Nagoya J. Med. Sci. 33: 189-202, 1970

INCLUSION BODIES IN DENERVATED SKELETAL MUSCLES OF MICE

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ABSTRACT

Three different kinds of inclusion bodies were found in denervated gastrocnemius muscles of mice. 1. A filamentous inclusion was found in fibers denervated for 18 days or longer and it consisted of a central mass and radiate filaments, one end of which constituted the surrounding normal myofibrils and the other end a part of the central mass. The central mass was composed of an aggregation of fine filamentous segments approximately 140 Å in thickness. The inclusion body or the cytoplasmic body was considered to be derived from disorganized myofibrils and the I band was the locus of origin. 2. An inclusion consisting of smaller cisternae, approximately 200 Å in width, was found in fibers denervated for 36 days or longer, and 3. that consisting of larger cisternae approximately 75 mμ in width was found in fibers denervated 156 days or longer. The latter two inclusions consisted of membranous structure, and were composed of parallel arrays of many cisternae which were considered to be derived or overdeveloped from the sarcoplasmic reticulums.

The presence of these inclusion bodies was considered as a specific characteristic of the injury of nerve fibers in any regions.

INTRODUCTION

In 1962, Engel[1] first described "cytoplasmic body" in human abnormal skeletal muscle fibers and chick embryo skeletal muscle fibers grown in tissue culture. He also found the body in skeletal muscle fibers in case of denervation and myogenic disease especially myotonic dystrophy. He suggested histochemically that the bodies were composed of degenerative myofibrils. Since then the cytoplasmic bodies have been found electron microscopically by several authors[2)3)4)5)6)7)], in human skeletal muscle fibers of various neuromuscular diseases.

The fine structure of the experimentally produced inclusion bodies has not been described yet[8)]. For understanding of the biology of denervated skeletal muscle, it is important to make detailed observations of the bodies. In this paper, the author investigated the ultrastructure of various inclusion bodies

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Received for publication April 1, 1970.

in muscle fibers of denervated skeletal muscles of mice and the results are presented here, comparing them with the bodies that have been found in human skeletal muscle fibers of various neuromuscular diseases, and the significance of the inclusion bodies is discussed.

MATERIALS AND METHODS

Fifty young adult male white mice (SMA strain) weighing about 30 to 40 grams were used for the experiment. The animals were fed freely. The hair was cut from the right buttock and leg. A posterior lateral incision was made without anaesthesia from a point just distal to the posterior superior iliac spine to the greater trochanter of the femur. The whole right leg was denervated by removing the sciatic nerve about 0.5-0.7 mm in length, high in the thigh. At intervals of three days for the first 36 days, subsequently at 42, 48, 54, 66, 75, 90, 105, 120, 156, 180, and 210 days after the operations, the gastrocnemius muscles of both legs were removed and weighed. The caput longus of both gastrocnemius muscles was used for the experiment. In each case the left gastrocnemius muscle served as a control. For fixation of the muscles three different methods were used: (a) The tissues in resting state in situ were fixed for 2 hours in cold 2% solution of glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) and post fixed in cold 1% Millonig's phosphate-buffered[9] osmium tetroxide solution. Some of the tissues were fixed directly in cold 1% Millonig's phosphate-buffered osmium tetroxide solution. (b) The two gastrocnemius muscles in adequately streched state were fastened with a pin on a board and were shaken in a solution of cold 2% glutaraldehyde of 0.05 M phosphate buffer (pH 7.4) and some tissues of the caput longus of the left gastrocnemius were post fixed in cold 1% Millonig's phosphate-buffered osmium tetroxide solution for 2 hours. (c) The lower extremities were fastened with a pin on a board for both gastrocnemius muscles being streched as intended. Then a ventral median incision through the peritoneum was made. Perfusion through the vena cava inferior directed peripherally with a warmed (37.0°C) mixture of 2.5% glutaraldehyde and 2% paraformaldehyde of 0.05 M phosphate buffered solution (pH 7.4)[10,11] was carried out. The two gastrocnemius muscles were fixed simultaneously for 3-5 hours. The tissues of the caput longus of the muscles were post fixed in cold 1% Millonig's phosphate-buffered osmium tetroxide solution for 2 hours.

These tissues were dehydrated in graded concentrations of ethanol and embedded in Epon 812[12]. At first 0.5 μ sections were cut with a Porter-Blum microtome and stained with Giemsa. After examination of these sections under the light microscope, the blocks were retrimmed and thin sections of the selected area were prepared for electron microscopy. The observations and the electron micrographs were made with a Hitachi HU-11A electron

microscope operating at 75 KV.

