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VIRUS PARTICLES (LEUKOSIS/SARCOMA GROUP LIKE VIRUSES) IN THE PERIPHERAL NERVE AND THE SKELETAL MUSCLE TISSUES WITH MAREK'S DISEASE (FOWL PARALYSIS)

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Two chickens with Marek's disease (MD) (Fowl paralysis) and one apparently normal chicken, which were diagnosed by clinical features and histopathology, were observed by electron microscopy. One bird with lymphoid leukosis was also examined for comparison.

Association and multiplication of virus particles (VP) in the peripheral nerves and the skeletal muscles accompanied by degenerative changes were unexpectedly observed.

Mature VP measuring from 80 to 100 mμ in diameter, covered with double membranes (inner and outer), had an electron opaque central nucleoid measuring 40 to 55 mμ and had numerous peripheral knobs on the outside of the particles. In the cytoplasm, there was viroplasm consisting of immature small VP (virospheres, from 70 to 80 mμ) surrounded by granules of the size of ribosomes (virosomes), viral matrix consisting of aggregates of electron opaque granules, and gray bodies, involving a few VP, surrounded by a single membrane. These VP matured by budding from the cytoplasmic membrane. Numerous VP were found in the lumen and intercellular spaces of the capillaries, in the endothelial cells, in the pericytes, in the intracytoplasmic vesicles of emigrating macrophages, in the axon, in the basement membrane of the unmyelinated nerve fibers, in the nerve cells, in the intercellular spaces of satellite cells, and in the sarcoplasm and basement membranes of the skeletal muscles.

These VP at present under investigation seemed to be similar to those of the leukosis/sarcoma group of viruses, because of their morphology and mode of multication.

INTRODUCTION

It has been suggested that the etiological agent of Marek's disease (MD) is a cell-associated, herpes-type virus which has been demonstrated in vitro by several authors (CHURCHILL & BIGGS 1967, NAZERIAN et al. 1968, SOLOMON et al. 1968). Recently, however, virus particles (VP) of this type were found in the epithelial cells of the kidneys of a chick which had contracted MD by natural transmission (SCHIDLOVSKY et al. 1969). On the other hand, SIMPSON (1969) observed VP, by electron microscopy, which appeared to be similar to those of the leukosis/sarcoma group of viruses in the irises of chickens with spontaneous

ocular leukosis. Although there were some reports on viral association in the chick tissues affected with MD (BERNHARD 1958; CAMPBELL 1966; WIGHT et al. 1967; GLASER et al. 1969), no reports have been found on the appearance of either virus of the leukosis/sarcoma group and herpes type in the nerves and the skeletal muscles.

During the electron microscopic investigation of 12 cases of MD and 4 cases of apparently normal chickens which were diagnosed by histopathology, we could find out VP which were similar to the leukosis/sarcoma group of viruses in the peripheral nerves and the skeletal muscles from 2 cases of MD and one case of an apparently normal chicken. This report describes the fine structure of the VP and the virus cell relationships by electron microscopy.

MATERIALS AND METHODS

The materials were taken from chickens of the following three groups: two cases of so-called "classical MD" (case Nos. 1 & 2), one case of apparently normal chicken (case No. 3) and one case of lymphoid leukosis (LL) (visceral lymphomatosis) (case No. 4) to act as a comparison. All birds were female White Leghorns (Heisdorf line). Two cases of MD were 60 days old (case No. 1) and 147 days old (case No. 2), respectively. One case of an apparently normal chicken (case No. 3) was 72 days old, and one case of LL (case No. 4) was 213 days old. Chickens affected with MD (case Nos. 1 & 2), LL (case No. 4) and the apparently normal chicken were cases from some poultry farms in the suburbs of Sapporo.

Although the birds of MD showed paralysis of the legs, the other two birds (case Nos. 3 & 4) did not show any motor disturbances. The materials for histopathology were obtained from as many parts of the various visceral organs and nerve tissues as possible. All materials were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin and sometimes Luxol-Fast-blue stain for myelin sheath. For electron microscopy, the materials were taken from the peripheral nerves (lumbosacral plexus, brachial plexus, cervical and lumbar spinal ganglia) and the skeletal muscles (biceps femoris, gastrocnemius muscle and peroneus longus). In the case of LL (case No. 4), the liver, the spleen and the kidneys were examined. The materials were fixed in 1% osmium tetroxide containing 0.054 g. sucrose per ml, buffered with phosphate, adjusted to pH 7.4 (MILLONG), and after dehydration in graded alcohols, they were embedded in a plastic mixture (MOLLENHAUER 1964). Sections were made with a Porter-Blum ultramicrotome equipped with glass knives, mounted on platinum grids, stained successively with uranyl acetate and lead citrate, and examined by JEM-7 electron microscope. Thick sections of approximately 1 μ were stained with toluidine blue for orientation by light microscopy.

OBSERVATIONS

1) Gross lesions

The peripheral nerve trunks, especially the lumbosacral plexus and the brachial plexus of chickens with MD (case Nos. 1 & 2) were edematous and swollen. In one part of the

nerve fibers, loss of striation was demonstrated. Skeletal muscles of the same case were thin and relatively pale cloudy, and sometimes accompanied by petechial hemorrhages. Although no remarkable changes were observed in the visceral organs of the apparently normal chicken (case No. 3), the subcutaneous tissues and the skletal muscles around the tibio-metatarsal joints were greenish, and there were edema and hemorrhages. In the case of LL (case No. 4), the liver and the spleen were highly enlarged and numerous minute white foci were distributed. The kidneys were accompanied by scattered millet-sized white foci. However, no remakable changes were found in the peripheral nerves.

2) Microscopic lesions

Case No. 1 The most remarkable distribution of cellular lesions in the peripheral nerves was seen in the brachial plexuses and their dorsal root ganglia, and then, observed less remarkably in the lumbosacral plexuses (fig. 1) and their dorsal root ganglia. In the brachial plexuses, perivascular cuffs with a focal accumulation of small or medium sized lymphocytes and histiocytes were seen. A diffuse infiltration of lymphocytes and plasma cells was also seen between the neurites, and edema and loss of nerve fibers were associated in one part of the nerves. In the dorsal root ganglia, focal lymphocytic cell accumulations, loose cellular infiltration, and shrinkage or tigrolysis of nerve cells were sporadically observed. In addition, autonomic nerves of the visceral organs had slight cellular infiltration. On the other hand, no remarkable changes were mentioned in the visceral organs, except vascular cuffs composed of small lymphocytes. Fresh or old desolative changes (degeneration and coagulative necrosis) of muscle fibers accompanied by hemorrhages were sometimes observed in the leg and thoracic muscles. Muscle fibers in the fresh lesions showed loss of striations, swelling, hyalinization, coagulative necrosis with fragmentation and liquefaction or lysis. In more advanced lesions, diffuse proliferation of histiocytic cells was seen in the interstitial tissues as a cellular reaction. The sarcolemmal nuclei apparently increased in number in some parts of the muscles and regenerative processes of the muscle fibers were occasionally pointed out. In one part of the leg muscles, some of the muscle fibers showed severe atrophy.

Case No. 2 One part of the lumbosacral plexus was completely destroyed and replaced by a massive proliferation of pleomorphic lymphoid cells of various sizes (fig. 2). The nerves were accompanied by proliferation of Schwann cells and axonal swelling in some parts. Lesions of the other kinds of peripheral nerves were almost similar to those in case No. 1. In the skeletal muscles, the most severe lesions were observed in the superficial pectoral muscles. Severe hemorrhages, coagulative necrosis with fragmentation of muscle fibers and intermuscular proliferation of histiocytic cells were markedly observed. In the other kinds of muscles, generally fresh desolative changes (degeneration and coagulative necrosis) were sporadically observed and perivascular lymphocytic accumulations were mentioned in one part of the muscles. There were no remarkable changes in the other visceral organs.

Case No. 3 Almost all of the peripheral nerves were normal except that a slight loss and degeneration of nerve fibers was recognized in one part of the brachial plexus. Some of the nerve cells of the ventral horns of the spinal cord showed tigrolysis. Lesions of the skeletal muscles were almost similar to those in case Nos. 1 and 2 (fig. 3). No

remarkable changes were observed in the other visceral organs.

Case No. 4 The most severe lesions were seen in the liver (fig. 4) and the spleen. The liver consisted of multiple, irregularly shaped, nodular cell foci of various sizes. They occupied almost all of the liver and the liver parenchyma remained only as a lace appearance with vacuolar degeneration. The cells composing these foci were uniform lymphoid cells (hemocytoblastic). They were large in size, with large nucleoli and basophilic cytoplasm possessing irregular cytoplasmic projections. The nucleus had a vesicular appearance as a whole. Mitotic figures were often scattered in the proliferative areas. Proliferated lymphoid cell masses surrounded by thin connective tissue capsules were numerously scattered in the liver. The liver lesions were of a distinctly nodular character and sometimes had a follicular appearance. The same kind of cellular foci were numerously observed in the spleen, and were also found in the kidneys, and the ovaries. The peripheral nerves were almost normal, except that a slight loss of nerve fibers was observed in one part of the brachial plexus and vagus.

