# Electron Microscopic Observations in Subacute Sclerosing Panencephalitis Brain Cell Cultures: Their Correlation with Cytochemical and Immunocytological Findings

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Three cell cultures established from brain tissue obtained by biopsy of patients with subacute sclerosing panencephalitis (SSPE) were studied with the electron microscope in an attempt to correlate ultrastructural changes with those found by cytochemistry and immunocytology. These cells contained a large number of nucleocapsids resembling those of a paramyxovirus concentrated in the nuclear inclusions, but also seen free in the nucleus and occasionally in the cytoplasmic inclusions. Nuclear bodies associated with the nucleocapsids and granular filaments occupied a vast area of the cytoplasm. The nuclear inclusions containing nucleocapsids corresponded to the eosinophilic and fluorescent nuclear inclusions. The areas occupied by granular filaments corresponded to the diffuse cytoplasmic fluorescence. The ultrastructural changes were similar to those seen in the original brain biopsies. In addition papova-like virions were noted in brain cell cultures derived from a biopsy but not in the brain tissue itself. Their relationship to SSPE remains undetermined.

Subacute sclerosing panencephalitis (SSPE) is characterized histologically by Cowdry type A inclusion bodies in neurons and oligodendroglia (5). The causative agent has been unknown until recently. The investigations that led to isolation of viral agents from patients with SSPE began with Bouteille et al. (2), who noted tubular structures resembling nucleocapsids of a paramyxovirus in the brain tissue of a patient with SSPE.

Subsequently, indirect evidence suggesting an association between SSPE and measles, or measles-like, virus has accumulated: elevated titers of antibody which reacts with measles virus in the sera and cerebrospinal fluids in most patients with SSPE (4, 10), specific immunofluorescence within SSPE brain tissue in the presence of measles antibody (4, 12), and the presence of nucleocapsid-like structures in the brain tissue. Moreover, in cells cultured from brain biopsies of patients with SSPE, an antigen reacting with measles antiserum in an immunofluorescence test was noted (13) and nucleocapsid-like structures were detected (3, 8).

Although no infectious virus could be isolated directly from the brain cells in culture (just as it had been impossible to isolate it from the brain tissue), recently three laboratories, including our own, have succeeded in deriving infectious virus resembling measles from these cells in culture by the technique of co-cultivation or fusion of these cells with susceptible indicator cells (1, 6, 7, 15).

Of the three cell lines we have studied (JAC, LEC, and ROB), only two yielded infectious virus after cell fusion. The third, ROB, continued to exhibit a cytopathic effect, but no evidence of measles antigen or infectious virus could be detected.

The purpose of the present report is to describe electron microscopic observations in these cell lines and to correlate them with the immunocytological and cytochemical findings reported by us previously (13).

## MATERIALS AND METHODS

The cell lines were derived from brain biopsies of three patients with SSPE. Their diagnosis was based on a typical clinical course and histological findings in the biopsied brain tissue. Cell cultures were derived from these brain tissues by a method described previously (13). They were grown in Basal Eagle's

Medium enriched with 10% fetal calf serum and split at a 1:2 ratio at weekly or biweekly intervals. Cells were examined by the electron microscope when they reached confluence at the following cell transfer levels: 8 to 9th, 12 to 13th, 18 to 19th, 22nd, 24th, 26th, 28th, 30th, and 35th. For this purpose, the cells in a monolayer culture were treated with 0.25% trypsin in a 0.1% ethylenediaminetetraacetic acid solution, suspended in phosphate-buffered saline solution, and pelleted by slow centrifugation. The pellets were then fixed in a 2% solution of phosphate-buffered glutaraldehyde at a pH of 7.3 for 1 hr and then postfixed for 1 hr in similarly buffered 1% osmium tetroxide solution. After dehydration through graded solutions of ethanol, they were embedded in epoxy resin.

In addition, several specimens at the late transfer levels were fixed in monolayer cultures grown in plastic petri dishes or flasks and embedded in situ as follows. The cell layer, after being washed with phosphate-buffered saline solution, was fixed with phosphate-buffered 2% glutaraldehyde solution for 30 min, followed by 1% osmium tetroxide in the same buffer solution for 1 hr; dehydrated rapidly with ethanol; and embedded in epoxy resin. The epoxy resin layer was made approximately 5 mm in thickness to assure its transparency. After polymerization of epoxy resin, the original plastic surface was removed by immersion in acetone. The embedded monolayer cultures were examined under a conventional light microscope or phase-contrast microscope. Selected small pieces of the areas containing giant cells with inclusion bodies were cut out, then re-embedded, and cut parallel to the plane of the cell surface in thin sections.

