ELECTRON MICROSCOPIC STUDY ON BENZENE INTOXICATED RAT BONE MARROW, WITH SPECIAL REFERENCE TO ITS RETICULO-ENDOTHELIAL STRUCTURE

BY

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Abstract

Subcutaneous injection of benzene, 2 ml/kg daily is performed for 21 days on male Wister rats.

Cellular changes of the bone marrow are studied serially in the light and electron microscopic levels.

As signs of cellular injury, swelling of the mitochondria, severe dilatation of the membrane system such as rough surfaced endoplasmic reticulum (rER), smooth surfaced endoplasmic reticulum (sER) and Golgi complexes, expansion of outer nuclear membrane, and swelling of the specific granules with clear content are revealed. Existence of membrane free ribosomes even in the mature leukocytes is also seen, and which phenomenon is considered as the maturation dissociation. Severely desintegrated haematopoietic cells are observed mostly caught within the phagocytic reticular cells.

The mechanism through which bone marrow are injured by benzene is discussed.

Reticulo-endothelial structure of the bone marrow in normal and benzene intoxicated animals is also studied. Reticulo-endothelial cells of the bone marrow are classified into three types, among which special attention is paid to the sub-sinusoidally lying reticular cells. This particular cells show lower phagocytic activity, and often transform into the fat cells.

Introduction

Since benzene poisoning on blood and blood-forming organs was described in 1897 by Santesson[22], innumerable clinical and experimental studies have been made so far about its chemical analysis and pathogenesis. Toxicity of benzene is, as is generally accepted, most prominent in haematopoietic organs. However, the mechanism through which benzene induces bone marrow damages has not yet been fully elucidated. Only a few reports are available on the cytological changes of the bone marrow in electron micrscopic level[15].

Although bone marrow changes caused by benzene intoxication are

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usually seen as a chronic disease in human being, the present author dealt with the experimental one of the rat caused by administration of large dosage for a short time, in order to study (1) cytological changes of the haematopoietic cells of the bone marrow, (2) normal structure of the reticulo-endothelial system in the bone marrow and its changes under such a condition.

Materials and Methods

The toxicity of benzene on the bone marrow is, generally, variable very much by several factors such as individuality, age and sex of animals, feeding condition including food, temperature, humidity, and protection against infections. Preliminary experiments elucidated that the following procedure was the most effective for our purpose to induce severe hypoplasia of the bone marrow.

After feeding male Wister rats, weighing 130–150 g, on commercial pelleted food for about 2 weeks without any treatment, daily subcutaneous injections of 4 ml/kg of 50:50 mixture of benzene and sterilized olive oil started. Total 3 rats, 2 benzene-injected and one control are sacrificed respectively on the 1, 2, 3, 4, 6, 10, 16 and 21st day.

Venous blood was taken from jugular vein, at the time of autopsy, for white and red blood cell count. Bone marrow smear was made from metaphyseal marrow of the femur. Light microscopic studies were made on femoral, sternal and vertebral marrow as well as other visceral organs, after fixation in 10% neutralized formalin.

A part of the femoral metaphyseal marrow of each animal was taken for electron microscopy, and was immediately put into s-Collidine buffered fixative containing 2% osmium tetraoxide (pH 7.4–7.6) prepared at 4°C[10]. After 10 minutes' fixation, the specimen was cut into tiny blocks, and returned back to the same fixative to be kept about 60 minutes. Dehydration was carried out in graded ethanol (50%—absolute) for not exceeding 60 minutes. Specimen was embeded in epoxy resin mixture and styrene-*n*-butyl-methacrylate mixture, using No. 00 gelatin capsule as a mould. Ultrathin sections were made, under the control of preliminary examination with methylene blue quick staining[21] and phase contrast microscopy, with Porter-Blum microtome using glass knife, and were double stained by uranyl acetate and lead hydroxide. Observation was made with HU-10 and HU-11-A electron microscope at the magnification ranging from 2,000 to 20,000 ×. The methylene blue stained preparations were used additionally for the light microscopic study.

Observations

A. *General observation of this experiment*:

After retardation in natural gain in body weight for several days, the animals gradually begin their body weight loss which continues till the end of this experiment (Fig. 1). Rapid fall occurs in number of white blood cell count of the peripheral blood, while the red blood cell count shows no remarkable decrease (Fig. 2). As benzene intoxication advances, relative lymphocytopenia and marked reduction of eoshinophile count appear in the peripheral blood (Fig. 3). It is highly interesting to observe many erythrocytes with polychromasia and basophilic stippling on around the 4th

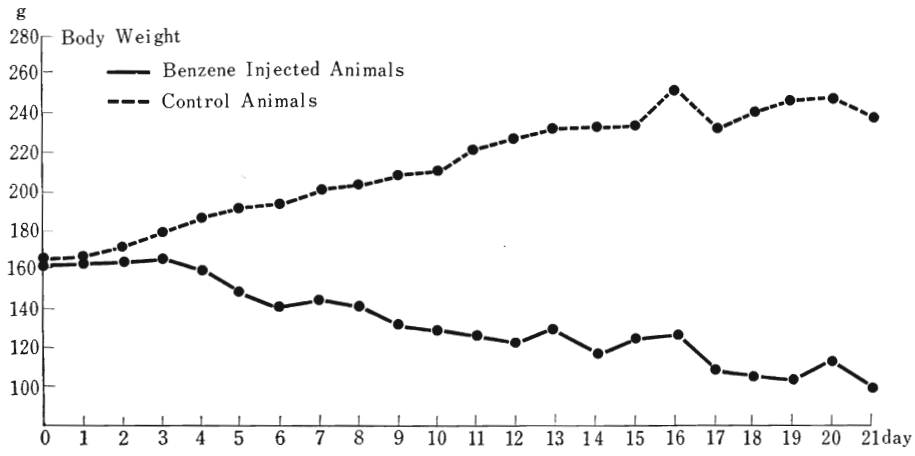


Fig. 1. Body weight change.

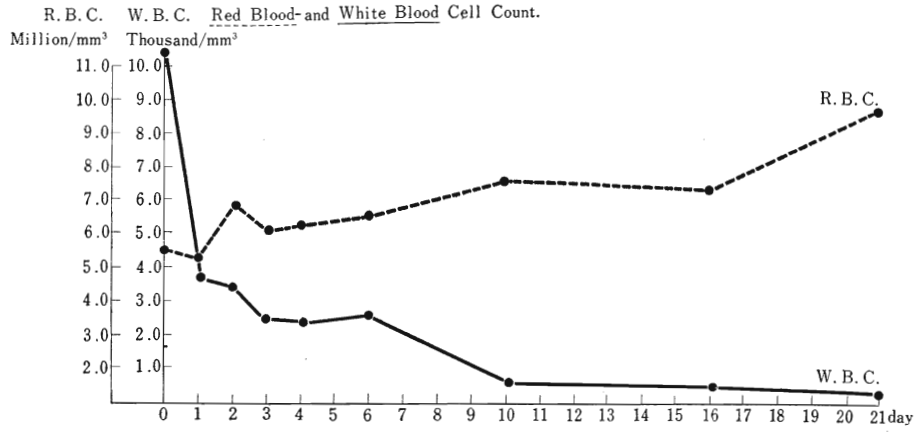


Fig. 2. Peripheral blood cell count.

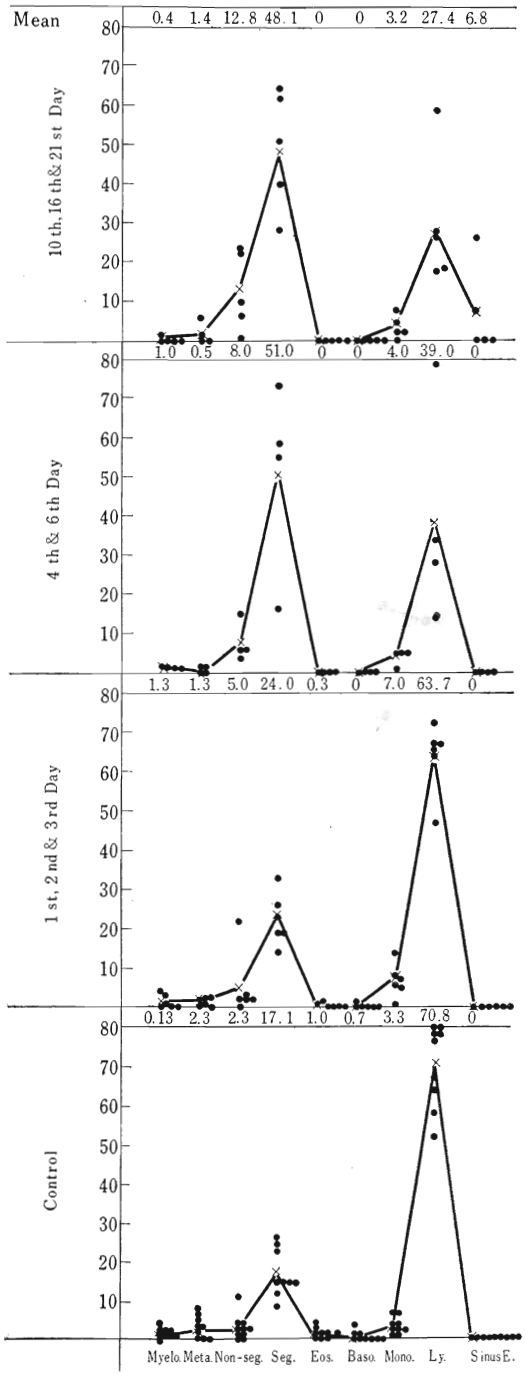
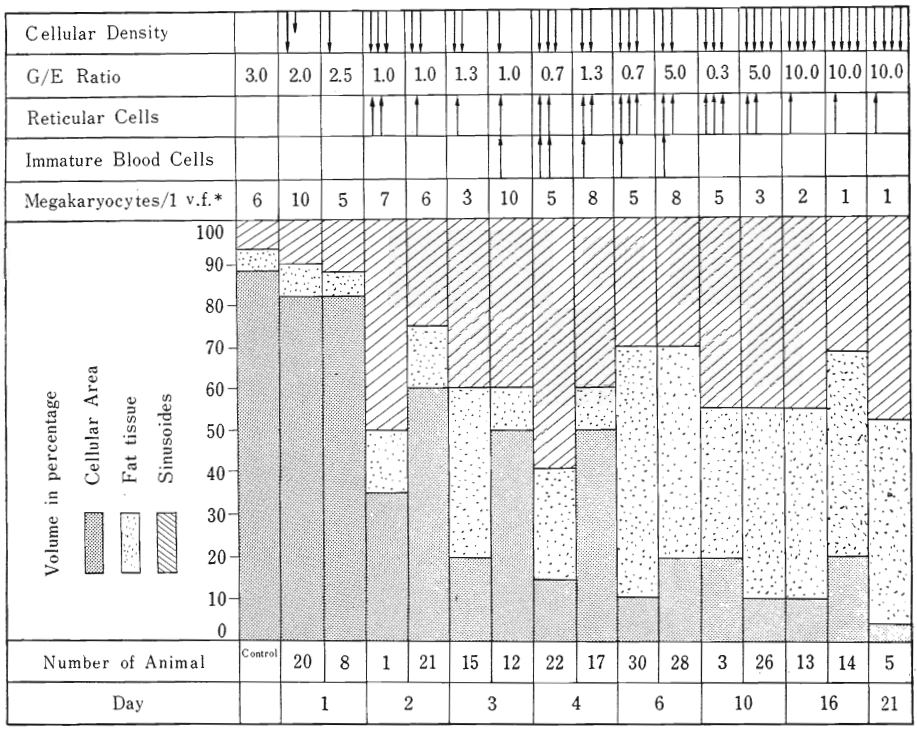