8) Electron microscopy

Peripheral nerves

Numerous VP were found in the lumen, in the intercellular spaces, and in the basement membrane of the endothelial cells of the capillaries in the peripheral nerves (brachial plexus, lumbosacral plexus and their spinal ganglia) of chickens affected with MD (case Nos. 1 & 2, fig. 5) and the apparently normal chicken (case No. 3). VP extracellularly emerged by budding from the cell membrane of the endothelial cells (case No. 2, fig. 5) and pericytes (case No. 1, fig. 6). Especially, in the endothelial cells, microvilli were obvious (case No. 2, fig. 5). A small number of VP were seen in the basement membrane of the unmyelinated nerve fibers (figs. 7 & 10), in the axon (fig. 10), and in the intracytoplasmic vacuoles of emigrating macrophages (figs. 8 & 9). Especially, in the myelinated nerve fibers of the lumbosacral plexus (case No. 1) which was associated with VP, myelin sheaths were severely disorganized and changed into various grades of myelin debris surrounded by a single limiting membrane (fig. 9). Numerous VP were seen in the intercellular spaces between the satellite cells which surrounded the nerve cells of the lumbar spinal ganglia of case No. 1. There were thickening and increased osmiophilia of the invaginated cell membrane in the satellite cell (fig. 11). It seemed that such thickening of the cell membrane might be attributed to pinocytosis. Gray bodies (viroplasts), which were surrounded by a single membrane and were contained with somewhat electron opaque amorphous materials, were found in the cytoplasm of adjoining nerve cell. Within the gray bodies, there were a few VP of varying size (fig. 11).

Skeletal muscles

In the same manner as was seen in the case of the peripheral nerve, numerous VP were observed in the lumen, in the intercellular spaces, and in the basement membrane of the capillaries in the skeletal muscles (biceps femoris, gastrocnemius muscle and peroneus longus) of chickens with MD (case Nos. 1 & 2) and the apparently normal chicken (case No. 3). It was pointed out that numerous VP emerged by budding from the cell membrane of the pericytes in the gastrocnemius muscle in case No. 1 (figs. 17~20). Amorphous

aggregates of electron opaque granules involving a virus-like spherical structure were seen in the cytoplasm of the pericyte (fig. 18). The above described gray bodies (viroplasts) were also found in the cytoplasm (fig. 19). VP were contained within the membrane-bound intracytoplasmic vesicles of emigrating macrophages. Pinocytosis was also seen. Many VP were embedded in the basement membranes of the skeletal muscles, especially near the sarcolemmal nuclei (fig. 12). Furthermore, mature VP numbering up to ten or more had accumulated in the dilated sarcoplasmic reticulum, and budding from the vesicular membrane was seemed to have occured from the findings in many of the muscle cells (figs. 13~16). Condensation of the sarcomere and myolysis was frequently observed in the muscle cells involved VP. Namely, muscular degeneration consisted of contraction of the sarcomere (case No. 2, gastrocnemius muscle, fig. 13), disintegration of Z-line, separation, fragmentation or disappearance of myofibrils and vacuolization of the sarcoplasmic reticulum (case No. 3, peroneus longus, fig. 14).

In the sarcoplasm of the gastrocnemius muscle in case No. 1, there was viroplasm without a limiting membrane, consisting of aggregates of immature small VP (virospheres) measuring about 70 to 80 mμ in diameter, with either an empty or a contained dense nucleoid, and their particles were surrounded by numerous small granules which were considered to be ribosomes (virosomes) (fig. 15). The size of these immature VP almost corresponded with those of the inner ring (inner membrane) of the typical mature VP to be explained later. The diameter of the central nucleoid of the immature VP was from 40 to 55 mμ.

Fine structure of the virus

VP, which were observed in 2 chickens of MD and the apparently normal one, had almost the same morphological characteristics and were considered to be the same kind of virus. Mature VP varied from 80 to 100 mμ in diameter. The particles had an outer membrane and this was covered by a series of many peripheral knobs. The particles appeared to possess an electron opaque central nucleoid measuring 40 to 55 mμ, surrounded by an inner membrane (inner ring), and there was an electron transparent zone between the inner and outer membranes (fig. 21). Some of the VP appeared to have tail-like projections (fig. 22).

VP in bird with lymphoid leukosis

In a bird which was diagnosed as LL by histopathology (case No. 4), numerous VP were found in the intercellular spaces among the proliferated lymphoid cells of the liver, in the intercellular spaces of the trabeculae of the spleen, and in the membrane-bound intracytoplasmic vesicles of the epithelial cells of the kidneys (figs, 23~26). Gray bodies (viroplasts) with double limiting membranes were also observed in the lymphoid cells of the liver (fig. 23). The size and fine structure of VP were identical to those of chickens with MD and the apparently normal one.

Discussion

In regard to the viruses associated with chickens of MD, Wight et al. (1967)

found VP, which were morphologically similar to the RNA viruses associated with the avian leukosis/sarcoma group of diseases, within the cytoplasmic vesicles of lymphocytes from birds affected with experimental acute MD (5/8) and spontaneous cases (2/2). Although, they found VP from one of six uninoculated control birds, they did not find out VP in the nerves, gonadal and renal tumors from spontaneous classical MD (0/3). CAMPBELL (1966) observed particles resembling the RNA virus associated with the avian leucotic conditions in the intracytoplasmic vesicles of lymphocytes from the buffy coat of experimentally transmitted MD, and did not find them in lymphocytes from the blood of clinically normal chickens. BERNHARD (1958) found VP resembling those of visceral lymphomatosis, in the spleen and the bone marrow affected with neurolymphomatosis. Recently, SIMPSON (1969) observed VP in the basement membrane of striated muscle cells, in membrane bound vesicles in muscle cells, in proximity to pigmented epithelial cells, and in the lumen and basement membrane of endothelial cells of the capillaries of the irises of seven chickens with lesions of spontaneous ocular leukosis. These VP were similar to those of the leukosis/sarcoma group of viruses.

On the other hand, GLASER et al. (1969) observed virus-like particles of approximately 100 mμ and of a dense nucleoid approximately 65 mμ in diameter, in plasma pellets prepared from birds with MD, and lymphocytes from infected birds contained particles in cytoplasmic vacuoles which were similar in morphology to those observed in plasma preparations. They described that the observed VP were not contaminants of the lymphoid leukosis group, because the birds used for experiment were all COFAL-negative. Recently COOK found a COFAL-negative syncytium-producing virus (ca. 100 mμ) which was different from herpes type virus in MD. CHURCHILL & BIGGS suggested the etiological agent of MD as a herpes type virus measuring about 100 mμ in diameter, having an internal nucleoid and external membrane, and accompanied by small particles measuring about 30 mμ in diameter, in sections of cell cultures. The VP were found only in the cytoplasm or extracellular spaces. EPSTEIN et al. (1968) found immature VP which were hexagonal, measuring about 85 mμ in diameter, and which were either empty or contained a central ring-shaped or dense nucleoid, in both the nucleus and the cytoplasm of chicken kidney cultures infected with MD. In addition, they observed larger mature particles, measuring about 130 mμ in diameter and surrounded by an outer membrane in the perinuclear space and within cytoplasmic membrane-bounded vacuoles. The immature particles matured by budding through cellular membranes and acquiring outer coats from these membranes as they passed through. Recently, SCHIDLOVSKY et al. (1969) observed a herpes type virus in the nucleus and cytoplasm of epithelial cells that line the

kidney collecting tubules obtained from a chick with MD. The chick had contracted the disease by direct contact transmission. However, the VP at present under investigation seemed to be similar to those of the leukosis/sarcoma group of viruses judging from the morphological point of view and the mode of multiplication (VOGT 1965).

Amorphous aggregates of electron opaque granules with virus like spherical structures, which were sometimes observed in the cytoplasm of the pericyte (fig. 18), seemed to be similar to those seen by DMOCHOWSKI (1964) in an erythroblast of the spleen of a chicken affected with erythroblastosis. From the morphological view point, these aggregates appeared to be an inclusion (viral matrix) containing immature VP in the developmental stage. With regard to the viroplasm, they had been observed in Rous sarcoma cells (BEARD 1963; DMOCHOWSKI 1964). Their structures consisted of aggregates of osmiophilic small particles of the size of ribosomes (virosomes)[12] and among them spherical structures (virospheres 650 Å)[10] of a size smaller than the complete VP. These structures have been encountered not only in Rous sarcoma cell, but also in tumor cells of erythroblastosis (BEARD 1963), visceral lymphomatosis (DMOCHOWSKI 1965) and nephroblastoma (BEARD 1963). Virospheres were considered as precursor material for the synthesis of the virus (HAGENAU & BEARD (1962). But, BEARD (1963) considered that the virospheres as incomplete virus particles may be formed in an abortive process of synthesis in the cytoplasm in the absence of constituents required for assembly of the complete particles. However, we cannot deny a possibility that virospheres will develop to a complete VP by budding of the cytoplasmic membrane, because the size of the virospheres corresponds to the size of the inner ring (inner membrane) of the complete VP.

The gray bodies, observed in the present investigation, were seen in other avian tumors (myeloblastosis, erythroblastosis, lymphomatosis, nephroblastoma, and so on)[7]. Gray bodies (viroplasts) have appeared to be the sites of virus synthesis in these avian tumors[1, 10]. DMOCHOWSKI (1965) described two possibilities for the existence of the gray bodies: The one possibility is a mitochondrial origin judging from the point of morphology, size and structure; the other possibility is that they developed from the granules or their precursors which appear in the cells of the myeloid series during their normal maturation to the granulocytic series of blood cells. HEINE et al. (1964) described that the derivation of structures with gray body morphology is not yet completely clear, nevertheless, most of the finding indicate a relationship to Golgi structures, which exhibit enzymatic activities similar to those of bodies regarded as lysosomes.

Mature VP within the sacks of the endoplasmic reticulum may be either eliminated to the extracellular spaces or taken into the cytoplasm reversely by

pinocytosis. Extracellular VP may be taken into vesicles of macrophage, and transported to an other place of the same living body, or VP themselves may be digested by macrophages. From the findings of direct elimination of VP from the cytoplasmic membrane of the endothelial cells of the capillaries, it may be assumed that the birds are in a viremia condition.

It is interesting that the cases at present under investigation revealed degenerative changes of the peripheral nerves and the skeletal muscles and viral association at the same time as SIMPSON described in their muscular lesions.