RESULTS

During the first 15 days after denervation the mouse gastrocnemius muscles decreased in weight very rapidly. At the 9th day the muscles decreased in weight to one-third, and at the 15th day to one-half. Subsequently, the decrease became much slower and at the 210th day by three-fifths. In earlier stages, all fibers were equally affected but in the later stages they came to vary in size. The fibers wasted primarily by losing their contractile material from each myofibril. In the denervated muscles glycogen granules were markedly increased in number. Observations were chiefly made on inclusion bodies of different kinds. They were classified into filamentous and membranous (smaller and larger) inclusions for convenience of description.

(*A*) *Filamentous inclusions*

Light microscope: Inclusion bodies were found in muscle fibers denervated for 18 day or longer. In the atrophied muscle, fibers containing the inclusions were common. The bodies were noted to be identical in staining reaction with Giemsa to normal muscle contractile fibrils (Fig. 1). The bodies showed almost always a disorder of the striations. They were found singly both in the subsarcolemmal region and in the depth of fibers. They were spheroid in shape and less than 2.5 μ in diameter, always with a light halo around them (Fig.

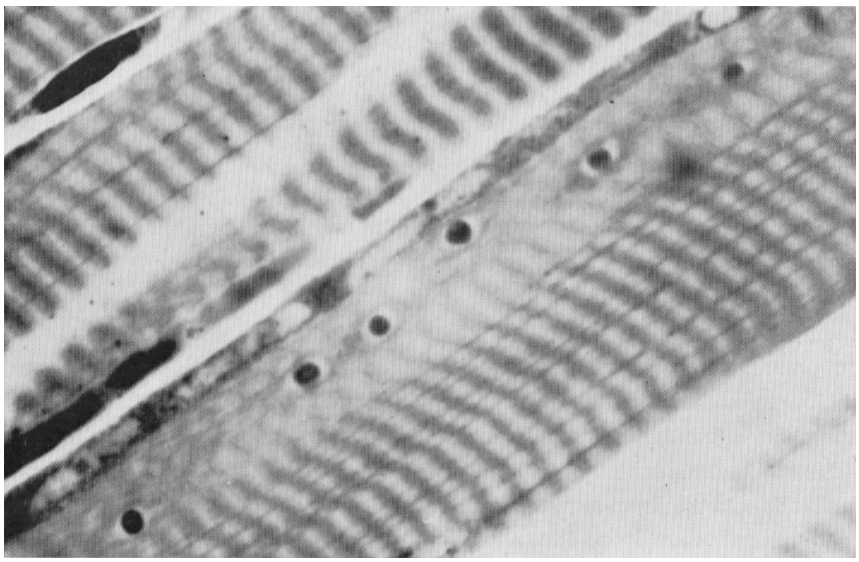

FIG. 1. Inclusion bodies in muscle fibers. Inclusion bodies are spheroid in shape, with light halo around them. Muscles denervated for 30 days. ($\times$1,700)

