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ELECTRON MICROSCOPIC STUDIES ON THE MATURATION PROCESS OF NEUTROPHILIC LEUKOCYTES

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Naoki KAIHOTSU. *Electron Microscopic Studies on the Maturation Process of Neutrophilic Leukocytes* Kobe J. Med. Sci. 13, 47-66, March 1967——Electron microscope was used to study the ultrastructure of neutrophilic leukocytes obtained from normal human bone marrow and peripheral blood of ten patients with acute myelocytic leukemia. The purpose of this report is to clarify the ultrastructure of maturation process of neutrophilic leukocytes and the forming mechanism of their specific granules. Appearance and increase of specific granules coincided with the development of endoplasmic reticulum and Golgi apparatus. They were found first adjacent to Golgi apparatus and later became to scatter and distribute widely throughout cytoplasm. By the above findings, it was concluded that specific granules were derived from Golgi apparatus, just same as lysosomes were produced by Golgi apparatus.

Leukemic myeloblasts could be differentiated from myeloblasts of normal human bone marrow by morphological characteristics such as, atypical configuration of nuclei, increased numbers of mitochondria and clustered ribosomes in the cytoplasm. The findings represented preparatory arrangements for enormous multiplication of cells.

INTRODUCTION

Electron microscopic observations on the fine structure of mature leukocytes have been carried out by Kautz and DeMarsh[1], Reinehart[2], Watanabe[4], Goodman[5] and others. Concerning the development of the intracytoplasmic organellae in the course of leukocytes maturation, studies on animal bone marrow by Watanabe[4], Ichikawa[6], and Pease[8] as well as on human bone marrow by Braunsteiner[7] and Capone[8] are available. Low and Freeman[9], Ito[10], Watanabe[11], Akasaka[12], Anderson[13], and others have described the characteristics of intracytoplasmic organellae in each type of leukemic cells. However, among various investigators there is divergence of opinions concerning the ultrastructure of intracytoplasmic organellae in the myeloblasts. The specific granules in neutrophilic leukocytes are believed to be originated from the mitochondria[2], from the endoplasmic reticulum[3,7,12,16] or from the Golgi apparatus[4,6,28]. In order to elucidate the maturation process of neutrophilic leukocytes, electron microscopic observations have been carried out on peripheral blood cells of patients with acute myelocytic leukemia and normal bone marrow cells.

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MATERIALS AND METHODS

Peripheral blood cells of ten patients with acute myelocytic leukemia and normal bone marrow cells were studied.

Clinical examinations are summarized in Table 1.

The specimens of blood cells for electron microscopic study were prepared by the following procedures: peripheral blood cells were obtained from leukemia patients by venipuncture using heparin, and bone marrow cells were obtained from patients, clinically free of hematologic disorders, by aspiration of the sternum.

Smear preparations were made for blood cell study with optical microscope and stained with May-Giemsa solution.

The separation of leukemic cells from peripheral blood cells were accomplished by the following methods. Five% Dextran-solution in physiological saline solution was added in siliconized glass tube at the rate of 1 ml to the 4 ml of heparinized peripheral blood. After standing for 30 minutes at room temperature the supernatant containing large number of leukemic cells was removed into a conical centrifuge tube treated with silicone, and then was centrifugated at 1500 r.p.m. for 10 minutes. The sedimented leukemic cells or the bone marrow cells were fixed in cold 1% phosphate buffered (pH 7.4) osmium tetroxide for half an hour to one hour. The usual graded ethanol series were employed for dehydration, followed by embedding in Epon 812.

Ultrathin sections, cut with glass knives on a Leitz type or Porter-Blum type ultramicrotome, were stained with uranil acetate for about 3 hours and lead nitrate for about 30 minutes. Micrographs were taken at original magnifications of 5000 with Hitachi HU-11 type electron microscope at 75 KV.

OBSERVATIONS

1) Myelocytic Leukemia Cells

Type I Myeloblasts

The nucleus was large and round or oval, occupying a major portion of the cell. It had usually one or two large nucleoli of which nucleolonema was not always clear. The nuclear membrane was two layered. The distance between the two layers was almost always constant.

In the narrow cytoplasm, free ribosomes and their numerous rosette-like arrangements (polysomes) were distributed more densely. The mitochondria visible in each section were few in number, and were not located at one pole of the cell but scattered in the cytoplasm. A few rough surfaced endoplasmic reticula (rER), which appeared to be in a flattened form, were scattered in the cytoplasm. The Golgi apparatus was slightly developed. The specific granules were not seen close to the Golgi area and in the cytoplasm.

Type II Myeloblasts

The myeloblasts of this type were usually larger in size and slightly abundant

in the cytoplasm than the type I myeloblasts. The nucleus appeared to be oval or kidney-like in shape and occasionally slightly indented. Each nucleus had one or two nucleoli, which were usually enormous and showed a dense convoluted structure of nucleolonema. The outer nuclear membrane was expanded and spacial gaps were occasionally seen.

Free ribosomes and polysomes were densely distributed in the cytoplasm, but slightly decreased in number than those of the type I myeloblasts. The mitochondria

Table 1 Clinical Examinations

Case No.	Age (y.)	Sex	Erythrocytes ($\times 10^4$)	Hemoglobin (g/dl)	Leukocytes ($\times 10^2$)	Myeloblasts (%)	Peroxidase Reaction
I	36	♂	213	6.8	856	97	+
II	35	♀	202	6.5	2076	97	+
III	24	♀	177	5.3	1306	62	+
IV	31	♂	252	7.2	2180	96	+
V	45	♀	349	9.8	1654	94	+
VI	24	♂	348	10.2	240	92	+
VII	22	♂	374	12.9	189	85	−
VIII	28	♂	303	8.9	1494	98	−
IX	36	♂	336	10.5	1100	95	−
X	15	♂	185	5.7	211	86	−

which generally appeared to be round or oval were fairly abundant in number and usually clustered in the cytoplasm opposite to the indented region of the nucleus. The flattened rER somewhat increased in number and were distributed in the cytoplasm. The Golgi apparatus was moderately developed and especially characterized by stacks of the Golgi membranes. A few specific granules, which had a uniform content of high density and measured 0.3 to 0.4μ in diameter, were mostly seen in the Golgi area but not widely scattered in the cytoplasm.

