PARAMYXOVIRUS-LIKE INCLUSIONS IN SYSTEMIC LUPUS ERYTHEMATOSUS

Electron Microscopic and Cell Culture Studies

Ken Hashimoto and Robert W. Chandler

From the Veterans Administration Hospital and the Department of Medicine (Dermatology and Rheumatology), the University of Tennessee School of Medicine, Memphis, Tennessee, USA

Abstract. The paramyxovirus-like inclusions that have been described previously in several connective tissue diseases were found in extracellular spaces bound in the unit membranes, in the nuclei of vascular endothelial cells, in dermal fibroblasts, in melanophages, in nerve cells and in epidermal keratinocytes of the lesions and normal skins of ten cases of acute systemic lupus erythematosus (SLE). Tissue culture studies of a typical discoid lesion of a chronic discoid LE patient who subsequently developed SLE revealed that more than 90% of the cells contained large inclusions whose ultrastructural characteristics were identical with those in the lesion. Both vascular endothelial cells and dermal fibroblasts grown in the tissue culture contained large inclusions free in the cytoplasm. Budding forms were also detected. Characteristics of these inclusions found both in the tissue and cultured cells were similar to those of many known paramyxoviruses.

The presence of paramyxovirus-like inclusions in the vascular endothelial cells and fibroblasts in the skin has been reported in discoid lupus erythematosus (DLE) (3), systemic lupus erythematosus (SLE) (10, 17), dermatomyositis (6) and a few other conditions (5). The exact nature of the inclusion has not been clarified. The relation between the inclusion and the activities of disease processes in DLE and dermatomyositis and its widespread existence in SLE, including the normal skin (17), were cited as evidence in favor of a viral nature of the inclusion. The presence of the inclusions within the rough endoplasmic reticulum, dimensions of tubular structures (200–250 Å) which are slightly larger than the known paramyxoviruses, absence of intranuclear inclusions and extracellular particles and widespread existence of the same or similar structures in apparently unrelated condition (8, 16), were, however, thought to contradict the viral theory (3, 10, 6).

Investigation was continued on acute SLE and on the inclusions in tissue-cultured cells grown from the biopsied material of a skin lesion of chronic SLE. This revealed membrane-bound inclusions in the extracellular spaces, intranuclear inclusions and intracytoplasmic inclusions in melanophages, nerve cells, epidermal keratinocytes and sebaceous gland cells. We wish to describe these new findings in this report.

MATERIALS AND METHODS

Case 1. A 59-year-old white male was admitted to the Memphis Veterans Administration Hospital in October, 1970, with erythematous macular lesions with early atrophy on exposed surfaces of the face, anterior chest and forearms. Hemoglobin was 9.2, WBC 4 100, LE cell tests negative and fluorescent antinuclear antibodies (FANA) weakly positive. Chest films showed a right upper lobe mass. Muscle enzymes and electromyograms were normal. He underwent a right thoracotomy with resectional biopsy of the right upper lobe mass. Histologic diagnosis was bronchogenic carcinoma, poorly differentiated.

Case 2. A 22-year-old negro female was admitted to the City of Memphis Hospitals in August, 1970, with fever, lethargy, cough and skin rash. On physical examination there were discoid lesions on the face and arms and hemorrhagic macular lesions on the palms and soles. Laboratory studies showed: Hemoglobin was 10; WBC 15 400; FANA positive at 1 : 256, LE cell tests negative; proteinuria 4 + ; strong basement membrane area and perivascular fluorescence of normal skin and of a lesion using direct fluorescent antibody technique. Acute renal failure developed. LE cell tests remained negative. FANA was positive at a titer of 1 : 1024. She failed to respond to high doses of steroids and expired April 24, 1971.

Case 3. A 14-year-old negro female was admitted to St Jude Hospital in Memphis, Tennessee in August, 1970, with complaints of weakness, generalized pain, fever and chest discomfort. On physical examination, she had purpuric and hyperpigmented macular lesions on the malar areas, palmar surfaces and elbows and had profound generalized muscle weakness. She had hepatosplenomegaly. Pertinent laboratory data included hematocrit 22.5, FANA positive at 1 : 1024, LE cell test positive, Coombs' test positive. Direct fluorescent antibody staining of specimens of normal skin and a lesion showed a strong fluorescence at the dermo–epidermal junction and in perivascular basement membrane.

Case 4. An 11-year-old negro male with generalized small pruritic vesicular eruptions of 6 months duration was admitted to the City of Memphis Hospitals in December, 1967. His father had suffered from pemphigus vulgaris for many years. Serum drawn for indirect immunofluorescence demonstrated basement membrane zone fluorescence with titer of 1 : 160. A glucose-6-phosphate dehydrogenase deficiency was found. In October, 1969, a severe arthralgia, butterfly erythema of the face, generalized lymphadenopathy and fever started. Biopsy from the cheek showed histopathological changes compatible with SLE. Shortly thereafter, a new crop of subepidermal blisters similar to the previous one appeared. Antinuclear factor and LE cell tests were positive. Patient was diagnosed as SLE and kept on prednisone, 15 mg/day, with remission. In July, 1970, acute exacerbation occurred and patient died. Autopsy confirmed SLE changes in many organs.

Case 5. This case was reported as case 3 of our previous DLE study (3). He was a 48-year-old negro male with typical DLE lesions for 12 years on the face, scalp and extremities. About 1 year prior to the present biopsy he developed SLE with positive FANA, left optic atrophy due to retrobulbar neuritis and aseptic necrosis of the femoral heads. (Aseptic necrosis was thought probably due to steroid therapy.) At the time of re-biopsy of the discoid lesion for cell culture studies, patient was on parenteral prednisone, 10 mg every other day.

Cell cultures were set up on March 19, 1971. After $3\frac{1}{2}$ months of good growth, during which the electron microscopic observations always demonstrated the "SLE inclusion body" the culture began to slow down.

Other cases. Six more cases of SLE with typical clinical pictures and courses and positive FANA and LE cells were included in this series. Specimens were obtained from typical lesions as well as from non-sun-exposed areas, mostly from the buttocks.

Electron microscopic methods

Several specimens were taken with a skin punch from each patient from involved as well as from normal skin, in most cases from the buttocks. The routine specimen preparation method of our laboratory (3, 6) was followed.

