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# ELECTRON MICROSCOPIC STUDY ON THE BEHAVIOUR OF MONONUCLEAR PHAGOCYTES IN THE LYMPH NODE AFTER SECONDARY ANTIGEN STIMULATION

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*Indexing Words*

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**phagocytosis;**
**Freund's adjuvant**

Tadashi SATANI *Electron Microscopic Study on the Behaviour of Mononuclear Phagocytes in the Lymph Node After Secondary Antigen Stimulation.* Kobe J. Med. Sci. 17, 35-57, March 1971——In immunological reactions the antigen uptaken by phagocytes may bear an important role in the antybody production by lymphocytes. To elucidate the mechanism, the observation was made on lymph nodes after secondary antigen stimulation in the animals previously sensitized with Freund's adjuvant containing bovine serum albumin with special reference to the macrophages and the immune cells using an electron microscope.

The secondary reaction was characterized by an intense phagocytosis of the antigen protein of mature phagocytes. Degeneration and destruction of some of the mature phagocytes were observed after a comparatively early stages. About this stage, immunoblasts were increased, then followed by a proliferation of plasma cells. Exactly at this stage, immature phagocytes came to appear in parenchyma of the lymph nodes. Along with the maturation of these cells, they changed to stimulating phagocytes. These cells then took up antigen protein, gradually transformed into ingesting phagocytes.

Scarcely any reaction of fixed reticulum cells was noted after antigen stimulus. Consequently the fixed reticulum cells do not probably represent the origin of the immature phagocytes seen in the early period following antigen challenge.

## INTRODUCTION

Recent studies have revealed the important participation of the antigen ingested by phagocytes in antibody formation by lymphocytes. Many noticeable reports are available on this point. Many reports have appeared recently on the process of transformation of lymphocytes, reacting to stimulation by antigens, into large blast cells followed by metamorphosis into plasma cells or small lymphocytes. When dead tubercle bacilli were administered along with a specific antigen (water-in-oil emulsion), the antibody response was definitely intensified, as noted by the many reports following the research works of Freund et al.[18] However, it was desired

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to explore more fully the mechanism of intensification of the immune reaction using some newer methods of cellular pathology. More specifically, the changes in lymph nodes during the secondary reaction following the administration of the antigen Bovine serum albumin (BSA) in animals which had been previously sensitized with Freund's complete or incomplete adjuvant with added BSA were studied ultramicroscopically. Emphasis was placed on the morphological picture and changes in the macrophages and immune cells.

## MATERIALS AND METHODS

*Group 1.* Twenty adult rabbits weighing approximately 2 Kg were used. Incomplete Freund's adjuvant to which had been added 0.5 ml of 10% BSA was intraperitoneally injected once a week for 3 weeks. Four weeks after the last sensitization, 0.5 ml of physiological saline containing 10% BSA was inoculated subcutaneously into the foot pad and the popliteal lymph node was resected after 24 hr, 48 hr, 72 hr, 5 days, 1 week, 2 weeks, 3 weeks, and 4 weeks, in that time sequence.

*Group 2.* Ten adult rabbits weighing 2 Kg were used. Sensitization was carried out as in Group 1 above, with complete Freund's adjuvant added BSA. Four weeks after the last treatment, 1 ml of physiological saline containing 1% BSA was inoculated subcutaneously into the foot pad. Popliteal lymph nodes were resected in the time sequence noted in Group 1 above.

*Group 3.* Ten adult rabbits weighing approximately 2 Kg each were used. One ml of physiological saline containing 1% BSA was inoculated subcutaneously into the foot pad. Popliteal lymph nodes were resected in the time sequence noted in Groups 1 and 2 above. Each material described above was prepared into small tissue fragments of approximately 1 cubic mm and placed in a mixture of 1 cc of phosphate buffer (pH 7.4) with added glucose and 1 cc of 2% osmic acid, for fixation for a period of 1½ hours at approximately 4°C. After dehydration with alcohol and embedding in Epon 812, the specimen was cut into thin sections using a Leitz or Porter-Blum ultramicrotome and subjected to double electron staining with uranyl acetate and lead nitrate for observation under a Hitachi HU-11 electron microscope. Part of the lymph node speciment was also fixed with 10% formalin and light microscopic findings were followed with H-E staining.

## RESULTS

### *A. Light microscope findings:*

In Group 1, dilatation of the lymph node sinus was observed 1 or 2 days after inoculation with the BSA. Appearance of monoculear phagocytes and the subsequent proliferation of these cells (so-called reticulum cell hyperplasia) was noted. Mononuclear phagocytes appeared and prolifereted into the nodules within the cortical parenchyma, especially around the follicles. After 5 days, the proliferation progressed further and continued for 2 more weeks. A gradual decrease was noted, however, and after 3 to 4 weeks only a small amount of phagocytes remained.

Three days after antigen administration, blast cells appeared within the cortical parenchyma. These cells had large nuclei with distinct nucleoli: They increased in size and the cytoplasm stained basophilically. After the 5th day, proliferation of the blast cells in the cortical parenchyma became even more distinct. Then proliferation of plasma cells and lymphoblasts followed. In the medullary cord, proliferation of large blast cells was noted markedly after 1 to 3 days and proliferation of plasma cells reached a maximum on the 5th day, followed by a gradual decrease. After 3 weeks, scarcely any were seen. Approximately the same series of events was noted in Group 2.

*B. Classification of various cell types according to electron microscopic findings:*

In Group 1 the amount of inoculated antigen was rather large in the secondary inoculation. As a consequence, the amount of ingested antigen within the phagocytes was especially abundant and degenerative and destructive changes in the phagocytes were especially pronounced in Group 1. However, no great difference was noted between Group 1 and Group 2 with regard to the fluctuation of the ingested antigen within the phagocytes, the time of appearance of various cell types, and their variation and morphology. Electron micrography of the main lymph node cells appearing in the course after the secondary stimulation were explained as follows:

(*1*) *Immunoblasts*

These cells were larger than immature phagocytes, with a size of approximately 10$\mu$. Nuclear substance showed no marked aggregation in the marginal zone of the nucleus. Although there were abundant polysomes, scarecely any Golgi apparatus was noted. Only a few rough surfaced endoplasmic reticulum (r-ER) could be found. On the contrary, in the immature phagocytes there were but a few polysomes and scarcely any slender r-ER as seen in the immunoblasts. However, the development of Golgi apparatus was more pronounced in the immature phagocytes than in the immunoblasts.