Type III Myeloblasts

The size of the cell, the volume of the cytoplasm and the shape of the nucleus were similar to those of the type II myeloblasts.

One or two nucleoli, which were generally enormous, were present and showed a densely convoluted structure. The outer nuclear membrane protruded, in parts, into the cytoplasm.

Free ribosomes and polysomes were densely distributed in the cytoplasm as

well as those of the type II myeloblasts. The numerous mitochondria were grouped in the cytoplasm close to the nuclear indentation. They were usually round or oval in shape, or occasionally rod-shaped. The rER showing flattened and vesicular appearance in the type III myeloblasts slightly increased in number than that of the type II, and were scattered in the cytoplasm. The Golgi apparatus was moderately developed as well as that of the type II myeloblasts. Some of the Golgi vesicles contained a homogeneous substance of low density. The specific granules with diameter of 0.3 to 0.4μ slightly increased in number, and were present in the Golgi area and also widely distributed in the cytoplasm. They showed a limiting membrane and a homogeneous content of high density, and intermediate type from the Golgi vesicles representing a homogeneous content of low density was seen and seemed to be closely associated with them.

Among those three types of myeloblasts described above, the type I myeloblasts were mainly obtained from the cases of peroxidase-negative myeloblasts, and, on the other hand, the type II and III myeloblasts usually from the cases of peroxidase-positive myeloblasts.

Promyelocytes

The promyelocytes showed a largest size of neutrophilic leukocytes.

The nucleus had usually a large nucleolus of which nucleolonema was clearly seen in most of the promyelocytes. The outer nuclear membrane protruded, in many parts, into the cytoplasm.

Free ribosomes were less densely distributed in the cytoplasm than those of the myeloblasts, and polysomes extremely decreased in number. Several mitochondria were scattered in the cytoplasm, and some of them were seen as rod-type. The cavities of the rER showing the flattened or vesicular appearance in the myeloblasts expanded into sinus-like spaces and were distributed abundantly in the cytoplasm. A homogeneous substance of low density was seen within the expanded cavities. The Golgi apparatus was well-developed and, as a rule, consisted of three components: Golgi membranes, Golgi vesicles and Golgi vacuoles. Especially, the Golgi apparatus was more characterized by stacks of the Golgi membranes and a number of Golgi vesicles, and increased markedly in size and became larger than that of myeloblasts. Clusters of the Golgi vesicles seemed to be in close association with the dilated ends of the Golgi membranes. A homogeneous substance of low density was rarely seen within the dilated ends. Most of the Golgi vesicles contained a homogeneous substance of low density similar to contents within the rER. The specific granules of high density with a limiting membrane were relatively abundant, distributed close to the Golgi area and scattered throughout the cytoplasm. Some of them showed a distinct space between the limiting membrane and the content. Most of the specific granules measured about 0.5μ in diameter. Those findings in the Golgi apparatus suggested that there might be a transition between the Golgi vesicles and the specific granules.

Myelocytes

The nucleolus was not usually found in the nucleus. The expanded spaces

of the outer nuclear membrane were seen in many parts.

A few mitochondria which closely resembled with those seen in the promyelocytes were scattered in the cytoplasm. The electron dense specific granules with a limiting membrane and the sinus-likely expanded rER were abundantly seen throughout the cytoplasm. But the expanded spaces of the rER had a tendency to contract and to decrease in number, when the specific granules increased following cellular maturation. Within the sinus-like rER, a homogeneous substance of low density was usually present. The specific granules measured 0.3 to 0.4μ in diameter. The Golgi apparatus generally contained three components and showed the maximal enlargement in the course of maturation of neutrophilic leukocytes. Several parallel rows of the Golgi membranes were arranged in the form of a ring encircling a centriole, and dilated at their extremities. A homogeneous substance of low density appeared to be rarely contained within the dilated ends of the Golgi membranes. The Golgi vesicles seemed to separate from the ends of the Golgi membranes by the pinching off of small vesicles containing a homogeneous substance of low density. A homogeneous substance of low density was seen within the Golgi vesicles. Therefore, a intermediate form was seen between the Golgi vesicles and the specific granules. By these evidences, it was evident that the specific granules might be formed within the Golgi apparatus and their ground substances might derive from the contents within the rER.

Metamyelocytes and Mature Neutrophils

Depending on each sectioning angle, the nucleus varied in shape and showed several segmented lobes. The expanded spaces of the outer nuclear membrane were not seen.

Free ribosomes in the cytoplasm were least in number of neutrophilic leukocytes. The mitochondria were reduced markedly and a few small rod-shaped ones scattered in the cytoplasm. The rER decreased in number and had returned to vesicular form instead of the sinus-like rER seen in the promyelocytes and the myelocytes. The Golgi apparatus degenerated, and consisted of a few Golgi membranes and vesicles which encircled a centriole. A number of specific granules of high density were distributed in the cytoplasm, however, the findings indicating a new formation of them were not seen. Their granules, 0.15 to 0.25μ in diameter, were usually smaller in size than those of immature cells, and represented a limiting membrane.

Throughout the maturation process of neutrophilic leukocytes, the smooth surfaced endoplasmic reticula (sER) were rarely seen in the cytoplasm.

2) Neutrophilic Leukocytes in Normal Human Bone Marrow

Type I Myeloblasts

The electron microscopic findings of this myeloblasts resembled to those of the type I myeloblasts of leukemic cells.