Cell culture methods

A two millimeter punch biopsy with sterile technique was performed on a skin lesion near the right elbow of the case 5 on March 19, 1971. The area was prepared with tincture of merthiolate followed by 95% ethanol. After transport to the laboratory in Hanks balanced salt solution (BSS) containing 100 units each of penicillin and streptomycin, the fragment of tissue was subjected to two 30-min trypsinizations in 0.2% trypsin (1 : 250) in BSS with gentle agitation using a magnetic stirrer. The epidermis was not removed prior to trypsinization. The free cells from each trypsinization were washed once by centrifugation at 400–500 rpm and resuspended in 12 ml of growth medium. This suspension was then placed in 250 ml square screw-cap bottles (Kimble no. 14250) and incubated with caps loose in 5% CO_2 and 100% humidity at 35–36°C.

Growth medium. Twenty per cent fetal bovine serum (Flow Laboratories, Lot No. 455352) and 100 units each of penicillin and streptomycin were added to medium 199 (Flow Laboratories) containing glutamine.

Cover glass cultures. Corning 22×22 mm cover glasses, No. $1\frac{1}{2}$ thinness, were washed in dilute Haemasol, well rinsed and stored in 95% ethanol. Prior to use, the cover glasses were individually flamed and placed in 35×10 mm Falcon petri dishes. A cell suspension was prepared by scraping of the culture bottles with a curved capillary pipette or a Teflon policeman. Three-tenths ml of the cell suspension, appropriately diluted in growth medium consisting of equal parts of conditioned and fresh medium, was then added to the cover glass. This volume, with reasonable care in handling, would not overflow the cover glass into the plate. The next day, after all cells had settled and adhered to the cover glasses, an additional 1.0 ml of media was added to the plate. Incubation was carried out at 35–36°C in 5% CO_2 and 100% humidity.

Preparation of cell cultures for electron microscopy. The growth medium was replaced with fresh medium one to two days before removal of the cells from the bottle. For removal, all but 1–2 ml of medium was decanted, and then the cells were scraped off the glass with a curved capillary pipette or Teflon policeman. The cell suspension was then mixed with 10 ml BSS in a 12 ml conical centrifuge tube and centrifuged at 400–500 rpm for 4 min. After removal of all but approximately 0.05 ml supernatant, 2 ml of cold 5% glutaraldehyde in 1/10 M cacodylate buffer, pH 7.2, was carefully layered over the small clump of cells. The fixation was allowed to continue for 1–2 hours at 4°C, and then floating cells were sedimented by low-speed centrifugation for 10 min to make a solid pellet. The pellet was divided into 1 mm^3 cubes and placed in the same buffer and rinsed overnight at 4°C and then post-fixed with 1% osmic acid in the same buffer for 30 min. After dehydration with 50% ethanol, the cell cubes were stained with 1% uranyl acetate in 50% ethanol for 15 min. Dehydration was carried out thereafter with higher concentrations of ethanol and propylene oxide and embedded in Araldite. Thin sections, 400–600 Å, were cut on a Porter-Blum MT-2 ultramicrotome and stained with 15% uranyl acetate in 50% methanol and then with Reynolds lead citrate (13). Stained sections were observed in an Hitachi HU-11C electron microscope.

Hematoxylin and eosin and methylgreen-pyronin stains. Cells grown on several cover glasses were stained with hematoxylin and eosin (11) as well as with the methylgreen-pyronin method for ribonucleic acid (12).
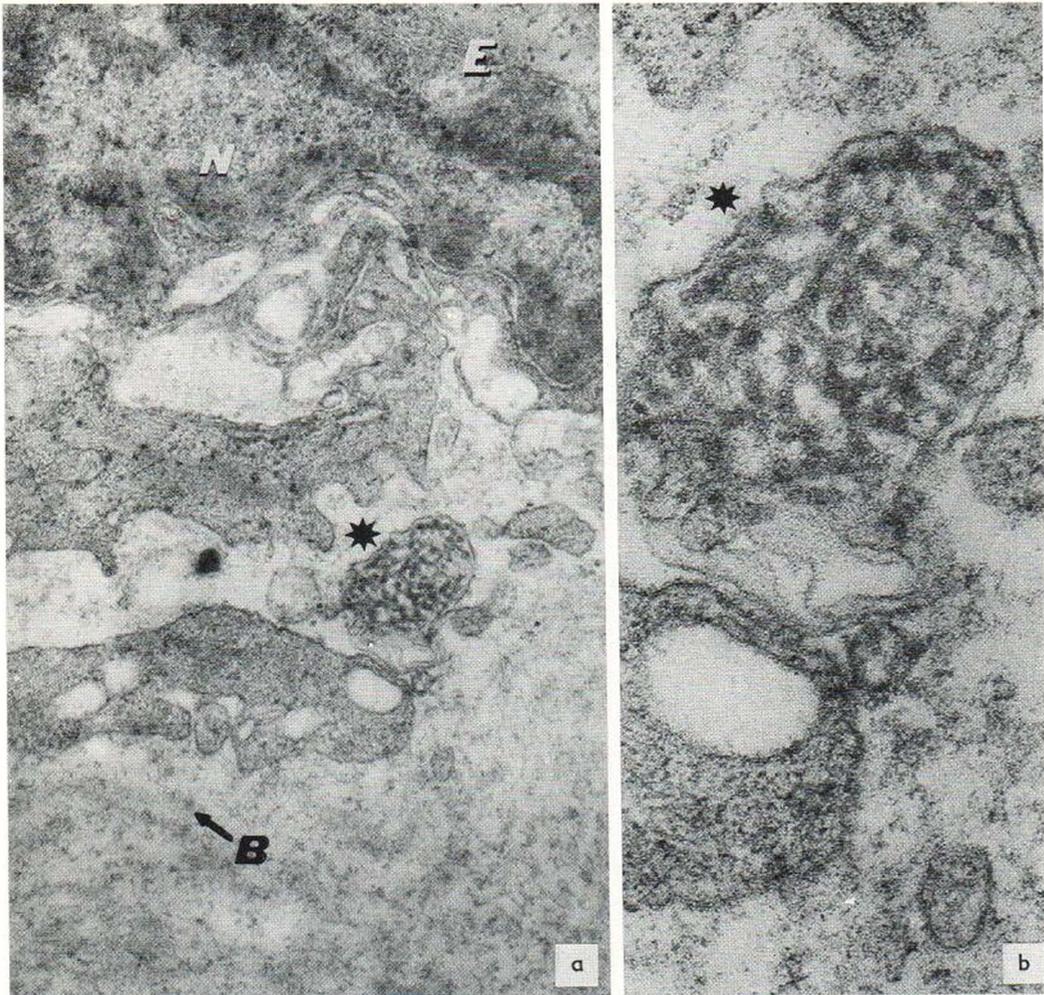
Fig. 1. Extracellular particle (*) containing tubular structures is seen in the vicinity of a vascular endothelial cell (*E*). Upon enlargement (*b*) this particle is seen surrounded by a membrane. *B*, Perivascular basal lamina; *N*, nucleus of an endothelial cell. Case 1, lesion. Left: × 93 250. Right: × 126 750.