(*2*) *Plasma cells of the mature type*

The size was 8–10$\mu$, with a circular or ellipsoid shape. The nucleus was round or oval, and occupied an eccentrically deviated position. The nuclear substance was relatively dense distributed, with an aggregation at the center or adjacent to the nuclear membrane. Small nucleoli were occasionally seen. The r-ER underwent remarkable development and was disposed in a lamellar arrangement surrounding the nucleus. The Golgi apparatus was also observed with a pronounced development in the central area. Round or elliptical mitochondria were scattered around the Golgi apparatus.

(*3*) *Small lymphocytes*

The diameter of the cytoplasm was 5-7$\mu$. Nuclei were circular with an occasional deep incision and a marked aggregation of nuclear substances was observed. Nucleoli were indistinct. The cytoplasm was narrow and only a few isolated ribo-

somes were seen. Mitochondria were small and round, being distributed at several sites. There were scarcely any r-ER. Golgi apparatus was also poorly developed.

(*4*) *Immature phagocytes*

They had the size of approximately 8$\mu$ in diameter. Their cytoplasm was somewhat narrow and the nucleus was either centrally located or eccentrically located, and a shallow incision was occasionally seen. Nuclear substances were fine and distributed loosely with little aggregation of the nucleus margine, one or two nucleoli being occasionally observed. Intracellular organella were poorly developed. Ribosomes were diffusely distributed taking the form of the polysomes in general, but a few smooth-surfaced endoplasmic reticulum were also noticed. Scarcely any r-ER were seen. Mitochondria were somewhat round, large and few in number. The Golgi apparatus was only occasionally seen and body structures with a high density, probably representing lysosomes or minutes phagosomes, were also occasionally seen.

(*5*) *Stimulating (mature) phagocytes (macrophages) and stimulating ingested phagocytes (macrophages)*

The size of the cytoplasm was 10-15$\mu$ The shape was irregular with many distinct cytoplasmic processes. The nucleus was located rather eccentrically with deep incisions. The nuclear substance was finely distributed without any marked aggregation at the marginal zone. Occasional nucleoli wer noted. Golgi fields were markedly enlarged, sometimes containing 2 to 3 groups of Golgi apparatus consisting of vesicles, vacuoles, and a membrane system. In the enlarged Golgi field, many small lysosome-like dense bodies with a high electron density, surrounded by limiting membranes, were scattered. Mitochondria were oval or rod-like in shape, small in size, and distributed mainly around the central area. Smooth surfaced endoplasmic reticula were increased and arranged in scattered or rosarybead-like fashion. The r-ER was also increased, and was arranged in lamella or whirls in the peripheral portion of the protoplasm. In the stimulating ingested phagocytes, middle-sized or small phagosomes with moderate or high electron density were seen around the central area. These phagosomes were circular in shape, with a maximum size of 1$\mu$ but were mostly below this size. The number of phagosomes contained was also smaller than that of large typical ingesting phagocytes and there was almost no lamellar body formation.

(*6*) *Large ingesting phagocytes*

This cell type was 10-20$\mu$ in diameter, the shape was irregular, and the cellular processes were most abundant. The nuclei were located eccentrically and were oval or irregular in shape, with occasional incisions. One or two nucleoli were occasionally seen. The nuclear substance was diffusely distributed with scarcely any aggregation in the marginal zone of the nucleus. A relatively wide Golgi fiield was seen in these cells, and the Golgi apparatus appeared in 2 or 3 groups. Development of r-ER was marked and the smooth surfaced endoplasmic reticulum was

scattered. Mitochondria were oval or short rod shaped. In the hypertrophied cytoplasm, Iarge- sized phagosomes containing amorphous substances of high electron density surrounded by one layer of limiting membrane were noted. The size of phagosomes was about $2\mu$ but occasionally $4\mu$ or more. This shape ranged from circuloid to an irregular type with great variation.

*(7) Fixed reticulum cells*

These cells give little reaction to antigentic stimulation. Their cytoplasm was about $10\mu$ in diameter and had an irreguler shape with many processes and long extensions. These prosesses and protoplasmic extensions were closely connected with fibers which were frequently seen even in the intra cellular spaces. The cytoplasm was relatively narrow and seldom contained small phagosomes less than $1\mu$ in diameter.

*C. Follow-up of the course after antigen inoculation by electron microscope observations:*

*(1) Findings in groups 1 and 2, one day after antigen inoculation*

Immunoblasts appeared in the medullary cord, but their appearance in the cortical parenchyma, especially in the area neighbouring the follicles was not distinct. Nodular proliferation of mononuclear phagocytes in the area neighbouring the follicles indicated loose collections of individual phagocytes, dispersed among other kinds of cells. At this stage, only a few immature phagocytes were seen. There were also a few stimulating phagocytes: Nodular proliferation of mononuclear phagocytes in the cortical parenchyma and of large macrophages within the sinus mainly consisted of ingesting phagocytes accompanied with occasional stimulating mature phagocytes. Under the electron microscope, the ingesting phagocytes showed a slight enlargement of nucleoli. Within their cytoplasm, many giant phagosomes were seen. Among these, an increase in dense lysosomes and r-ER was noted. In some ingesting phagocytes, there was noted some distinctive degeneration, in which enlargement with swelling of r-ER and mitochondria were distinctive. Stimulating phagocytes in the early stages were generally small in size, and the incision in the nucleus was not distinguishable. Their nuclear substance was distributed diffusely, however, nucleoli formation was occasionally found. Although their Golgi apparatus was still small in size, dilatation was occasionally seen. Lysosome-like dense bodies were also seen in some of them.

*(1') Findings in group 3 one day after inoculation*

Ingesting phagocytes with medium sized phagosomes were noted in the medullary cord, sinus and cortical parenchyma together with a few stimulating phagocytes. The number of these cells was smaller than in group 1 and 2 and the tendency towards degeneration of ingesting phagocytes was rather indistinct.

(2) *Findings in group 1 aud 2, during 3 to 5 post-inoculation days*

A distinct proliferation of immunoblasts and plasma cells was noted in the areas neighbouring the follicle in the cortical parenchyma and within the medullary cord. As for the nodular proliferation of large mononuclear phagocytes in the cortical parenchyma especially in the areas neighbouring the follicle, an increase of many ingesting and stimulating phagocytes was noted. Many immature phagocytes also appeared, and they developed in sequence to the mature types of phagocytes. In the ingesting phagocytes, the enlargement of nucleoli was distinct. Many giant phagosomes were also noted within the cytoplasm. The content of phagosomes indicated a high and low amorphous density in some and a small massive aggregation in others. In some phagosomes, a decrease of density was seen and a formation of a lamellar body was also noted in others. At this stage, many of the ingesting phagocytes revealed an increase in r-ER and small mitochondria, scattered all over the cytoplasm. In some ingesting phagocytes, enlargement and swelling of the r-ER and the mitochondria indicating the presence of degenerative and destructive processes were noted. On the 5th day, the tendency towards degenerative and destructive changes in ingesting phagocytes became pronounced, and the r-ER showed a marked enlargement. Swelling of mitochondria became more pronounced, and the appearance of fatty droplet was noted. In the intercellular space, many organella of the degenerated and destroyed cells were noted. As for the stimulating phagocytes, they originated from immature phagocytes and, in accordance with the development of intracellular organella an increase in various intracellular organella developed, which became the hallmarks of the morphological characteristics of mature phagocytes. There were also stimulating phagocytes with small phagosomes.