Fig. 3. Haemogram.

to 6th day, and degeneration in non-segmented or segmented nuclear leukocytes on 16th–21st day; i.e. oversegmented, partly pycnotic nuclei and isolated nuclear fragments are noticeable (Pict. 5) in the slightly basophilic cytoplasm with various sized azurophilic granules (Pict. 6). In the terminal stage, some large cells become to be found frequently which highly resemble the sinusoidal endothelial cells (Pict. 7).



*One visual field at the magnification of×400

Fig. 4. Approximately estimated proportion of cellular area, sinusoides and fat tissue measured in histological sections of vertebral marrow. Cellular density, G/E ratio, degree of prominence of reticular cells, inmature haematopoietic cells and megakaryocytes are also estimated.

Figure 4 shows approximately estimated proportion of cellular area, sinusoids and fat tissue measured in histological sections of vertebral marrow, using planimetry in several cases. Changing in cellular density, degree of prominence of reticular cells and inmature haematopoietic cells, and G/E (Granulopoietic cells/Erythropoietic cells) ratio at each period are also shown. Parenchymal marrow cells decrease both in spread and in density already in the 1st day of benzene injection, and gradual dilatation of sinusoides and increase of fat cells follow since the second or third day (Pict. 1).

Severe hypoplasia with fat cell hyperplasia is induced on the 16th and 21st day (Pict. 2). Reticular cells are tended to be prominent, especially around the sinusoidal wall from the 2nd day and reach maximum on the 4th–6th

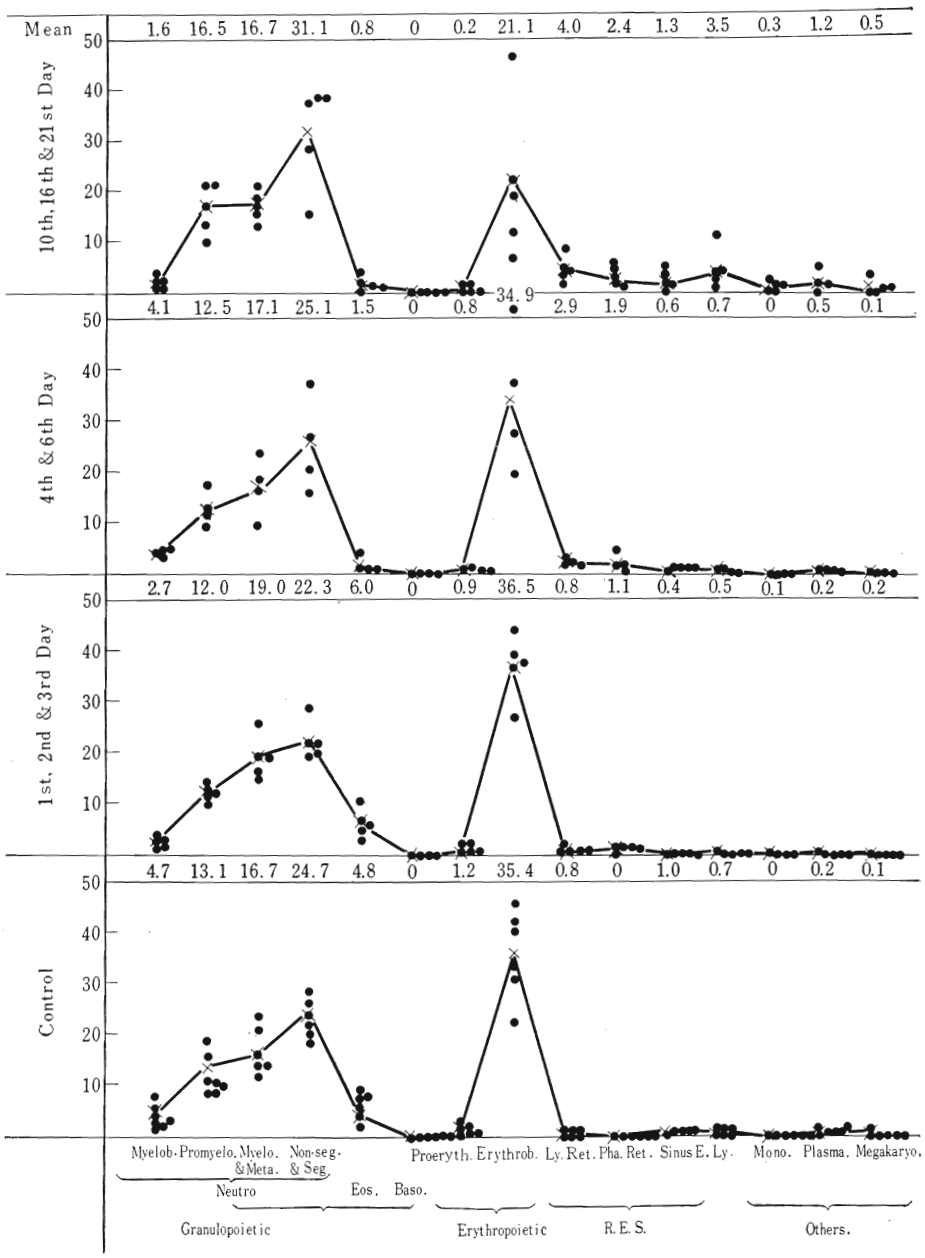


Fig. 5. Myelogram.

day. G/E ratio tends to decrease slightly during the first week and increase rapidly thereafter. Same results are obtained in the G/E ratio calculated in smeared specimen of the femur metaphyseal marrow (Table).

In the differential count of the myelogram, slight increase in ratio of promyelocytes, RES cells, plasma cells and lymphocytes are found, and slight decrease of eosinophiles are observed as intoxication advances (Fig. 5). Mitotic index increases rapidly and reaches its maximum up to about three times to the normal within several days (Table), and decreases gradually,

Table: Mitotic index and G/E ratio measured in smeared preparation.

Day		Control	1		2		3		4		5		10		16		21
Number of Animal		/	20	8	1	21	15	12	22	17	30	29	3	26	13	14	5
Mitotic Index %	Granulopoietic	8.7	4.0	26.0	26.0	24.0	26.0	16.0	24.0	18.0	12.4	20.0	18.0	6.0	16.0	10.0	14.0
	Erythropoietic	3.3	2.0	0	8.0	5.0	0	6.0	10.0	16.0	14.0	10.0	0	2.0	2.0	6.0	0
G/E Ratio		1.6	1.8	1.5	2.6	2.0	2.4	1.4	1.5	2.3	0.7	3.8	3.2	3.5	7.6	1.1	7.9

thereafter. Abnormal mitotic figures cannot be encountered in our present study, although their frequent appearance was reported by Koike and Kawai (1959)[6]. Normal mitotic figures of various phases are found.

B. *Cytological changes of the bone marrow*:

1. *Cellular changes observed in smeares and sections*:

Although bone marrow changes are variable in severity depending on susceptivity of animals to benzene, cellular changes of the haematopoietic cells tend to become prominent generally within several days of daily injection. Numerous small vacuoles appear in cytoplasm of the myelo- and metamyelocytes. On the other hand, slightly larger vacuoles are found, though scarce in number, in the myeloblasts and promyelocytes (Pict. 8). Frequently, marked dissociation in nuclear and cytoplasmic maturation occur and toxic granules come out in their cytoplasm (Pict. 9). Reticular cells increase in number from the 2nd day and their phagocytic activity reaches its maximum on around the 4th–6th day (Pict. 10). Around the 4th–6th day nuclear clumping occurs in erythroblasts (Pict. 11). On the 16th and 21st day, many number of the haematopoietic cells look abnormal showing various kind of the above described changes in variable degree.

2. *Cytological changes observed with electron microscope*:

1) *Granulopoietic cells*:

a. *Myeloblasts*:

No obvious changes occur in the cytoplasmic texture except for the fact that the cytoplasm tends to become clearer, though slight in degree, with decrease of free ribosomes. Short, saccular rough surfaced endoplasmic reticulums (rERs) are found remarkably dilated and increased in number. Spherical mitochondria are swollen, resulting in enlarged irregular contour, decreased density of matrix, and disarrangement of cristae (Pict. 12). Occasionally found are multivesicular bodies, irregular shaped fat droplets and small dense bodies. In the terminal stage, nucleus become rounded showing homogenized nucleoplasm. Nucleoli disappear completely.