Thin sections were cut on a Porter-Blum MT-1 microtome and stained with lead citrate and uranyl acetate. The examination was carried out under a Siemens Elmiskop 1 electron microscope.

For cytochemical analysis, the cells were seeded on cover slips and, when they reached confluence, fixed in 4% buffered formaldehyde solution (Veronal-acetate buffer, pH 7.2, for 24 hr). The cells were then stained with hematoxylin-eosin, cresyl violet, methylene blue, or Feulgen stain. After extraction in absolute ethanol, the periodic acid-Schiff stain was used for demonstration of carbohydrate components.

Fluorescent-antibody staining was performed on cells which had been seeded on cover slips, washed twice in phosphate-buffered saline solution, and fixed in acetone at room temperature. Indirect staining technique was applied by using sera from patients with SSPE or convalescent sera from patients with measles, followed by fluorescein-isothiocyanate-conjugated rabbit serum prepared against human immunoglobulin G.

## RESULTS

The abnormal intracellular components most frequently encountered in the 8th through the 12th transfer levels in the JAC and LEC cells and in the 22nd transfer level in ROB cells were nuclear bodies (Fig. 9, NB). They consisted of

central aggregates of dense particles surrounded by a ring structure of fibrillar material. At the later transfer levels, when a cytopathic effect characterized by the formation of syncytia and multinucleated giant cells had become pronounced (Fig. 1), many cells, particularly the giant cells, were seen to contain nucleocapsids. They were most frequently present in the nuclei, but occasionally they were also seen in the cytoplasm (Fig. 3–5, 7–8, 10–11).

Individual nucleocapsids, at a high magnification, had hollow structures (Fig. 8, arrows), which appeared more distinct in cross section, characterized by regular beads on the outer surface of the nucleocapsids approximately 4 nm apart (Fig. 8). The outer diameter of the nucleocapsids was approximately 15 to 17 nm, and the inner diameter was 5 to 7 nm. The length of the tubules was variable because of their random position in the thin section, but the longest measured more than 500 nm.

**Cytoplasmic changes.** In the cytoplasm, three major changes were noted: (i) nucleocapsid formation associated with what appeared to be granular substance, (ii) granular filaments, and (iii) occasional increase in lysosomes.

In most cases, the nucleocapsids formed either large aggregates (Fig. 3, NC) or bundles of a few nucleocapsids (Fig. 4, arrows) always located in the ground substance of the cytoplasm. They were never seen within the cytoplasmic vacuoles. The large aggregates were usually well demarcated from the rest of the cytoplasm by the rough and smooth endoplasmic reticulum surrounding them (Fig. 3), and bundles of the nucleocapsids were embedded in granular substance, which occasionally replaced the cytoplasm around the nuclei and beneath the plasma membrane (Fig. 4, G). Some cells appeared to be in the final stage of degeneration and contained fewer nucleocapsids which were sparsely scattered throughout the cytoplasm, loosely intermixed with the cytoplasmic organelles. The nuclear and plasma membranes were still well preserved and there was no evidence suggesting that the nucleocapsids had migrated into the cytoplasm from the nucleus.

The granular filaments were a constant feature of the cells that had nuclear or cytoplasmic nucleocapsids and they usually occupied a vast area of the cytoplasm (Fig. 5, GF). Their structure was essentially tubular and they resembled the nucleocapsids. However, their outer diameter, 20 to 25 nm, was larger than that of the nucleocapsids, and fine granular material attached irregularly to their outer surface made their appearance somewhat ill-defined (Fig. 5, insert).
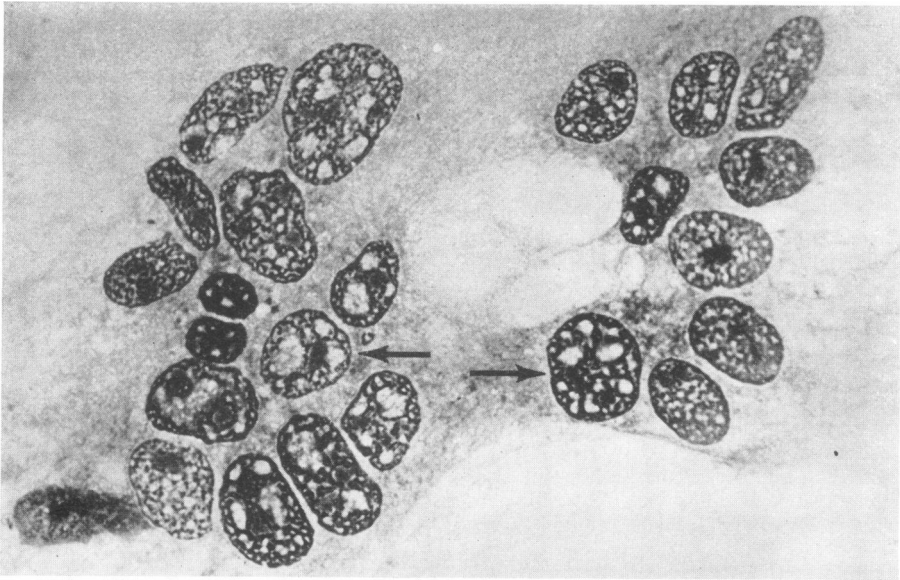
Finally, an increase in the lysosomes was noted