Among these cells, some were provided with medium or small phagosomes which had a relatively low density content or occasionally had lamellar structures. These represented an increase of r-ER, suggesting that there was an increase of intracellular synthetic action besides the phagocytic activity of the phagocyte.

(2') *Findings in group 3, during three to five days after inoculation*

The formation of blast cells and plasma cells was noted but they were less abundant than in groups 1 and 2. Many ingesting phagocytes were noted and some of the phagosomes showed formation of a lamellar body. In the ingesting phagocytes, degenerative and destructive changes were noted, but were not always distinct.

(3) *Findings in groups 1 and 2, one week after inoculation*

In the areas neighbouring the follicle within the cortical parenchyma and also in the medullary cord, immunoblasts and many plasma cells were noted. Nodular proliferation of phagocytes noted under light microscopy showed a loose collection of phagocytes dispersed among other cells under the electron microscope. Among the collection of phagocytes, lymphocytes were frequently seen. The prolif-

erating phagocytes at this stage mainly consisted of stimulating phagocytes. Along with a decrease in immature phagocytes, an increase of the stimulating type and the appearance of ingesting phagocytes with small phagosomes were noted. The ingesting phagocytes filled with giant phagosomes were decreased or almost gone. In many of the ingesting phagocytes development of the Golgi apparatus and an increase of r-ER and mitochondria were seen, together with circular medium-sized or small phagosomes. Some of these phagosomes were of amorphous and homogeneous structure, some contained small aggregated masses within low density vacoules, and the others contained lamellar bodies. Various stages ranging from high electron density to low electron density, and completely light vacuoles were observed. At this stage, the numbers of those with degenerative and destructive changes decreased. The stimulating phagocytes showed an increase of their protoplasmic processes with marked irregular indentation in the nuclei. Their Golgi apparatus also showed a marked development, lysosome-like dense bodies increased, the r-ER increased, and the mitochondria become small and increased in number The density of the cytoplasm was increased due to the presence of many polysomes and ribosomes.

(*3'*) *Findings in group 3, one week after inoculation*

Marked proliferation of plasma cells was seen in the medullary cord and cortical parenchyma. While ingesting phagocytes were decreased in the sinus, they were found abundantly in the cortical parenchyma. The content of phagosomes showed a decrease in density in some of them, and the formation of lamellar bodies was somewhat increased. Proliforation of the stimulating phagocytes was milder than in groups 1 and 2.

(*4*) *Findings in groups 1 and 2, 2 weeks after inoculation*

Immunoblasts and plasma cells were decreased. Proliferating phagocytes were seen around the follicles in the cortical parenchyma mainly consisting of the stimulating type ones. Ingesting phagocytes were somewhat decreased, and the immature phagocytes were further decreased. In the ingesting phagocytes at this stage, the number of phagosomes was decreased. In cells with large- or medium-sized phagosomes, a decrease in density of the contents was noted. In cells with dense medium-sized or small phagosomes, were increased and vesicular or long tubular r-ER and mitochondria, and a mild increase of polysomes were also increased mildly. Enlargement and dispersion of the Golgi apparatus was also noted. There were many stimulating phagocytes: Their cytoplasm underwent hypertrophy, and the enlargement of the Golgi field became even more pronounced, along with increase of lysosome-like dense bodies distributed around it. Mitochondria were small in shape and increased in number, frequently distributed around the Golgi field. The r-ER was also increased, and showed lamellar or whirl-like distributions. Some of the medium-sized or small phagosomes seen in the stimulating and ingesting phagocytes showed a decrease in protoplasmic density. However, no formation of lamellar body was seen in the ingesting phagocytes.

(4') *Findings in group 3, 2 weeks after inoculation*

Phagocytes were already decreased. A decrease of the density of phagosomes content in ingesting phagocytes was seen, and the degree of increase was milder than in groups 1 and 2. The size was somewhat smaller and the outline was relatively simple. The growth of organellae was less pronounced than in the sensitized animals. A few immature phagocytes were noted.

(5) *Findings in groups 1 and 2, 3 to 4 weeks after inoculation*

No immunoblasts were noted. Plasma cells and proliferating cells were markedly decreased. The stimulating phagocytes and a few ingesting phagocytes remained. In ingesting phagocytes, phagosomes were medium or small in size, some of them contained dense material, while the content of others was of low density. Increase of r-ER and enlargement of the Golgi field were noted. Polysomes were also increased in number and the whole cell gave a dense appearance. Occasionally, large cells with large phagosomes ingesting debris of destroyed cells were noted. The ribosomes of the cytoplasm of stimulating phagocytes were decreased, with an increase of vesicle formation. General cytoplasmic density was decreased. After 4 weeks, the cytoplasm appeared markedly light in general. Sometimes a rosary bead-like arrangement of vesicle along the outer perimeter of the cytoplasm was noted.

## DISCUSSION

White et al. administered ovine albumin together with Freund's complete adjuvant, and confirmed the proliferation of macrophages at the site of injection, a morphological abnormality of these cells (formation of epitheloid cells), a marked stimulation of the RE system all over the body, and extensive proliferation of the plasma cells in the lymph nodes, spleen, and liver.[40]

Whether such proliferation of macrophages is of local origin or bone marrow origin presents an extremely interesting problem with a long historical background. In our laboratory, reticulum cells and large phagocytes were studied using the light and electron microscopes especially on the origin and the formation of tuberculous epithelioid cells in various organs.[32,33]