In the present investigation, association and multiplication of leukosis/sarcoma group like virus were seen in two cases of MD and one of an apparently normal chicken. This indicates that infection of this type of virus may usually exist in the wide range of field cases of apparently normal chickens independent of the existence of cellular lesions. As it has been strongly suggested that the etiological agent of MD is a cell-associated herpes type virus, it is interesting to observe whether viral infection at present described may play some role in the factor of pathogenesis of MD or only contaminants in the field cases.

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Explanation of plates

Plate I

Fig. 1 Case No. 1 Lumbosacral plexus
Perivascular cuffs with a focal accumulation of small or medium sized lymphocytes and histiocytes, and a diffuse infiltration of lymphocytes and plasma cells between the neurites H-E × 470

Fig. 2 Case No. 2 Lumbosacral plexus
Neoplastic proliferation of lymphoid cells, and swelling of axons H-E × 470

Fig. 3 Case No. 3 Gastrocnemius muscle
Loss of striations, swelling, hyalinization, coagulative necrosis and liquefaction of muscle fibers H-E × 118

Fig. 4 Case No. 4 Liver
Proliferated lymphoid cell masses which are surrounded with thin connective tissue. Arrow (↓) indicates mitotic figures H-E × 470

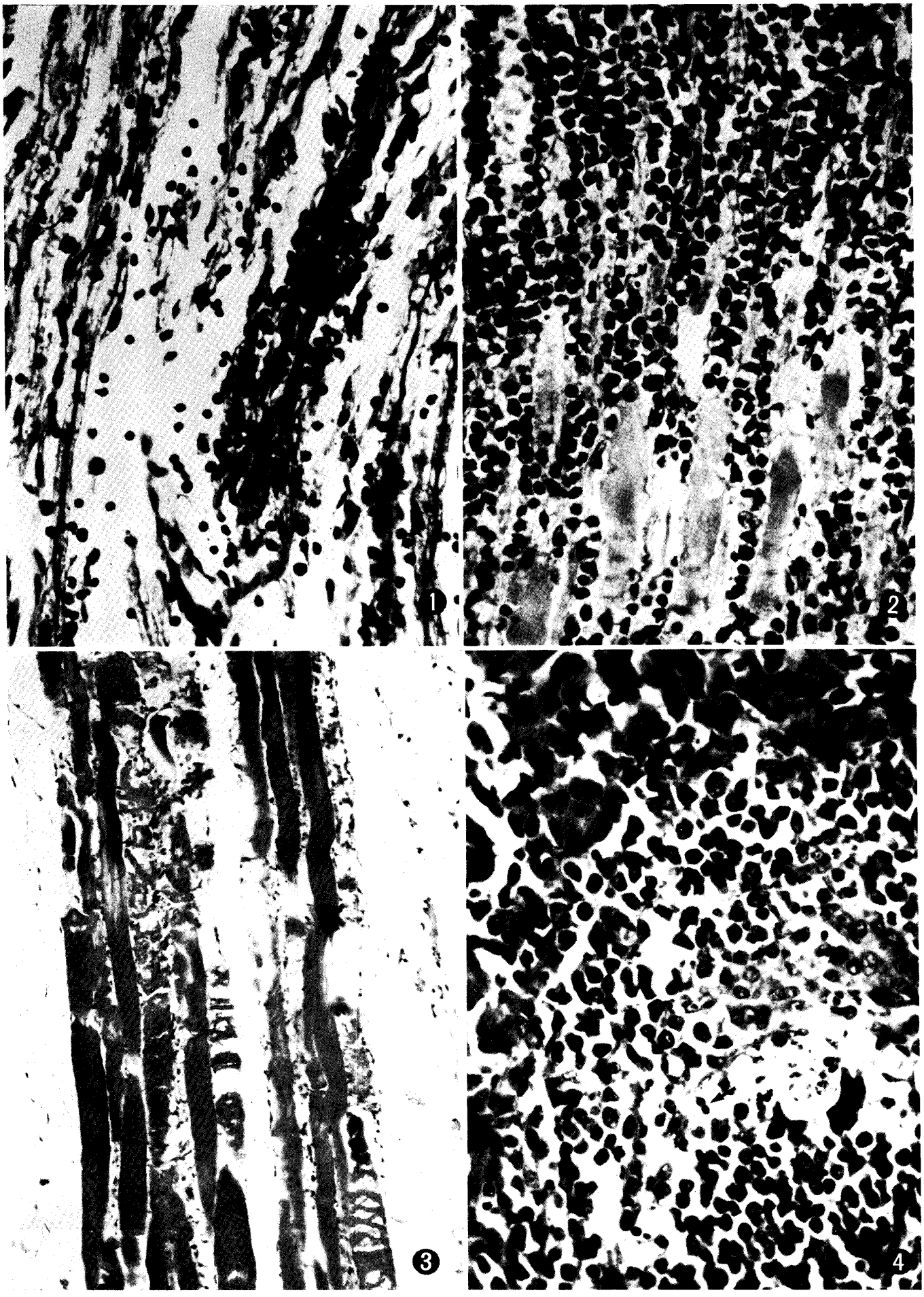


PLATE II

Fig. 5 Case No. 2 A capillary in the cervical spinal ganglia
Numerous VP (↓) are found in the lumen, in the intercellular spaces, and in the basement membrane of the endothelial cells of a capillary.VP (b) seen to emerge as buds from the top of the microvilli in the endothelial cell × 16,000

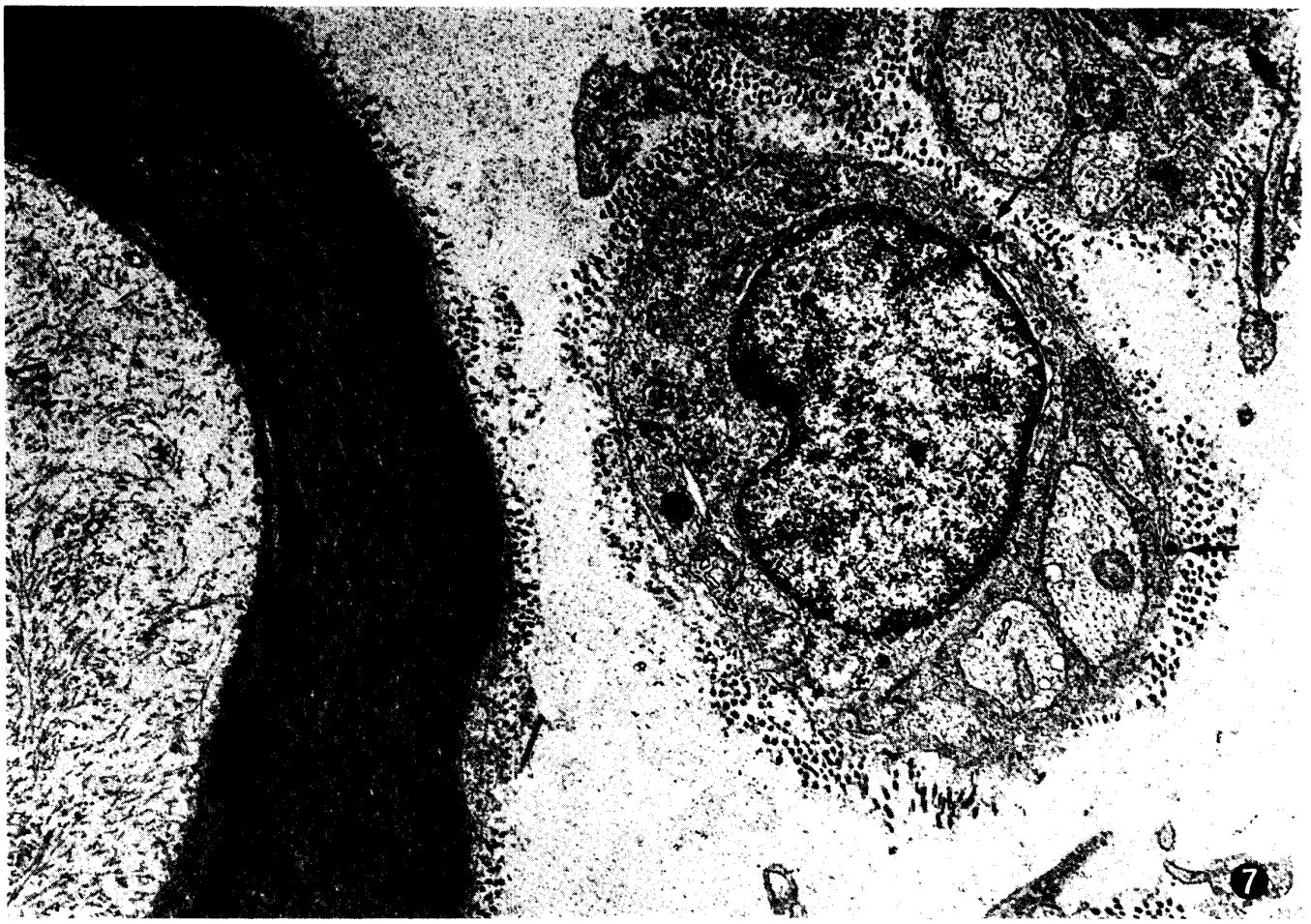
Fig. 6 Case No. 1 Cervical spinal ganglia
VP emerge by budding from the cell membrane of the pericyte of a small blood vessel × 60,000


b
b
6
5

PLATE III

Fig. 7 Case No. 1 Brachial plexus
VP (↓) are seen in the basement membrane of an unmyelinated nerve fiber × 11,700

Fig. 8 Case No. 1 Lumbosacral plexus
VP (↓) are seen in the axon and in the intracytoplasmic vacuole of an emigrating macrophage × 8,000