Not frequently are found cells which are very small in size due to severe condensation of entire cellular body, in which remarkably swollen mitochondrion, rERs and Golgi vesicles are still existing (Pict. 13).

b. *Promyelocytes, myelocytes and metamyelocytes*:

In intoxicated state, free ribosomes are found rich throughout the cytoplasm of the metamyelocytes as well and even in the non-segmented leukocytes, while these disappear usually in the myelocytes of the control animals. This ultrastructural finding corresponds to basophilism observed microscopically with May-Giemsa satining (cf. B-1). Short and long saccular rERs, seen most numerously in the promyelocytes and inmature myelocyte, are remarkably dilated, containing hydrous amorphous content and sometimes communicating each other forming irregular channels in the cytoplasm (Pict. 14). Frequently, these dilated rERs closely located to the nucleus show communication with partially expanding outer nuclear membrane which is attached by ribosomes. It must be noted here that rERs of eosinophiles are seen dilated frequently even in the control animals. The spherical mitochondria with well arranged cristae and dark matrix are found remarkably swollen, resulting in irregular contour and disarrangement of cristae. Mitochondrial changes seem to appear later than the one of rER. Irregular-shaped fat droplets, multivesicular bodies and cytolysomes are found occasionally in the cytoplasm.

Granules of low and high electron density, which are surrounded by distinct limiting membrane, are identified among the specific granules of the neutrophiles. The former and the latter respectively correspond to A and B type granules reported by Watanabe (1956)[29] who studied bone marrow of man and guinea pig. Ito (1958)[2] reported transitional form between those two in human leukemic cells. Specific granules of eosinophiles differ from one of neutrophiles in respect of the fact that the former are larger in size and contain central disk. In this experiment using rats, disk-

like structures are observed not infrequently in the neutrophilic granules, too. As benzene intoxication advances, these granules become swollen in which small vacuoles come out. Finally, they show irregular contour and are filled with electron lucent materials, sometimes including various shaped substances of high electron density. These granules remind the author in some respects of C-type granule reported by Watanabe. These changes of the specific granules are recognized both in the neutrophiles (Pict. 15) and eosinophiles (Pict. 16). It might be considered, in our present study, that these changes are the expression of some degenerating process occurring in the specific granules.

c. *Mature leukocytes*:

There scattered, not infrequently, free ribosomes in mature leukocytes of intoxicated animals. Specific granules showing degenerative changes above described are found considerably increased in number, especially around the Golgi area which seem to be rather well developed comparing with that of control mature leukocytes. Small, saccular rERs are present markedly dilated and in larger number than ones of control animals. Mitochondria are found slightly rounded and increased in number near the nucleus. Sometimes found in the cytoplasm are irregular shaped fat droplets, multivesicular bodies and ovoid dense bodies resembling cytolysomes[13] or mitochondria-containing vacuoles[1]. Cytoplasmic islands are occasionally found, as described by Watanabe (1963), in the nucleus of the mature leukocytes surrounded by a thin rim of nucleoplasm. It is not clear whether this phenomenon is associated with abnormal cell division or with direct injury of the nucleus. Nucleoplasm tends to become homogenous with indistinct chromatin condensations along the nuclear membrane.

2) *Erythropoietic cells*:

a. *Proerythroblasts*:

The proerythroblasts and myeloblasts are also found prominent beneath sinusoidal and arteriolar wall on around the 4th to 6th day. Their dark cytoplasmic texture filled with free ribosomes seem to lose their density as benzene intoxication advances. Large, rod-shaped mitochondria are swollen considerably developing clearness of their matrix and disarrangement of their cristae. These cells can not be easily identified in the bone marrow in case of severe intoxication.

b. *Erythroblasts and erythrocytes*:

In control animals, erythroblasts and reticulocytes are found grouped in the bone marrow. The more they differentiate, the more decrease is seen in their size and cytoplasmic width, on one hand, and the more increase occurs in their cytoplasmic density and nuclear chromatin condensations

especially along the nuclear membrane, on the other.

The reticulocytes of various electron density containing obvious mitochondria and rERs are found clustered together close to the sinusoidal wall and often in the sinusoidal lumen. Among those cells, are present frequently the ones which have prominently homogenized nuclei showing partial break up of the nuclear membrane. And it is not rare to see the dense nucleoplasm pouring into the cytoplasm from the break[33] resulting in diffusion of nuclear materials in rather clear cytoplasm without distinct border or limiting membrane. Neither bare nuclei nor isolated ones covered with a part of erythroblastic cytoplasm[16] can be detected in our electron microscopic specimen either of the control or intoxicated rat bone marrow.

In benzene intoxicated animals, the cytoplasm of the erythroblast decreases their textual density, and sometimes it swells remarkably to locate the nucleus excentrically in the cytoplasm. In such cases, swollen mitochondria gather around the nucleus (Pict. 17). Multivesicular and small dense bodies appear, not infrequently, in the cytoplasm. The nucleoplasm tends to become homogenous with loss of chromatin condensations. Erythroctyes are often found rounded spherically filling the enlarged sinusoidal lumens.

C. *Reticulo-endothelial structure and its changes under benzene intoxication*:

1. *RES structure of the control animals*:

In the light microscope sections, the width of sinusoidal vessels are variable, though not so dilated usually. On the sinusoidal wall, flat sinusoidal endothelial cells are found which show cytoplasm of variable thickness. There scattered among haematopoietic cells, though scarce in number, reticular cells of abundant pale cytoplasm with round and clear nuclei containing prominent nucleoli, occasionally showing phagocytosis. Such reticular cells are identified in the smeared preparation as large pale cells with indistinct cell border, round homogenous nuclei and vast pale cytoplasm containing prominent azurophilic granules and various kinds of phagocytized materials. In smeared preparation, the sinusoidal endothelial cells are found often in cluster entangled with reticulins, their nuclei are spindle in shape surrounded by faintly visible pale cytoplasm.

Under the electron microscope, besides sinusoidal endothelial cells and phagocytic reticular cells, the non-phagocytic reticular cells can be identified frequently locating under the sinusoidal endothelial cells. In this respect, special discussion is to be made on the reticular cells which are found beneath the sinusoidal endothelial cells. In the control animals too, the reticular cells seem to constitute a kind of mesh-work between the haemato-

poietic cells connecting each other with their long and slender cytoplasmic processes attached by reticulins.

Sinusoidal endothelial cells appear in considerably variable forms, as they are, showing a remarkable tendency to be flattened out as a sheet of cytoplasm less than 0.1 micron in thickness, remaining a part rich in cytoplasm including single nucleus. Their cytoplasmic extension seems to surround completely the sinusoidal lumen, usually in single and occasionally in double layers (Pict. 18). The texture of the cytoplasm is rather clear. The luminal surface displays marked pinocytotic activity. Large, flattened rERs are often seen, though not so numerous, running along the long axis of the cells and frequently attached to and partly covering the mitochondria. They often contain amorphous ground materials. Small vesicles of smooth wall are abundant. Only occasionally membrane-free ribosomes are noticeable. Round or short eliptical mitochondria are present, rather scarce in number, mostly around the nucleus. They are rather smaller than that of phagocytic reticular cells and with a few, disarranged simple cristae, and electron lucent ground. Golgi complexes are found forming two or three clusters close to either pole of the eliptical nucleus. They are composed of well developed small vesicles and poorly lamellated smooth membranes. Round bodies in various size with homogenous dense material are occasionally found in the cytoplasm. As far as these cells are concerned, phagocytosis can not be recognized, in general. The nuclei are long eliptical in shape, almost always displaying several indentations along nuclear membrane on their basal surface. Nucleoli are seldom observed. Nucleoplasm is clear and homogenous, as they are, and it seems to have a slight tendency of forming nodular chromatin condensations upon the nuclear membrane. At the junction of these cells, there frequently observed a structure of tight junction resembling desmosomes (Pict. 19). They are often present between cytoplasmic extensions which are found running alongside the sinusoidal lumen partially in double layers. These structures have not been recognized either in the bone marrow of albino rabbit (Weiss 1961)[31], of guinea pig (Zamboni & Pease 1961)[35] or in the splenic sinusoides of the rat (Weiss 1957)[30], or in the lymphatic sinusoides of the mouse (Tanaka 1957)[27], of rat (Seon 1961)[23] and of white rabbit (Sorensen 1961)[25].

Underlying the sinusoidal endothelial cells, dense amorphous ground materials are observed in various density and thickness, often in close contact with the cell membrane (Pict. 20). Although they frequently contain apparently fibrillar structures, no such a cross striations as is observed in the collagenous fibers can be detected. They in certainty correspond to the reticulins. Such a structure of the basement membrane as is found around the vascular endothelial cells has not been detected around the sinusoides

either in lymphnode of the rat[3] and mouse[27], or in the splenic red pulp of the human and the rat[30], or in the liver of the rat[28].

Among non-phagocytic *reticular cells,* those cells are especially interesting, which are found, in many places, under the sinusoidal endothelial cells (Pict. 3). Electron microscopically they are highly variable in form, usually having polyhedral contour with rather narrow cytoplasm around the nucleus and short cytoplasmic processes which often insinuate themselves between the haematopoietic cells and occasionally distend themselves under the sinusoidal endothelial cells. Their short cytoplasmic processes often interdigitate with that of the phagocytic reticular cells in the parenchym. The cytoplasmic texture is rather dark and rich in free ribosomes in comparison with that of the phagocytic reticular cells. Small, but occasionally long, saccular rERs are also well developed in the cytoplasm. Only a scanty sERs are seen in the cytoplasm except for Golgi areas. Mitochondria are scarce in number and small in size, and diffusely scattered throughout the cytoplasm. They are variable in shape from round to short rod and with electron lucent ground and poorly developed, randomly arranged cristae. Golgi complexes are poorly developed and situate close to the nucleus, in one or two clusters. They are consisted of poorly developed lamellae of smooth membranes and small vesicles. Dense bodies of smaller diameter than that of mitochondria are found sparcely in the cytoplasm. No phagocytic activities are seen. The nucleus is located at the center of the cytoplasm and is spherical in shape often displaying several indentations. Nucleoplasm is usually clear and homogenous and nucleolus is often prominent.

The Phagocytic reticular cells are found, not so numerous, in the control animals. They are distributed among the haematopoietic cells, having often close contact with the sinusoidal wall and the subsinusoidally lying reticular cells through their long cytoplasmic processes. They are different from the subsinusoidally lying reticular cells in respect of the presence of phagocytic vacuoles which are contained rarely in the latter. They have considerably voluminous cytoplasm around the nucleus and also have long cytoplasmic processes and small digitations. The cytoplasmic texture is clearer than that of haematopoietic cells.