Type II Myeloblasts

The nucleus was usually round and had one or two nucleoli showing a

relatively dense convoluted structure of nucleolonema. The projection of the outer nuclear membrane was not seen.

Free ribosomes and polysomes were numerous and densely distributed in the cytoplasm, and showed a finding similar to those seen in the type II myeloblasts of leukemic cells. A few mitochondria and flattened rER were scattered in the cytoplasm. The Golgi apparatus was moderately developed. A few specific granules of high density, which measured 0.2 to 0.3μ in diameter, were seen close to the Golgi area but not seen in the cytoplasm.

Type III Myeloblasts

The nucleus was generally round and showed no irregular indentation. It had one or two nucleoli, which were large in general and showed a convoluted structure. There were no expanded spaces of the outer nuclear membrane.

Free ribosomes and polysomes were densely distributed in the cytoplasm as well as those of the type II myeloblasts. The mitochondria were a few in number, representing a clear contrast with their abundant appearance in the leukemic type II and III myeloblasts. A few flattened rER were scattered in the cytoplasm. The Golgi apparatus was moderately developed. A few specific granules of high density, which measured 0.2 to 0.3μ in diameter, were seen in the Golgi area and the cytoplasm.

Promyelocytes, Myelocytes, Metamyelocytes and Mature Neutrophils

The features of the intracytoplasmic organellae of these cells were similar to those of leukemic cells.

DISCUSSION

Electron microscopic studies on leukemic cells and normal human bone marrow cells have been reported by many investigators, with description on the characteristics of intracytoplasmic organellae.

Myeloblasts in acute myelocytic leukemia identified under optical microscope show various variation in their intracytoplasmic organellae. Based on the amount of the specific granules and the morphological features of endoplasmic reticulum, Ito[14] recently classified myeloblasts into 3 stages and Fuginaga[15] into 4 stages. On the other hand, many investigators have a different opinion concerning the existence of the specific granules: it was posturated by Watanabe,[4] Kondo,[16] Fukuhara,[17] Capone,[8] and Bainton[18] that the specific granules were not seen in the stage of myeloblasts, while according to Amaki,[30] Akasaka,[12] Ito,[10] and Toshima[19] they might occasionally appear in a small number of myeloblasts, and to Pease[3] and Sanada[20] they could always exist in myeloblasts.

With regard to the formation of the specific granules, several opinions have been expressed so far: they are originated from the sER (Pease[3] and Braunsteiner[7]), from the rER (Akasaka [12] and Kondo[16]), from the mitochondria (Reinehart [2]), and from the Golgi vesicles. However, as described by Ichikawa,[6] Watanabe,[4] Ito,[23] and Bainton,[18] it seems to be more reliable that the Golgi

apparatus plays an important role in the formation of the specific granules. There may be a close correlation between the development of the Golgi apparatus and the increase of the specific granules. The latter is actually seen, at first, close to the former and then they become to scatter widely in the cytoplasm.

Toshima[19] and Capone[8] described a typical ring-like arrangement of the Golgi membranes in the myelocytes. The findings may surely suggest that the Golgi apparatus show a maximal activity and is producing enzymatic substances of the specific granules by adding of proteinous substances formed in the neighboring rER.

The localization of peroxidase reaction of human leukocytes (Senda[21]) and of leukemic cells (Kondo and Enomoto[22, 25]) was investigated with electron microscope, and fein particles showing positive reaction were demonstrated in the specific granules of high density.

According to Ichikawa[6] and Kondo,[16] the well-developed and expanded rER were considered to be an origin of azurophilic granules markedly seen in the promyelocytes. Bainton and Farquhar[18] attributed the formation of azurophilic granules to the Golgi apparatus. It was described by them that azurophilic granules were formed from high dense granules at the proximal face of the Golgi apparatus, and, on the other hand, the specific granules from low dense granules at the distal face of it.

In the present studies on the maturation process of neutrophilic leukocytes with electron microscope, profound attention has been paid to every morphological feature of cells, especially nucleus as an indicator of cell multiplication and intracytoplasmic organellae as an indicator of cell differentiation. Morphological features in each stage of neutrophilic leukocyte by the present investigation are summarized in Table 2. According to the results of the present observation, it is evidently deduced that the rER and the Golgi apparatus play an important role in the formation of the specific granules. In the type I myeloblasts, the rER and the Golgi apparatus show poor development and no specific granules are seen, while in the type II and III myeloblasts a gradual development took place. Simultaneously, appearance and increase of the specific granules close to the Golgi area and in the cytoplasm are noted. A homogeneous substance of low density is seen in the Golgi vesicles, and they show a transition to the specific granules. Such relationship between the Golgi vesicles and the specific granules has been already described by Ichikawa,[6] Watanabe,[4] Ito,[23] and Bainton.[18] From enzymological studies,[24,25,26] it was proved that the specific granules obtained by centrifugation contained various enzymes. Judging from these findings, it can be surely induced that the proteinous substances are transported into the Golgi apparatus, elaborated and concentrated there, and reliesed as the specific granules in its neighborhood, as secretary granules and lysosomes are formed in the Golgi apparatus of glandular cells.[27,28,29] In addition, the rER show sinus-like dilation and the cavities are generally filled with a homogeneous substance of low density at the stage of the promyelocytes and myelocytes. These evidences suggest an increased production of proteinous substances in the rER. However, there are no evidences supporting the views in which direct transition from the substances in the dilated rER to the specific granules is contended by Akasaka[12] or to azurophilic granules by Ichikawa[6] and Kondo.[16]

