A CYTOLOGICAL STUDY OF BONE MARROW IN PHAGOCYTIC ASPECT

KAZUO YAIDA, TSUTOMU ITO AND TOYOKI ICHIKAWA

1st Department of Internal Medicine, Nagoya University School of Medicine
(Director: Prof. Susumu Hibino)

Since the practical application by Arinkin of bone marrow aspiration, the new staining method for smear specimens has brought about brilliant results in the whole area of cytology related to myelocytic and erythrocytic cell systems, of which bone marrow parenchyma is composed.

While Aschoff and others[1)-3)] threw light on the function and morphology of RES, no remarkable advance has been observed in the study of this cell group in the bone marrow smear specimen, in spite of the unusual efforts of Rohr *et al.*[4) 5)] In our country, the recent review by Akazaki *et al.*[6)] on the cytology of RES has gone into details, employing the supravital staining method and others.

In this connection, employing various cytological and histochemical staining methods, we made further observations of this cell system. We kept in mind to mediate the contrast between sectioned specimens and smear ones. It is hoped that the writers might make some contributions to the morphology and classification of RES in bone marrow, and to the difficulty of its differentiation usually felt in the readings of myelogram.

MATERIALS AND METHODS

The laboratory animals used were albino rats, rabbits, and dogs. Studying the cell morphology of RES, we identified the cell group by its phagocytic activity or affinity to vital staining, which, since Aschoff, has been considered as one of the main functions of RES. India ink was chosen among others as materials for phagocyting, because it remains unaffected in staining of specimens and gives a satisfactory contrast to the common dye colors. Concentration and quantity of India ink may give rise to various problems. The ink was prepared by Tanabe's method.[7)] When a small quantity of India ink was injected, most the particles were usually phagocytised only by the sinus endothelium of the bone marrow, and the appearance frequency of India ink phagocytizing cells in smear specimens was not so high. Therefore the following conditions were considered.

Albino rats (B.W. 70–100 g) were injected per the vein of the tail once with 2 ccm and examined 30 minutes after injection. Rabbits (B.W. 1–2 kg) through the auricular vein, 3 ccm every day, for 3 days, and examined 24 hours after the last injection, or once with 10 ccm, and examined 30 minutes after injection. Dogs (B.W. 3–4 kg) through the vein of the extremity, 10 ccm, per day,

Received for publication February 27, 1954.

for 3 days, and examined 24 hours after the last injection.

The section and smear specimens of the femur marrows (red marrow) of these animals were examined. In the case of sectioned specimens stained by hematoxylin-eosin, we observed that India ink was taken up in the cytoplasm of the reticulum cells or histiocytes in the bone marrow parenchyma, not to mention sinus endothelium cells. Sometimes we noticed a hemorrhage in the parenchyma.

In this connection, we made a cytological and histochemical study as follows of India ink phagocyting cells (regarded as part of RES cells) appearing in smear specimens of these bone marrows.

1) Supravital staining method with Janus green and neutral red (Morita's method[8] or Forkner's method).[9]
2) Giemsa staining.
3) Phase microscopic observation.
   I observed the materials under conditions of dark medium or bright medium.
4) Stabile oxidase reaction (Schultz's method) and peroxidase reaction (Graham's or Sato and Sekiya's method).
5) RNA] (Unna-Pappenheim's staining method and treatment with ribonuclease).[10]
6) Glycogen and other polysaccharide (Hotchkiss's periodic acid-Schiff staining method and digestion with saliva).[11]-[13]
7) Protein (Yasuma and Ichikawa's alloxan- or ninhydrin-Schiff staining method).[14]
8) Phosphatase activity (Gomori's method).[15]
9) Sudanophile substances.
   (Goldmann's staining method for substances considered to be lipid, and Demel's supravital staining method for substances considered to be neutral fat).[16]
10) Papanicolaou's staining (by moist fixation with Champy's fluid).[17]
11) Altmann's granules (Altmann's staining method by moist fixation with Champy's fluid).[18]
12) Nucleolus (0.5-1.0% brillant cresyl blue supravital staining and Heidenhein's iron-hematoxylin staining in moist fixation with Zenker-acetic acid fluid).[19]
13) Golgi apparatus (modified Kolatshef's method).[20]