In the periphery of the cytoplasm, membrane-free ribosomes and long flattened rERs are prominent. Spherical and rod-shaped larger mitochondria are distributed rather centrally around the nucleus, having uniformely dense matrix and well arranged cristae. The entire cytoplasm is filled with well developed vesicular sERs which are variable in density. Golgi complexes are found well developed in two or three clusters close to the nucleus. They consist of well developed vesicles and closely lamellated smooth membranes. The nucleus is spherical to eliptical in shape, with rather smooth nuclear

contour and locates centrally in the cells. Nucleoplasms is rather clear and have a tendency of nodular chromatin condensation along the nuclear membrane. Large nucleolus is located centrally in the nucleus revealing prominent nucleolonema.

Many discussions have been made about various organs, as for the nature of reticulins[3,4,23,25~27,30~32] and their impregnability with silver[5]. The same reticulins as described above with sinusoidal wall are often found also alongside the long cytoplasmic processes of the phagocytic reticular cells and sometimes infolded by cell membrane[3,4,23] (Pict. 21). In the bone marrow, the reticular cells surround the muscle layer of arteriolar wall with their long slender cytoplasmic processes (Pict. 23).

2. *RES changes due to acute poisoning of benzene given in large dosage*:

As the haematopoietic cells reduce their number, the sinusoidal lumens are dilated remarkably. Sinusoidal endothelial cells are found frequently lying against the megakaryocytes and fat cells which increase in number as benzene intoxication advances.

In electorn microscopy, *the sinusoidal endothelial cells* are found often incomplete in their continuity and many erythrocytes are interposed in the disrupted portion of the sinusoidal endothelium, and somewhere the disruption is covered with mural thrombosis containing fibrin and thrombocytes (Pict. 24). Whether the disruption occurs at the junction of endothelial cells or in the flattened portion of these can not be decided at present.

Among *reticular cells,* subsinusoidally lying reticular cells tend to become prominent in comparison with that of control animals. Among them, those cells are found occasionally which contain more or less lysosome-like bodies in the considerably enlarged cytoplasm, and seem to be transitional form moving to the phagocytic reticular cells (Pict. 25). Some of them are found insinuated themselves between haematopoietic cells and attached with one elongated portion of the cytoplasm to the sinusoidal wall (Pict. 26). They are, in many respects, coming to resemble the phagocytic reticular cells. Occasionally, their cytoplasm are flattened out, remaining a scanty cytoplasmic portion which contain rather inmature nucleus, and lying closely under the sinusoidal endothelial cells (Pict. 27). Those cells are also found which contain a lot of fat droplets surrounded by a distinct limiting membrane and are considered to be transitional form to the fat cells (Pict. 28). Those rather small cells can be recognized, too, of which narrow cytoplasm are rich in free ribosomes and remarkably poor in the cytoplasmic organells, and which have a large nucleus with inmature nucleoplasm.

Phagocytic reticular cells become very obvious among the reduced haematopoietic cells. Usually, the cytoplasm are clearer and more plump,

with broad cytoplasmic processes insinuated themselves between the haematopoietic cells. They are often connected each other with long cytoplasmic procsses in a manner of interdigitation or only of a touch. They are found closely to the sinusoidal wall and connect to the sinusoidal endothelial cells with their cytoplasmic processes, interposed by reticulins (Pict. 22). Various shaped phagocytic vacuoles are seen in the cytoplasm, especially around the nucleus[12,19]. Besides spherical small dense bodies of various electron density which are surrounded by distinct single limiting membrane, lysosome-like bodies of various size and shape are seen. They contain various electron dense materials and occasionally show prominent myeloid figures and ferritin granules (Pict. 30 & 31). They have a tendency to be found gathering near the nucleus, not infrequently having close contact with the Golgi complexes. They show phagocytic activity and this reaches its maximum on around the 4th to 6th day, when many plumped phagocytic reticular cells are filled with variety of phagocytized haematopoietic cells which are undergoing destruction of various degree. Severely desintegrated cells are observed almost exclusively in the phagocytic reticular cells (Pict. 30).

The fat cells appear strikingly increased in number along the sinusoidalwall (Pict. 4 & 29), partly in accordance with the observation of Shimamine[24] (1952) who studied first appearance of the fat cells in the bone marrow of the human and rabbit embryos. They vary in shape from ones which contain fat droplets of various size in the cytoplasm, to ones in which large fat vacuoles occupy almost entire cytoplasm, leaving only a thin rim and semilunar space of cytoplasm where elongated spherical nucleus is seen. These fat droplets are distinctively surrounded by limiting membrane. It seems highly possible that small fat droplets fuse together to form larger ones. The cytoplasm of the fat cells contains moderate number of oval to rod shaped mitochondria with dense matrix. Free ribosomes and rERs are seen rich in the cytoplasm.

Discussion

A. *Discussion on toxic action of benzene*:

As has long been accepted, benzene has toxic effects on the blood and blood forming organs. However, the mechanism through which this toxic substance induces severe myelotoxicity has not yet been fully clarified. Especially, numerous discussions has hitherto been made on the problem whether benzene itself or its oxydated metabolites such as phenols, hydroquinones, pyrocathecols[9,11,18] etc. act on the tissue, and whether benzene acts as mitotic poison[6,9,20] or causes direct injury to the cells[6,9]. On the chronic poisoning in man, innumerable reports are available, however,

it is so complicated and none of them has offered the full answer to the problem[7,9,17].

On the basis of our findings, it may be valid to make a conclusion that daily injection of benzene in large dosage, such as 2 ml/kg, causes direct destruction of the haematopoietic tissue in the bone marrow[6,14]. The evidences are: (1) Degenerating changes and phagocytosis of desintegrating cells are recognized both in the smeared preparations and electron microscopic sections of the marrow, as early as on the first day. Furthermore, reduction of parenchymal marrow cells also occurs very soon, one day after injection of benzene, and continues thereafter (Fig. 4). (2) Mitotic index increases rapidly and reach its maximum within several days of benzene intoxication, however, gradually decrease thereafter. Various phases of mitosis can be recognized in every case (Table). Remarkably abnormal figures and degenerative changes can not be observed in the cells under mitosis. However, possibility of the mitotic poisoning of benzene, administrated in large dosage, can not fully be denied in our present study, so it might be needed further cytokinetic investigations in order to clarify this problem.

B. *Reticulo-endothelial structure in the normal and benzene intoxicated bone marrow*:

It has hitherto been known, in the light microscopic level, that the myeloid stroma consist of sinusoidal vessels to which closely connected the mesh work of primitive and phagocytic reticular cells supported by the argylophile fibers. The sinusoidal walls were lined by flattened endothelial cells which had indistinct cell border and were in direct connection with similar cells of the stroma. They were said not to be the typical endothelium, but rather flattened part of the mesh work of the stromal reticular cells. In some specific conditions, for example in vital staining with carmin or indian ink, they could be easily rounded off and appeared as free macrophages in the sinusoides. Thus, the sinusoidal lining cells and stromal reticular cells have been believed to have the same origin[8].

Recently, several studies on these problem, at the electron optic level have been made. Zamboni & Pease[35] (1960) observed the bone marrow of the normal and aminopterin intoxicated guinea pig. They reached the conclusion that it was in vain to look for reticular cells unassociated with sinusoides, and that reticular cells of the bone marrow were almost entirely confined to the walls of vascular channels. Weiss[31] (1960) reported, after observation of normal bone marrow of albino rabbit, that almost all reticular cells in the bone marrow took part in forming vascular sinuses, while phagocytic reticular cells unassociated with sinuses, which were small

in number in the marrow, might be only the remnants of endothelial cells.

On the strength of our present study, it may be valid, as was described above, to divide the reticulo-endothelial cells of the bone marrow into following three types, especially from the view point of their localisation, morphological distinctions and functional speciality. These differences become clearer, as acute benzene injury on the bone marrow increases its severity. (1) Sinusoidal endothelial cells: They are found lining the sinusoidal wall with attenuated cytoplasm. They are endowed with such a morphological distinctions as described before in the observation (cf. C-1 and 2) and with such a functional speciality as having cellular polarity and vigorous pinocytotic activity. (2) Phagocytic reticular cells: They are present distributed among the haematopoietic cells constituting a kind of mesh work connecting each other with their long cytoplasmic processes associated with reticulins. These polyhedral cells have enormously vast cytoplasm showing characteristic cytological distinctions of which details are described above, also (cf. C-1 and 2). As benzene intoxication advances, they are found more frequently and their phagocytic activity becomes more distinct. (3) Among non-phagocytic reticular cells, the subsinusoidally lying leticular cells are found, almost always, just beneath the sinusoidal endothelial cells, occasionally connected with phagocytic reticuar cells by cytoplasmic interdigitations. Their polyhedral contour resembles that of phagocytic reticular cells, however, their cellular size and cytoplasmic processes are smaller than that of the latter, and they have different cytological distinctions as was described before in the observation (cf. C-1 and 2). Although their functional speciality is not fully elucidated, the possible role of those cells is to be discussed later.

Concerning the classification of RES cells on electron optic level, numerous studies have been brought about so far with other organs, i.e. on the lymph nodes[23,25,27] and on the spleen[4,30,32]. Besides phagocytic reticular cells in those organs, some investigators have described two kinds of non-phagocytic reticular cells, namely, mature or resting ones (Yamori 1963)[34] on one hand and inmature (Yamori) or proliferative ones (Tanaka 1958)[27] on the other. Possibility of their differentiation of the latter form, especially towards haematopoietic cells, has long been discussed seriously and not yet clarified. In the present study those reticular cells are detected frequently which lie mostly under the sinusoidal endothelial cells. They are rather inmature in cytological characteristics. They may be called "subsinusoidal reticular cells". They show phagocytosis in only a rare instance throughout our experiment, even though transformation to the phagocytic reticular cells are found occasionally. On the other hand, they are found not infrequently transforming in situ into fat cells accumulating fat droplets in their cyto-

plasm.

Possibility should be described here that they transform into sinusoidal endothelial cells, considering from their localisation, however, no direct evidence is available in our present study. The author is still far from being able to consider whether they are related with haematopoietic cells or not in their cytogenesis. Accordingly, further studies are required, in the future, to determine the ultimate character of these cells.

Acknowledgement

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Explanation of Pictures

Pict. 1. Paraffin section of vertebral marrow. Parenchymal marrow cells decrease both in spread and in density as early as 2nd day, followed by dilatation of sinusoides and gradual increase of fat tissue. H.E. (× 200).

Pict. 2. Severe hypoplasia in parenchyma with fat cell hyperplasia is induced on the 16th day. Parenchymal cells are seen remained only around the trabeculae of bone. H.E. (× 200).