Table 2 Ultrastructures of Neutrophilic Leukocytes

	Myeloblast			Promyelocyte	Myelocyte	Mature Neutrophil
	I	II	III			
shape of nucleus						
L. C.	round or oval	oval or kidney	kidney	slightly indented	slightly irregular	segmented
N. C.	round	round	round	round or oval	round or oval	segmented
size of nucleolus						
L. C.	large	enormous	enormous	large	not seen	not seen
N. C.	large	large	large	large	not seen	not seen
ribosome	markedly numerous	markedly numerous	markedly numerous	numerous	moderately decreased	markedly decreased
polysome	markedly numerous	numerous	numerous	markedly decreased	not seen	not seen
rER shape	flattened	flattened	flattened and vesicular	dilated	dilated	vesicular
rER number	a few	a few	a few	numerous	numerous	moderately decreased
number of mitochondria						
L. C.	a few	numerous	numerous	several	a few	a few
N. C.	a few	a few	a few	a few	a few	a few
Golgi apparatus	slightly developed	moderately developed	moderately developed	well-developed	most well-developed	poorly developed
number of specific granules	not seen	a few close to Golgi area	a few close to Golgi area and in the cytoplasm	moderately increased	increased	most increased

cf. L. C. : myelocytic leukemia cells
N. C. : normal bone marrow cells

Concerning the formation of azurophilic granules markedly seen only in the promyelocytes, it is impossible under electron microscope to demonstrate the presence of granules going through an entirely different process of development from that of the specific granulus.

As shown in Table 2, the development of both the rER and the Golgi apparatus occure simultaneously with the formation of the specific granules. Consequently, it is deduced that in early stage after the formation of the specific granules in the Golgi apparatus they may be strongly basophilic and represent azurophilic granules seen in the promyelocytes under optical microscope. The specific granules may subsequently combine with proteinous substances in the expanded rER, and result in neutrophilic nature at the stages of myelocytes to mature neutrophils, because of their reduction of stain ability to basic azure dye of Giemsa solution. From the

above supposition, the specific granules are divided into two types, one is azurophilic and the other neutrophilic. However, clear distinction between neutrophilic granules and azurophilic granules is not seen under electron microscope, as far as human materials are concerned.

Following difference is noted between the myeloblasts in normal human bone marrow and the myeloblasts in acute myelocytic leukemia, although cells of other stages are free of such distinction. The former show no marked aggregation of the mitochondria and no irregular shape of the nucleus. Irregular shape of the nucleus in leukemic myeloblasts can be easily understood as a sign of enormous proliferation of cells.

However, increase and marked aggregation of the mitochondria in leukemic myeloblasts seem to remain almost unsolved. In order to elucidate it reasonably, the function of the mitochondria of immature cells should be recalled. Mitochondria and increasing amount of free ribosomes, predominantly as polysomes were already present in the undifferentiated structure from single cell stage to the gastrula change of triturus helveticus (Sasaki and Büchner[81,82]). It was also presented by Büchner and Hara[83] that in the various stages of the neurula of triturus helveticus hypoxia of 24 hours led to a severe inhibition of the incorporation of H^3-thymidin into DNA. From both evidences, it is obviously concluded that intracellular respiration mediated by mitochondria should be directed principally to the synthesis of DNA in the nucleus throughout early development of cells. It can be also deduced that marked aggregation and increasing amount of mitochondria in leukemic myeloblasts in contrast with a few of them in myeloblasts of normal bone marrow may be a consequence of elevated intracellular respiration for accelerated DNA synthesis which is necessary for enormous multiplication of leukemic myeloblasts.

SUMMARY

Leukemic cells and normal human bone marrow cells were investigated with electron microscope.

Myeloblasts were classified in the three types by the features of intracytoplasmic organellae.

By the aid of intracellular respiration of mitochondria in myeloblasts, DNA synthesis was elevated and consequently multiplication of cells was revealed. Abundant polysomes were also seen in myeloblasts.

Leukemic myeloblasts were discriminated by indentation of the nucleus and aggregation of numerous mitochondria which may result from the demand of elevated DNA synthesis in the nucleus in contrast with a few mitochondria in myeloblasts of normal bone marrow.

Promyelocytes and myelocytes were chiefly directed to differentiation of the cells, that is, to the formation of the specific granules by elaboration of the well-developed Golgi apparatus and the expanded rER.

Leukemic promyelocytes and myelocytes could not be distinguished from those of normal human bone marrow by fine structures.

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EXPLANATION OF PLATES

Acute Myelocytic Leukemia Cells (Fig. 1-6)

Fig. 1 Type I Myeloblast. In the narrow cytoplasm, a few mitochondria and rER are scattered, but the specific granules are not seen. ×9000

Fig. 2 Type II Myeloblast. The nucleus is oval in shape and slightly indented. The Golgi apparatus is moderately developed, and a few specific granules are seen close to the Golgi area. The mitochondria are enormously increased and located at one pole of the cell. ×9000

Fig. 3 Type III Myeloblast. The eccentric nucleus shows a kidney-like form. The Golgi apparatus is moderately developed and a few specific granules are seen close to the Golgi area and in the cytoplasm. A few flattened and vesicular rER are seen. The numerous mitochondria are grouped in the cytoplasm. ×9000

Fig. 4 Promyelocyte. The sinus-like rER with a homogeneous substance of low density, the specific granules and the mitochondria are distributed in the cytoplasm. The Golgi apparatus is well-developed, and a transition is seen between the Golgi vesicles with a homogeneous substance of low density and the specific granules. ×9000

Fig. 5 Myelocyte. The nucleus is not seen. The sinus-like rER with a homogeneous substance of low density, the numerous specific granules and a few mitochondria are distributed in the cytoplasm. The Golgi apparatus is most well-developed of neutrophilic leukocytes and the Golgi membranes are arranged in the form of a ring encircling a centriole. A transitional form is seen between the Golgi vesicles and the specific granules. ×9000

Fig. 6 Mature Neutrophil. The rER decrease in number and show a vesicular appearance. The Golgi apparatus degenerates. ×9000

Normal Human Bone Marrow Cells (Fig. 7-12)

Fig. 7 Type I Myeloblast. A few mitochondria and flattened rER are scattered in the cytoplasm, but the specific granules are not seen. ×9000

Fig. 8 Type II Myeloblast. A few mitochondria and flattened rER are scattered in the cytoplasm. A few specific granules are seen close to the Golgi area. The nucleus is round in shape. ×9000

Fig. 9 Type III Myeloblast. A few mitochondria and flattened rER are scattered in the cytoplasm. A few specific granules are seen close to the Golgi area and in the cytoplasm. The nucleus is round in shape. ×9000

Fig. 10 Promyelocyte. ×9000

Fig. 11 Myelocyte. ×9000

Fig. 12 Mature Neutrophil. ×9000

The features of intracytoplasmic organellae of Fig. 10, 11 and 12 are similar to those of Fig. 4, 5 and 6.