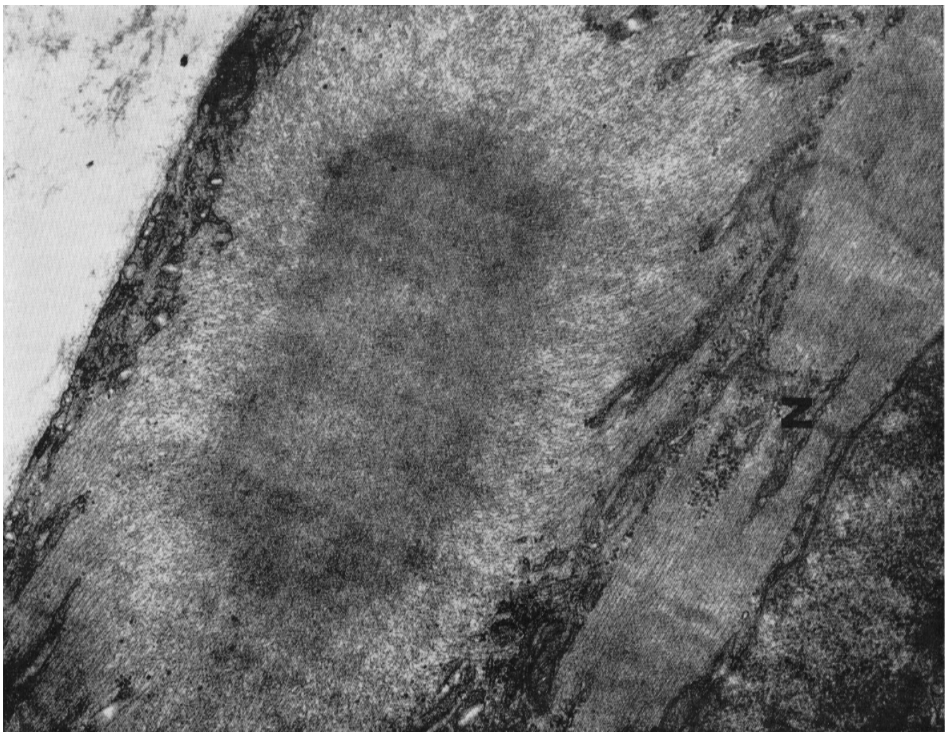

FIG. 2. An inclusion body in a longitudinal section of a muscle fiber. The continuity with surrounding myofibrils is observed. T system tubules are conspicuous around the inclusion. Muscle fiber denervated for 180 days. Z: *Z* band (×22,000).

1). The perimysial fibrous and fatty connective tissue were slightly increased in amount.

Electron microscope: The individual inclusion body showed a fairly uniform structure in each stage after the denervations (Figs. 2, 3, and 4). The body was always situated between the myofibrils and located in the area corresponding to the site of I band. It consisted of a central mass of higher electron density and filaments arranged radially to the central mass. The central mass was always composed of compact irregularly-running fine filaments 140 Å in thickness and showed nearly the same electron density as the surrounding Z band material (Fig. 4). The radiate filament, one end of which constituted the surrounding normal myofibril and the other end constituted a part of the central mass, was invariably observed. No limiting membrane separated the bodies from the surrounding myofibrils. The surrounding myofibrils showed a normal structure, with decrease in number of filaments and with some dislocated Z bands.

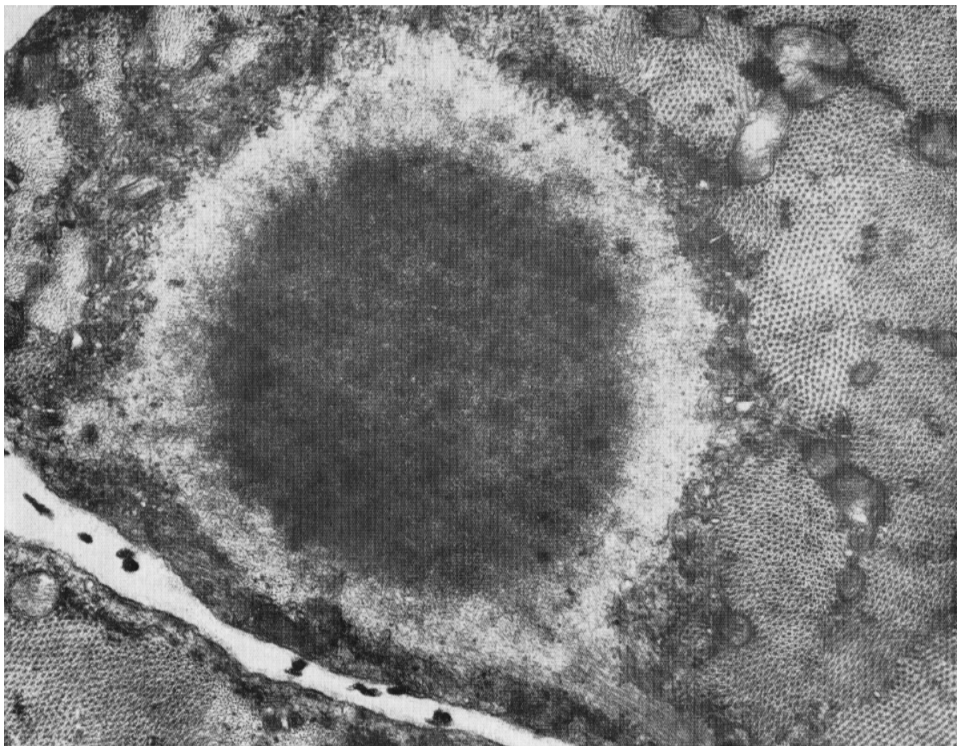

FIG. 3. An inclusion body in a cross section of muscle fibers. The arrays of myofilaments surrounding the inclusion are intact. Muscle fibers denervated for 180 days (×27,000).