Pict. 3. Methylene-blue staining epoxy-resin section of control animal. The sub-sinusoidally lying reitcular cells are found around the sinusoides and are indicated by arrows. (× 1,500).

Pict. 4. Fat cell is seen underlying the sinusoidal endothelial cells. (× 1,500).

Pict. 5–7. Peripheral blood smear of benzene intoxicated animals. May-Grünwald-Giemsa. (× 1,500).

Pict. 5. Pycnotic nucleus with isolated nuclear fragment in the mature leukocyte.

Pict. 6. Slightly basophilic cytoplasm of the neutrophilic mature leukocytes, containing toxic granules. Cytoplasmic islands are seen in the nucleus.

Pict. 7. The cell resembling the sinusoidal endothelial one is observed in the terminal stage.

Pict. 8–11. Bone marrow smear of the benzene intoxicated animals. May-Grünwald-Giemsa. (× 1,500).

Pict. 8. Vacuoles are seen in the cytoplasm of the myeloblasts and promeylocytes.

Pict. 9. Neutrophilic granulopoietic cells. Dissociation in nuclear and cytoplasmic maturation are recognizable and toxic granules come out in the cytoplasm. Note the over-segmented nucleus in the mature leukocytes.

Pict. 10. Phagocytic reticular cells.

Pict. 11. Nuclear clumping is seen in the erythroblasts. Erythroblasts are seen caught in the phagocytic reticular cells.

Pict. 12. Electron microscope of the myeloblast of benzene intoxicated animals. Note dilatated short saccular rERs which are found increased in number (× 7,000).

Pict. 13. Dark cells reveals remarkably swollen mitochondria, rERs and Golgi vesicles in the dark cytoplasm (× 7,000).

Pict. 14. Severely degenerated promyelocyte in benzene intoxicated animal. Note the remarkably dilated rERs and swollen mitochondria and specific granules. Spherical nucleus shows marked homogenization of the nucleoplasm (× 6,000).

Pict. 15. A cytoplasmic part of the mature leukocyte of benzene intoxicated animal.

Swollen specific granules, cytolysomes, multivesicular bodies and fat droplets are seen in the cytoplasm where free ribosomes are observed (× 14,000).

Pict. 16. Swollen specific granules in the eosinophilic metamyelocyte of the benzene intoxicated animal (× 7,000).

Pict. 17. Degenerated erythroblast shows swollen cytoplasm to locate the nucleus excentrically. Swollen mitochondria gather around the nucleus (× 3,600).

Pict. 18. Sinusoidal endothelial cell. Note pinocytotic vesicles along the luminal surface of the clear cytoplasm. Evidence of cellular polarity is seen as well as in the nuclear shape (× 8,000).

Pict. 19. At the junction of the sinusoidal endothelial cells, there observed the structure of tight junction resembling desmosomes (arrow) (× 42,000).

Pict. 20. Underlying the sinusoidal endothelial cells, reticulins are found well developed and are consited of dense amorphous materials and distinct fibrillar structures (× 24,000).

Pict. 21. Reticuline are sometimes infolded by cell membrane of the reticular cells (× 24,500).

Pict. 22. Phagocytic reticular cells connect with the sinusoidal endothelial cells through their long cytoplasmic processes, interposed by reticulin (arrow) (× 16,000).

Pict. 23. The reticular cells surround the muscle layer of arteriolar wall with their long slender cytoplasmic processes (× 3,000).

Pict. 24. The disruption of the sinusoidal endothelium are covered somewhere with mural thrombosis containing fibrins and thrombocytes. Arrow shows disrupted portion of the sinusoidal endothelium (× 8,000).

Pict. 25. The subsinusoidally lying reticular cells contain occasionally lysosome-like bodies (arrow) and are coming to resemble the phagocytic reticular cells (× 7,000).

Pict. 26. Some of them (Pict. 25) are found insinuated themselves between the haematopoietic cells, however, they are still attached with one elongated cytoplasm to the sinusoidal wall (× 7,000).

Pict. 27. The subsinusoidally lying reticular cells are found extending their long cytoplasmic portion closely under the sinusoidal endothelial cells (× 7,000).

Pict. 28. The subsinusoidally lying reticular cells are found containing a lot of fat droplets, surrounded by distinct limiting membrane in their cytoplasm (× 14,000).

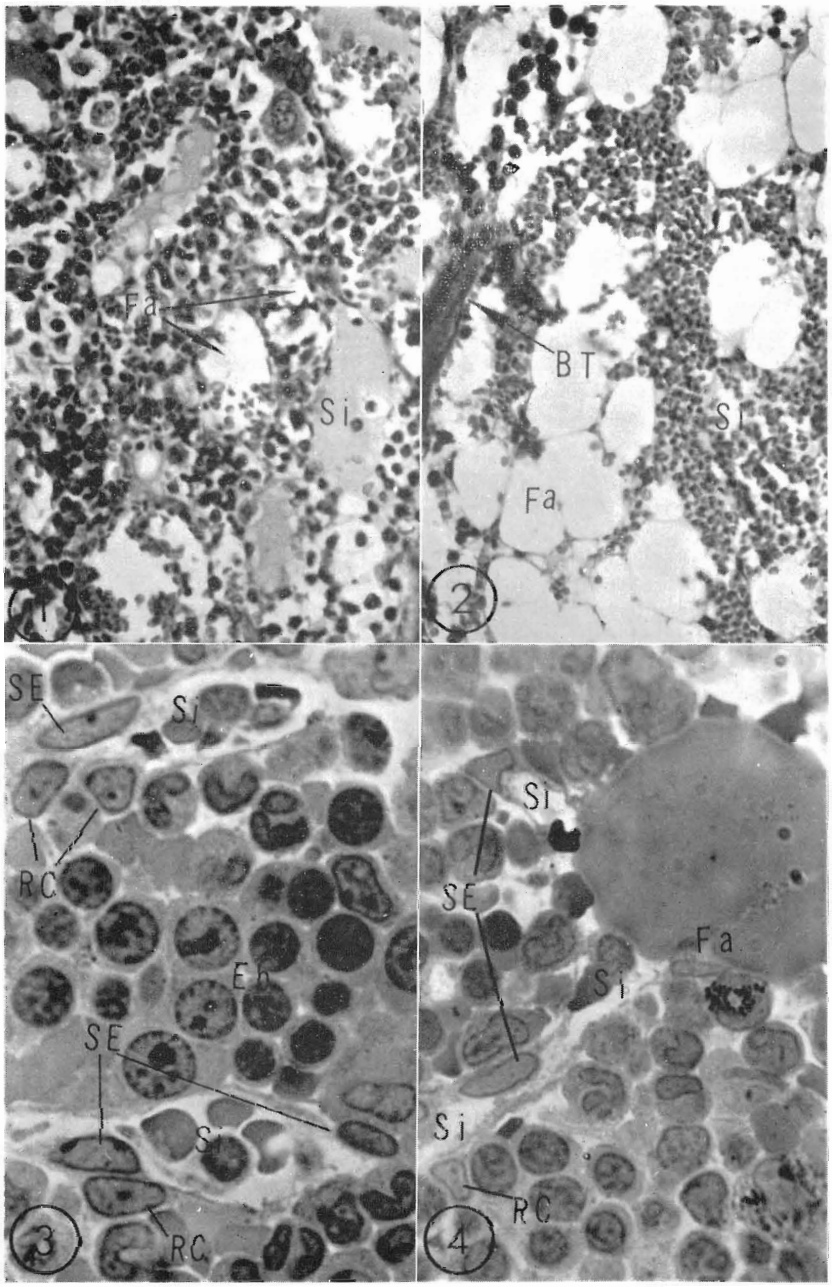
Pict. 29. The fat cells appear along the sinusoidal wall. It seems possible that small fat droplets fuse together to form larger ones (× 7,000).

Pict. 30. Severely desintegrated haematopoietic cells are observed in the phagocytic reticular cells (× 3,600).

Pict. 31. Various shaped phagocytic vacuoles are seen in the cytoplasm of the phagocytic reticular cells, especially around the nucleus (× 7,000).

Key to Abbreviation (Pict. 1–31)

BT: Trabecula of bone, Cy: Cytolysome, Eb: Erythroblast, EGr: Eosinophilic granule, En: Endothel, ER: Endoplasmic reticulum, Fa: Fat cell, FD: Fat droplet, Fi: Fibrin, Go: Golgi complex, LB: Lysosome-like body, Lu: Lumen, M: Mitochondrion, Mb: Myeloblast, Mu: Multivesicular body, NGr: Neutrophilic granule, Nl: Nucleolus, Nu: Nucleus, PH: Phagocitized haematopoietic cell, PRC: Phagocytic reticular cell, PV: Phagocytic vacuole, Re: Reticulin, RC: Reticular cell, SE: Sinusoidal endothel, Si: Sinusoid, SM: Smooth muscle, Th: Thrombocyte.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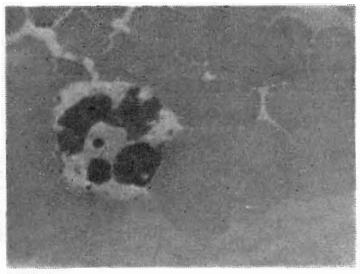
Pict. 5.