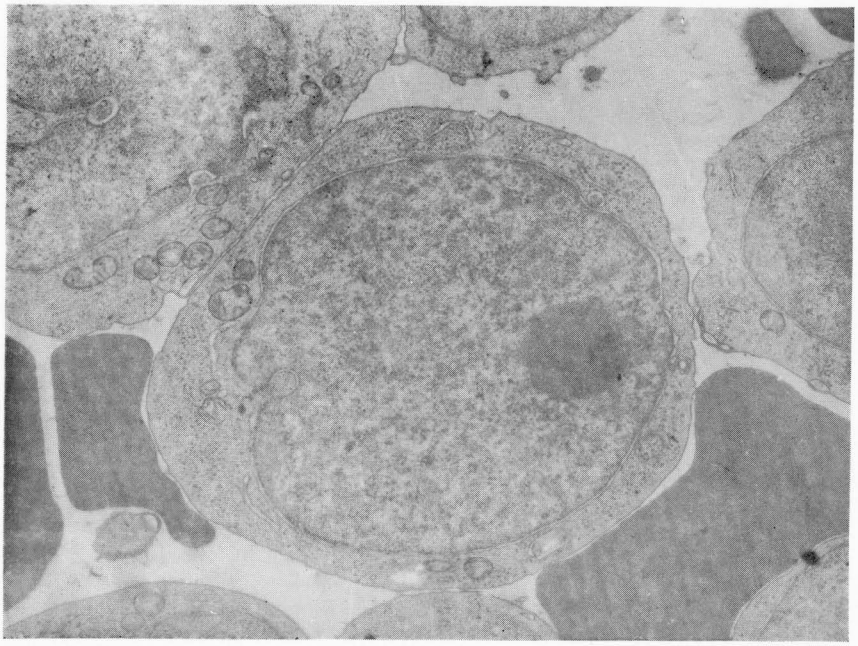

Fig. 1

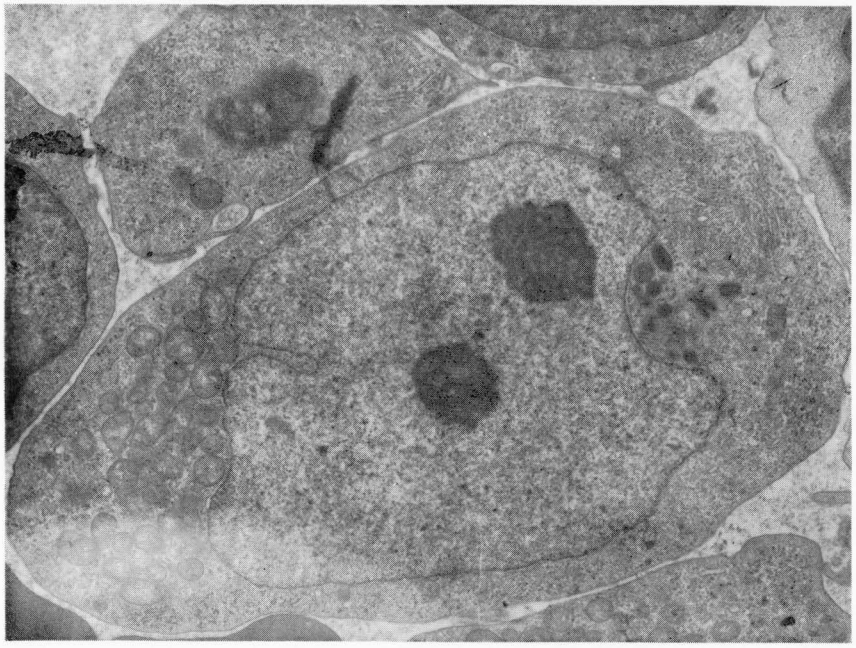

Fig. 2

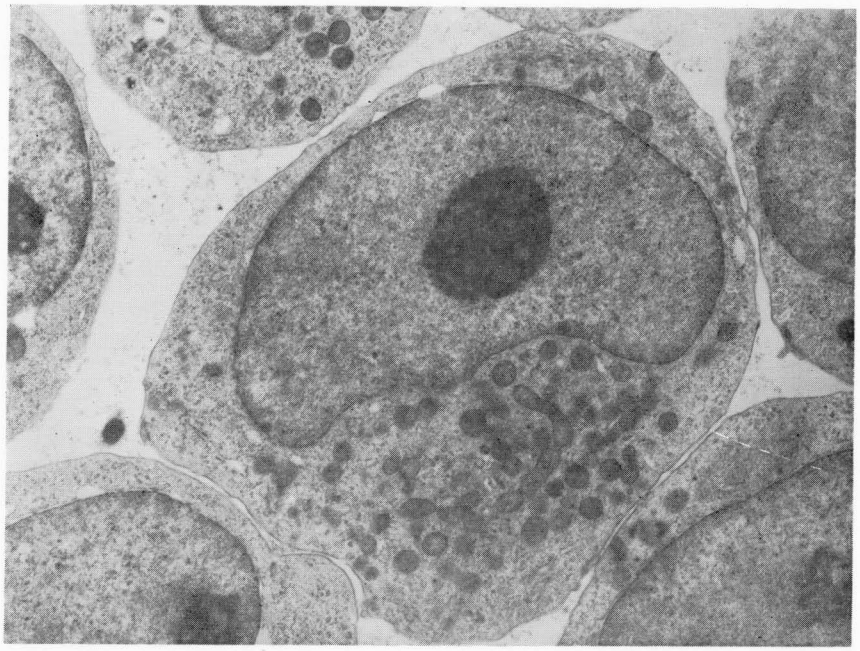

Fig. 3