(B) Membranous inclusions

The sarcoplasmic reticulum and T-system tubule appeared to show no picture of decrease or increase in number in case of denervation of mouse, although there were noted apparently membranous inclusions cosisting of smaller and larger cisternae. They were found in denervated muscle independently of the changes of the myofibrils. Both inclusions could be observed only by the electron microscope.

(1) Inclusions consisting of smaller cisternae

Inclusions consisting of parallel arrays of smaller cisternae (Fig. 5), were observed in muscle fibers denervated for 36 days or longer after the operations and they gradually increased in number. These structures were found not only in severely atrophic muscles but also in comparatively normal muscle fibers. In longitudinal sections to the long axes of the inclusions, they were observed to be composed of about three to six cisternae in earlier stages and frequently ten or more in later stages. Although the diameter of the individual cisterna was variable, the width of sarcoplasma between the cisternae was

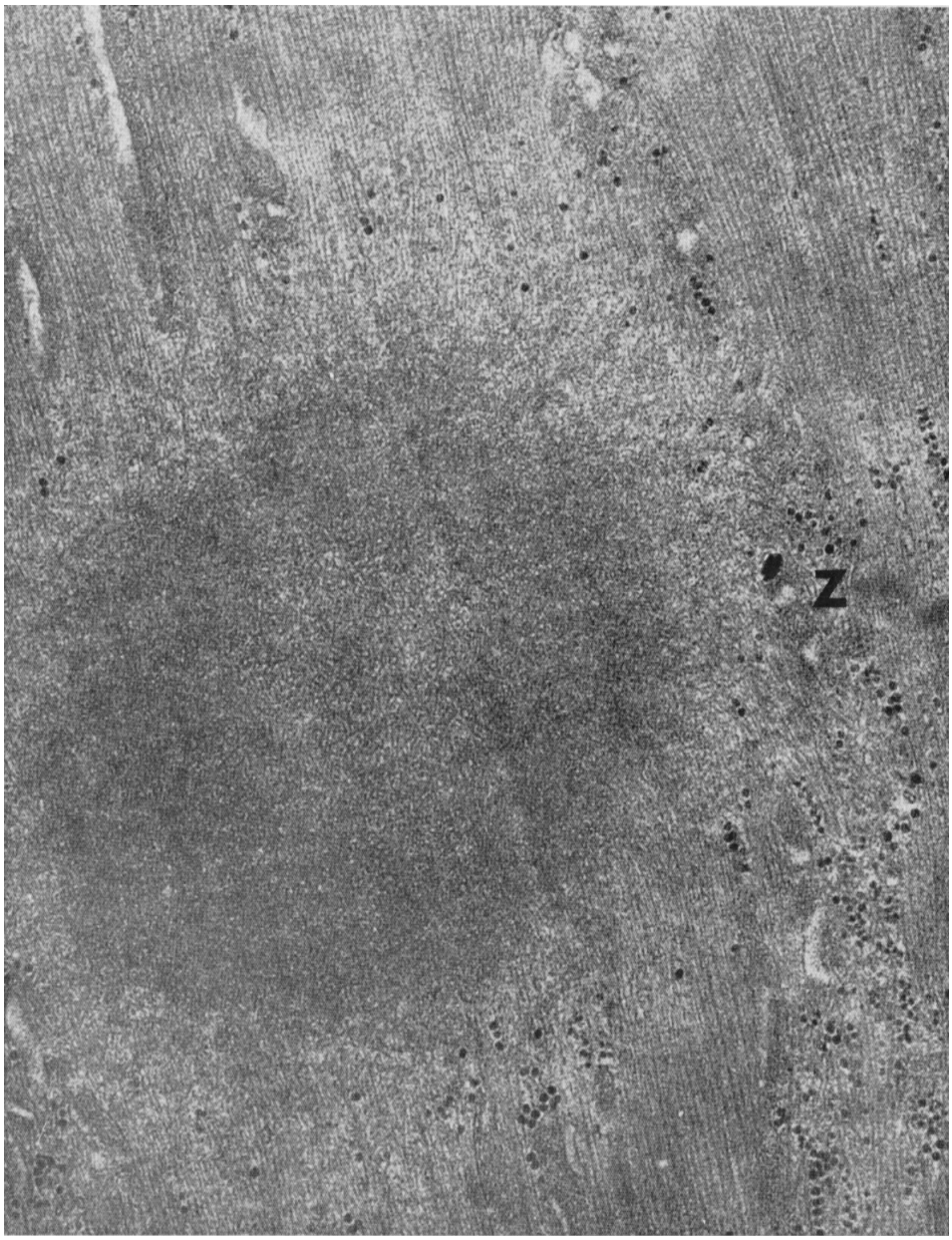

FIG. 4. An inclusion body of higher magnification. The central mass is composed of compact irregularly-running fine filaments 140 Å in thickness. Continuity between the body and surrounding myofilaments is clear. Glycogen granules are increased in number. A muscle fiber denervated for 36 days. Z: Z band (×42,000).