RESULTS

I. Cytology of India ink phagocyting cells

A) RES cells

1) Supravital staining with Janus green and neutral red

Cytoplasm of RES cells is irregular or fusi form in shape, and phagocytized India ink particles in various quantities, which appear as fine granules, clumps or both. The shape of nucleus is ovoid or round. Many neutral red granules, large and brown, lie soattered in the cytoplasm---socalled neutral red vacuoles.

Some of them are small in size. But these granules do not form the typical rosette such as is found in the case of monocytes. Janus green granules are scarcely found in those cells which phagocyted India ink particles in large quantities, however, a small number of fine granules sometimes appear round the nucleus or are found scattered in the cytoplasm, when India ink particles were phagocyted in small quantities.

2) Giemsa staining

Usually the cytoplasm of RES cells is extensive and vaguely shaped, ranging from spindle to irregularly swollen shape. Cytoplasm of this cell group is not so basophilic, and sometimes contains vacuoles of all sizes. India ink particles are phagocyted to various degrees as found with supravital staining.

The nucleus varies in shape, and is, round, oval, fusi form etc. The nuclear chromatin shows mostly a coarse net-structure such as described by Rohr. Sometimes, however, we noticed pycnosis or fine meshes. One or two nucleoli, round and violet-blue, are distinctly observed.

3) Phase microscopic picture

Observed under the phase microscope, RES cells reveal somewhat rough and massive nuclear structure, and nucleoli of irregular shapes. Whereas the myelocytic cells contain a diffuse nucleus and fine nucleoli, erythroblastic cells show a dense massive nuclear structure and contain more irregular nucleoli. On the other hand, cytoplasm of RES cells are observed to include vacuoles and phagocyted foreign matters, but no distinct views have been obtained with reference to the structure of cytoplasm.

4) Stabile oxidase and peroxidase reaction

The cellular reaction of RES cells is mostly negative; when positive, rare as it is, presumably it is because of the positive reaction of the phagocyted substances *e.g.* granulocytes.

5) RNA

RNA in this cytoplasm generally is weak positive. The nucleolus will be referred to later.

6) Glycogen and other polysaccharide

There are PAS staining positive masses of various sizes in cytoplasm of this cell group. They are presumably polysaccharides other than glycogen, because they are not digested by saliva treatment. As far as we have observed, these masses were distinctly seen in cases when we tried to destroy the parenchyma cells of bone marrow, for example, by the injection of nitrogen mustard. I presume that the presence of these polysaccharide masses in cytoplasm is one of the characteristic signs of RES cells. We are now trying to demonstrate whether they are related to phagocyted substances or a process of metabolism in the cell.

7) Protein

By the Alloxan-Schiff method, cytoplasm of this cell group can hardly be stained. No prominent features have been observed.

8) Phosphatase activity

With acid phosphatase, only the nucleus is stained. With alkaline phosphatase, are lightly stained both nucleus and cytoplasm. However, no remarkable features have been noticed.

9) Sudan III stainable substances

Several Demel's granules, believed to stain neutral fat, appear in cytoplasm. They are presumably identical with the vacuoles obtained by Giemsa staining method. With Goldmann's staining method coarse granules were sometimes observed, and most RES cells showed negative reaction.

10) Papanicolaou's staining

This method furnishes beautifully dyed nucleus and nucleolus. The outstanding feature observed in this case was that cytoplasm was dyed dark green with these dyes, especially, with, "light green." This is most distinctive as compared with other blood cell elements.

11) Altmann's granules

With respect to the cells that phagocyted small quantities of India ink particles, several fine bacilliform Altmann's granules are observed around the nucleus, which, I presume, are identical with Janus green stainable mitochondria.