It is the conclusion of the studies in our laboratory that the epithelioid cells were transformed from young histiocytes, originating from the most primitive cells of the same system in the tissue (immature histiocytes or immature reticulum cells). The immature histiocytes resembled lymphocytes which always showed scanty organella in the cytoplasm. Unlike lymphocytes, the aggregation of nuclear substance was decreased, with an increasing of their fine diffuse distribution. Under many circumstances, nucleoli became distinct, followed by a further enlargement. Within the cytoplasm, distribution of ribosomes was seen more abundantly than in lymphocytes. In the course of maturation, cytoplasm was increased, together with the development of a Golgi apparatus, an increase of vesicles, the formation of lysosome-like dense bodies, r-ER, and frequently phagosomes. In the stimulated spleen, Yamori and Mori

observed blastic young cells which showed abundant ribosomes and polysomes in the cytoplasm, and diffuse distribution of fine nuclear substance, with an occasional nucleoli in the nucleus, and considered them to be macrophages originating from reticulum cells. For this reason, these were called reticuloblasts. However, since discovery of these cells was far less frequent than the multiple appearance of macrophages in the stimulated spleen, these workers subsequently doubted whether these blastic young cells originated from fixed reticulum cells in the spleen.[41,42] In fact, the reticulum cell system in the lymph nodes represents only a slowly proliferating population, as is evident from the studies of Ricke et al. using labelled tritiatated thymidine. These may also be only very slowly reproducing cells closely related to body growth. Consequently, the local fixed reticulum cells most probably do not represent stem cells in the formation of blastic young cells and in the appearance of many macrophages upon antigenic and other inflammatory stimulation.[35]

Harui in our laboratory demonstrated recently that fixed reticulum cells played mainly a supportive role, attaching to the reticulum fibers on one side and never proving a marked phagocytosis. This was done using electron microscopic observation of the spleen.

According to him, the fixed reticulum cells belonged to a system different from the large phagocytes (macrophages) with intense phagocytic activity.[25] Similar properties of fixed reticulum cells were confirmed in lymph nodes in the present study. However, the direct origin of large phagocytes (macrophage) has not been made clear, though it was made apparent that the macrophage was not principally derived from the local reticulum cells in the lymph nodes.

The origin of large inflammatory phagocytes from blood cells was pointed out by Ebert and Florey quite a long time ago.[10] Amano and his associates in Japan also pointed out that tissue macrophages originated from monocytes in the blood.[2] Recently, there have been many studies and much progress in this area.

Using labelled $H^3$-thymidine in rats Cronkite et al. inferred an origin of the granuloma macrophages to be derived from blood born cells.[7,8] Specter doubly labelled leukocytes in blood with both $H^3$-thymidine and colloidal carbon and concluded that the phagocytes in inflammatory exudates originated from monocytes in the blood based on the close proportional appearance of labelled cells which were seen in blood and subcutaneous tissues.[36]

Volkman, Gowans and others observed macrophages using the skin window method technique and the cover slip method and came to the conclusion that macrophages occurred through mitosis and transformation of precusor cells originating from blood cells. Such precursors would surely proliferate at the sites other than the inflammatory area, to be released into the blood stream. Emigration of macrophages was not suppressed in the observation in rats from which lymphocytes were deprived by thoracic duct fistule formation. By transfusion of the cells obtained from the thoracic duct lymph, lymph nodes, thymus, spleen and bone marrow containing radio-actively labeled cells and by the investigation of the labeled macrophages seen in the skin window or cover slips in the recipient animal, they were able to conclude a bone marrow origin for the tissue macrophages.[37,38]

Kosunen et al. on the other hand, ascribed the macrophage response in the inflammatory lesion under various states of delayed reaction to the mitosis of medium lymphocytes as the precursor cells, and not to transformed small lymphocytes.[28, 29]

Gough et al. labeled small lymphocytes with $H^3$-thymidine and cultivated these bloood cells. Since macrophages with radioactive markers were noted in the course of blood cell culture, transformation of the small lymphocytes to phagocytes was assumed.[22]

Berman et al. conducted blood cell culture using a special medium to induce slow growth to study the process of maturation of macrophages. In the initial stage the lymphocytes gave a monocyte-like appearance with basic lymphocytic characteristics. Nuclear substance gradually became finer. On the second day, altered lymphocytes with phagocytosis appeared. On the third day, the size became enlarged with the appearance of cytoplasm with large vacuoles and granules. The nuclei resembled those of histiocytes and reticulocytes, and came to be indistinguishable from these. The size subsequently increased further, along with an intensification of basophilia and the appearance of nuclei. On the 17th day macrophages increased in number and their nuclei became eccentric in location. From the 12th to 25th day, the changes from mononuclear phagocytes to polynuclear giant cells were noted.[4] These observations in blood culture might probably show the occurence and the development of macrophages from blood cells in vivo to some extent. Concerning the differentiation phagocytes, the studies of Cohn et al. are noticeable. They investigated granule formation in the intraperitoneal phagocytes and the relationship to the production of hydrolytic enzymes, changing the concentration of calf serum in the calture medium. As a result, granules of phagocytes were rapidly formed under high concentrations of calf serum. The size was large and hydrolytic enzymes were abundant. On the contrary, in culture medium with calf serum at low concentrations, granules were small in shape and few in number, and consequently hydrolytic enzymes scarcely increased.[5] These findings would indicate a participation of some substance and its concentration in the maturation of phagocytes.

The intracellular digestion of the substance ingested by phagocytes and development of immunity will be considered next. In the initial stages of investigation of the role of phagocytic action of phagocytes on immunity, ingestion and digestion of antigenic foreign bodies were considered to cause the immune reaction. In recent studies, however, the significance of such intracellular treatment ingested antigens in the phagocytes, on immunity, has gradually been elucidated. According to Nossal and Mitchell, phagocytosis of radioactively labeled soluble protein and flagellar particles was surprisingly ineffective in inducing immunity occurrence in newborn rats for several days after birth. In newborn animals antigenic material mostly spread diffusely in the lymphatic tissues to be present outside of the phagocytes for a long time. No immunity was induced, but on the contrary tolerance against these antigens appeared. According to these authours the reasons were surely due to the fact that the antigens were not phagocytized and present for long duration continuously on or in the lymphocytes.[34] The experiments of Frei et al. also clarified the significance of phagocytosis on the formation of immunity. $I^{131}$-labeled BSA was injected intravenously into normal adult rabbits to obtain serum

after clearance of the phagocytizable particles from the blood stream. Such serum samples containing biologically "filtered" antigen were administered to other rabbits for induction of immunity. In these animals, unlike the control animals, no immune response was induced, but, on the contrary, tolerance against BSA was induced in some animals.[17]

The studies of Fishman et al. on the treatment of antigen by phagocytes are of extreme interest. The phenol extract of phagocytes which had ingested $T^2$-phage was found to have a factor which induced immunity. This factor had a low molecular weight, a species specificity, and a sensitivity to RNase. According to their report the RNA formed in the phagocytes would act on lymphocytes, playing an important role in antigen formation, as the author pointed out.[11, 12] However, Askonas et al. later reported that his phenol extract (RNA) contained antigen or antigenic fragments and that the newly formed RNA was not the responsible agent.[1]

