nucleocapsids has been the major problem encountered in the purification procedure, and finding that the addition of a small quantity of BSA stabilized the nucleocapsids to dialysis and CsCl centrifugation was an important step toward their ultimate purification. BSA also prevented much of the aggregation of the nucleocapsids in the presence of CsCl, as has been reported for Newcastle disease virus (15) and measles virus (23).

The isotope dilution data obtained during purification (Table 3) suggest that the nucleocapsid was highly purified after the second CsCl density gradient centrifugation. The data also suggest that most of the material labeled by

FIG. 4. Electron micrograph of SSPE virus nucleocapsids obtained from CsCl density gradient. $\times 88,000$.

^3H -uridine after virus infection was not nucleocapsid material, since most of this material was removed by centrifugation through the 30% sucrose solution.

The various physical properties of the SSPE-specific nucleocapsids showed that the nucleocapsids were quite similar in structure to most of the paramyxoviruses studied so far. Their density in CsCl of 1.31 g/cm^3 is comparable to the densities of 1.31, 1.297, and 1.305 of nucleocapsids Sendai virus (15), simian virus 5 (6), and measles virus (23), respectively, and the estimated RNA content of 4.3% is also similar to that reported for simian virus 5 nucleocapsid (6), tobacco mosaic virus (3), and measles virus nucleocapsid (26). The morphological characteristics observed under electron microscopy of negatively stained preparations of SSPE-specific nucleocapsids revealed their similarity to the nucleocapsids of paramyxoviruses (6, 11, 14, 18) with a length of $1.3 \mu\text{m}$, a width of $18 \pm 1 \text{ nm}$, a central core of $5 \pm 1 \text{ nm}$, and a periodicity of the nucleocapsid protein structures of $6 \pm 1 \text{ nm}$.

The SSPE agent was originally classified as measles virus or measles-like virus by Horta-

Barbosa et al. (16) and others (5) for two principle reasons. The first was electron microscope observation of paramyxovirus-like nucleocapsids in both the brain tissues and derived cell cultures of SSPE patients. The second was the high measles-antibody titer found in the sera of patients suffering from the disease. Although this presumptive evidence was accepted clinically, no critical tests to determine the exact relationship between SSPE and measles have yet been performed. Several experimental tests can be employed to define this relationship. First, the production of monotypic antibody against purified nucleocapsids of the two viral agents would permit serological tests. Secondly, the isolation of viral nucleocapsids should permit the molecular characteristics of the RNA genomes of these two viruses to be compared. Ultimately homologous and heterologous RNA hybridization trials, with RNA extracted from measles and SSPE nucleocapsid and complementary strand RNA found in paramyxovirus-infected cells (2, 8), would be the most definitive measure of relatedness. The development of the purification procedure for SSPE virus nucleocapsids reported here should lead to

FIG. 5. Partial extension and uncoiling of SSPE virus nucleocapsids and examples of end-on views of the helix. $\times 132,000$.

FIG. 6. Distribution of the length of SSPE virus nucleocapsids released from CV-1 cells.

fruitful research on the relatedness of SSPE to measles and on the growth and development of SSPE in brain tissue.

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