FIG. 1. *Multinucleated giant cell, showing intranuclear eosinophilic inclusion bodies (arrows). Hematoxylin and eosin stain.* × *640.*
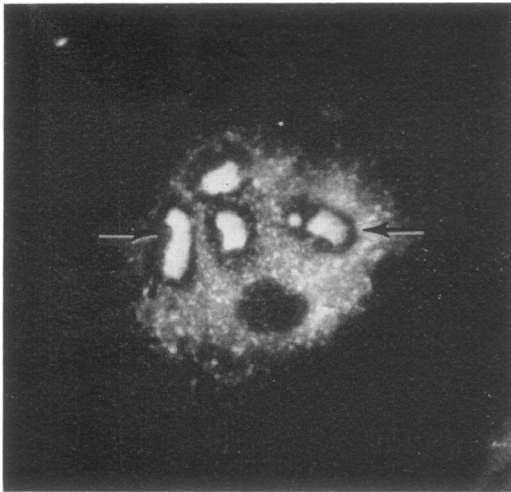


FIG. 2. *Multinucleated giant cell, showing fluorescent intranuclear inclusion bodies (arrows).* × *400.*

(Fig. 5, L). In some cases, lysosomes were scattered throughout the entire cytoplasm among the nucleocapsids and granular filaments.

In the ROB cells in the early cell transfer levels, nucleocapsids, nuclear or cytoplasmic, were readily observed. However, by the 28th cell transfer level, they were no longer seen. ROB cells at the 24th to 26th cell transfer level showed an accumulation of papova-like virions (1) in the cytoplasm (Fig. 6). The individual particles consisted of capsomere substructure without a

limiting membrane. They measured approximately 60 nm in diameter.

**Nuclear changes.** The intranuclear nucleocapsids in most instances formed an inclusion that occupied almost the entire nucleus, sparing only narrow areas beneath the nuclear membrane and around the nucleoli (Fig. 7). The nucleocapsids were arranged in a random manner, tightly or loosely packed, with occasional formation of parallel arrays of the nucleocapsids in the sections (Fig. 8). The nucleocapsids in the nuclei of mononuclear cells were not organized as inclusion bodies but formed foci of tangles or bundles of nucleocapsids scattered within the karyoplasm. Some of them were surrounded by the same ring structures of fibrillar material as were seen in the nuclear bodies. In rare cases, typical nuclear bodies contained the nucleocapsids in bundles inside the ring structures (Fig. 10, arrows; 11). Some cells showed swelling of the nuclei with a marked reduction of chromatin granules; the nucleocapsids were diffusely and sparsely scattered throughout them, but the nuclear membrane was well preserved.

In all of the cells examined under the electron microscope, no particles suggesting virions of a paramyxovirus could be recognized. Nor was there any feature of budding of viruses on the surface of the cells with nuclear or cytoplasmic nucleocapsid formation.