Friedman et al. also pointed out that it was not macrophage RNA but an antigen-RNA complex that stimulated antibody formation in lymphocytes. According to the results of these studies, the factor stimulating the antibody formation of lymphocytes supposed not to be the m-RNA formed in the phagocytes.[19] According to Bishop et al., the biological activity of such macrophage RNA was retained in the 6 to 10s region.[3] According to Gottlieb et al., this RNA was precipitated between 4s and 6s and would contain a proteinaceous substance which resulted in a decrease of immune ability after incubation with pronase.[21]

According to Campbell et al., the antigenic substance ingested by phagocytes was at first digested and antigenic fragments were formed. These combined with the soluble RNA within the phagocytes to form a complex which became active as an immunogenic substance.[6] According to Levine and Benacerraf, moreover, the immune tissue of the "responder" guinea pig with immune response against hapten-poly-L-lysine conjugates and of the "non-responder" guinea pigs without such a response were both capable of disintegrating the poly-L-lysin conjugates into small molecular fragments, but none of these animals were able to break up hapten-D-lysine conjugates and cause an immune reaction.[31] This study would indicate that antigens are broken into small fragments with antigenic determinants in the immune tissue for immune induction and that it is necessary for the split product to pass through certain specific additive metabolic steps. In non-responders, the latter process being lacking, immune induction was reported impossible.

According to Janeway, $^{125}$I-D-amino acid synthetic polypeptides, administered to mice, were maintained in the subcapsular sinus of the lymph nodes and especially among the phagocytes within the medullary cord. $^{125}$I-L-amino acid synthetic polypeptide was at first found within these phagocytes then in the germinal center. Both induced immunity upon treatment with complete Freund's adjuvant. However, administration of the former in saline caused an immunological paralysis but similar administration of the latter did not induce any paralysis, but rather immunity. Under such circumstances, circulating antibody was noted in correlation to the appearance of autoradiography by $^{125}$I-L-polymer in the germinal centers.[27] This report was of profound interest, since the relationship between immunity and di-

gestability of an antigen, between immune paralysis and immunity, and between immunity and complete Freund's adjuvant was discussed.

The mechanism of proliferation of stimulating phagocytes will be considered next. When antigenic stimulus is given, especially when the antigen contains a specific cell wall component such as the wax D of the tubercle bacilli, some active agent acts hematogenously on the bone marrow, inducing the proliferation of stem cells which should later leave the bone marrow and be mobilized to the tissue via the blood and grow in that tissue to form phagocytes. These phagocytes were found in the lymph nodes after antigen stimulations. After stimulation with complete Freund's adjuvant containing antigen, the proliferation of these cells was pronounced, and their appearance remarkably intensified, especially in the secondary reaction. After primary stimulation, the reactive appearance of the phagocytes in the lymph nodes was present to a small extent and resulted in a preparatory state for secondary stimulation where proliferation of the precursor cells in the bone marrow, their mobilization and maturation to phagocytes in the tissues occurred in an accelerated manner. This would be readily deduced from the studies of Willoughby et al., who demonstrated the continuous appearance of monocytosis in the lymph node upon inoculation of complete Freund's adjuvant and some other preparation into the lymph nodes.[39]

Finally formation of lymphocytes and plasma cells will be discussed. Damshek named the large primitive cell immunoblast and considered it to be derived from small lymphocytes or RE cells exposed to antigen stimulation and to be functionally identical with stem cells for plasma cells and lymphocytes precursor and proliferation roles[9]. However, the concept that pyroninophilic blast cells originate from the reticulm cell, supported since Fagraeus, has recently been losing advocators[13, 14] No demonstration of a transition from a RE cell to a blast cell has recently been made. However, the transition from a pyroninophile cells to plasma cells has been confirmed by many observations. In our laboratory, Fukumizu[20] and Ikuhashi[26] confirmed these findings by electron microscopic observation. The noteworthy studies of Gowans et al. in which it was concluded that pyroninophilic blast cells are formed through transition from small lymphocytes, have been supported by many investigators. According to Gowans, administration of syngenic small lymphocytes in rats without immunological reactivity through deprivation of lymphocytes resulted in a recovery of the immune reaction. They pointed out the important role of lymphocytes in the immune reaction, and demonstrated the transformation of lymphocytes labeled with radioactive markers into large pyroninophilic cells upon contact with antigen, followed by a further transition into immunologically active lymphocytes in their experiments with chimaeric mice.[15, 16, 23, 24].

Kubota in our laboratory, on the other hand, administered yeast protein and yeast RNA to rats as the antigen to investigate the changes in lymph nodes by the examination using an electron microscope. The first findings after antigen inoculation were the activation of the nuclei of the small lymphocytes in the lymph nodes. Nucleoli became distinct and further enlarged. Aggregation of the nuclear substance was decreased, and the nuclei showed a diffuse distribution of a fine nuclear substance. In the cytoplasm, an increase of ribosomes and a formation

of polysomes were noted. Moreover, such activated small lymphocytes changed into large blast cells or immunoblasts. Subsequently, these cells were steps such changed into a respective adult form of the plasma cells or lymphocytes via initial as a plasmablast or lymphoblast along with the accumulation of antibody within the r-ER, as shown in the detailed description by Kubota.[30] This observation was also confirmed in the present study.

## CONCLUSION

After sensitizing adult rabbits with complete and incomplete Freund's adjuvant containing BSA, BSA was inoculated subcutaneously into the foot pad and popliteal lymph nodes, and the fate of antigen and cellular changes were investigated with regard to time. The ultrastructural changes of various types of phagocytes appearing during the period following stimulation were clarified. The relationship be tween formation of immunoblasts, plasma cells, lymphocytes and morphological changes of the phagocytes was studies. The following results were obtained. In the lymph nodes immediately after antigen stimulation in a secondary response, active phagocytic ingestion of antigen protein by pre-existing mature (stimulating type) phagocytes occurred in the lymphatic sinus, and the cortical parenchyma especially in the areas neighbouring follicles. In the ingesting phagocytes, Golgi fields were enlarged, and an increase of mitochondria and r-ER was noted. However, in some specimens, degenerative changes such as swelling and enlargement of r-ER and mitochondria, and appearance of fatty droplets were observed. Subsequently, degenerated phagocytes were destroyed. Such degeneration and destruction became most pronounced 3 to 5 days after antigen administration. At about this stage, immunoblasts increased and a transition to plasma cells was noted. Exactly at this stage, appearance of immature phagocytes in the neighbouring area of the follicles and cortical parenchyma of lymph nodes began. Along with their maturation, a transition to stimulating (mature) phagocytes was noted: Enlargement of the Golgi field, formation of lysosome-like dense bodies, and an increase of r-ER came to be noted. Such stimulating phagocytes further ingested debris of destroyed cells and the antigen protein released into the intercellular space. These cells were gradually transformed into ingesting phagocytes. In the sensitized animals, a marked appearance of imature phagocytes was noted, along with an intense proliferation of stimulating (mature) phagocytes. The period of the most intense appearance of immature phagocytes was from 5 to 7 days after antigenic stimulation. The increase of stimulating phagocytes reached a maximum after 2 weeks. Proliferation of large mononuclear macrophages in cortical parenchyma especially the area neighbouring the follicles, was interspersed with lymphocytes and other cells and exhibited some gradual change in cell types during the time course. These represent a loose collection of immature type, stimulating (mature) type and ingesting type in accordance with the progression of stages after antigen stimulation. 3 to 4 weeks after stimulation, the increase of phagocytes ceased, and the remaining mature stimulating phagocytes and ingesting phagocytes gradually decreased. The fixed reticulum cells were intimately related with the reticulun fibers without any marked reactivity during antigen