Controls. Fibroblasts were cultured from two normal skin biopsies and one skin lesion of a patient with primary systemic amyloidosis. The same media and the techniques described above were employed.

RESULTS

Tubular inclusions were positive both in the lesions and in the normal skins of all 10 cases except case 5. The involved tissue, however, usually contained more inclusions than the normal skins. Sizes and fine structural characteristics were the same as those described in other connective tissue diseases (3, 10, 6). The distribution of the inclusions was mainly in the vascular en-

dothelial cells, perivascular cells and dermal fibroblasts but in some cases also in the perineurial cells, dermal phagocytes, including melanophages, epidermal keratinocytes and sebaceous cells of the sebaceous gland. In general, the more acute disease processes were associated with a greater number of inclusions in both the lesion and the normal skin.

Extracellular inclusions

In the lesion of acute SLE, membrane-surrounded tubular structures were observed in periendothelial spaces (Fig. 1), in the vicinity of dermal fibroblasts and within the vascular lumen. In
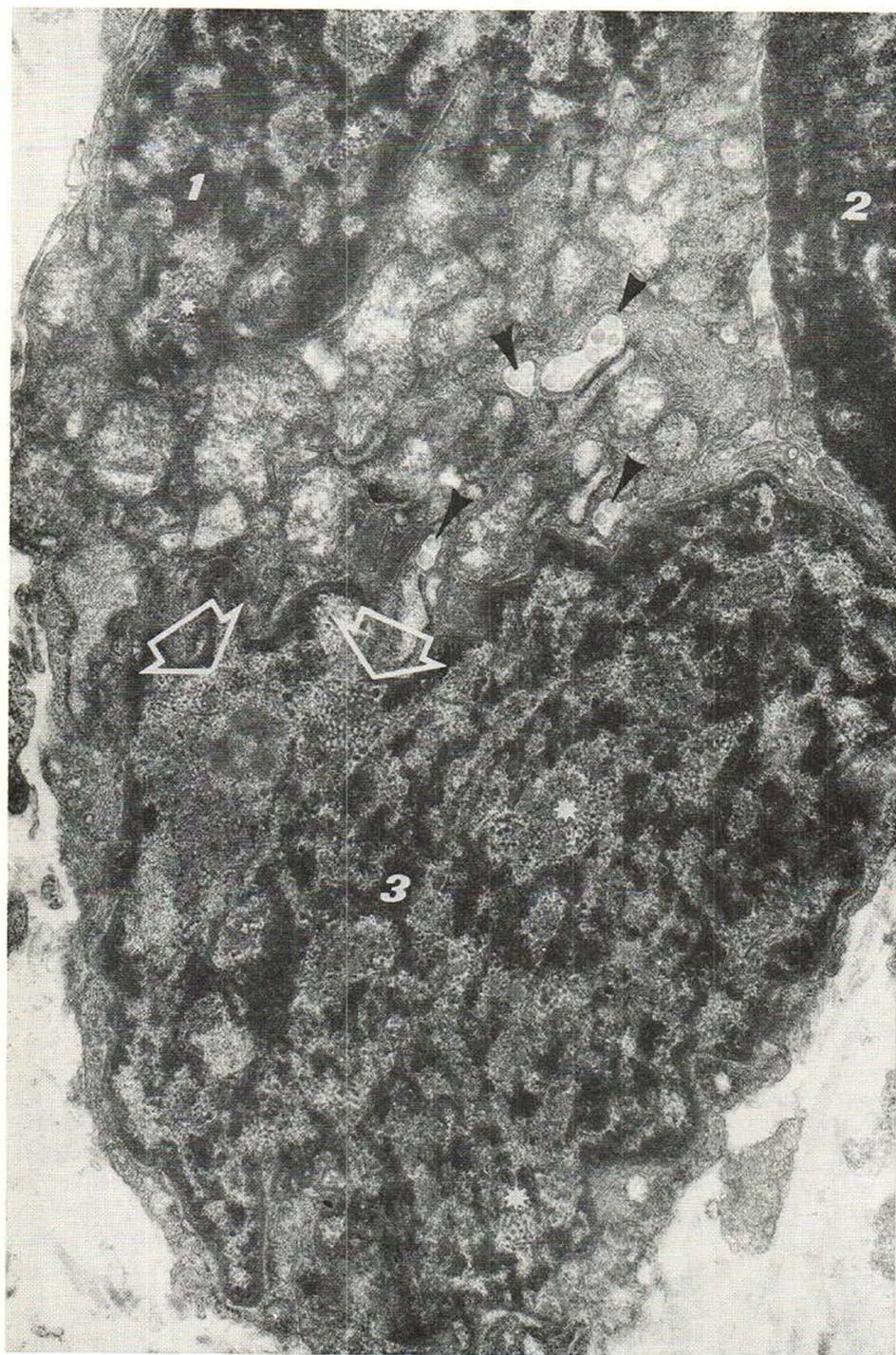
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Fig. 2 a. A multinucleate cell contains three nuclei (1, 2, 3). They may represent cross-sections of a convoluted nucleus or, more probably, nuclei of three separate cells whose cytoplasm fused to form a syncytium. The presence of collagen fibrils (arrowheads) within the cytoplasm favors this interpretation. Another possible interpretation would be that cytoplasmic division did not follow the nuclear division. A number of intranuclear tubular inclusions are seen (*). Case 2, lesion × 25 550.