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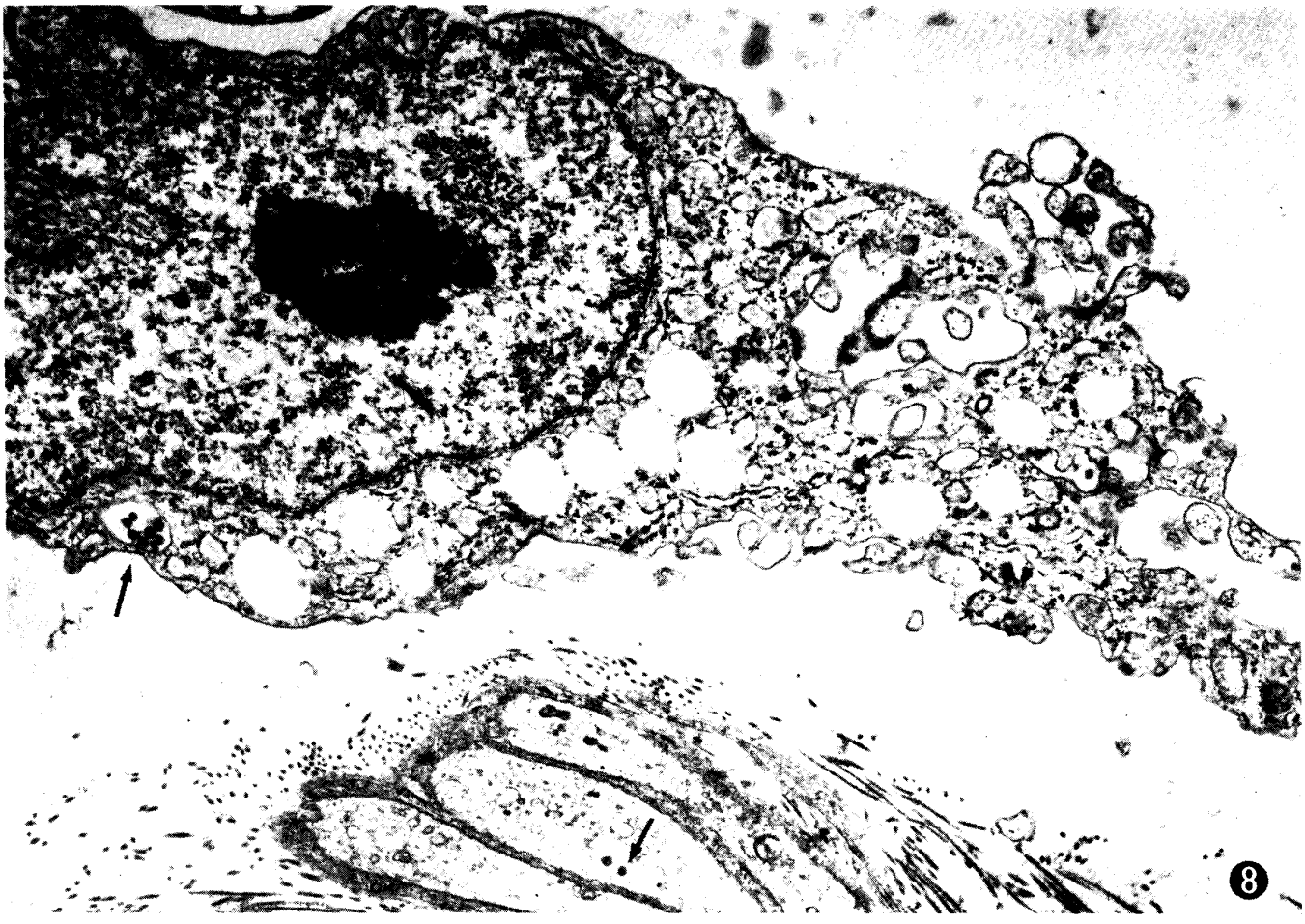

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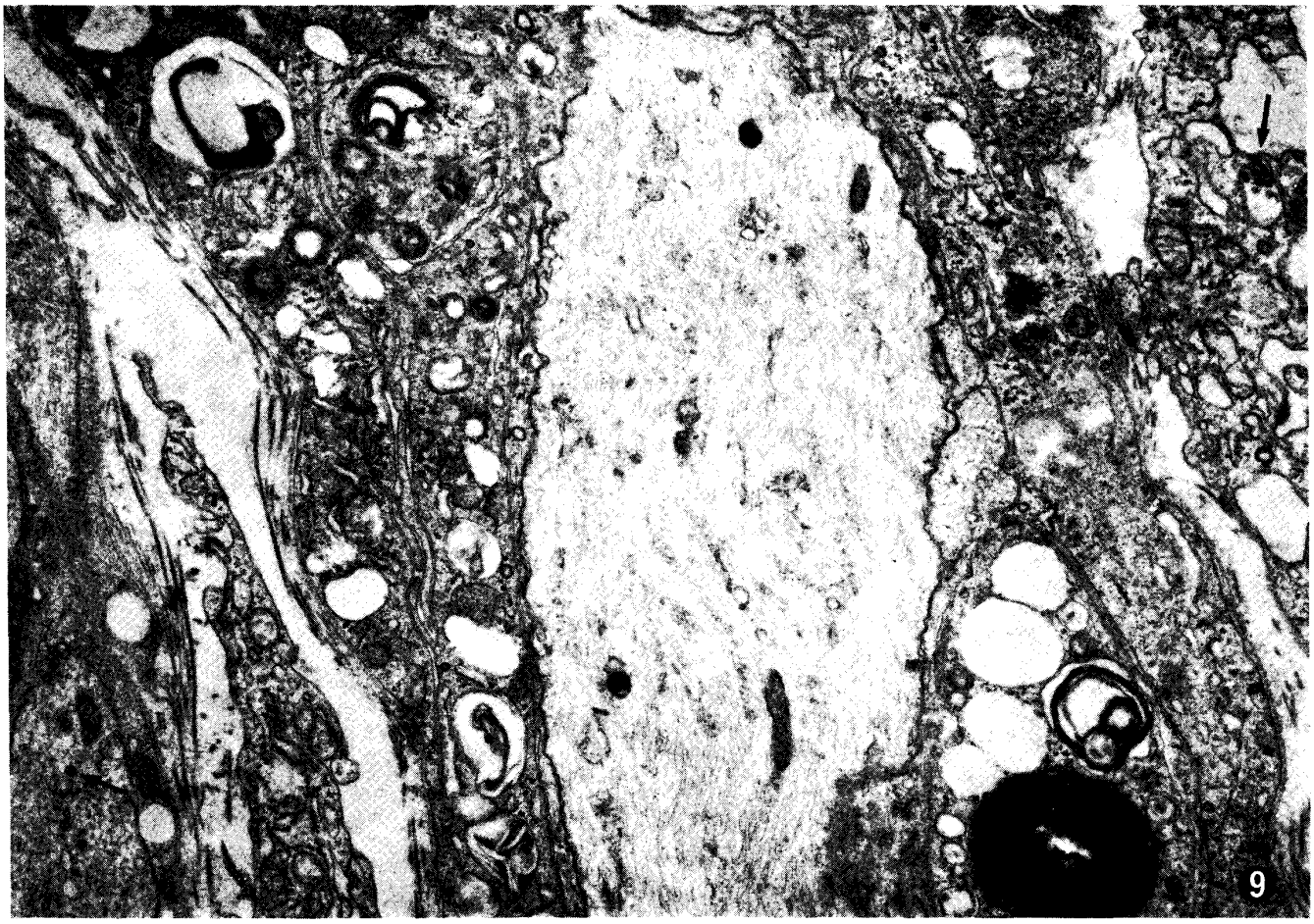
PLATE IV

Fig. 9 Case No. 1 Lumbosacral plexus
Myelin sheaths are severely disorganized. Arrows (↓) indicate VP. × 8,000