**Correlation with cytochemistry and immunocytology.** The relationships among the cytochemical, immunocytological, and electron microscopic
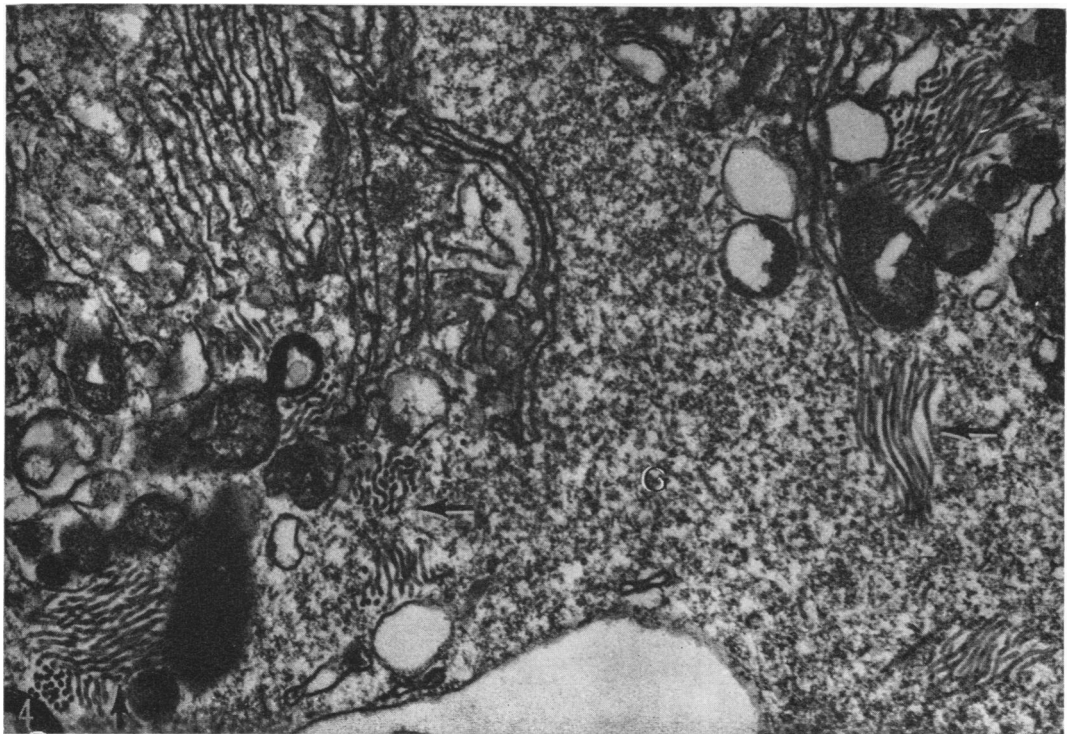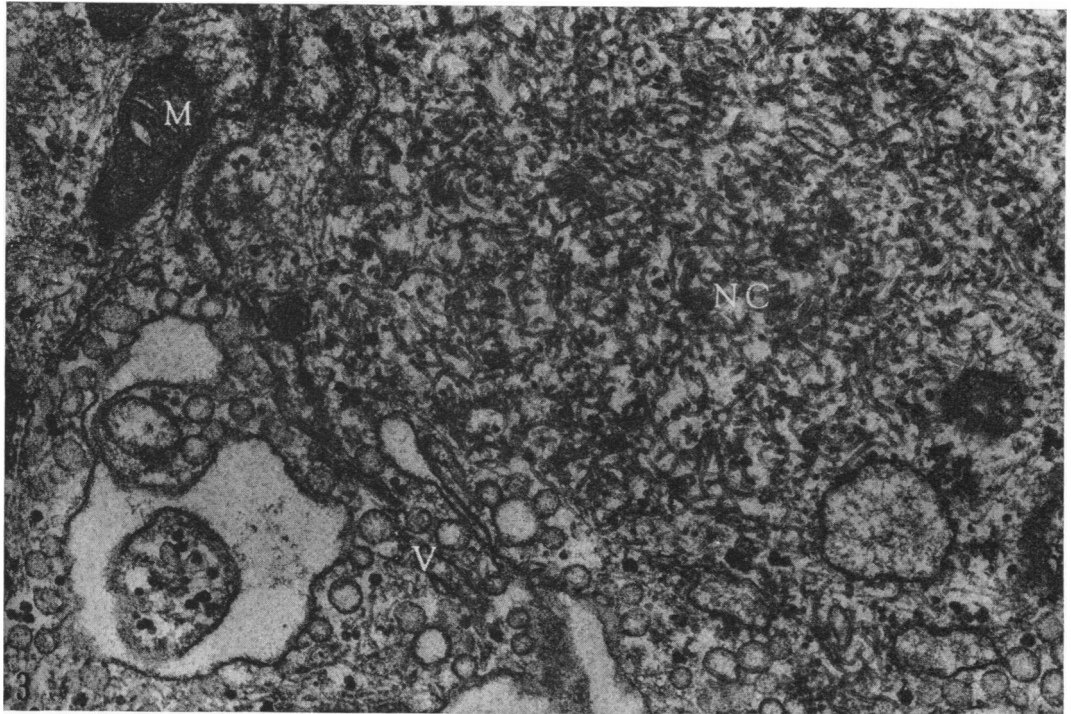
FIG. 3. *Aggregate of nucleocapsids forming an inclusion body in the cytoplasm. NC, nucleocapsid, V, cytoplasmic vesicle; M, mitochondria. JAC, 13th transfer level.* × *50,000.*