sfimulution. Their mitotic proliferation in the local tissue was extremely sparse in ordinary inflammation conditions such as antigen stimulation. Consequently, immature phagocytes appearing in the early period after antigenic stimulation do not appear to arise from the fixed reticulum cells.

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## LEGENDS

Fig. 1. Immature phagocyte
The fine chromatin is loosely distributed, showing no clear aggregation at the nuclear membrane. Organellae are poorly developed than those of mature phagocyte. Poorly developed Golgi apparatus, a few medium sized mitochondria and some times ingested dense bodies are seen. Five days after administration of BSA. × 8,000

Fig. 2. Stimulating phagocyte.
The fine distributed chromatin shows no aggregation around the periphery of the nucleus. Many lysosome-like dense bodies are scattered in the markedly enlarged Golgi area. Mitochondria, polysome and other organellae are increased in number. Two weeks after administration of BSA. × 10,000

Fig. 3. Stimulating phagocyte.
The well developed r-ER are arranged in whirls in the periphery cytoplasm. Two weeks after administration of BSA. × 20,000

Fig. 4. Stimulating phagocyte.
Higher magnification of r-ER of stimulating phagocyte.
Two weeks after administration of BSA. × 40,000

Fig. 5. Stimulating ingested phagocyte.
There are present medium sized, oval or round phagosomes at the periphery of enlarged Golgi area. Five days after administration of BSA. × 10,000

Fig. 6. Ingesting phagocyte.
In the cytoplasm many phagosomes with moderate or high electron density are noticed. Three days after administration of BSA. × 10,000

Fig. 7. Degeneration of ingesting phagocyte.
Degenerative processes are revealed by enlargement and swelling of r-ER and mitochondria. Three days after administration of BSA. ×10,000

Fig. 8. Degeneration of ingesting phagocyte.
Swelling of r-ER and rupture of cell membrane are seen.
Cytoplasmic organellae and phagosomes are present outside the cell. Three days after administration of BSA. × 10,000

Fig. 9. Degeneration of ingesting phagocyte.
Degenerative changes are smillar to those of Fig. 8.
Three days after administration of BSA. × 10,000

Fig. 10. Fixed reticulum cell.
Fixed reticulum cells with reticulum fibers are hardly responsed to antigenic stimulation. Five days after administration of BSA. × 10,000

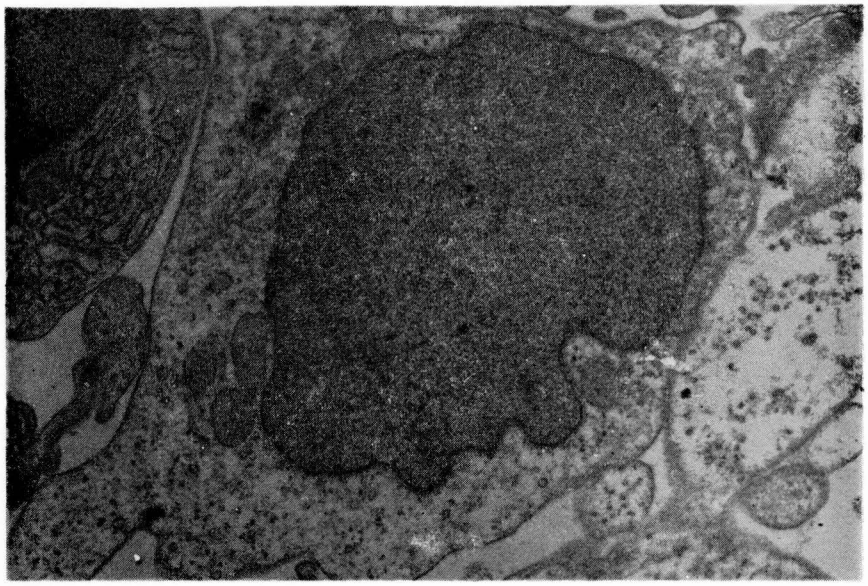
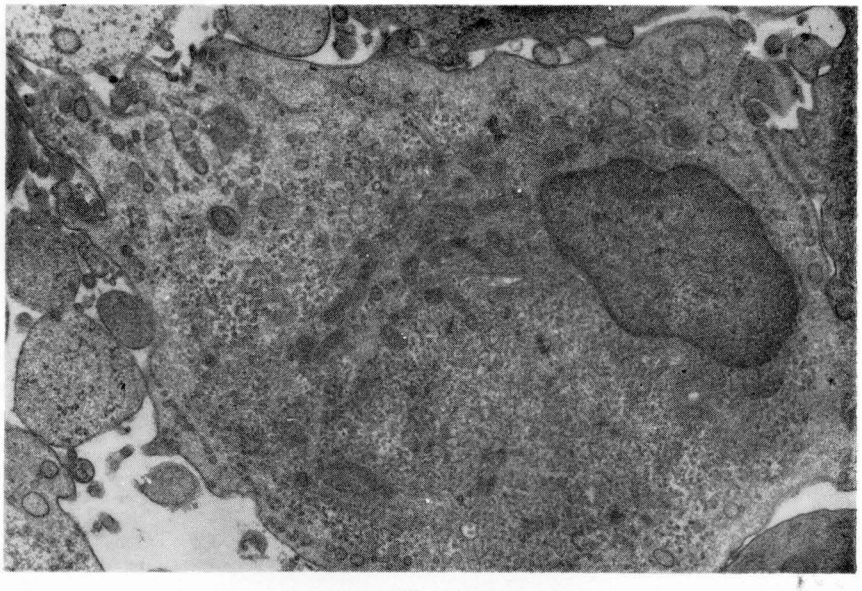