Fig. 10 Case No. 1 Lumbar spinal ganglia
VP (↓) are seen in the vesicle of the axon and in the basement membrane of an umyelinated nerve fiber × 32,000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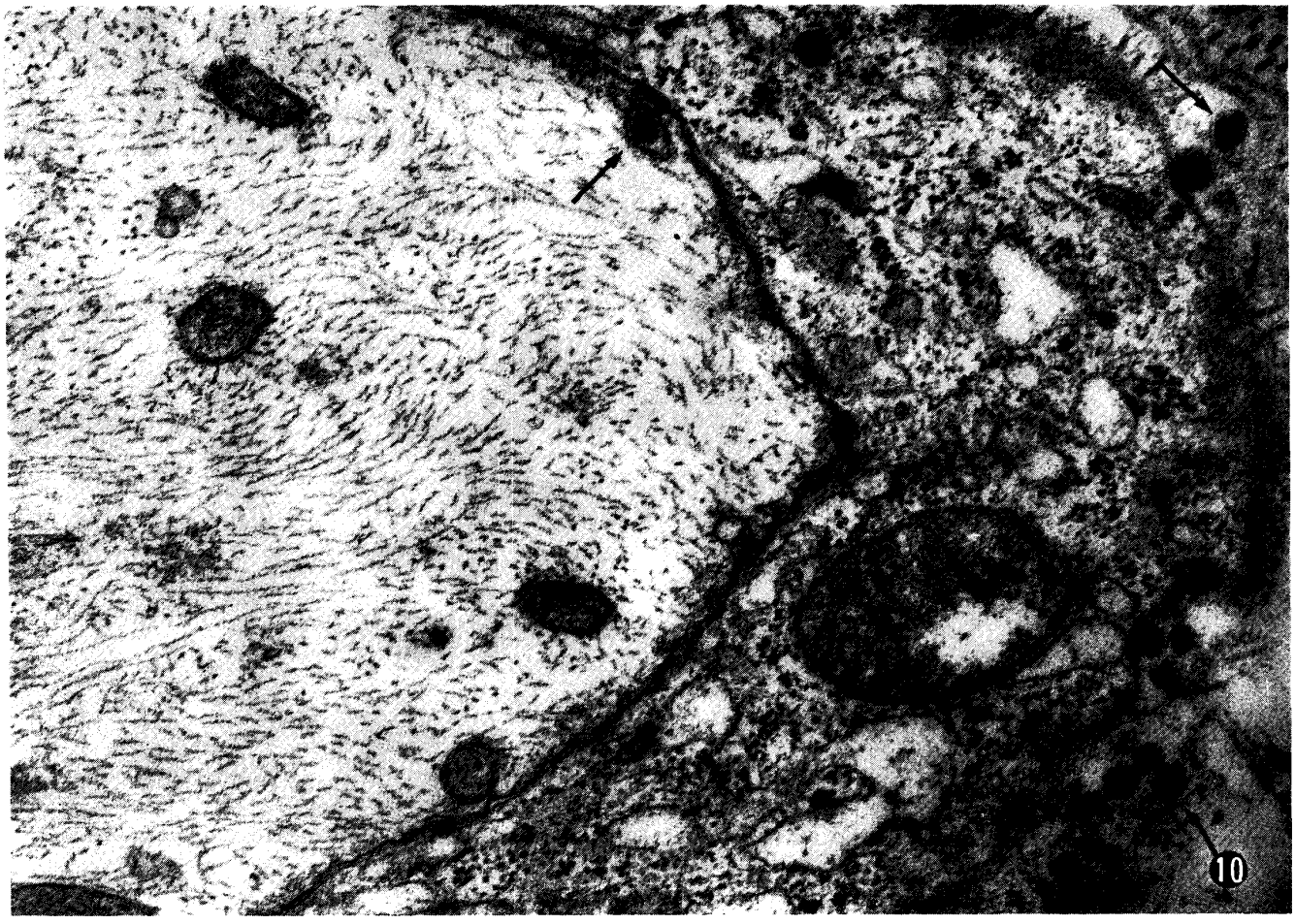
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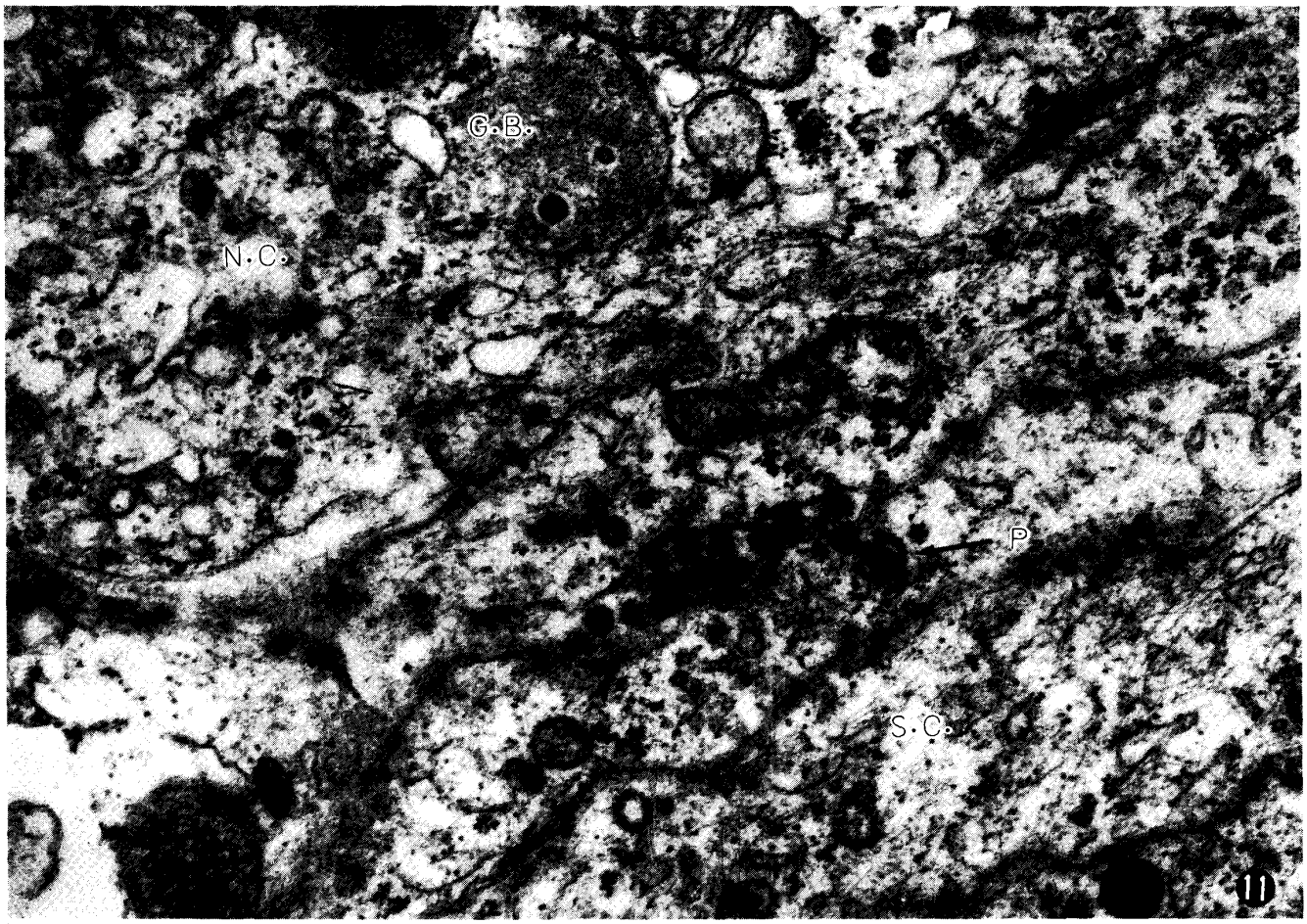
PLATE V

Fig. 11 Case No. 1 Lumbar spinal ganglia
VP are seen in the intercellular space between satellite cells (S.C.) which surround nerve cell (N.C.). P: pinocytosis Within the gray bodies (G.B.), a few virus like particles of various sizes are seen × 37,000

Fig. 12 Case No. 2 Gastrocnemius muscle
VP are embedded in the basement membrane of the skeletal muscle, especially near the sarcolemmal nucleus × 33,000


G.B.
N.C.
P
S.C.
11

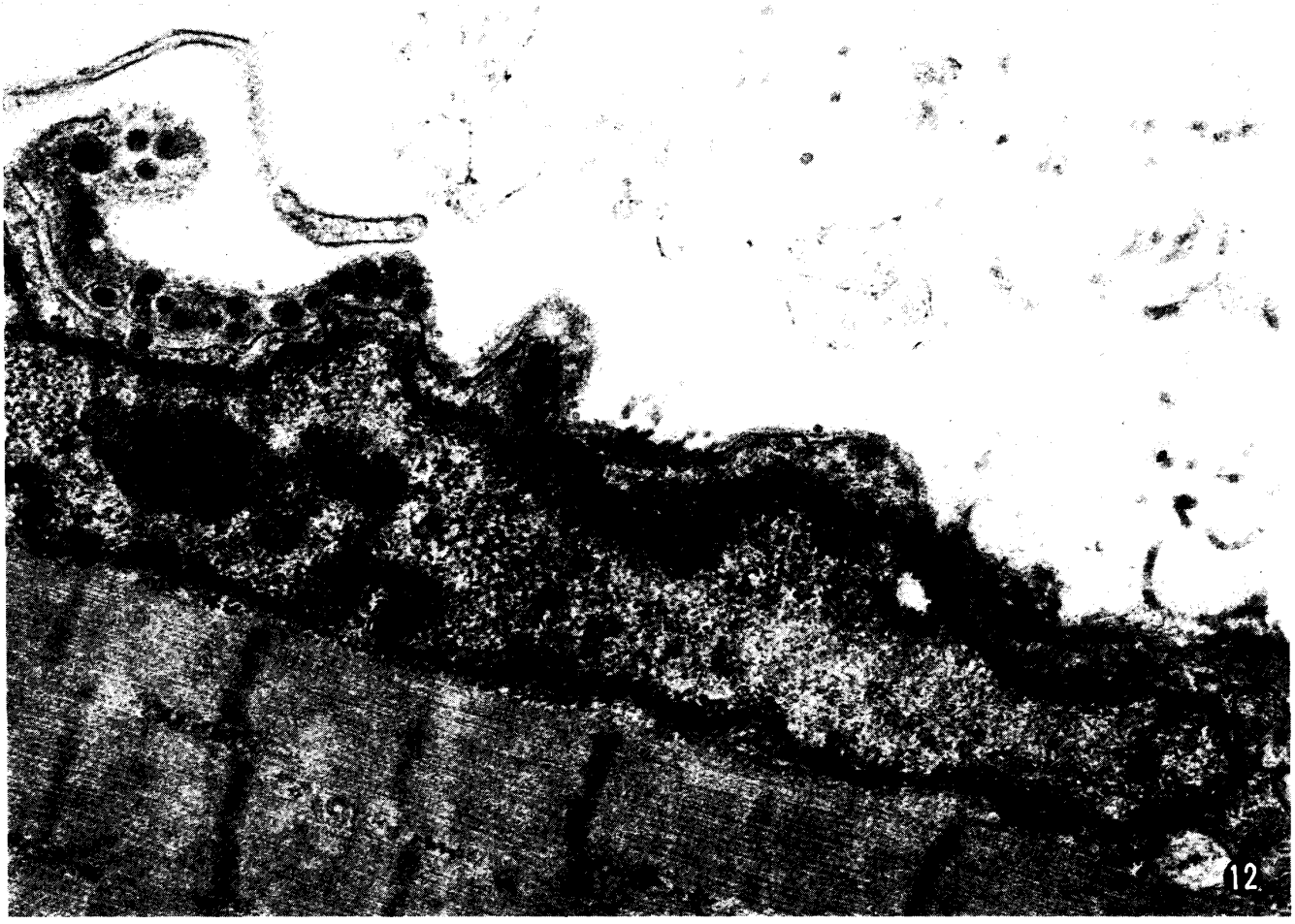

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PLATE VI

Fig. 13 Case No. 2 Gastrocnemius muscle
Contraction of the sarcomere is seen in the muscle cell involving VP (↓) × 32,000