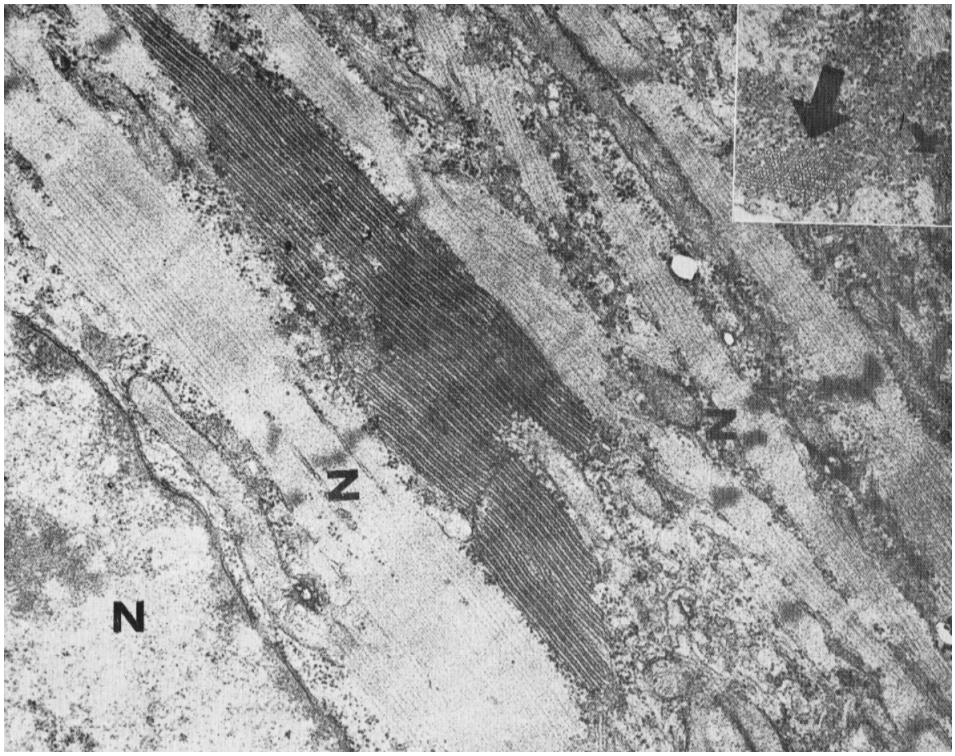

FIG. 5. An aggregation of smaller cisternae in a longitudinal section of a muscle fiber. Z: Z band N: sarcolemmal nucleus (×22,000). Inset: these structures in cross section (arrows) (×22,2000).

approximately from 175 Å to 250 Å. Sometimes, there were glycogen granules in the sarcoplasma between the cisternae. In a transverse section the inclusion appeared as an aggregation of many small vesicles variable in diameter, and the sarcoplasm between the vesicles was approximately 200 Å in width (Inset of Fig. 5). In some regions, the transitions were demonstrated between these cisternae and the comparatively normal sarcoplasmic reticulums. Dimensions of the inclusions were varied and never exceeded 2 sarcomeres. The long axes of the inclusions took random directions.

(2) Inclusions consisting of larger cisternae

Inclusions consisting of parallel arrays of larger cisternae were observed in muscle fibers denervated for 156, 180 and 210 days. Their long axes were always parallel to that of myofibrils. The inclusions were found everywhere in the muscle fiber viz., between the myofibrils, in the subsarcolemmal and perinuclear regions (Figs. 6 and 7). In transverse section, they consisted of vesicular structures from several to several thousands in number. They were observed in the muscle fibers, independently of degenerative changes of myo-