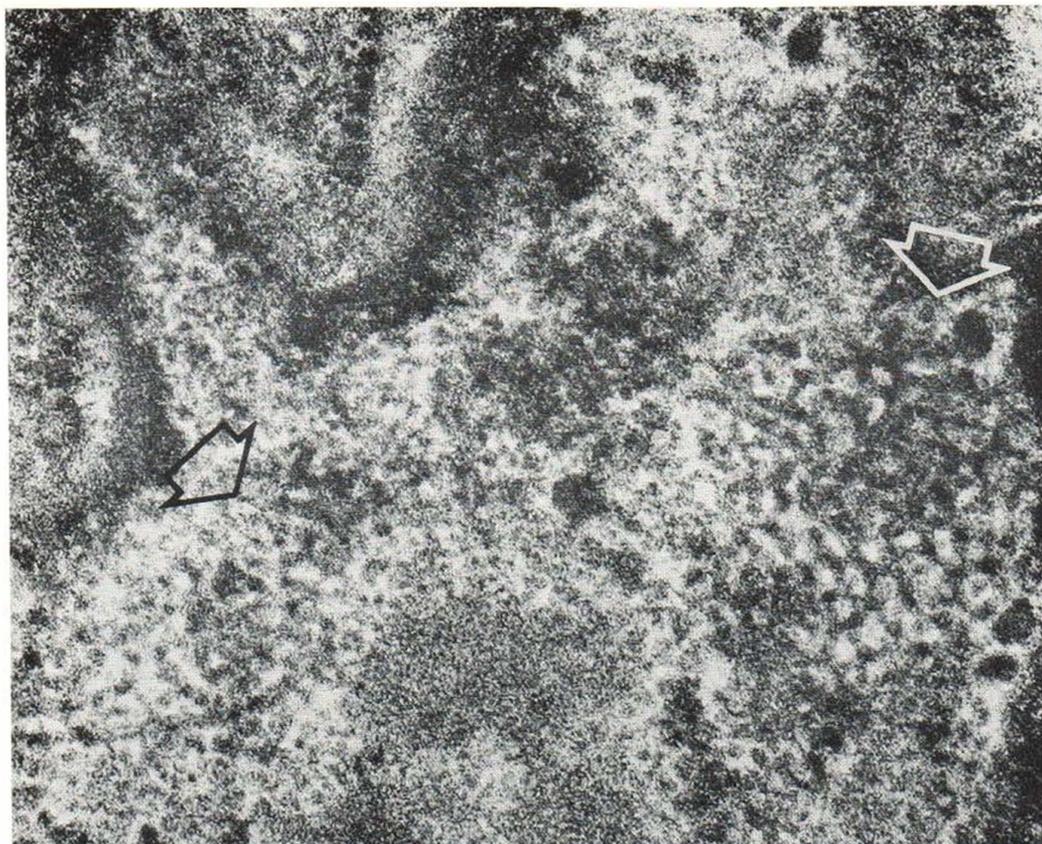*Fig. 2 b.* Those tubular structures marked by outline arrows are enlarged. Case 2, lesion × 119 750.

some instances there were many lysosomes and degenerative changes of endothelial cells and other cells were evident; it was therefore assumed that the release of inclusions was a concomitance of cellular degeneration. On the other hand, the membrane that surrounded the tubular structures was always smooth in contrast to the ribosome-studded rough endoplasmic reticulum which enveloped the intracellular inclusions. It was thought, therefore, that most of the extracellular inclusions were produced by budding from the host cell, and the surrounding membrane represented the plasma membrane (Fig. 1). In the normal skin these extracellular particles were found less frequently.

Intranuclear inclusions

Inclusions were found within the outer and inner nuclear membranes, i.e., in the perinuclear cisternae. Intranuclear inclusions without communication with the perinuclear cisternae were frequently found in the vascular endothelial cells and also in the dermal fibroblasts (Figs. 2, 3). Some inclusion-positive dermal cells were multinucleated (Fig. 2). Since collagen fibrils were enclosed within the cytoplasm of some of these cells, it was thought that they were produced by the coalescence of individual cells and, therefore, represented syncytial giant cells (Fig. 2). The intranuclear inclusions were also found in the vascular endothelial cells and dermal fibroblasts in the normal skin of all SLE patients. The inner nuclear membranes of inclusion-positive nuclei were often thickened (Fig. 3). Similar thickening of the inner nuclear membrane was observed in some cells which did not contain inclusions. Obviously, it was difficult to rule out the possibility that at some level other than that of the section, such inclusion-negative cells might actually contain inclusions.