Fig. 1



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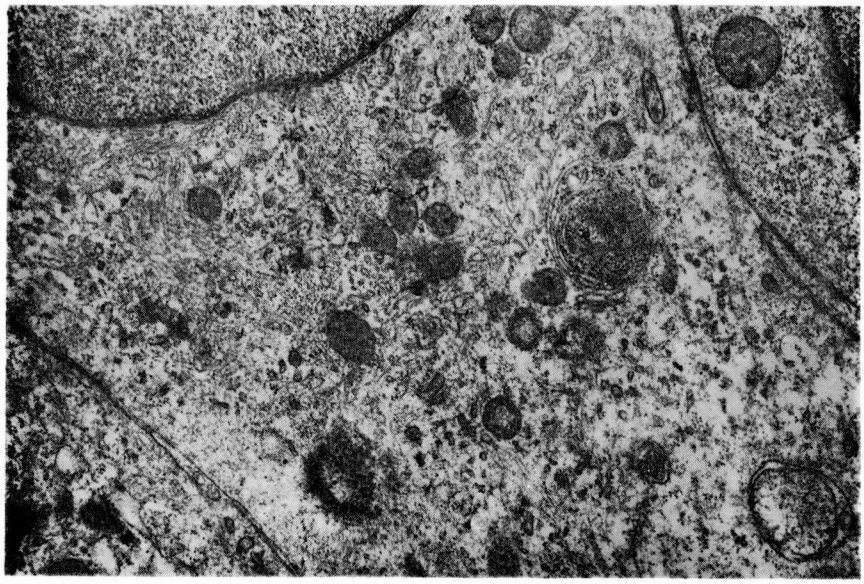

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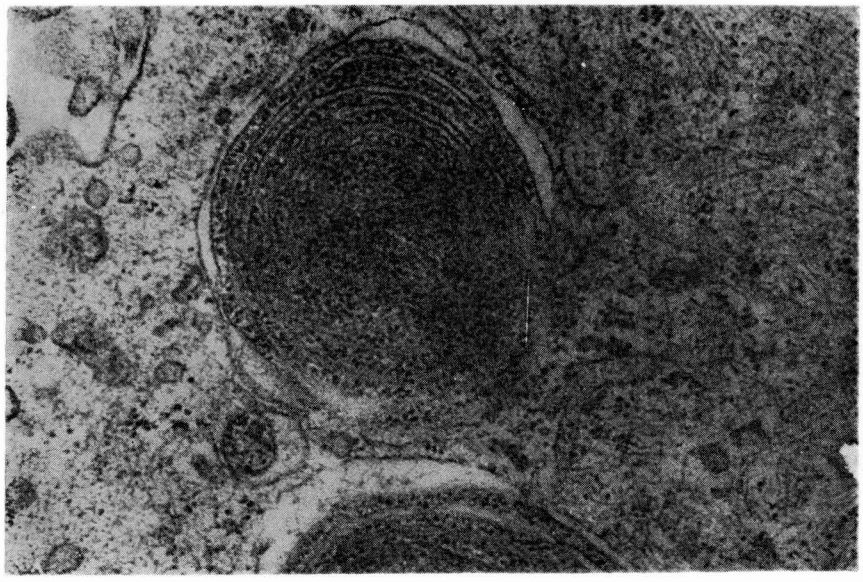

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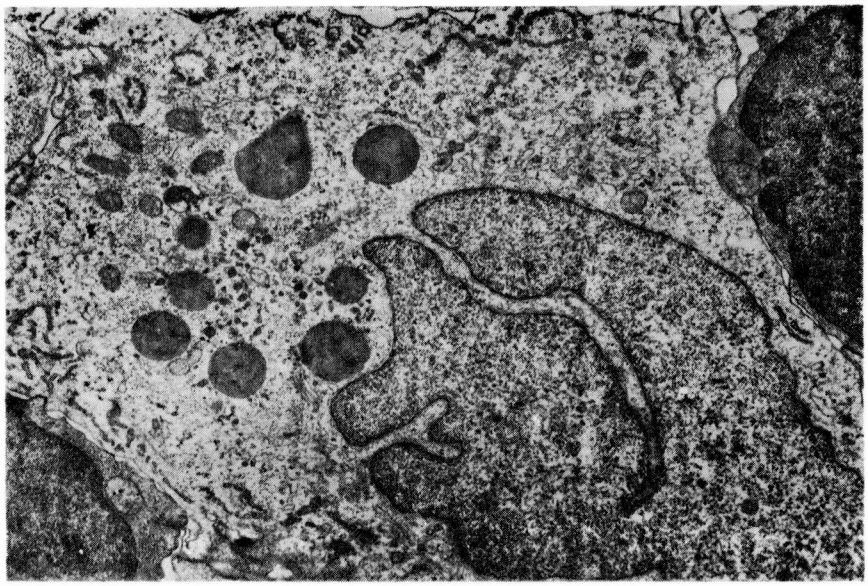

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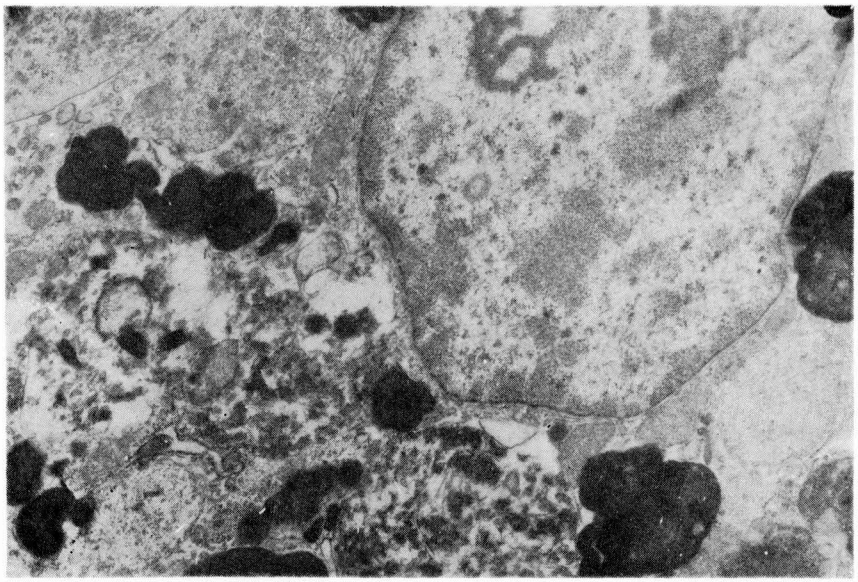