Besides, in the foregoing example of nitrogen mustard injection, amorphous fuchsinophile substances were observed appearing in clusters in cytoplasm.

12) Nncleolus

There are discernible in nucleus of this cell group 1-3 nucleoli of slightly irregular shape and of medium size. Nucleoli in the cell with a fusuform nucleus are rather small in size; but nucleoli in irregularly swollen cells show dense pigmentation around themselves. Some of them suggest a certain structure within the nucleolus.

13) Golgi apparatus

RES cells have typical complex forms of Golgi apparatus or sometimes Golgi-rest form.

B) Monocytes

When the injection of India ink or vital staining with lithium carmine are performed, we, as in the case with RES, encounter the fact that menocytes also have phagocytic activity, weak as it is. We conducted researches into the cytological features of ordinary blood monocytes and those which phagocyted India ink particles—the latter, though small in quantity, being observed in blood when India ink was injected. By this means we intended to differentiate cytologically, monocytes in bone marrow from RES cells. Comparatively few blood monocytes phagocyte the particles, when India ink is injected from vein of animals. With Janus green-neutral red supravital staining, monocytes show a typical rosette. While, when India ink particles are phagocyted, the rosette sometimes causes diffusion. Moderate Janus green granules cluster around the nucleus, which are sometimes hard to stain when they phagocyte India ink particles. With Giemsa staining, monocytes were proved to show cytoplasm of clear-cut

border and a few vacuoles of small size, which, however, are not so distinctly observed as those in RES cells. Each of their nuclei shows characteristic concavity and coarse chromatin net. They have one or two nucleoli of small size and round shape. Oxidase and peroxidase reactions of a large percentage of monocytes are positive in the case of rats, as many investigators have so far demonstrated. The reaction of Goldmann's granules is positive. As mentioned above, monocytes, appearing in peripheral blood and bone marrow, show considerable different cytological characters from those of RES cells. So far as our experiment are concerned, it has not been so difficult to differentiate those monocytes which phagocyted India ink particles from RES cells.

C) Endothelium cells proper

Since this cell group exhibits weak phagoytic activity under forcible vital staining, and none observed under usual conditions, it has been considered by Ashoff to belong to the RES in a broard senes. Furthermore, it is located contiguous to the sinus endothelium. These two facts necessitated us to demonstrate the cytological features of this cell group. Endothelium cells proper appear as a long serial cell band in the case of smear specimens of bone marrow. The cytoplasm is slender, along its longer axis and the nucleus is slender, too. The chromatin net is dense. One or two very fine nucleoli are found. Janus green granules appear as a row along the perpendicular axis of a cell, which can be demonstrated by Altmann's staining method. Demel's granules, Gold-

TABLE 1. Cytological Characteristics of RES Cells and Others

	RES cells	Blood monocytes	Endothelium cells proper
Giemsa staining	Nucleus: rough net work Cytoplasm: unclear or extensive	Nucleus: characteristic concavity Cytoplasm: distinct limit	Nucleus: slender, dense chromatin Cytoplasm: slender
Jg-Nr supravital staining	Small or large neutral red vacuoles, a few Janus green granules	Neutral red rosette, moderate Janus green granules	Janus green gran. in a row, a few neutral red granules
Phase microscopic view	Rough and massive nuclear structure	Diffuse nuclear structure	Dense nuclear structure
Stabile oxidase and peroxidase reaction	Negative	Positive in large percentage	Negative
Glycogen and other polysaccharide	Polysaccharide masses	Glycogen positive or none	Slightly positive for polysaccharide
Goldmann's staining	Coarse granules	Positive	Negative
Demel's supravital staining	Several granules	Several granules, rather finer	Negative
Nucleolus	Of middle size, somewhat irregular shape, dense pigmentation around nucleolus	Small and round	Very fine
RNA in cytoplasm	Weakly positive	Weakly positive	Slightly present
Altmann's gronules	Fine bacilli formed, amorphous fuchsinophile masses	Moderate, around nucleus	Fine, ranged in a row

mann's granules, oxidase- and peroxidase reaction were all negative. Polysaccharide reaction was slightly positive with PAS staining.