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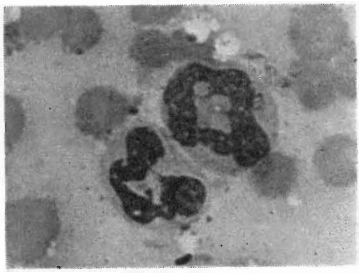

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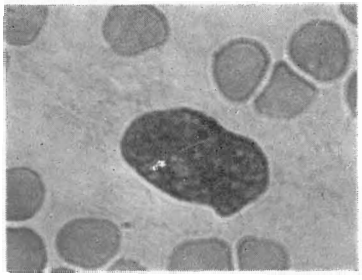

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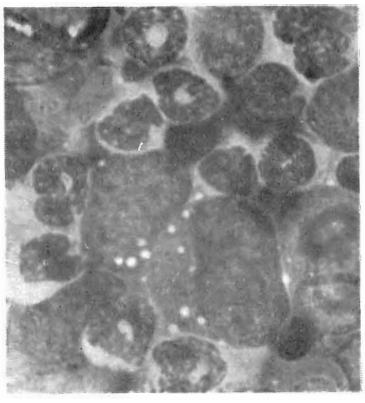

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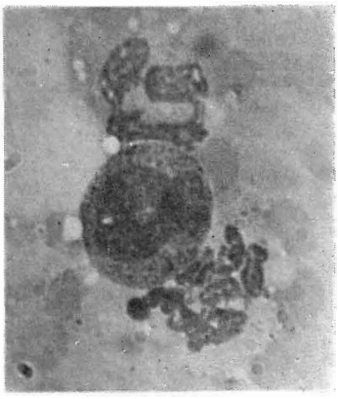

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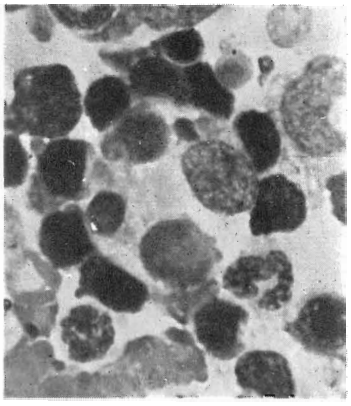

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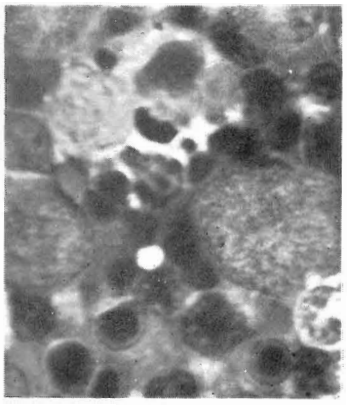