Fig. 3. Two tubular inclusions (arrows) are seen in the nucleus of a fibroblast. The inner nuclear membrane is thickened (between triangles). C, Collagen. Case 2, ×93 250.
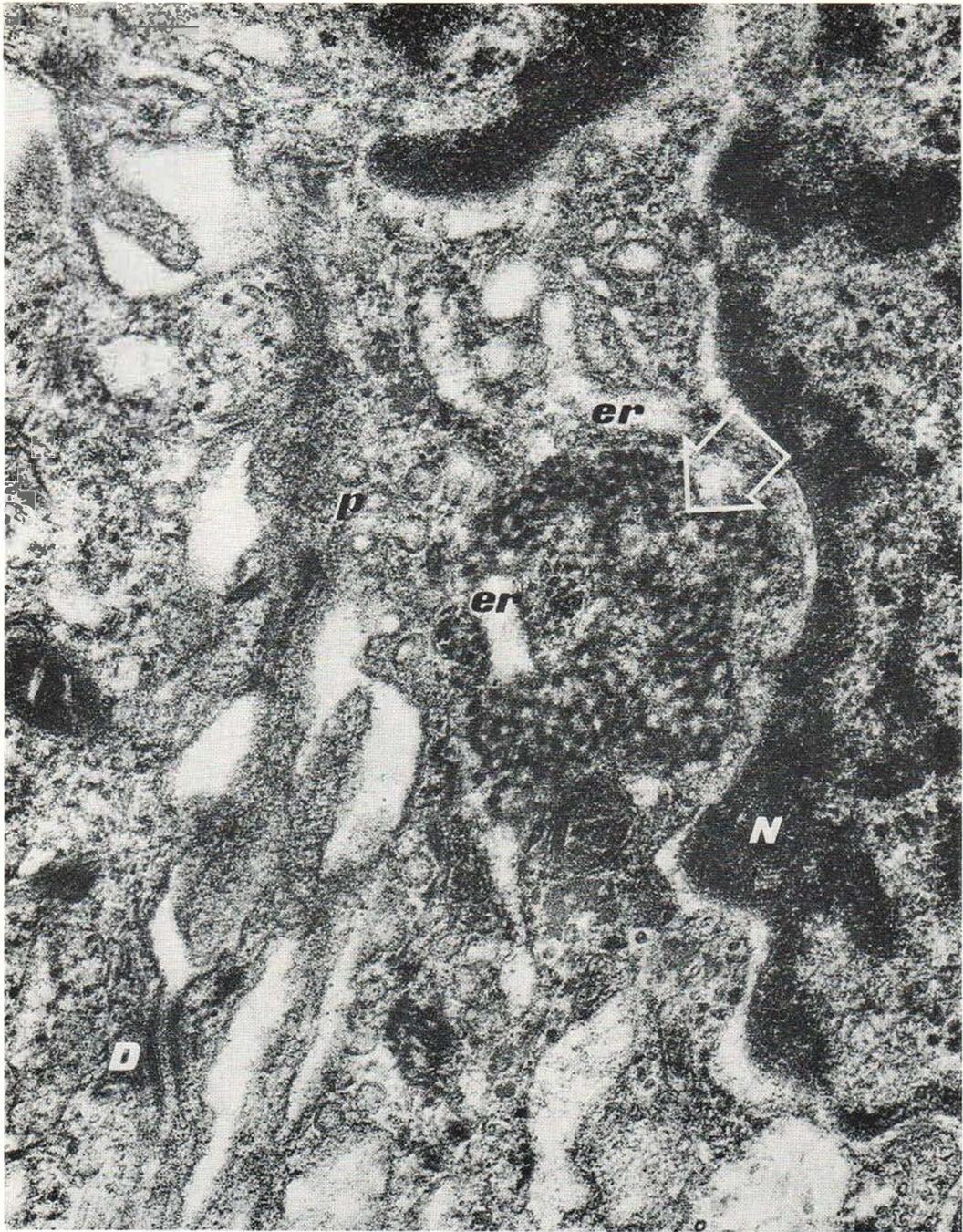
Fig. 4. A tubular inclusion (arrow) is present in a *Malpighian cell* of the epidermis. It is apparently located outside of rough endoplasmic reticulum *(er)*. *D*, Desmo-some; *N*, nucleus; *p*, pinocytotic vesicle. Case 2, lesion × 75 000.
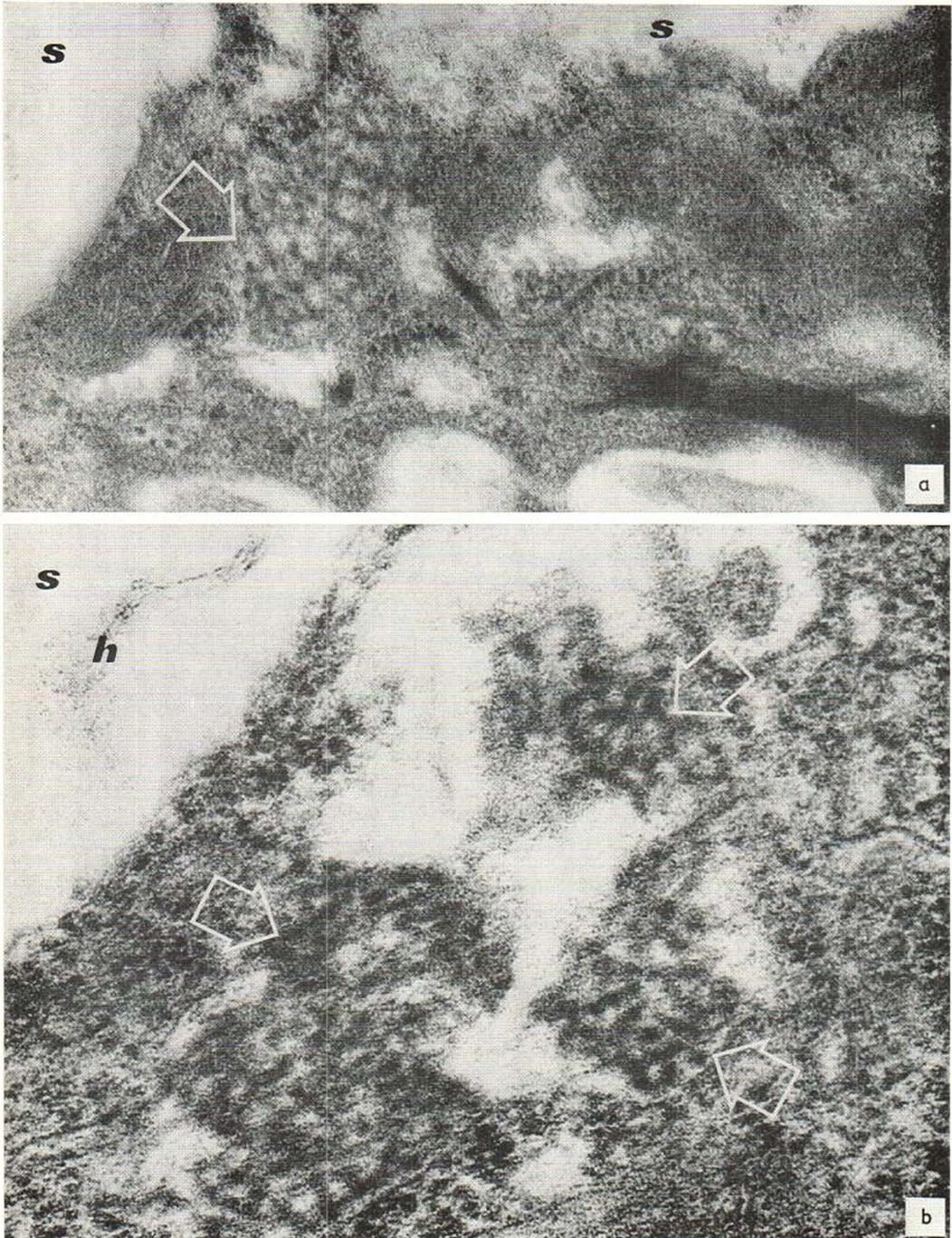
Fig. 5. Tubular inclusions (arrows) in sebaceous cells are located free in the cytoplasm. h, Husk; s. sebum. Case 2, lesion (a) × 74 440. (b) × 98 750.