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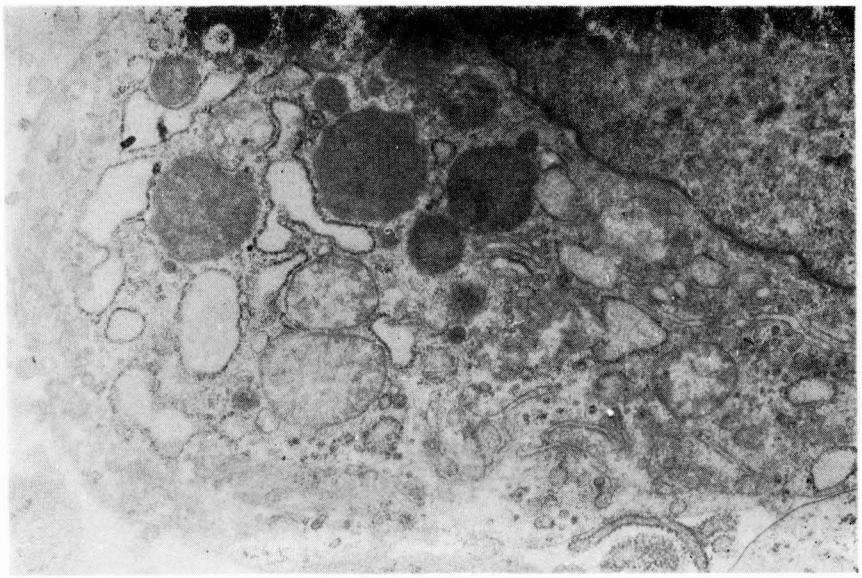

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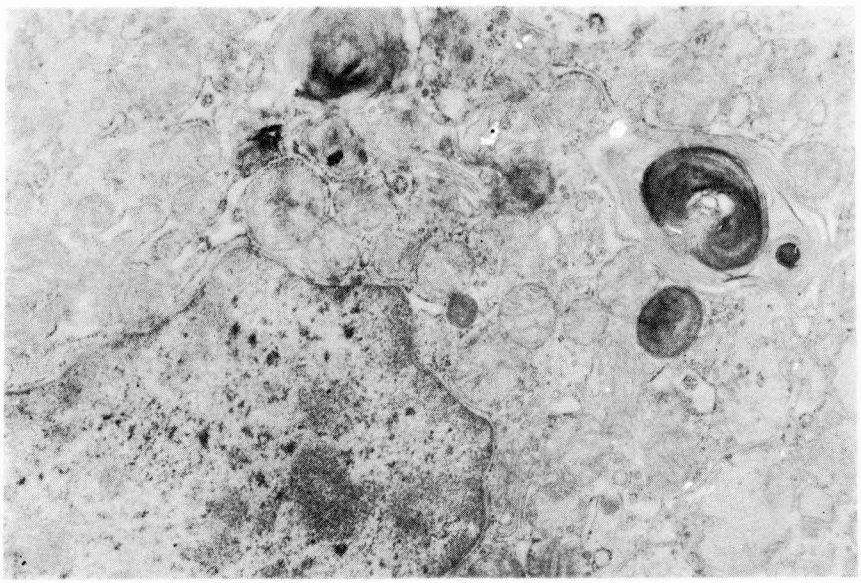

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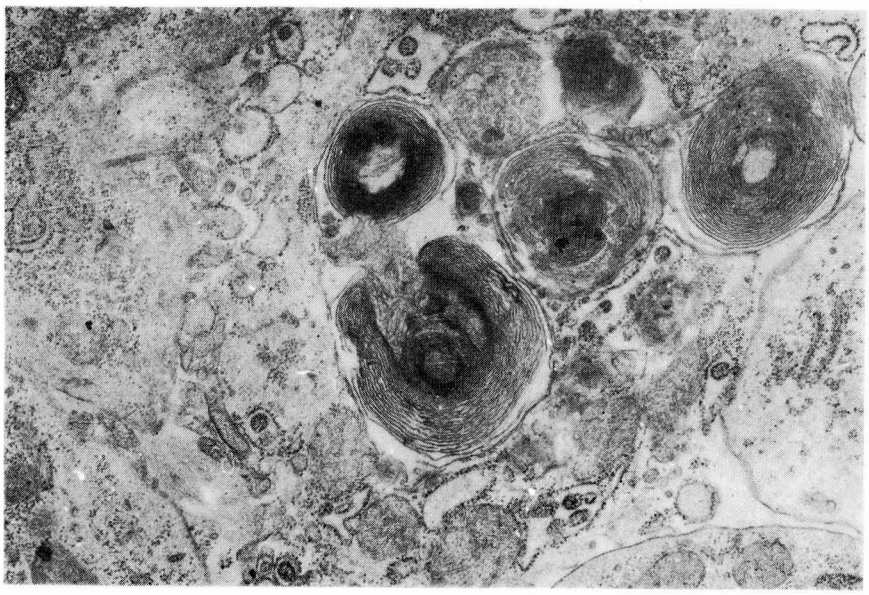

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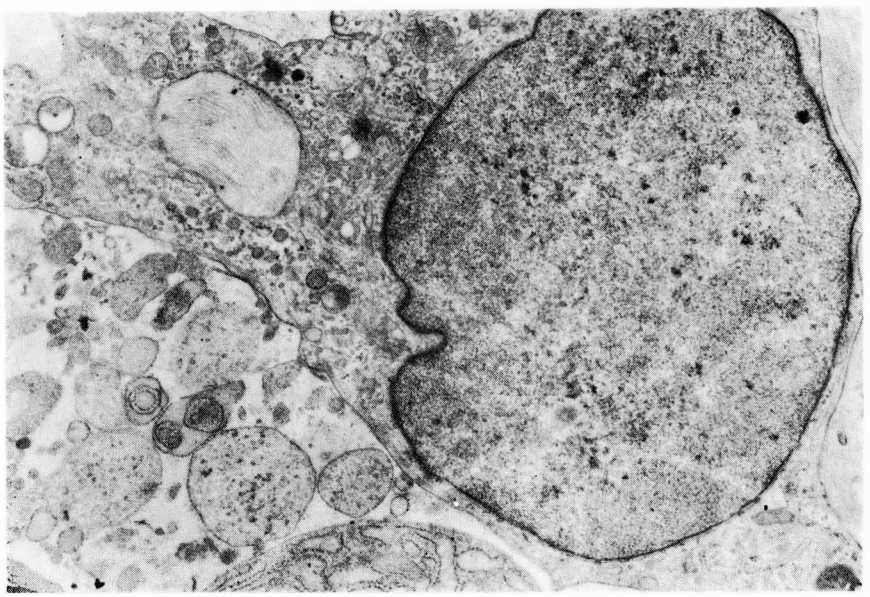

Fig. 10.