D) Fat cells

In reference to this cell group, Rohr[4] furnished details. We, however, carreid out Sudan III staining. The cell of this group is identified as one filled with fat droplets stainable by Demel's supravital staining, and is located contiguous to a large fat drop. Although Rohr regarded the cells filled with boyryoidal fat droplike vacuoles of equal size, as RES cells that phagocyted fat droplets, we could not always stain these vacuoles with Demel's staining.

II. Injection of India ink and lithium carmine

Secondly we studied the distribution of India ink and lithium carmine, injected in rabbits and dogs. Our experiments with rabbits injected daily with 3 ccm of India ink for 3 days, showed that reticulum cells or histiocytes in parenchyma as well as sinus endothelium cells of bone marrow, phagocyted India ink particles. On the other hand, we observed that only sinus endothelium cells phagocyted carmine particles, in the case of rabbits injected daily with 5 ccm of 2.5% lithium carine for 5 days continuously. Hereupon we presumed that concurrent performance of these two experiments might enable us to differentiate sinus endothelium cells from reticulum cells or histiocytes in parenchyma by means of phagocyted particles. On the foregoing assumption I conducted the following experiments.

With rabbits, we injected daily 3 ccm of India ink for 3 days, succeeded by 5 ccm of 2.5% lithium carmine daily for 5 days. The result was examined 24 hours after the last injection. With dogs, we injected daily 10 ccm of India ink for 3 days, then 10 ccm of 2.5% lithium carmine for 5 days, and the result was examined as in the former case.

Our investigation was made upon sectioned and smear specimens of femur marrow of these experimental animals. The observation of these sectioned specimens by means of hematoxylin single staining shows that carmine particles are predominantly phagocyted only by sinus endothelium cells, while India ink particles are phagocyted not only by sinus endothelium cells, but by some cells in parenchyma. This enabled me to differentiate sinus endothelium cells phagocyting both carmine and rough India ink particles from reticulum cells or histiocytes phagocyting only India ink particles as dustlike or coarse granules.—Comments: In regard to the latter, we tried concurrently to stain argyrophile fibers by means of silberimpregnation method. However, I am not confident in differentiating fixed reticulum cells from free histiocytes, because discoloration of carmine is brought about by the application of Pap's method, and India ink particles tend to be confounded with silver precipitate.—It is learned by observation that sinus endothelium cells have linear cytoplasms and ovoid nuclei, and their chromatin nets are generally condense. While, cytoplasms of reticulum cells or histiocytes are somewhat irregularly swollen with India ink particles, their nuclei being larger and well rounded, the chromatin nets, in this case, are bright and rough. The cells which phagocyted both carmine and India ink,

and those which phagocyted India ink only, are in the ratio of 2.9% and 1.6% to 1 000 nucleus containing cells of boue marrow.

Giemsa staining (in this case, carmine particles were dyed blue,) and hematoxylin single staining were employed to make clear the above mentioned relations with smear and imprint specimens.

The thickly smeared portion of specimens of these two cell groups presents features such as that observed in the case of sectioned specimens. In the thinly smeared portion, familiar to us in microscopic observation, the cells presumed to be sinus endothelium cells are devoid of their characteristic slenderness and show nuclei and cytoplasms of irregular shape. In this connection, only the nuclear structure calls for debate in differentiation; but it supplies no decisive proof in the case of observation by smear specimens.