Inclusions in the epidermis and sebaceous glands

The tubular inclusions identical with those found in the vascular endothelial cells and dermal fibroblasts were present in the basal cells and Malpighian cells of the epidermis (Fig. 4) and in the sebaceous cells of the sebaceous glands (Fig. 5) of case 2. The main localization was free in the cytoplasm and only occasionally in the rough endoplasmic reticulum. Inclusions were also found in the nuclei of these cells.

Phagocytes and perineurial cells

Dermal phagocytes, including melanophages, also contained intracytoplasmic as well as intranuclear inclusions (Fig. 6). Less frequently, perineurial cells of the cutaneous nerves also contained the inclusions (Fig. 7).

Cell culture of skin lesion

The skin lesion of case 5 cultured *in vitro* did not show attachment and spreading for 4 days. Initial multiplication was observed at the sixth day. Of the cells obtained from trypsinization of approximately 1–2 mm³ tissue, only 5–10 cells originally survived and multiplied. Many cells were spread extensively over the cover glass (Fig. 8). When observed with the light microscope, approximately 30% of these cells contained 0.5–1.0 μ sized intracytoplasmic inclusions which stained greyish with hematoxylin and eosin and pinkish with the methylgreen-pyronin method for ribonucleic acid. Some cells contained more than one inclusion. Intranuclear inclusions were occasionally observed.

Electron microscopic examination of cells taken at 7th week from a monolayer of the original culture bottle revealed that the majority of the cells were fibroblasts of well developed rough endoplasmic reticulum, extended cytoplasm with numerous peripheral villi (Fig. 9) and the presence of amorphous and thin filamentous material in the vicinity of the cells (Fig. 9). Within such material thicker fibrils were admixed (Fig. 9). The amorphous component formed a basal lamina-like layer surrounding some cells, and such cells often formed half desmosome-like densities along their plasma membranes (Fig. 9). However, these cells could be fibroblasts since fibroblasts in embryonic mesenchyme (4) and in amyloid tissue
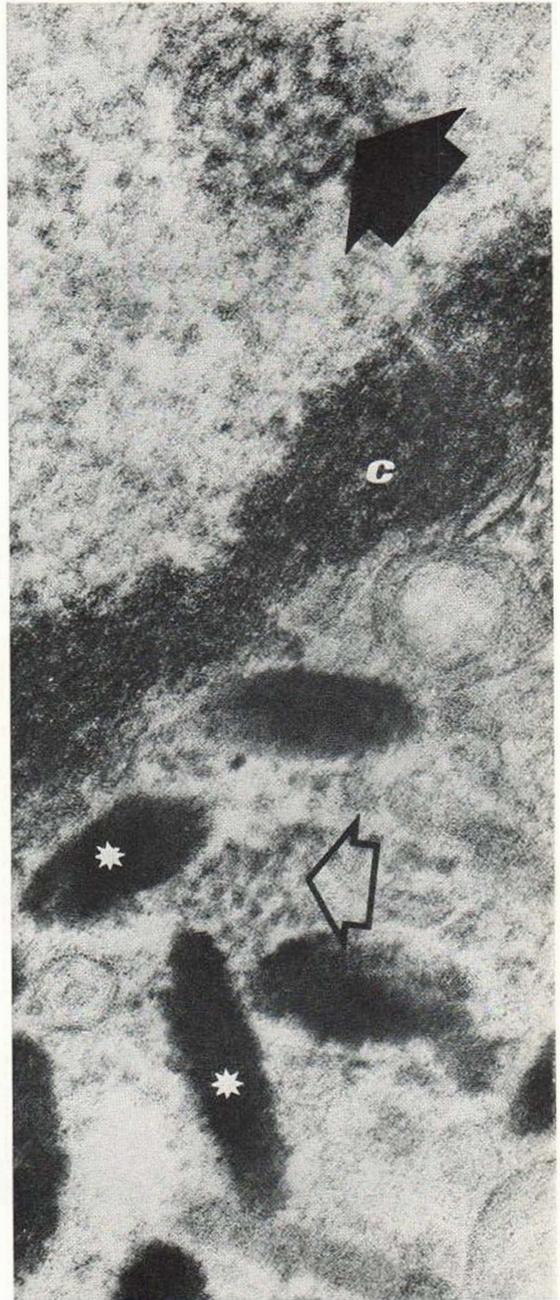

Fig. 6. Intranuclear (solid arrow) and cytoplasmic (outline arrow) inclusions are seen in a dermal melanophage which phagocytosed a number of melanosomes (*). *c*, Heterochromatin. Case 1, lesion × 102 000.

(7) can form desmosomes and half-desmosomes. Other cells were connected to each other with desmosome-like junctions, and were considered to

Fig. 7. A perineurial cell of myelinated (*M*) peripheral nerve in the dermis contains an inclusion composed of fairly straight tubules (*). Case 2, lesion × 44 250.

have derived from vascular endothelial cells. When checked with the electron microscope, approximately 90 % of the cells harvested from the primary culture contained one or more inclusions of various size. The average diameter of the tubular component of these inclusions measured uniformly about 200 Å (Fig. 10). Intranuclear

inclusions of similar dimensions were occasionally observed in the cultured cells. These tubules were frequently branched and anastomosed with each other. The morphology of the tubular structure found in the cultured cells was, therefore, the same as that of the inclusions in the lesion. The inclusions of cultured cells were, however, mainly

located in the cytoplasm without direct relation to either rough or smooth endoplasmic reticulum (Figs. 9, 10), whereas in the biopsied lesion of DLE (3), SLE (10) and dermatomyositis (6), the majority of the tubular structures were enclosed within the rough endoplasmic reticulum. A possibility was considered that these cells grown in culture derived from the epidermis or the sebaceous gland where the inclusions are usually free in the cytoplasm (Figs. 4, 5). However, these cells contained neither tonofibrils nor sebum.