Fig. 14 Case No. 3 Peroneus longus
Disintegration of Z-lines (Z), separation, fragmentation or disappearence of myofibrils and vacuolization of the sarcoplasmic reticulum. Arrows (↓) indicate VP × 32,000

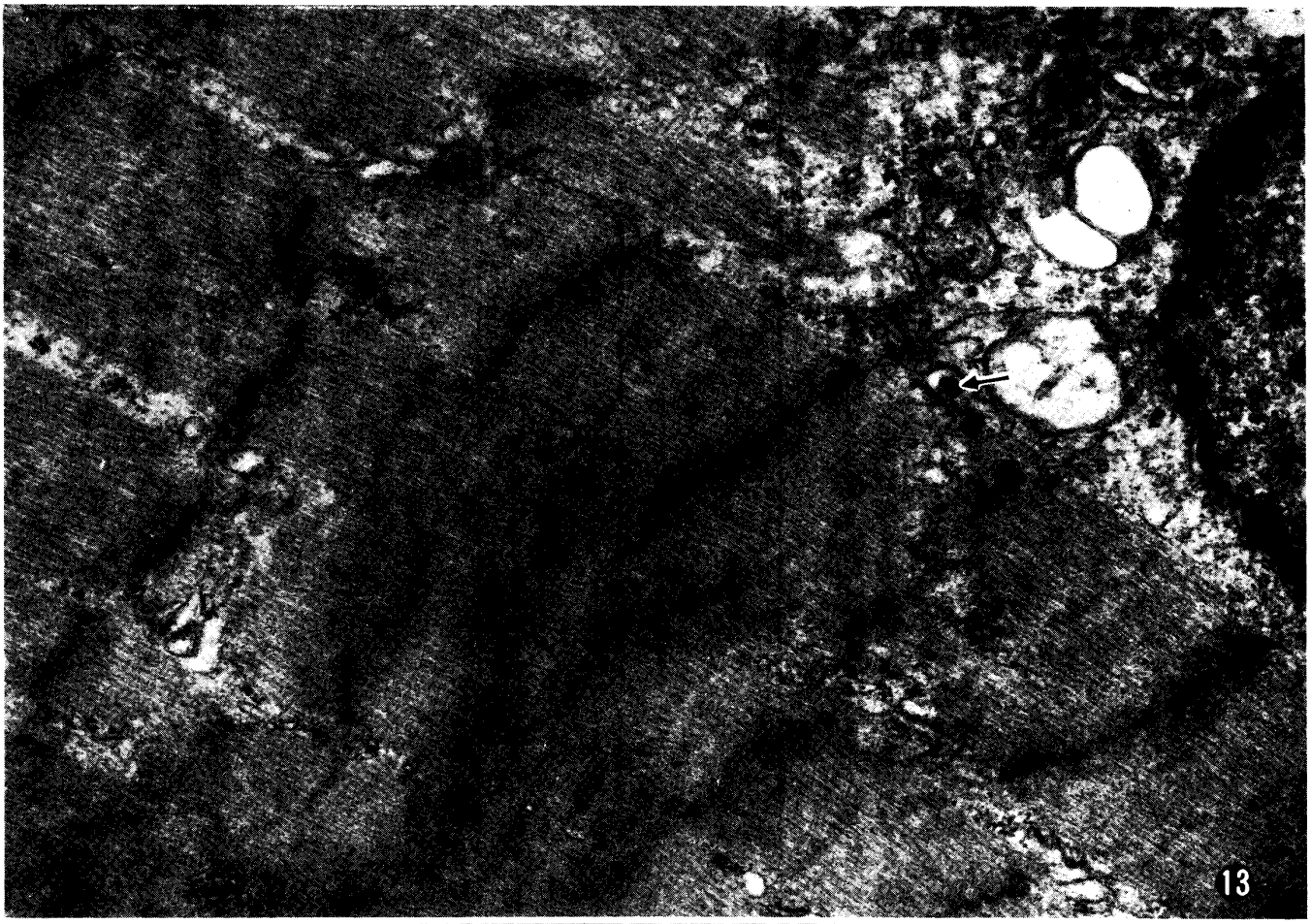
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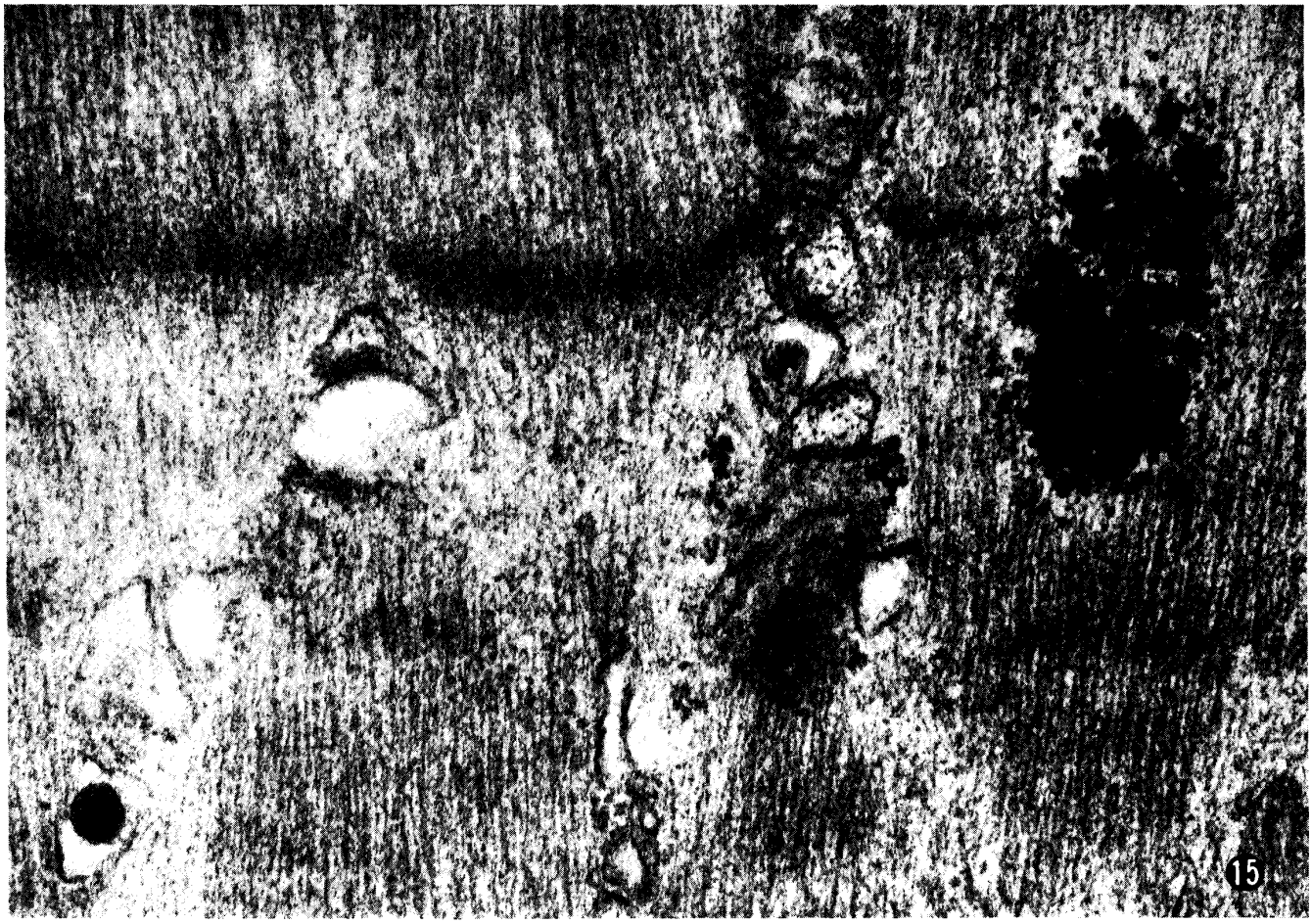
PLATE VII

Fig. 15 Case No. 1 Gastrocnemius muscle
Viroplasm, consisting of aggregates of immature small VP surrounded by numerous small granules which were considered as ribosomes is seen on the right side of the plate. VP are seen on the left side of the plate shows mature form × 64,000