FIG. 4. *Nucleocapsids in an array, embedded in the granular substance. G, granular substance. ROB, 24th transfer level.* × *40,000.*
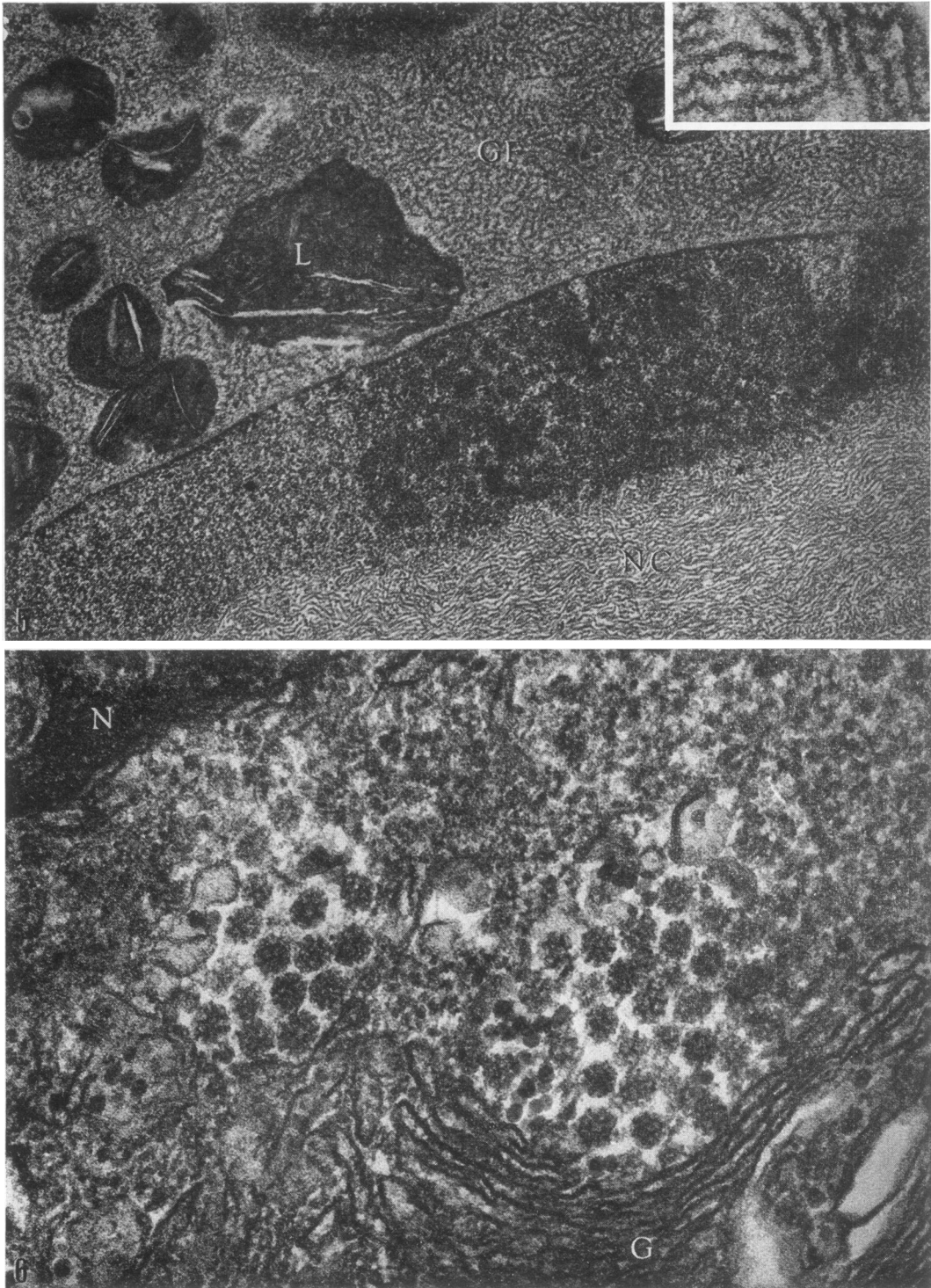
FIG. 5. *Nucleocapsids in the nucleus and the granular filaments in the cytoplasm. Note granular and ill-defined appearance of the granular filaments. NC, nucleocapsid; GF, granular filaments; L, lysosome. JAC, 19th transfer level. Embedded in situ.* × 20,000. *Insert: high magnification of granular filaments. Note granular material attached to the surface of nucleocapsids. Embedded in situ.* × 80,000.