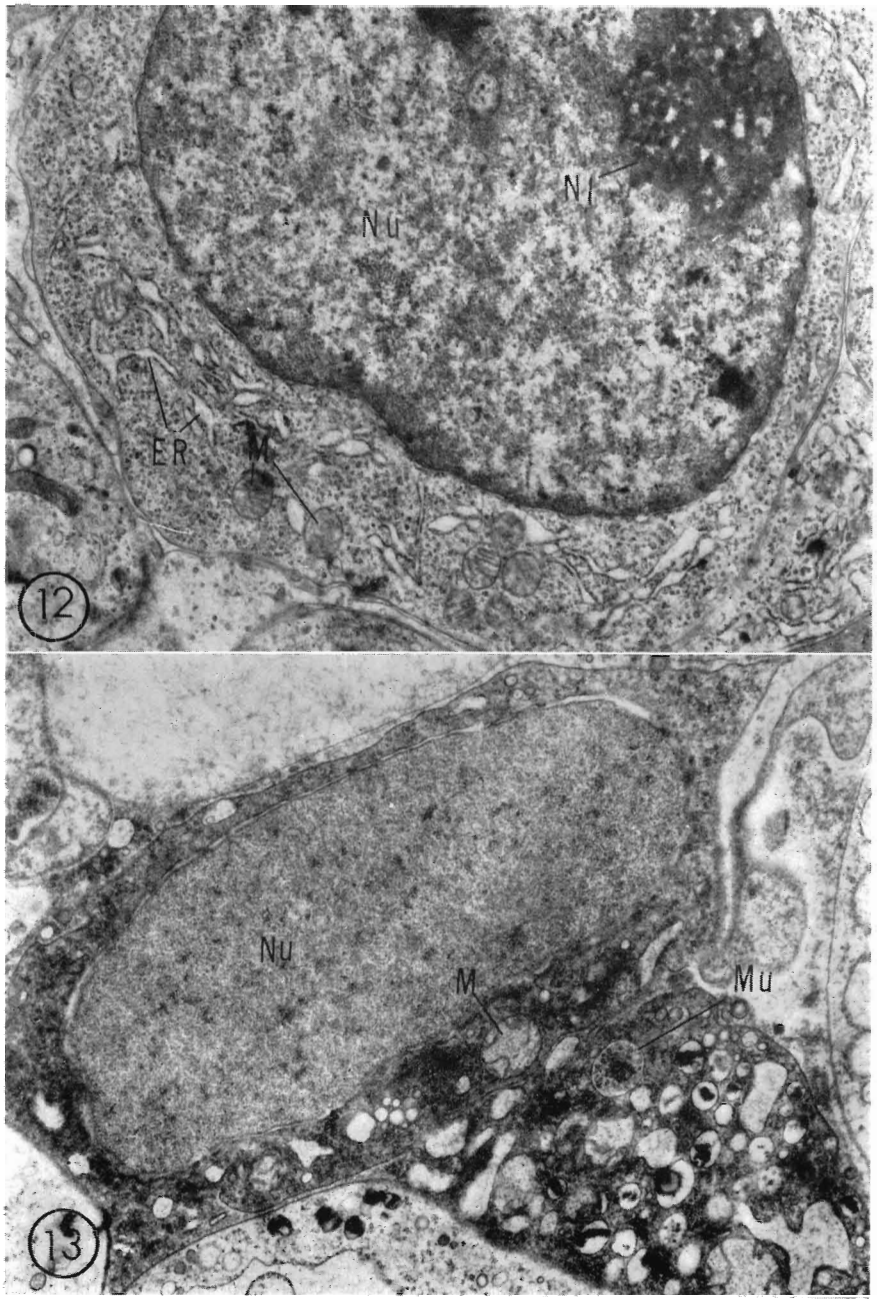
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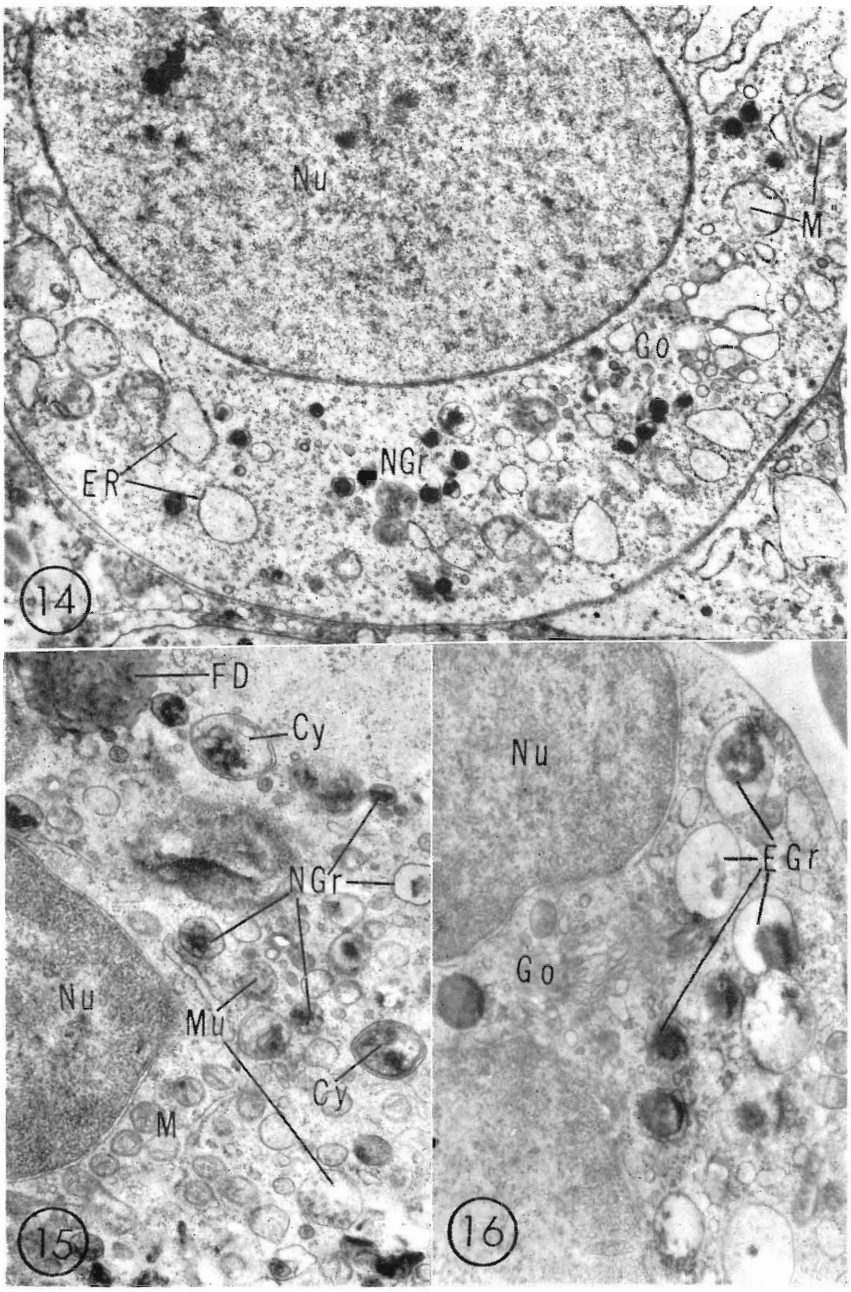
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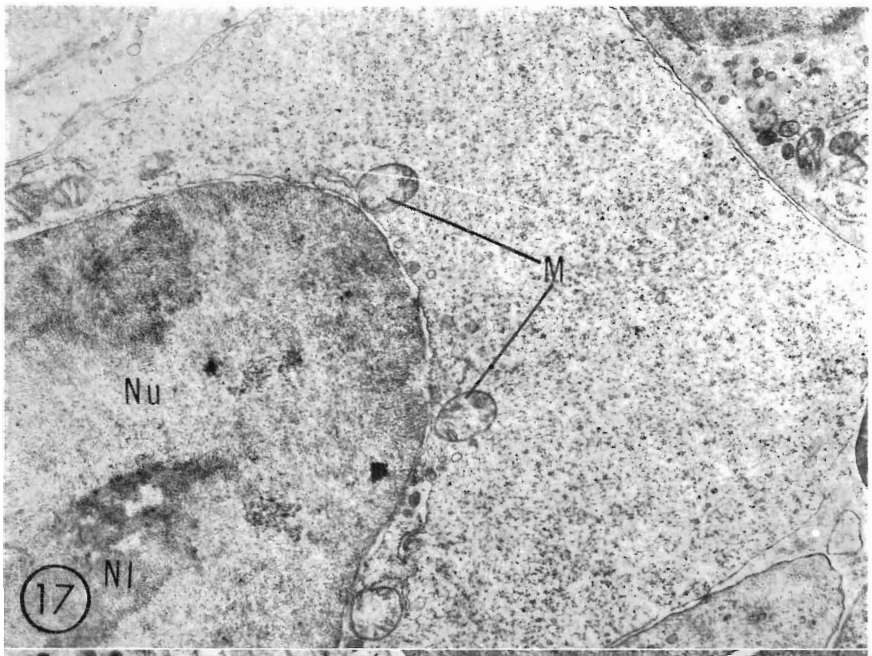
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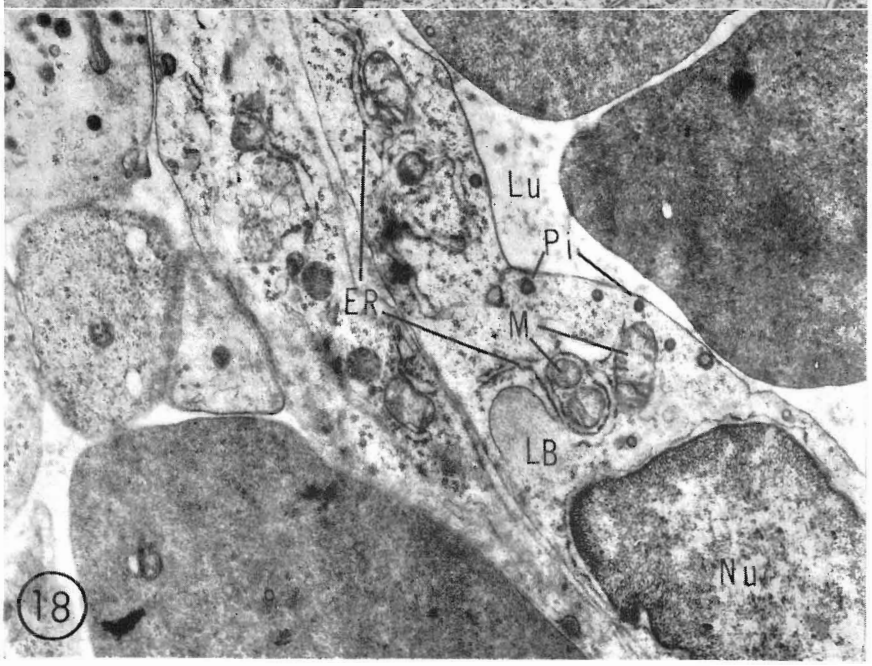
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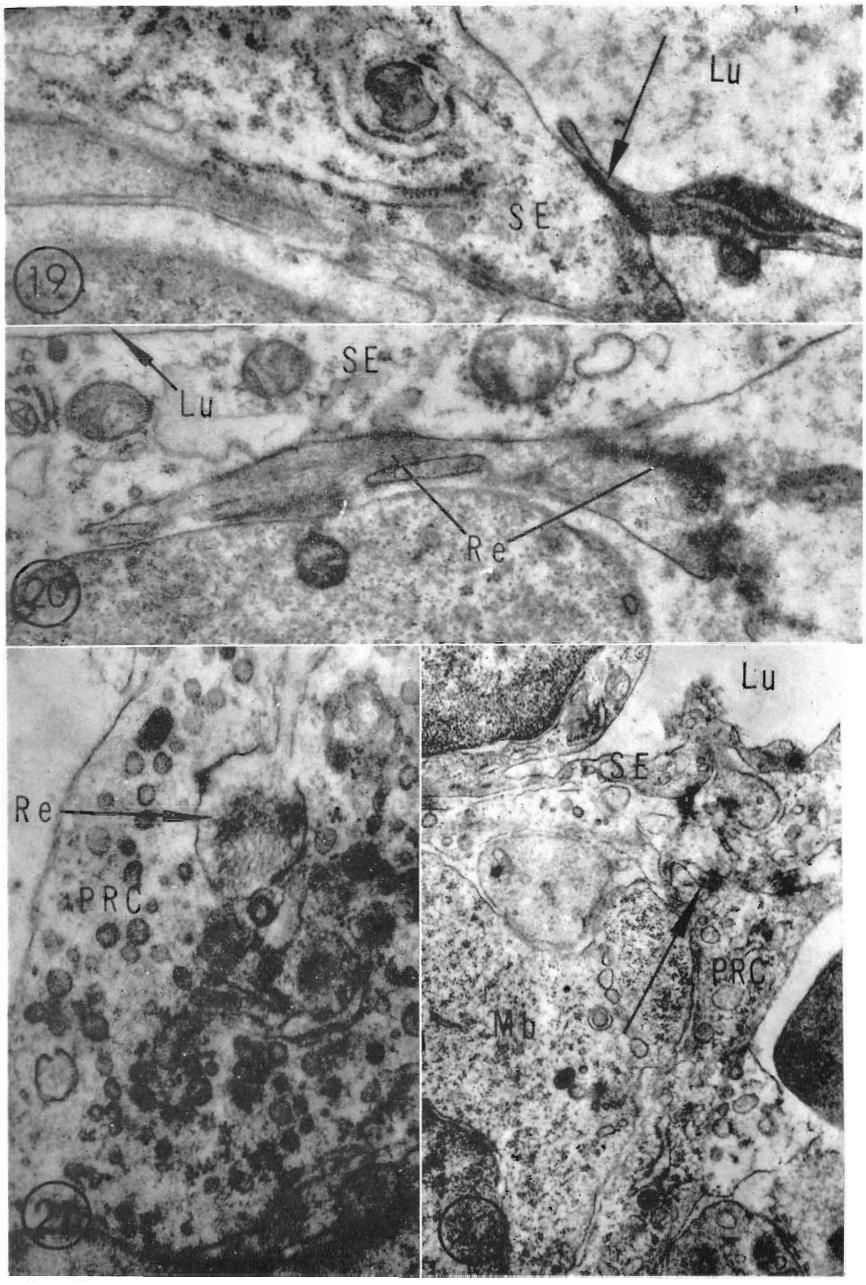
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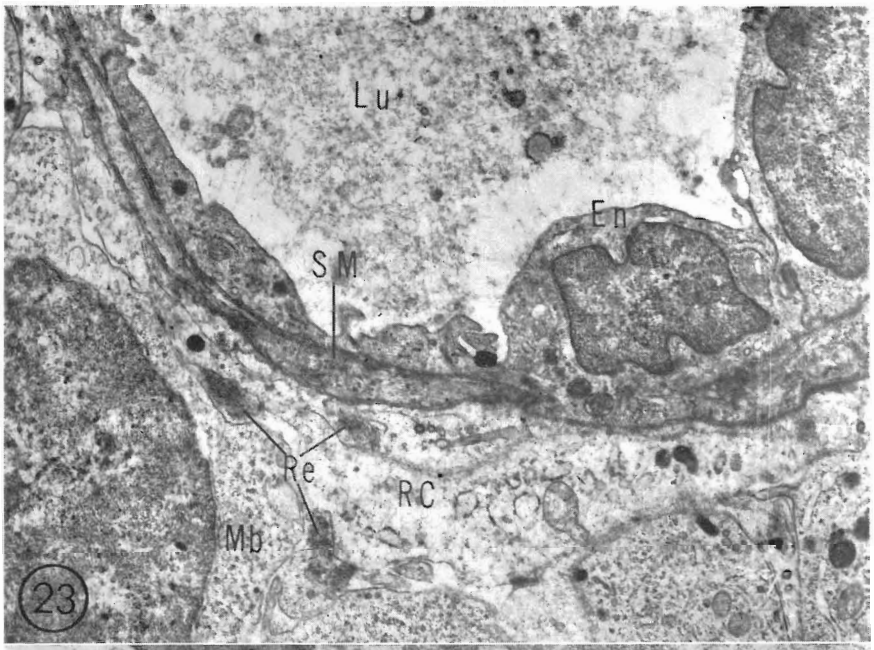