The majority of the tubular structures in cultured cells seemed to be derived from the free ribosomes in the cytoplasm (Fig. 10) and only occasionally from the rough endoplasmic reticulum by budding into the cytoplasm (Fig. 10). In contrast, in the biopsied lesions, tubules were formed by budding and elongation of the wall of the rough endoplasmic reticulum into the cisternae (3, 6).

Budding of inclusions through the plasma membranes into the extracellular spaces was observed (Fig. 10). The budding proliferation was often contiguous with the intracytoplasmic inclusion (Fig. 10). Free-floating particles containing tubular structures were also detected.

DISCUSSION

Inclusions in lesions

The viral nature of the paramyxovirus-like tubular inclusions in many connective tissue diseases has been postulated (15). If these structures are truly viral in nature, one would expect to see intranuclear inclusions, budding forms produced at the plasma membrane and propagation in suitable tissue culture media, since typical known paramyxoviruses exhibit these features (2, 9). Although our previous efforts (3, 6) as well as those of others (10) failed, we have now succeeded in the demonstration of these features in SLE.

The presence of the identical tubular structures in the epidermal keratinocytes may well be taken as evidence of infectious spread by means of the extracellular "infective" particles rather than as a cellular reaction product. Schmitt et al. also showed similar inclusions in the epidermal keratinocytes of a SLE (?) patient. In the epidermal keratinocytes, rough endoplasmic reticulum is poorly developed, in contrast to that of the vascular endothelial cells and dermal fibro-
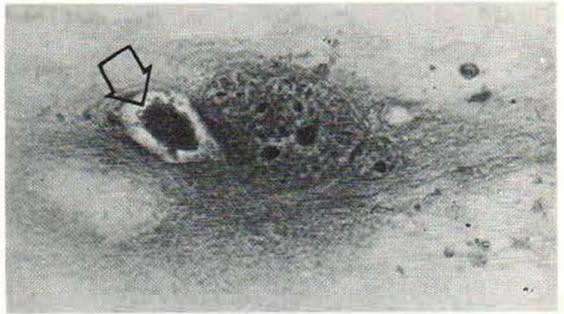

Fig. 8. Hematoxylin and eosin stained cultured cell shows a fairly large (about 0.5–1.0 μ in long diameter) cytoplasmic inclusion (arrow). Case 5, lesion × 1 100.

blasts. It is hard to conceive that they produced a large number of inclusions from this organelle, as other types of cells did, in response to a common stimulus. We do not believe that all of the hydropic degeneration of the basal cells of the epidermis, hair follicle and sebaceous gland are due to the presence of the inclusions; but in acute SLE, at least some of the epithelial changes as seen by the light microscope could be caused by the inclusions.

We do not deny that at least some of the extracellular particles could have been released from degenerated host cells. However, the majority must have been formed through budding since the intracellular inclusions are enclosed in rough-surfaced endoplasmic reticulum and are fairly large in size (3, 6). In contrast, the extracellular particles as demonstrated in this study, were all enveloped by smooth trilaminar membranes, presumably the host cell membranes (Fig. 1). Also, they were much smaller than the average intracellular inclusions.

The presence of these inclusions in the normal, unexposed skin may indicate the widespread nature of this "infection." On the other hand, the fact that such "infected" skin does not manifest clinical symptoms until exposed to ultraviolet rays or other stimuli points out three important possibilities: First, the body may not recognize the "virus" as such, and therefore allows "infection" to become widespread. Second, ultraviolet rays may stimulate this "virus" as in the case of herpes simplex infections. Third, ultraviolet rays may rupture the lysosomal membrane, release hydrolytic enzymes and initiate the vascular, connective tissue and epidermal damages.

Fig. 9. Several tubular inclusions (hollow arrows) are seen free in the cytoplasm of a cultured fibroblast (*F*). Inclusion-positive cell in the left upper corner is surrounded with amorphous (*a*) and fibrillar (*f*) material. Amorphous material forms a basal-lamina-like layer (arrow) in close apposition to the wall of this cell. The cell forms half desmosome-like membrane densities (*). This cell, therefore, shows characteristics of endothelial cells. Case 5, lesion, × 29 400.
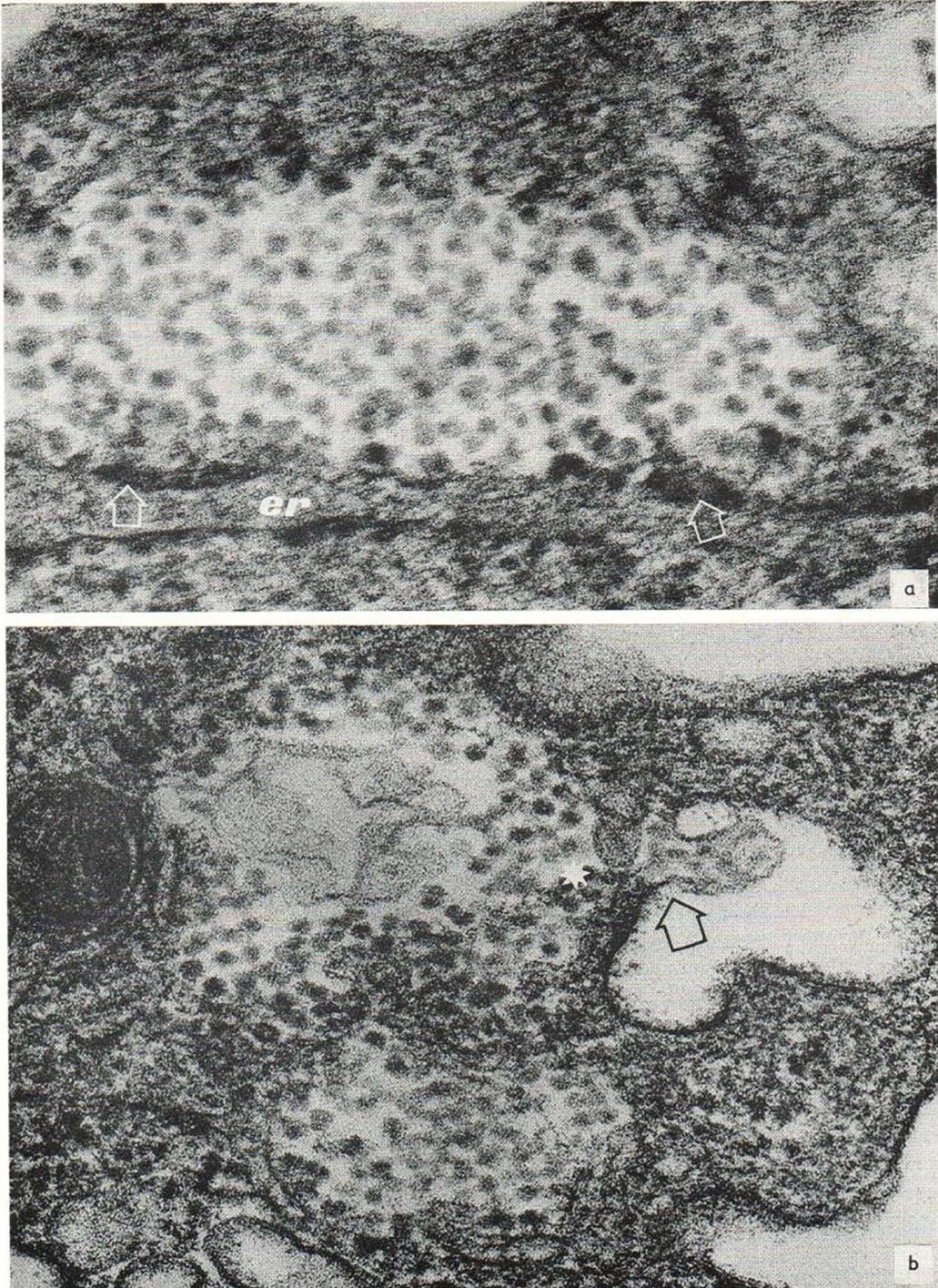
Fig. 10. (*a*) Tubular structures are being produced by budding into the cytoplasm (arrow) from the ribosomes on the rough-surfaced endoplasmic reticulum (*er*). × 122 200. (*b*) Tubular inclusions are seen budding through the plasma membrane into the extracellular space (arrow). This inclusion is contiguous with that in the cytoplasm (*). Case 5, lesion × 131 000.