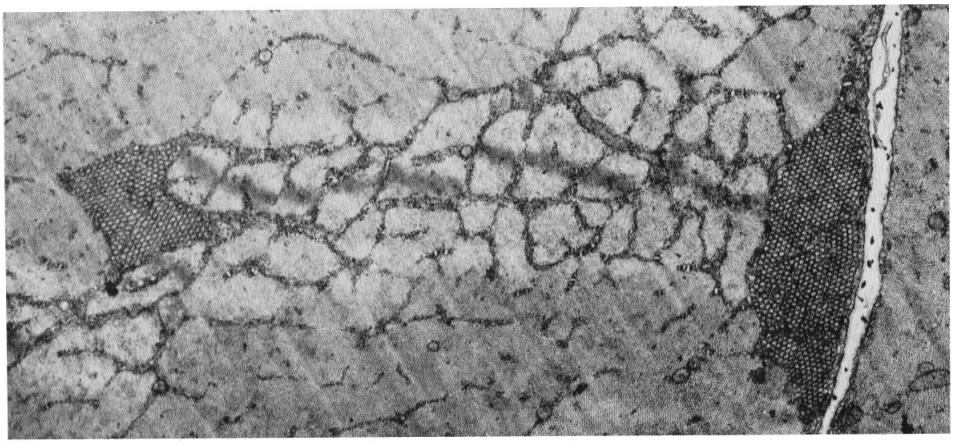

FIG. 6. Two aggregations of larger cisternae in a cross section. Low magnification (×9,700).

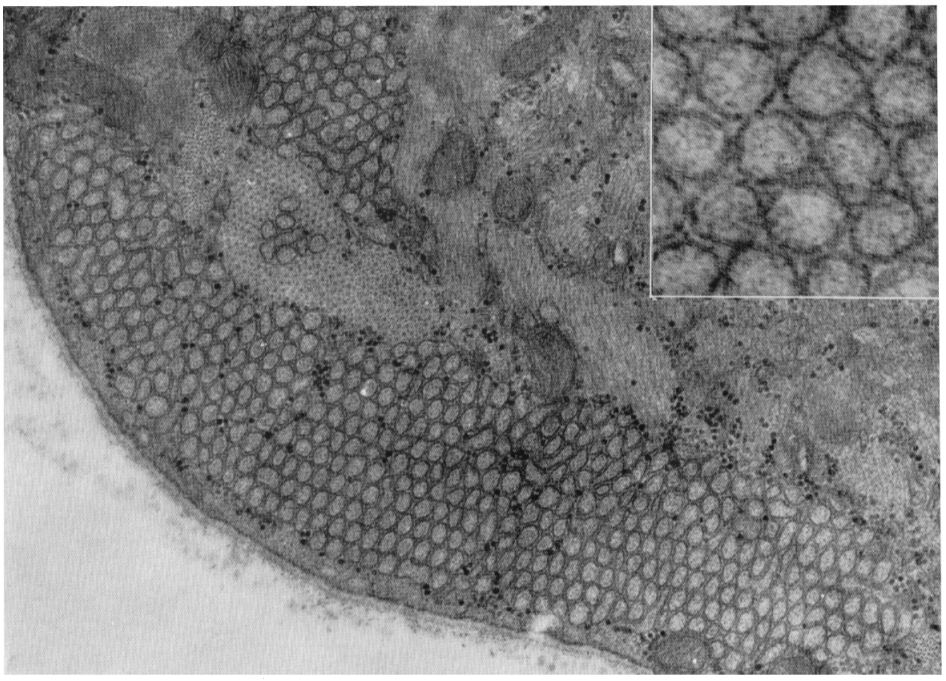

FIG. 7. Aggregations of larger cisternae in a cross section. Glycogen granules found in sarcoplasma between the vesicles (×32,000). Inset: higher magnification of the vesicles. A unit membrane is distinguishable (×90,000).

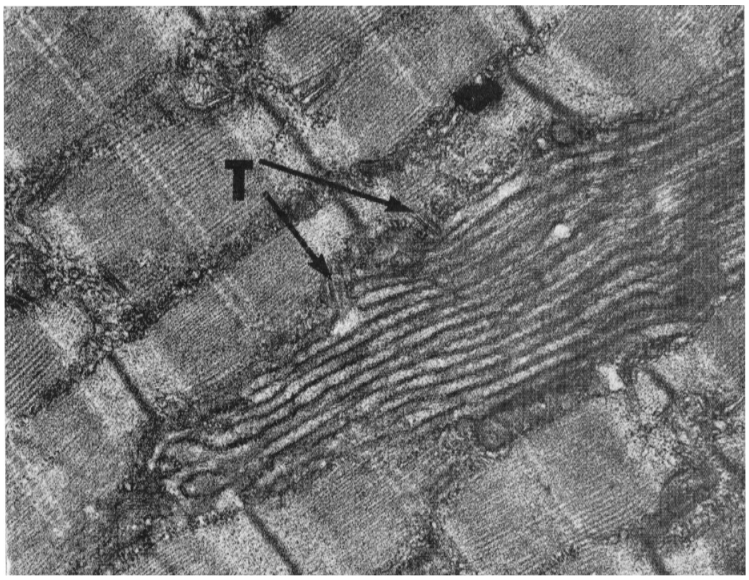

FIG. 8. An aggregation of larger cisternae in a longitudinal section. Some of the ends of the cisternae are in close contact with T system tubules (T). Arrows show transverse tubules (×22,000).