Fig. 16 Case No. 1 Gastrocnemius muscle
VP in the dilated sarcoplasmic reticulum
Arrow (↓) shows VP which is budding from the vesicular membrane × 64,000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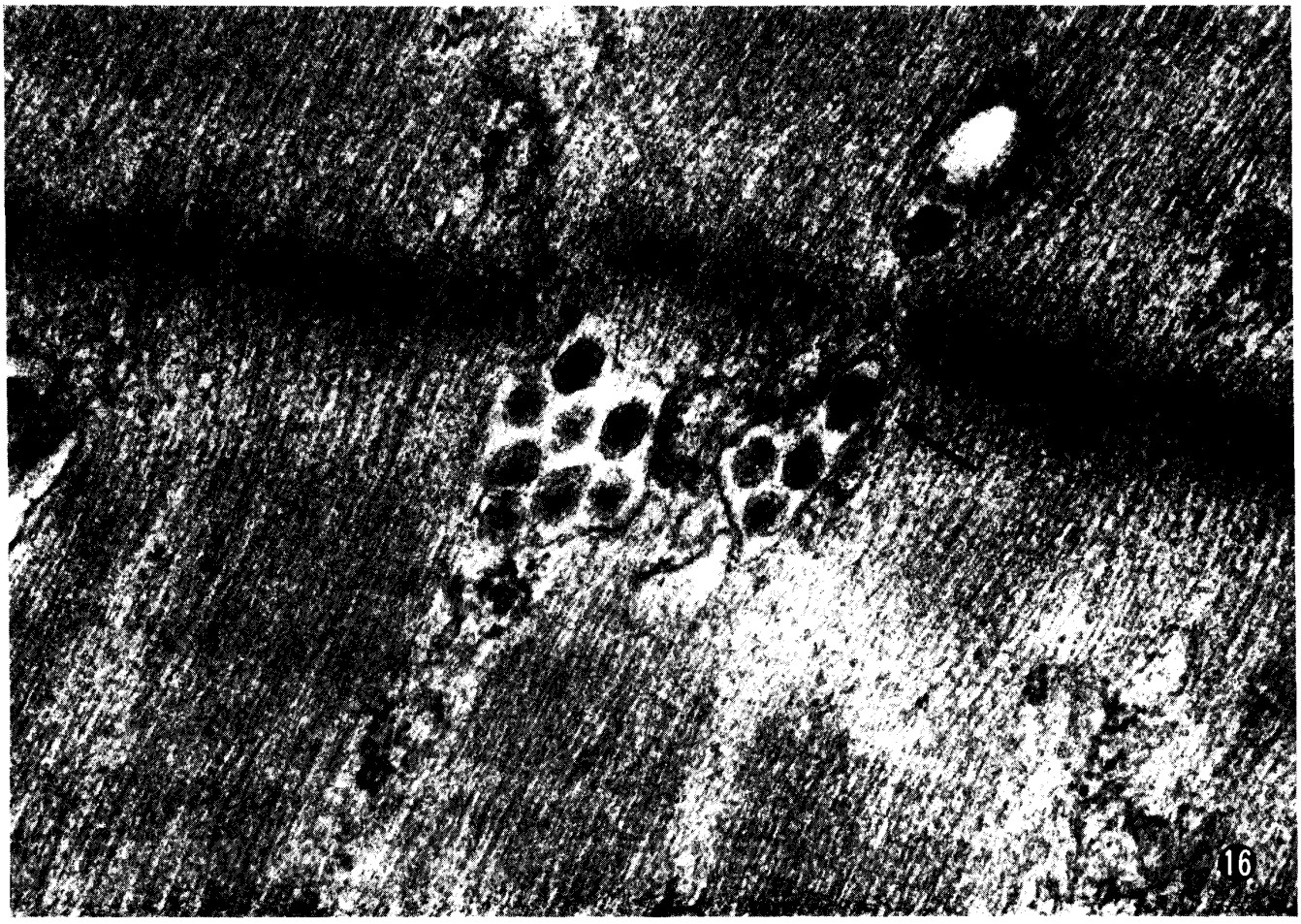
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PLATE VIII

Fig. 17 Case No. 1 Gastrocnemius muscle
VP are seen in the extracellular space around a pericyte of a small blood vessel. Numerous VP are emerging by budding from the cell membrane of the pericyte × 16,000

Fig. 18~20 A part of fig. 17 at higher magnification
Budding in the pericyte × 56,000
Viral matrix (Fig. 18), involving a virus like spherical structure (↓)
Gray body (Fig. 19), surrounded by a single membrane

Fig. 21 Case No.1 Gastrocnemius muscle
VP have an outer membrane with many peripheral knobs, inner membrane (inner ring) and central nucleoid × 120,000

Fig. 22 Case No. 1 Lumbosacral plexus
A VP have tail-like projection × 120,000

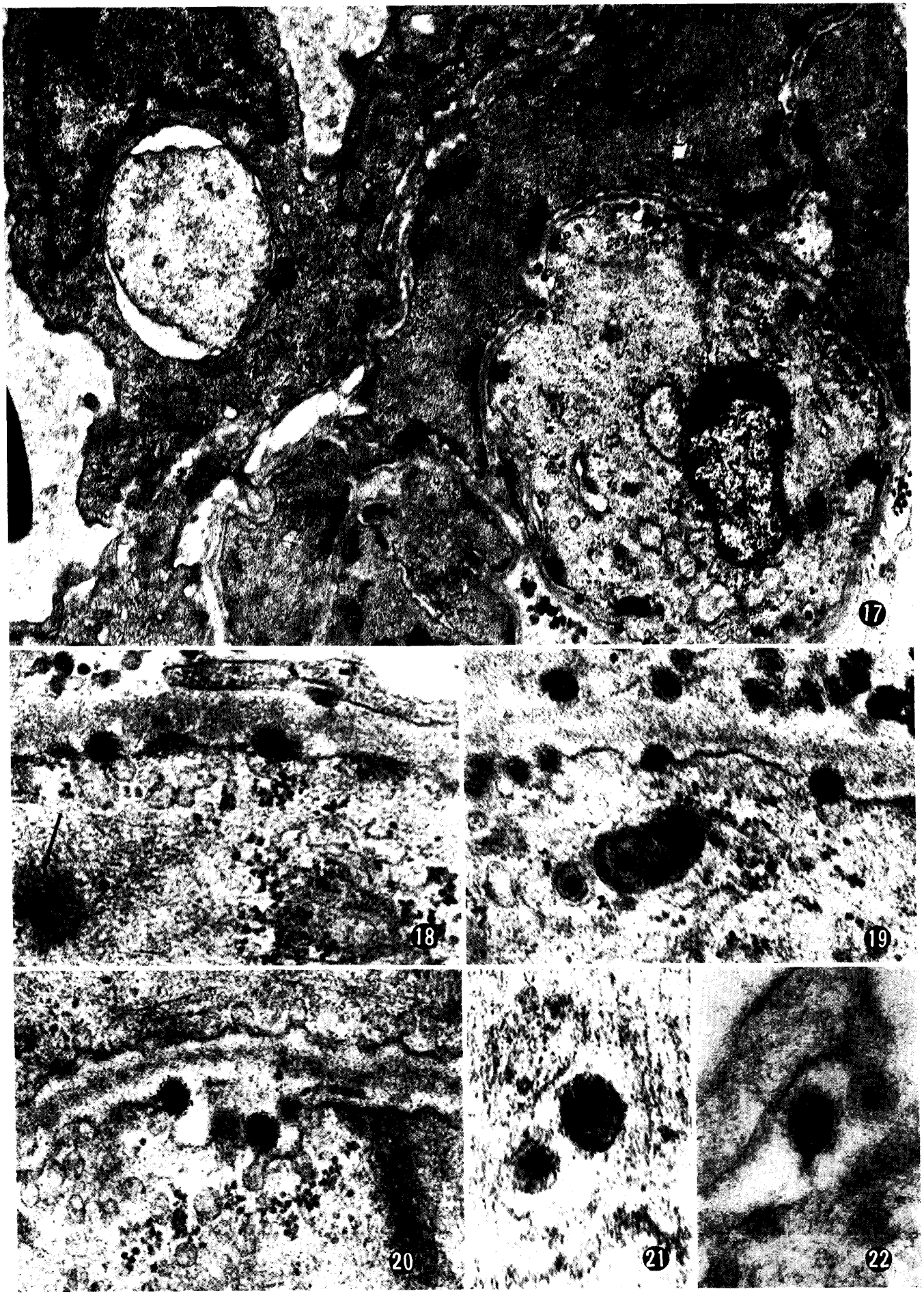
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PLATE IX

Fig. 23 Case No. 4 Liver
Gray bodies in the proliferated lymphoid cells × 32,000

Fig. 24 Case No. Spleen
VP are seen in the intercellular spaces of the trabeculae × 32,000