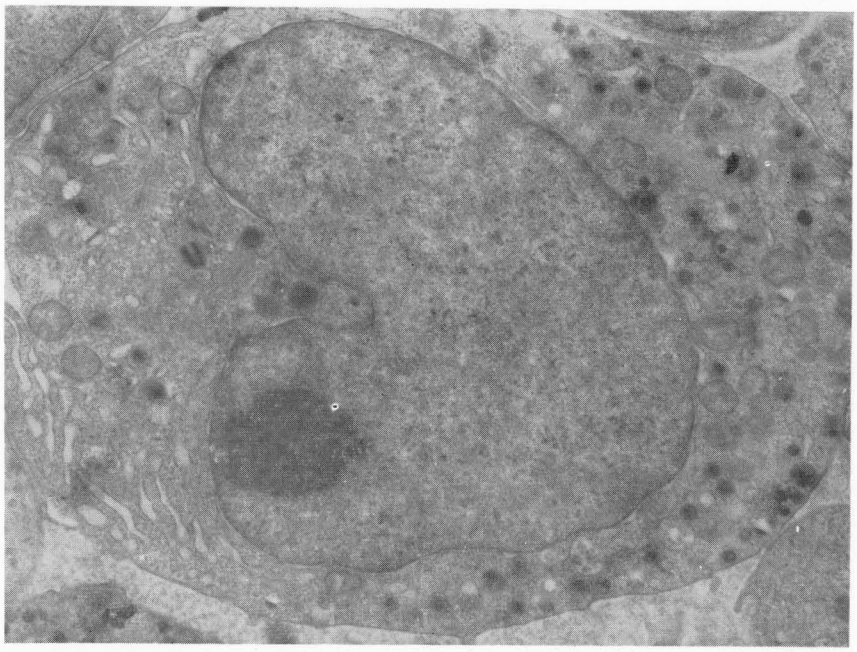

Fig. 4

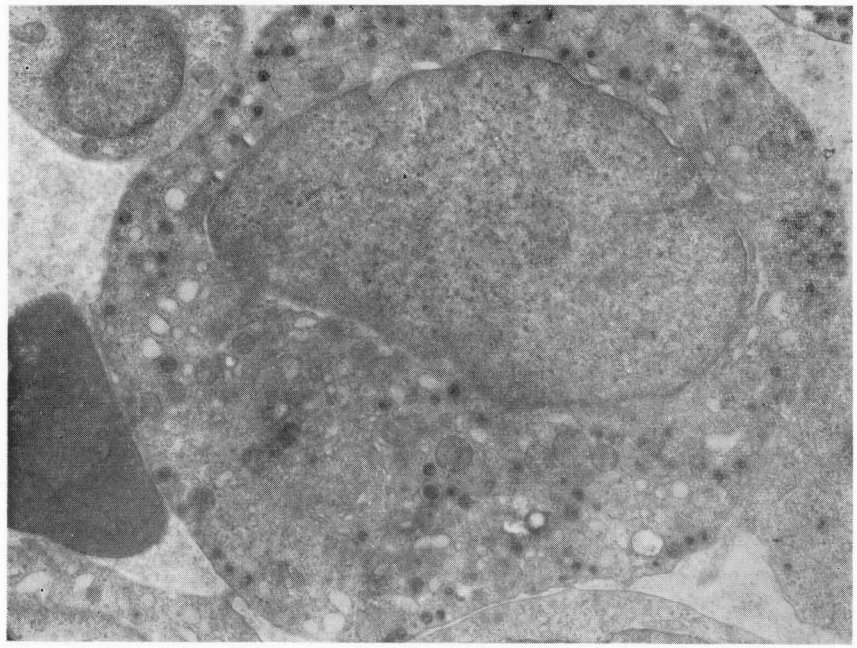

Fig. 5

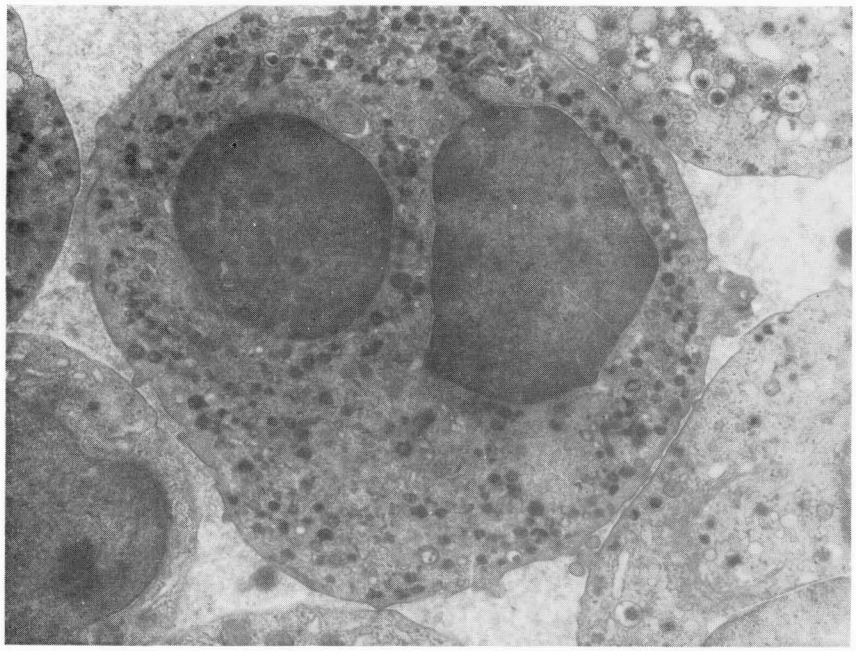

Fig. 6