FIG. 6. *Papovavirus-like particles. They consist of capsomere-like subunits without a limiting membrane. N, nucleus; G, Golgi apparatus. ROB, 26th transfer level.* × 100,000.

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FIG. 7. *Aggregates of nucleocapsids forming nuclear inclusions in a multinucleated giant cell. Their configuration is comparable to that of the eosinophilic nuclear inclusions seen under light microscope. NC, nucleocapsids; NO, nucleolus. Embedded in situ. ROB, 26th transfer level.* $\times$ *20,000.*
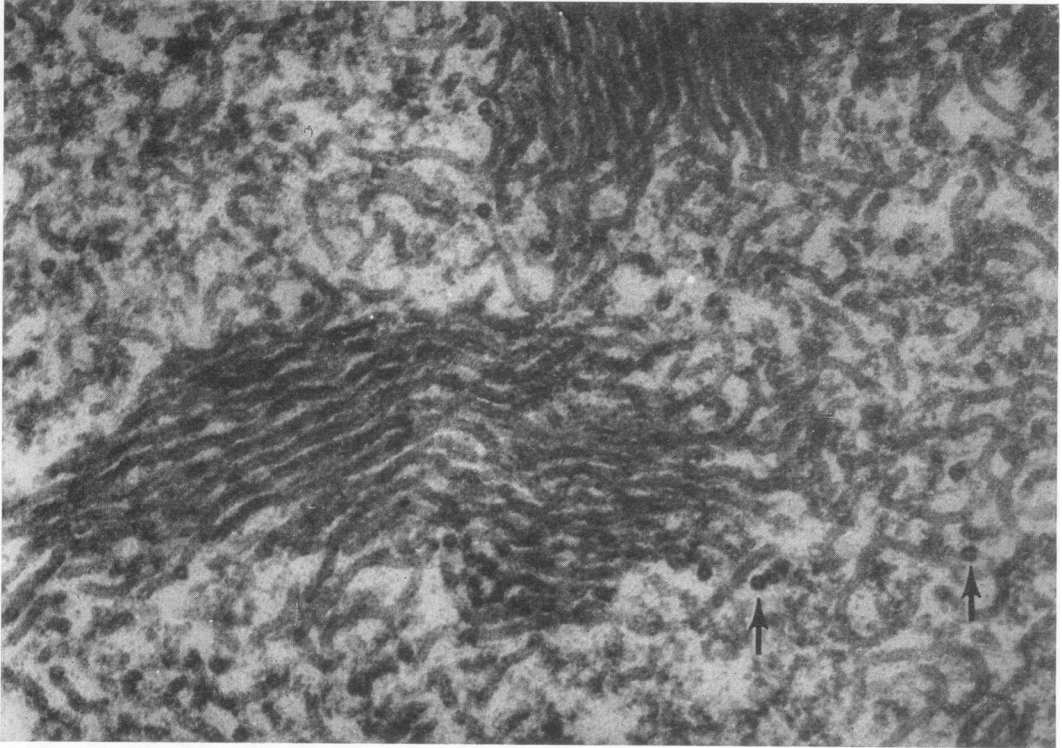
FIG. 8. *Parallel arrays of nucleocapsids in a nuclear inclusion. JAC, 13th transfer level.* × *120,000.*

observations are summarized in Table 1. The nuclear inclusions containing nucleocapsids were of the same configuration as the inclusions in which intense fluorescence was seen in the nuclei of the cells by the immunofluorescence technique (Fig. 2, arrows). The same areas contained eosinophilic inclusions (Fig. 1, arrows) and ribonucleic acid (RNA)-, protein-, and periodic acid-Schiff stain-positive inclusions. There were no deoxyribonucleic acid inclusions. The granular filaments seen by electron microscopy corresponded to the areas characterized by diffuse fluorescence. The cytoplasmic nucleocapsid inclusions corresponded to the areas where periodic acid-Schiff stain-, protein-, and RNA-positive inclusions were seen by cytochemistry.

## DISCUSSION

Evidence of the presence of tubular structures resembling nucleocapsids in the nuclei of cultured brain cells derived from SSPE brains was reported recently by Chen et al. (3) and by us (8). Their morphological identity with those seen in the brain tissue from which the cells originated was also pointed out in these reports. Subsequent investigations of the two cell cultures reported by

us (JAC and LEC) and a third culture (ROB) revealed that the nucleocapsids were present not only in the nuclei, but also in the cytoplasm, and that there were other cytoplasmic filaments. Infectious viruses were isolated from the JAC and LEC cells after these cells were fused with African green monkey kidney cells (AGMK) in the presence of inactivated Sendai virus (1). Subsequently, the AGMK cells were infected with the SSPE viruses and they contained the same type of nucleocapsids as described previously (1).