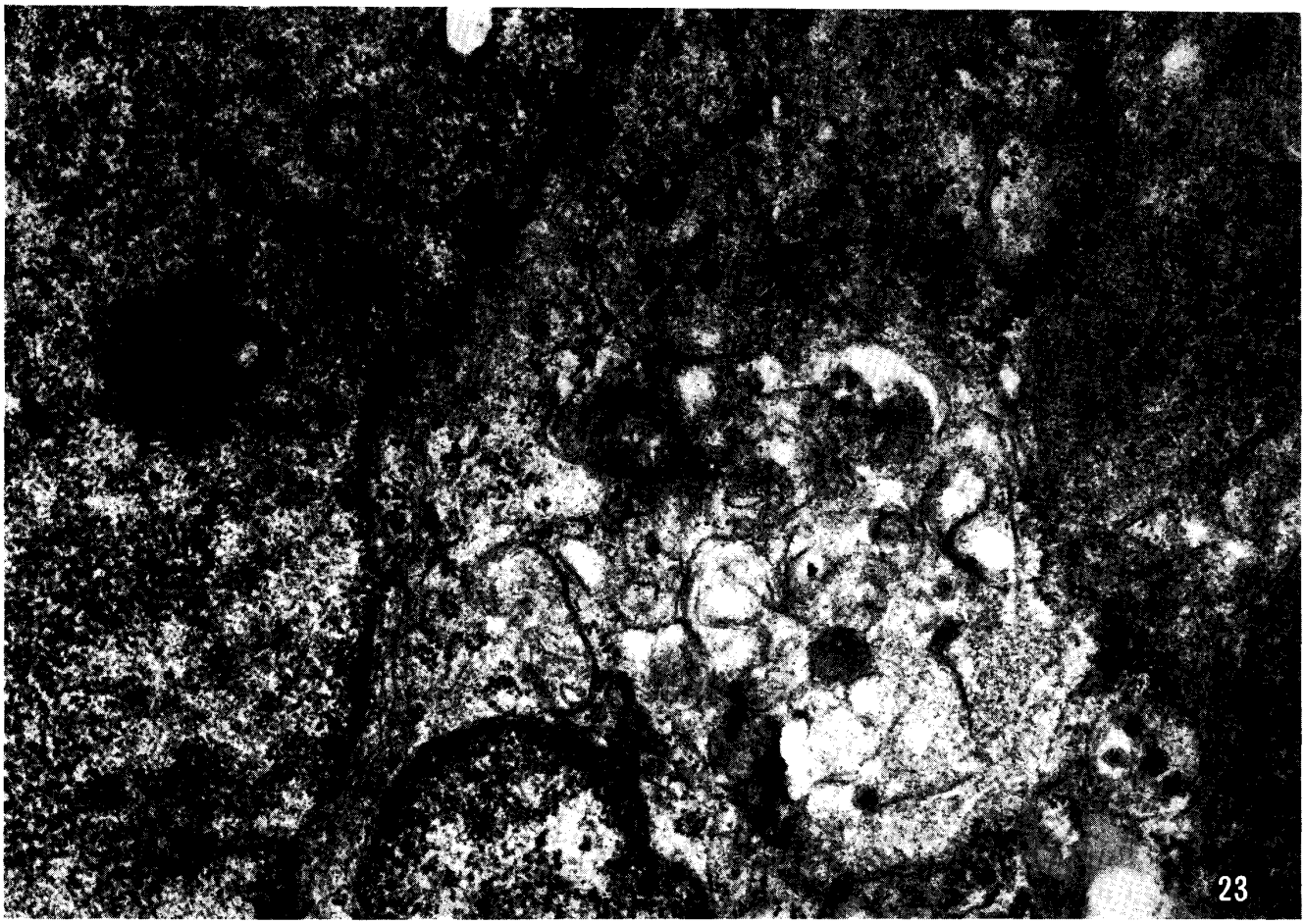
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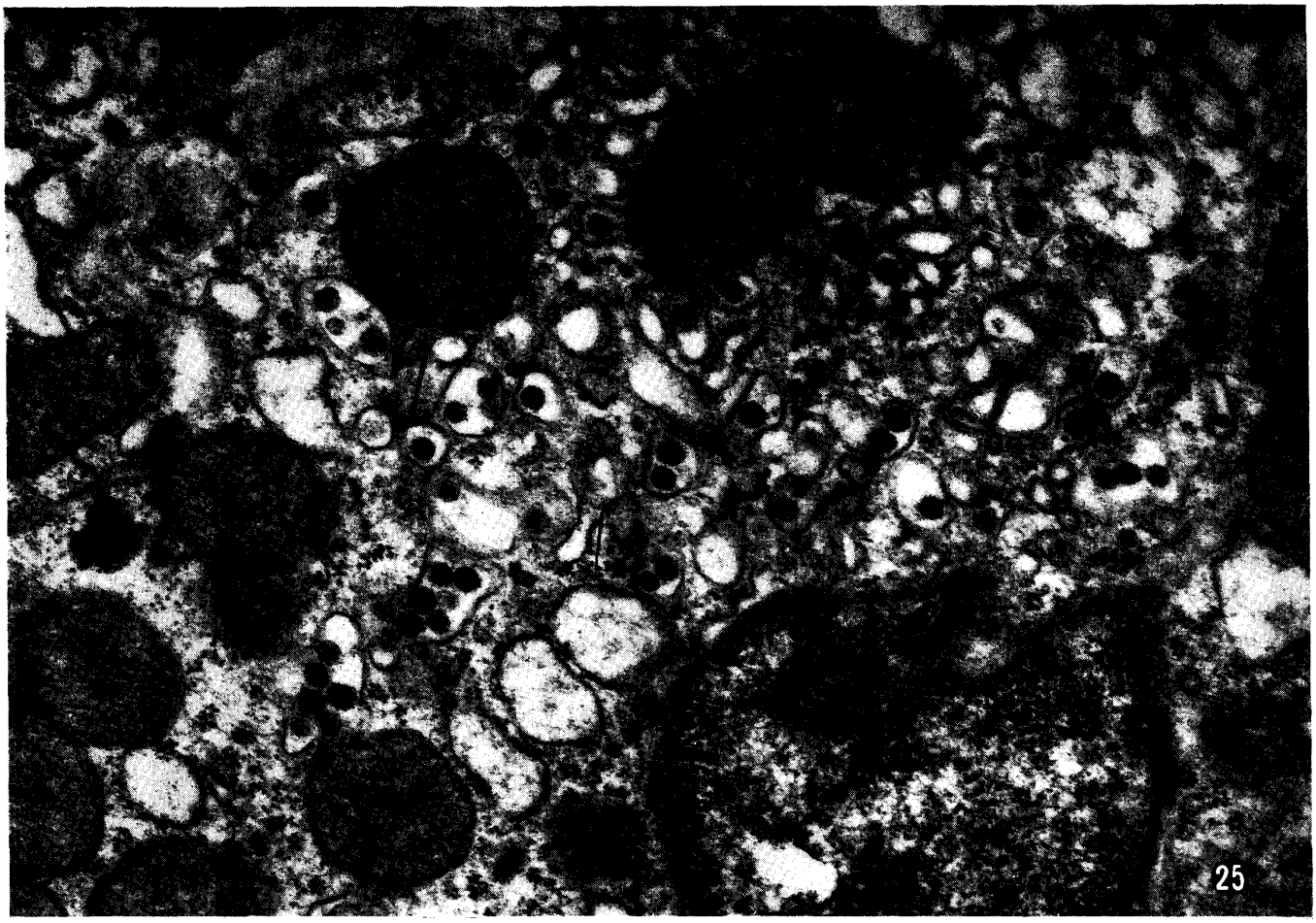
PLATE X

Fig. 25 Case No. 4 Kidney
VP are seen in the intracytoplasmic vesicles of the epithelial cell
× 32,000

Fig. 26 Case No. 4 Spleen
VP are morphologically identical to those of case Nos. 1~3
× 50,000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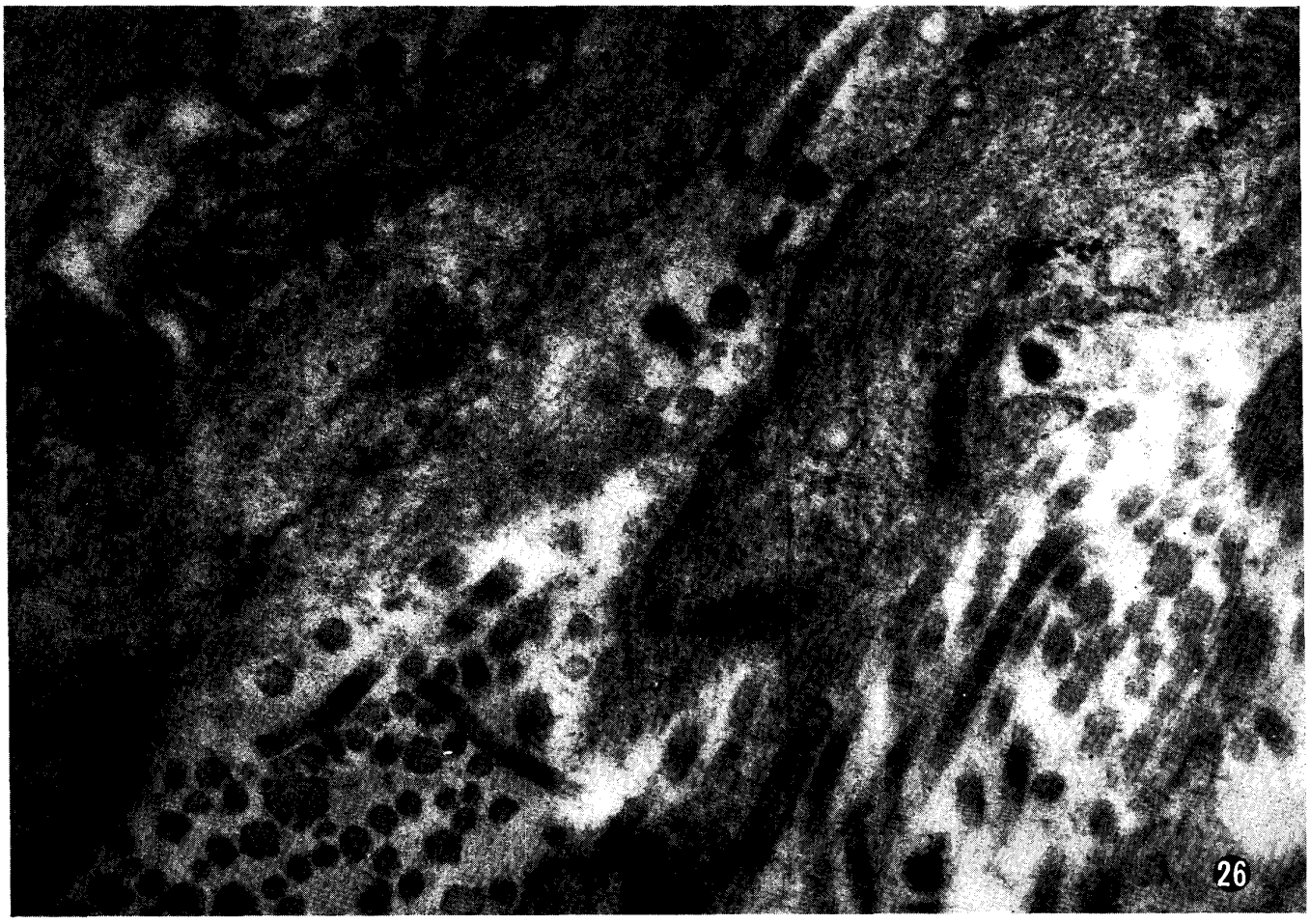
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