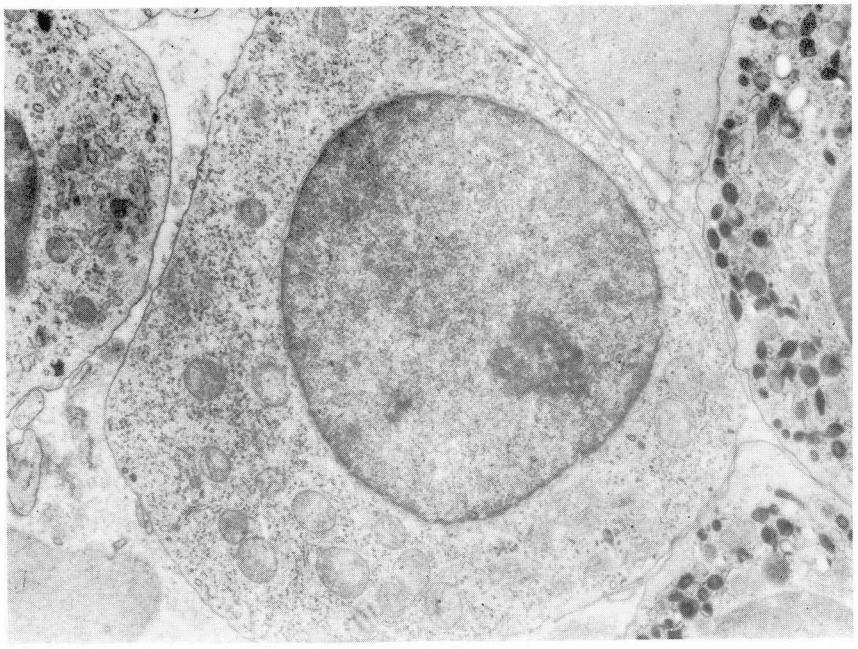

Fig. 7

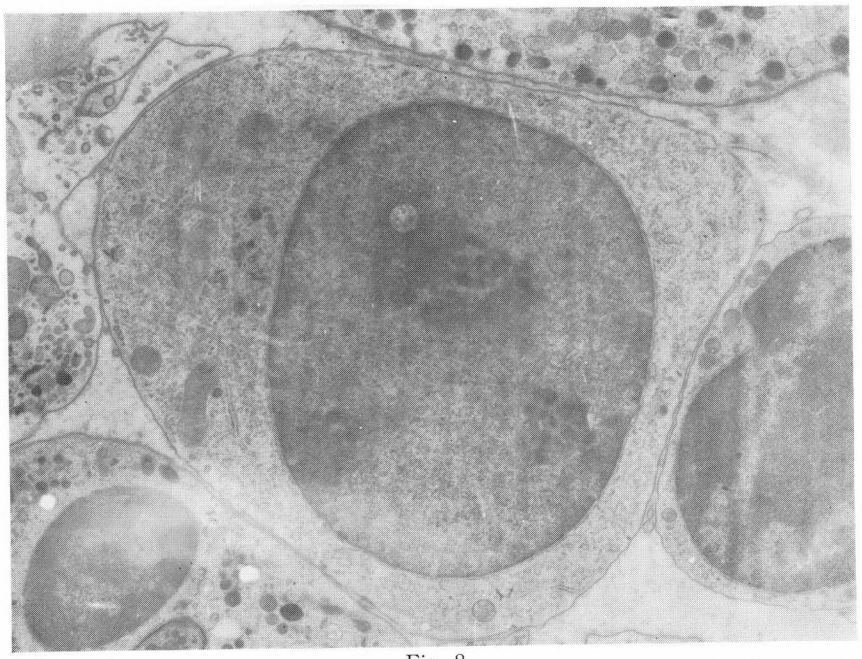

Fig. 8

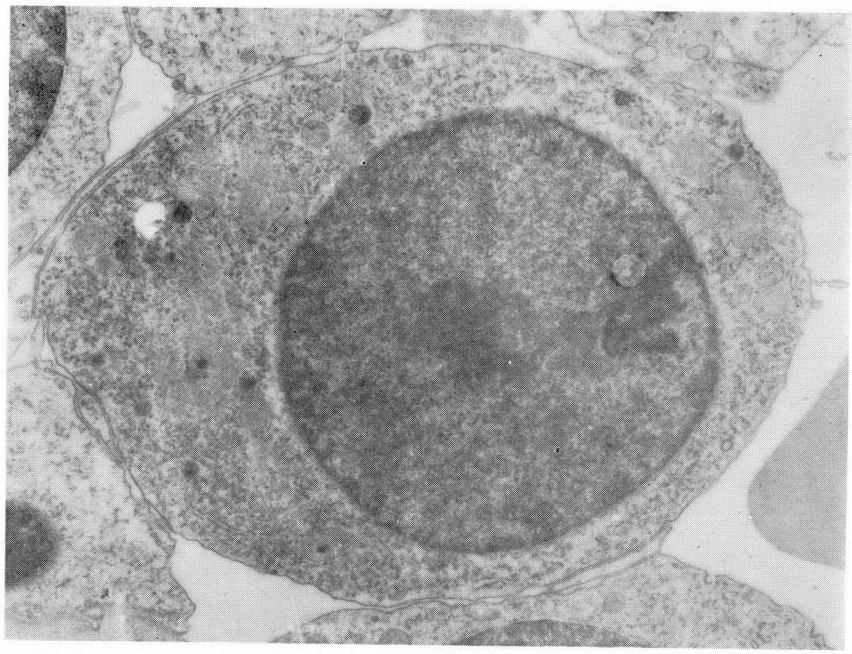

Fig. 9