fibrils surrounding them (Figs. 6 and 7). The limiting membrane of the vesicles was composed of a unit membrane (Inset of Fig. 7). These vesicular structures were approximately 75 mμ in diameter and gathered to make an aggregation with narrow sarcoplasma between them. In a transverse section of an inclusion it showed a honeycomblike arrangement. In the sarcoplasma between these vesicular structures there were noted a few glycogen granules (Fig. 7). In the longitudinal section of the inclusion body, the cisternae ran parallel to each other and were very long and extended over 5 to 6 sarcomeres (Fig. 8). The cisternae were approximately 45 to 90 mμ in width and separated from each other by narrow sarcoplasma. Some ends of the cisternae seemed to be in close contact with T tubules which were perpendicular to the long axes of the cisternae (Fig. 8). The individual cisterna was similar to the normal terminal cisterna in shape, size and electron density (Fig. 8).

DISCUSSION

Filamentous inclusion

With the light microscope, Engel[1] found an inclusion in human skeletal muscle fibers of denervation and myotonic dystrophy, and called it a "cytoplasmic body". He stated that the cytoplasmic bodies were uncommon, but were found in denervated muscle fibers in more advanced stages of atrophy

and usually occured less frequently in myopathic fibers. He considered that their presence was indicative of muscle fiber abnormality but not of specific diagnostic significance. He suggested that the "cytoplasmic body proper" consisted of altered myofibrillar material.

Later, Shafiq *et al.*[2] fouud an inclusion or a cytoplasmic body in muscle fibers of patients with polymyositis. With the electron microscope, they suggested that the body represented remnants of degenerated myfibrils, and that it could be found in fibers otherwise of normal morphology, as well as in those in which considerable dissolution of myofibrils had occurred. D'Agostino *et al.*[3], in a case of familial myopathy with abnormal muscle mitochondria, described the presence of a large dense mass surrounded by radially oriented myofilaments. They described its presence with the Z band alterations, and suggested that the large dense mass might represent an accumulation of Z band meterial, but since a transverse periodicity was not observed their exact origin was not known. Nakashima *et al.*[6] reported an ambiguous case (whether neurogenic or myogenic) with numerous inclusion bodies in skeletal muscle fibers, and the possible origin and formation process of the inclusions were discussed. They suggested that the aggregations of filamentous segments of the inclusions originated from disorganized myofibrils. They classified the inclusion into two types by shape, and one of which consisted of a central, highly electrondense mass with filaments radiating from the central mass. These inclusions described by them were very similar to those found in this experiment. The inclusions of human cases, reported by Odor *et al.*[4], Macdonald *et al.*[5] and Nakashima *et al.* (type d)[6], were oval and no filaments radiating from the central mass.

From the researches on denervated skeletal muscles of the rat[15)16)], it has been ascertained that the Z band is the earliest structure involved and the alterations of the Z lines cause disorder of filaments and subsequent collapse of myofibrillar structures. But on the fine structures of an inclusion body experimentally produced in animals, no investigations have been carried out.

In this experiment, the filamentous inclusion bodies were frequently found at a brief period of 18 days, against expectations. The bodies were similar in shape to those of the above mentioned human cases but smaller in size, approximately one-fourth. The bodies showed continuity with the surrounding normal myofilaments and were always located in the area corresponding to I band. The bodies had no limiting membrane. From these facts, although an initial change and a formation process of the filamentous inclusion body were not demonstrated successively in this experiment, it was suggested that the inclusion body was composed of disorganized myofibrils and the locus of origin might be I band.

The filamentous inclusion bodies were found in human skeletal muscle fibers of various types of myopathy and denervation. Experimentally, it was

produced in chick embryo skeletal muscle in tissue culture[1] and in denervated skeletal muscle as shown in this study. From these facts it can be deduced that this abnormal structure is not specific and is one type of degeneration of myofibrils, or that this structure was specific for denervation—in the human cases described previously as myopathy, nerve fibers as well as the muscle fibers were affected at the peripheral regions by some agent. These abnormal structures were easily distinguishable from nemaline bodies which are characterized by transverse periodic streakings perpendicular to the bodies.