In various skin diseases which have been suspected to be of viral etiology, such as keratoacanthoma (18), the intranuclear inclusions were related to nucleoli. Schmitt et al. (14) also demonstrated tubular inclusions in nucleolus of a fibrocyte from the lesion of SLE (?). They were not uniformly tubular, rather irregular in shape and dimensions, and not always present; whereas those found in this study were identical with the intracytoplasmic tubular inclusions. It is noteworthy that most of the known paramyxoviruses pass through the nuclear phase during their growth cycle (2, 9). The formation of syncytial cells (Fig. 2) is also one of the characteristics of paramyxovirus-infected cells (1).

Tissue culture inclusions

One may argue that the inclusions demonstrated in cultured cells represented the original inclusions carried over from the biopsied material. However, if the dilution factor involved in the multiplication (about 5 cells to at least several millions) and the fact that over 90 % of these cells contained large inclusions were considered, such an argument is untenable. Furthermore, the inclusions of cultured cells were found naked in the cytoplasm instead of within the rough endoplasmic reticulum. We may conclude, therefore, that small numbers of inclusions transferred to the daughter cells during mitosis have subsequently grown in the cytoplasm, as typical paramyxovirus particles do, or "infective" particles produced by budding indeed infected the daughter and neighboring cells. Although the third possibility would be that the latent form of "virus" which was eclipsed and invisible in some cells *in vivo* manifest in the tissue culture system, this factor should not have played a major role since the cells surviving in the original culture were very small in number. One of the great advantages of tissue culture studies was that so many cells containing inclusions could be prepared together and observed in the same section. In this system we could confirm those various stages of development and release of this "virus" in the same preparation, whereas they had been seen only in the lesions from several patients. We are reasonably certain that those phenomena observed in the lesion were not artifacts; fibroblasts from the normal skins and from an amyloid lesion cultured in the same media did not contain the inclusions.

Another obvious advantage of tissue culture study is that we could observe the characteristics of this "virus" in a system separated from the immunological influences and other defense mechanisms of the host and in a cell-line most suited for their growth. The fact that the inclusions were still present in the cultured cells indicates that influences of body metabolism, disease processes, etc. were not inducing these inclusions, i.e., these inclusions were most likely not the product of cellular toxicity, but rather inherent agents in these cells. Thus, a small number of fit cells originally survived the vigorous preparative procedures and began to harbor a large number of "virus" inclusions. The localization of the inclusions free in the cytoplasm instead of being confined within the endoplasmic reticulum may, therefore, reflect the true growth pattern of this virus. In known paramyxoviruses, the inclusions are also present naked in the cytoplasm rather than in rough-surfaced endoplasmic reticulum (9).

The tubular structures may contain ribonucleic acid, as all known paramyxoviruses do, if the inclusions stained with methylgreen-pyronin method are the same as those seen by the electron microscope. Derivation of tubular structures from free ribosomes (Fig. 10) which are predominantly composed of ribonucleic acid supports these histochemical findings. It was previously demonstrated that the inclusions in the biopsied lesions were susceptible to ribonuclease and stained heavily with uranyl acetate (6).

In summary, we believe that this study provided a few additional pieces of evidence in support of the viral theory of these inclusions. In order to relate these "viral" inclusions to the etiologies of the diseases, we must be cautious because even if they are true viruses, it is still possible that they could be passenger viruses which do not cause the diseases.

ACKNOWLEDGEMENTS

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Ken Hashimoto, M.D.
The Veterans Administration Hospital
1030 Jefferson Avenue
Memphis, Tennessee 38104
USA

Addendum:

One inclusion-positive culture from case 5, whose growth rate markedly diminished at $3\frac{1}{2}$ months, is still being maintained with three subcultures and shows inclusions after 12 months. The other two positive cultures continue to contain inclusions at 5 and 6 months and the negative ones have remained so for 6–8 months. These observations seem to further support the vertical transmission hypothesis of these inclusions and rule out the toxic product theory.