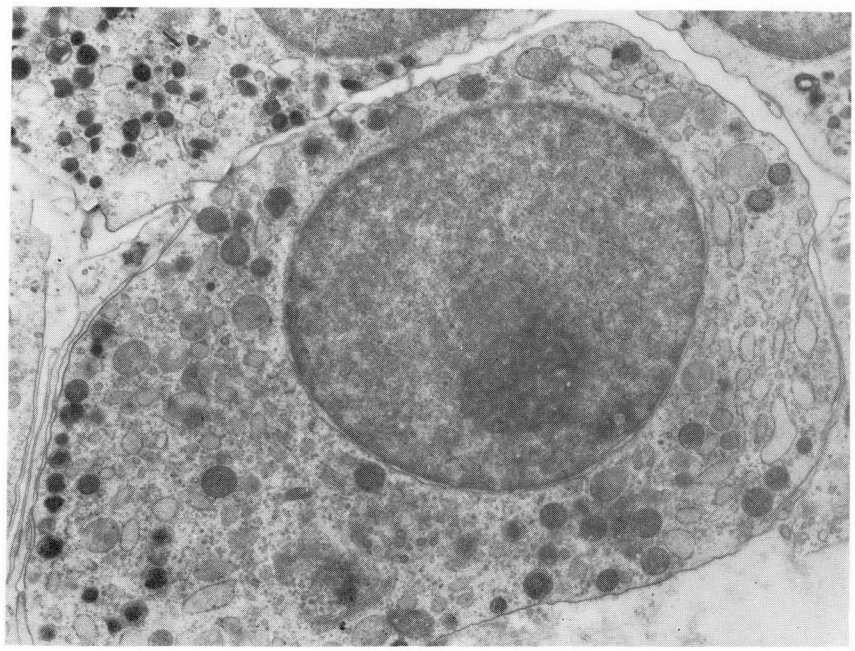

Fig. 10

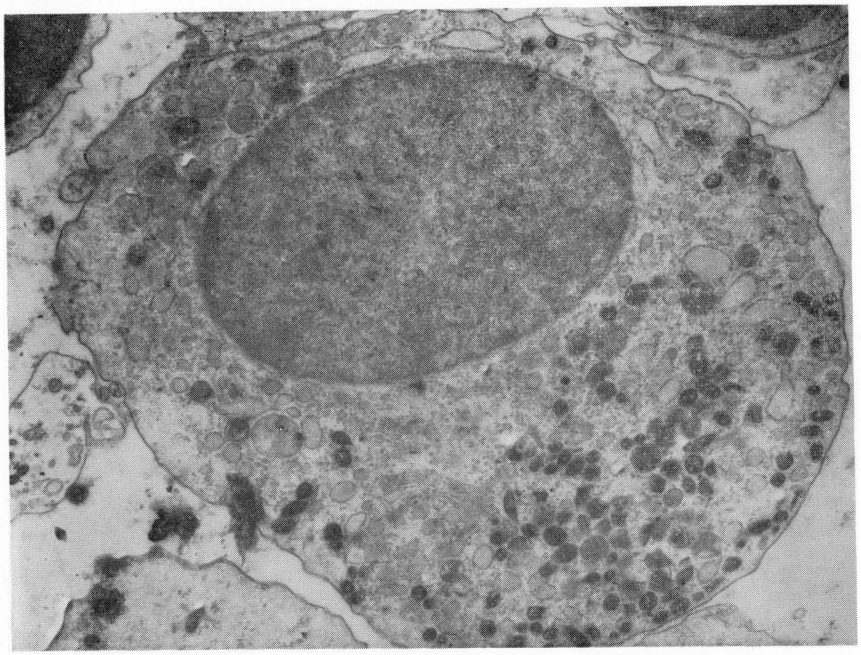

Fig. 11

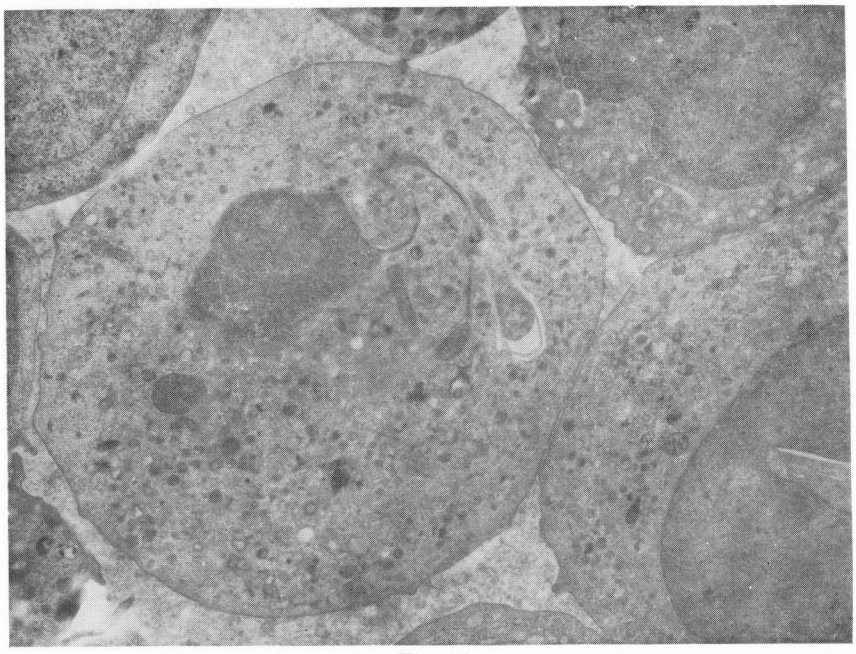

Fig. 12