Membranous inclusions

There have been many descriptions dealing with changes of membrane-structure, especially sarcoplasmic reticulum and T system, of denervated rat [7,9,15,16,17], pigeon[18], frog[19], chick embryo in tissue culture[21], and pathological human skeletal muscle fibers[4,20]. Some authors described that in the early stages of denervation atrophy of skeletal muscle, the contractile material underwent continuous reduction while the sarcoplasmic reticulum and T system underwent absolute or/and relative increase in number[7,15,17,18]. In this experiment sarcoplasmic reticulum and T system did not show a positive picture of decrease or increase. However, two kinds of membranous inclusions, which consisted of parallel arrays of cisternal structures, were conspicuously noted. Discussion was limited to the membranous inclusions in order not to deviate from the subject of this paper.

In the gastrocnemius muscle of a rat, Schrodt and Walker[7] observed lameller arrays of membrane-enclosed cisternae in fibers denervated for 2 months or longer. They suggested that the structure could be derived from elements of the sarcoplasmic reticulum, because the structure of the cisternae and the location of glycogen particles to the limiting membrane of the cisternae in denervated muscles, resembled those of the sacoplasmic reticulums in normal muscle. The inclusions of smaller cisternae in this experiment were similar to the lamellar structures shown by Schrot and Walker, and we agree with their opinion on the origin of the inclusion from elements of the sarcoplasmic reticulum.

The cisternae making up the inclusion of smaller cisternae varied in number from a few to several tens, in size and in direction. No interrelation between the number of the cisternae and the duration of denervation after the operation was found. In some places these abnormal structures continued to normal sarcoplasmic reticulums. It would be possible that the sarcoplasmic reticulums being left behind were reorganized after the myofibrils collapsed. In human cases there have been found no reports on this abnormal structure as far as we are aware.

Odor *et al.*[4], in a case of periodic paralysis, described the presence of aggregates of tubules approximately 73 mμ in diameter. The aggregates were

very similar to the inclusions of larger cisternae in this study, but their limiting membrane was single-layered. They believed that the structure was derived from the sarcoplasmic reticulum. But they described that it was undetermined whether the tubular aggregates were a specific characteristic of some cases of familial periodic paralysis or a nonspecific degenerative change. Nakashima *et al.*[6] reported staggered double rows of vesicles approximately 0.1 μ in diameter in Figs. 7 and 8. These structures were analogous to the inclusions of larger cisternae. Price *et al.*[8], in rat skeletal muscle following extreme injury by cold, described clusters of tubule-like structures defined by a single membrane, which were similar to the inclusions of larger cisternae. They interpreted these structures as damaged vestiges of the sarcoplasmic reticulum.

The inclusion of larger cisternae in this study consisted of an aggregation of tubular structures, and the unit membranes of them were clear. These structures were considered to be derived from sarcoplasmic reticulum, since they showed frequent continuity with the structures similar to normal sarcoplasmic reticulum or terminal cisternae. The present author believes that these abnormal structures are indicative of abnormal overdevelopments of the sarcoplasmic raticulum, but because of some differences in the architecture and staining reaction there may be difference in quality between the larger cisternae and the normal sarcoplasmic reticulums. This third type of inclusion has been found to coexist with the filamentous inclusion in human cases[4)6)] as well as in this study. It remains a question whether these regularly arranged cisternal structures, such as the inclusions of smaller and larger cisternae, are merely an expression of degenerative changes of muscle fibers or specific findings of denervation.

There would be two discordant opinions about the significance of the inclusions above mentioned, namely, these inclusion are indicative of muscle fiber abnormality but not of specific significance on one hand, and the presence of each inclusion represents the injury of nerve fibers in any region and may be characteristic of a nerve injury on the other hand. From the results obtained in the present study, the present author is inclined to agree with the later opinion.

In this study three different methods were used for the fixation of the muscle. Perfusion through the vena cava infeior in a peripheral direction was the most recommendable for gastrocnemius muscles. The muscles were fixed in a streched state as intended, and stained evenly.

ACKNOWLEDGEMENT

I wish to thank Professor H. Tauchi, M.D. and Dr. H. Watanabe for their encouragement and advice during the course of this work and for their critical readings of the manuscript. The technical assistance of Mr. J. Aoki and Miss.

H. Asai is greatly appreciated.

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