The ultrastructural characteristics of the cells in culture were identical with those previously seen in the original brain tissues (S. Oyanagi, L. B. Rorke, and M. Katz, *in preparation*) that gave rise to these cultures. The major site of nucleocapsid concentration was the nucleus, but complete virus particles were not seen. Nuclear bodies appeared only occasionally. In this regard, these cells resembled one type of measles-infected cells, primary rhesus monkey kidney cells, described by Nakai (14), in which intranuclear nucleocapsids predominated but no budding viral particles were seen and the titer of infectious virus was low ($10^4$ plaque-forming units/ml).

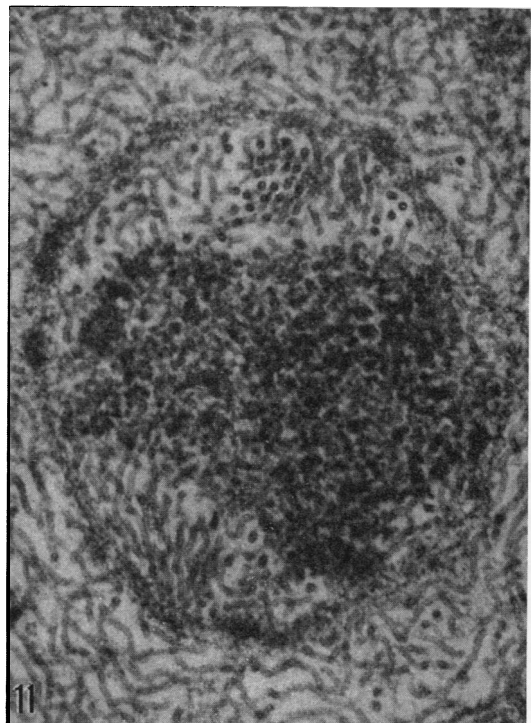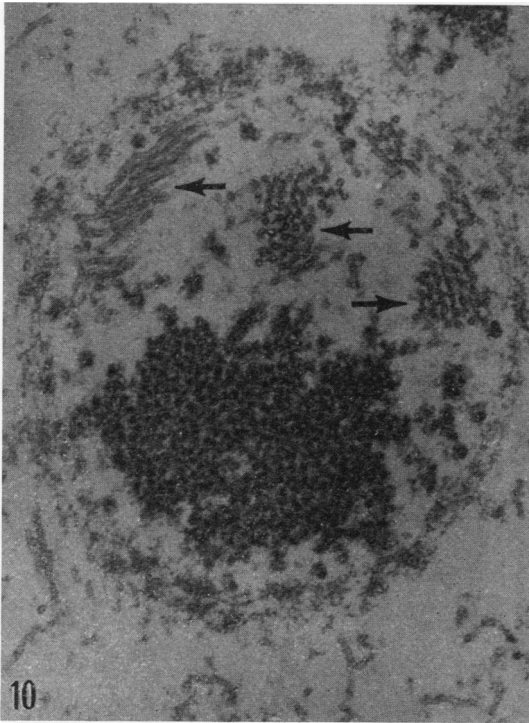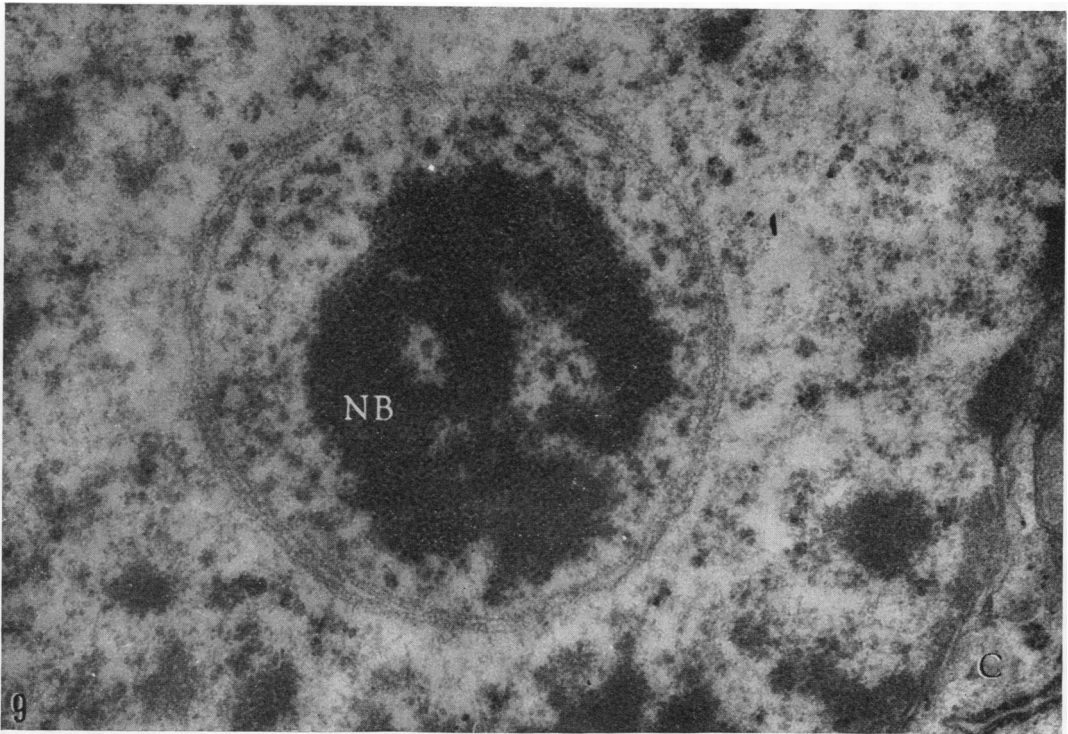Nuclear bodies seen especially often in the 8th