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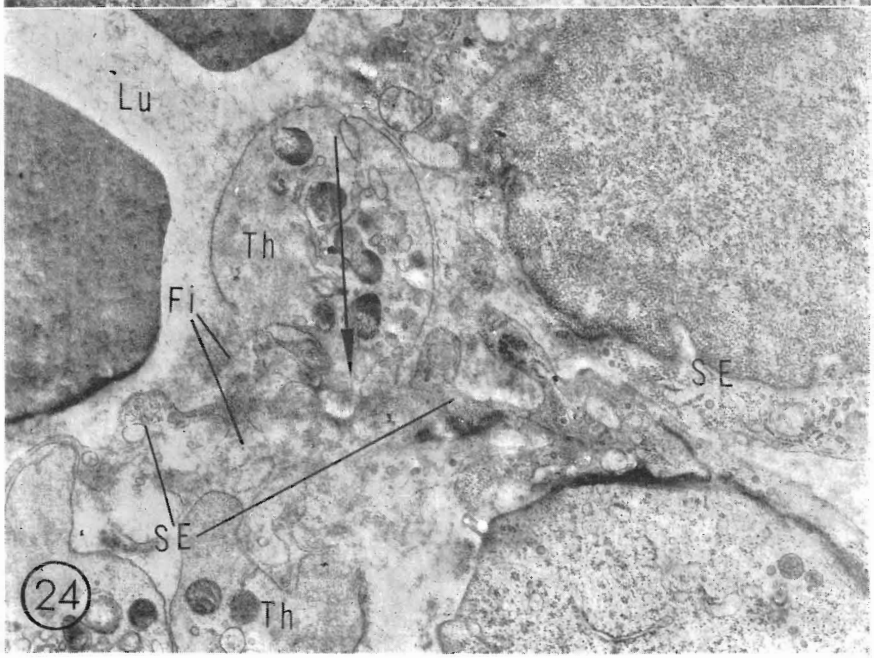
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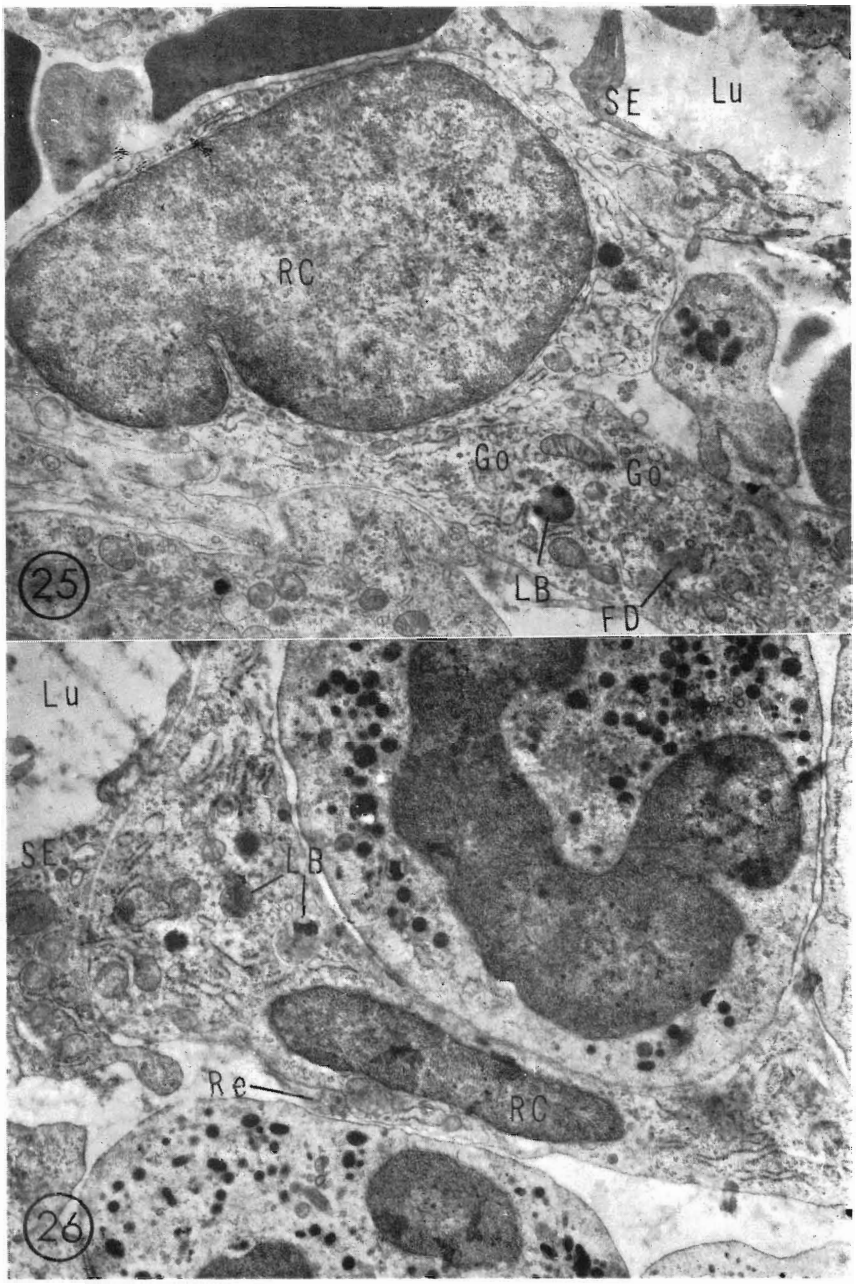
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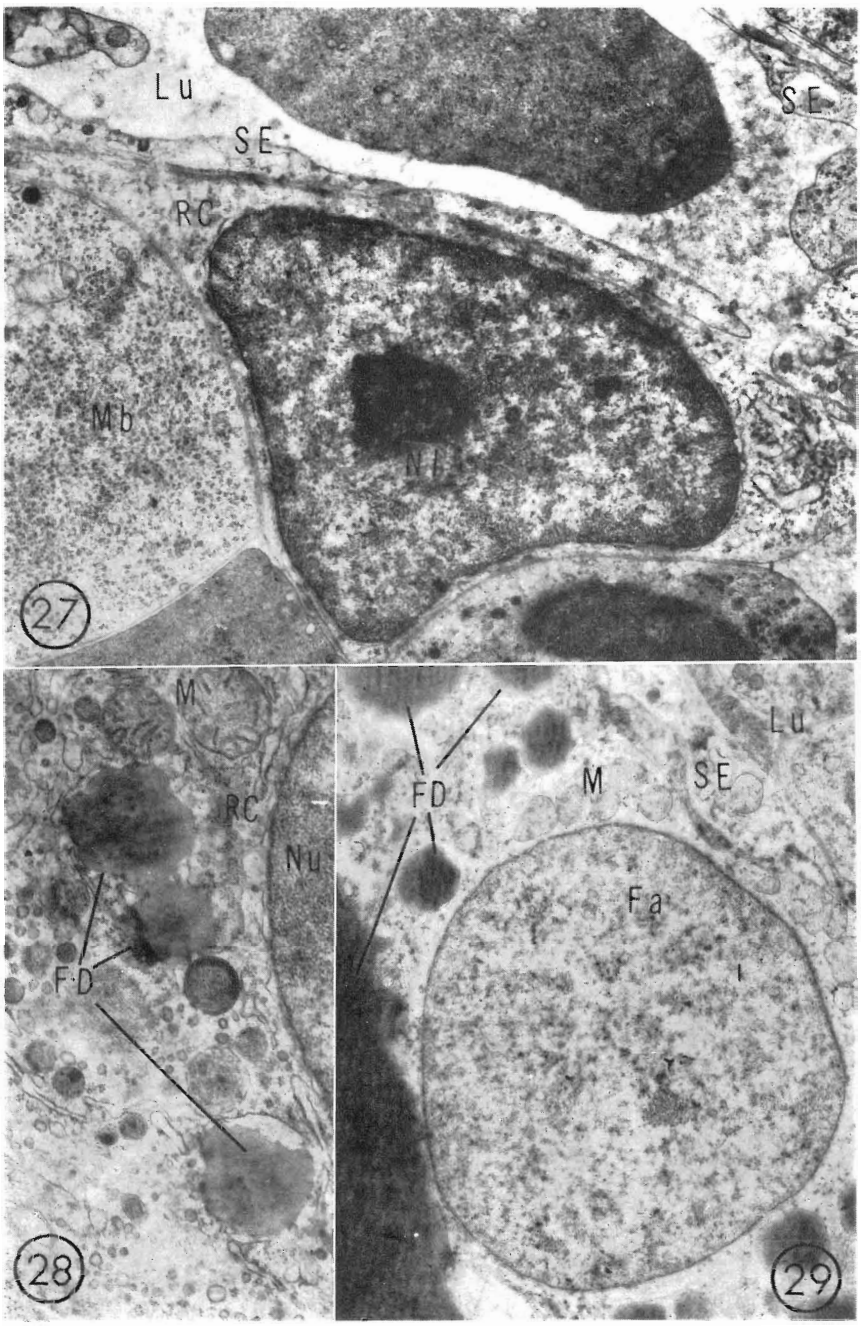
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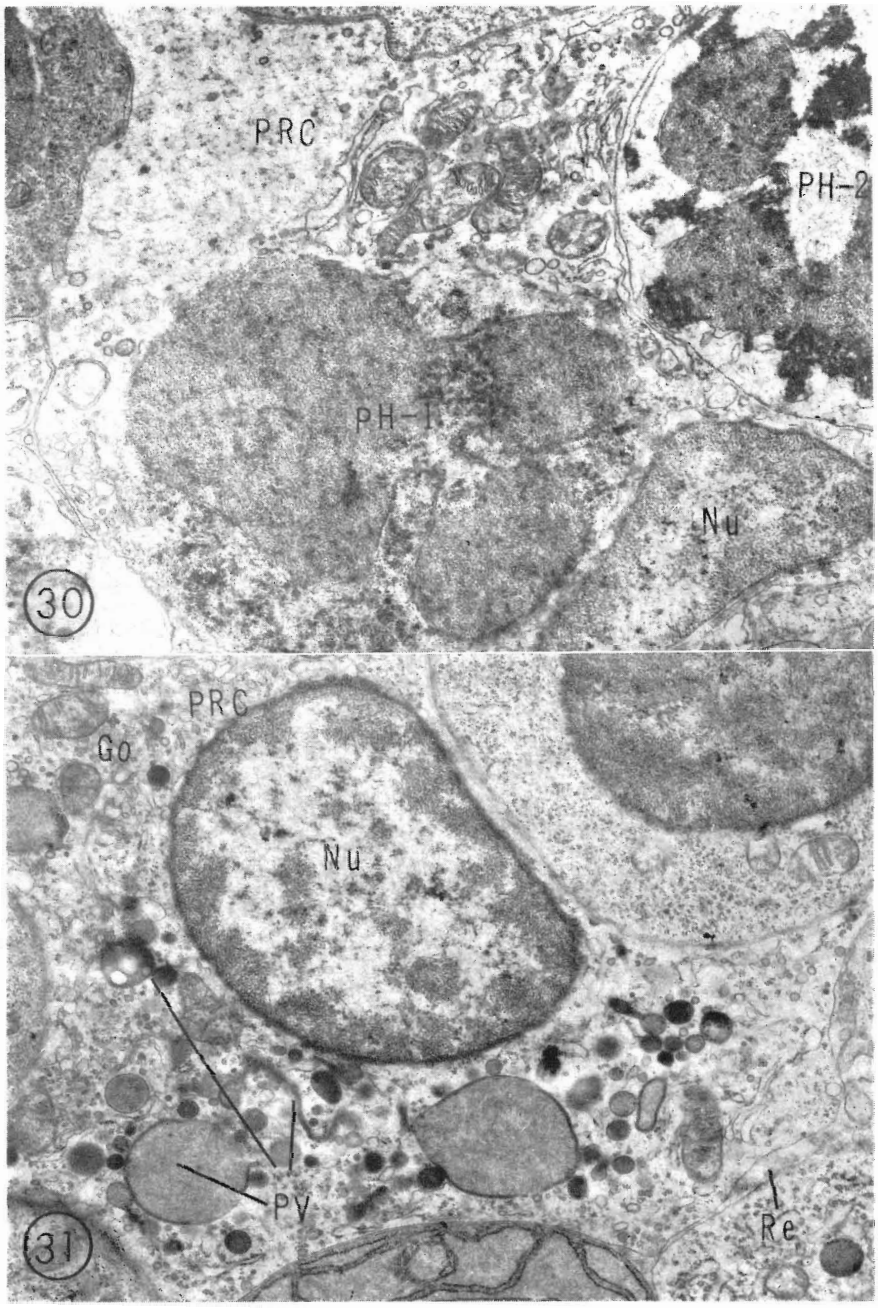
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