FIG. 9. *Nuclear body seen at the 9th cell transfer level in JAC. It consists of a central aggregate of electron-dense granules and fibrillar material surrounding it. NB, nuclear body; C, cytoplasm.* × 30,000.

FIG. 10. *Nuclear body containing nucleocapsids in arrays inside the ring structure. JAC, 13th transfer level.* × 47,000.

FIG. 11. *Nuclear body within an inclusion, showing its association with the nucleocapsids. JAC, 19th transfer level. Embedded in situ.* × 60,000.

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Table 1. *Correlation of electron microscopic observations with cytochemical and immunological findings*[a]

| Giant cells | Electron microscopy | Cytochemistry | Fluorescence microscopy[b] |
|---|---|---|---|
| Cytoplasm | Nucleocapsids<br>Granular substance<br>Granular filaments | Inclusion of PAS +, protein +, RNA +, DNA −<br>Diffuse staining of PAS, protein, RNA | Diffuse cytoplasmic staining |
| Nucleus | Nuclear bodies<br>Nucleocapsid inclusion<br><br>Nuclear bodies with nucleocapsid | Inclusion of PAS +, protein +, RNA +, DNA − | Fluorescence within inclusion bodies |

[a] Abbreviations: PAS, periodic acid-Schiff stain; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

[b] In the presence of serum of a patient with SSPE.

to 9th transfer levels of the cells, before the appearance of nucleocapsids, have also been reported in SSPE brains (16) and were found in the brain tissue of our three cases (S. Oyanagi, L. B. Rorke, and M. Katz, *in preparation*). It may well be that they represent early foci in the formation of nucleocapsids, since our present study showed nuclear bodies containing nucleocapsids inside of the ring structures.

When the nucleocapsids were observed in the cytoplasm, forming primarily large aggregates, tangles, or bundles, they were embedded in granular substance. It may be that this substance is formed as the earliest event and that this is followed by the appearance of the nucleocapsids. Ultimately, the whole matrix may be replaced by the nucleocapsids. The fact that no virions could be found in the cells correlates with our failure to isolate infectious virus from these brain cell cultures.

The granular filaments seen in the cytoplasm were larger in diameter and more granular in appearance than the nuclear or cytoplasmic nucleocapsids. This relationship was also observed in Vero cells infected with the Edmonston strain of measles virus, as demonstrated by Matsumoto (11). In their tubular structures, these filaments resemble the nucleocapsids but are larger. Since these structures were the predominant change in the cytoplasm of the cells and since they consistently filled the cytoplasm, it is possible that they represent the antigenic material detected by immunofluorescence.

The correlation of the presence of nucleocapsids within the inclusions in the nucleus, positive immunofluorescence of these inclusions, and the cytochemical detection of RNA and proteins within them supports our assumption.

The relationship to SSPE and the nature of the papova-like virions, observed by us in brain cell cultures but not in the brain tissue itself, remain a matter of speculation (9) and are the subject of our current studies.

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