Therefore, it is difficult to draw an absolutely morphological distinction between those cells which presumably are sinus endothelium cells and what presumably are reticulum cells or histiocytes in parenchyma. Accordingly, the only distinction lies in the variety of phagocyted substances. These observations are worthy of special attention if we take into consideration that Kupffer's cells in liver have well-rounded nuclei and cytoplasms in the case of smear specimens but oval or fusiform in the case of sectioned specimens. The cells which phagocyted both carmine and India ink and those which phagocyted only India ink are in the ratio of 0.9% and 0.5% to 1 000 nucleus containing cells of bone marrow. Relative rate of the ratio of the former to the latter is similar to what was observed in the case of sectioned specimens. However, their appearance is less frequent, decreasing to 1/3.

Furthermore we observed the femur marrows of rabbits, which were injected dailly with 3 ccm of India ink for 3 days, succeeded by 5 ccm of 2.5% lithium carmine daily for 13 days, and slaughtered 24 hours after the last injection. In this case, not only sinus endothelium cells, but reticulum cells or histiocytes in parenchyma were observed to phagocyte a large amount of carmine particles, and phagocytic activity of the latter almost exceeded that of the former, as described by Onishi.[21] As sinus endothelium cells phagocyted India ink and more carmine particles than what was observed in the previous experiment, so the cytoplasm of this cell group varied from slender to somewhat swollen in form, with the nuclei changing in shape accordingly, although not so different from the typical morphologically. It happens on here that some cells ingest both India ink and lithium carmine, some India ink only and others, though small in number, only carmine in parenchyma, because the reticulum cells or histiocytes phagocyte carmine particles. The cytoplasms are extensive and swollen, their nuclei well rounded and their chromatin nets appearing bright. Some, strong in thier phagocyting activity, show pyknosis.

Under these experimental conditions therefore, we found difficulty in differentiating one cell element from another by means of smear specimen, because neither morphological observation nor phagocyted substances served our purpose.

These two experiments, however, suggested the possibility of differentiating them by phagocyted substances and according to morphologically fundamental type, if a certain experimental condition can be provided. So, we traced next

how the morphological features of cells observed in smear specimens changed in the case of sectioned specimens.

III. Injection of trypan blue

Our observation was made on femur marrows of rabbits injected daily with 10 ccm of 1.0% trypan blue solution for 4 days. On this occasion, reticulum cells or histiocytes in parenchyma, as well as sinus endothelium cells, phagocyted dye particles markedly. Presumably it is because trypan blue possessed destructive activity for endothelium, and accordingly it brings about the permeation of dye into the parenchyma without difficulty.

IV. Injection of bacilli

Injections of various bacilli were also attempted, besides the above mentioned dyes. We employed typhus vaccine, pertussis vaccine, staphlococcus, streptococcus, BCG and bacterium coli as bacilli. The results were that bacterium coli proved best for observations of phagocytic activity, BCG coming next.

V. Injection of heterogeneous erythrocytes

Human erythrocytes suspended in physiological saline solution in the ratio of 10% were employed as heterogeneous erythrocytes. Rabbits were injected with this erythrocyte suspension 10 ccm on the first day, succeeded by 20 ccm for 3 days, and the result was observed 24 hour after the last injection. No reference may here be made to the condition of erythrocytes, ingested or destroyed in phagocytic cells, as they vary with experimental conditions, and present various problems difficult to solve. The cells which phagocyted erythrocytes were observed distinctly in the sectioned specimen of seleen, but not so distinctly in bone marrow. Employing the iron staining method (Berlin blue staining method), we observed that sinus endothelium cells of bone marrow were dyed bluish, while some large cells in bone marrow parenchyma were seen to stain deep blue. The cells which proved positive with iron staining method appeared in greater numbers in the treated groups than in the untreated ones. Employing various substances, our experiments showed that the phagocytic activity of sinus endothelium cells and reticulum cells or histiocytes depend upon the experimental conditions.

VI. Frequency of appearance of phagocytic cells in smear specimen

It interested me to find out whether or not our ordinary method of bone marrow aspiration can throw a light on the entire aspect of the RES. It has been argued that bone marrow aspirated materials of animal injected with erythrocytes marked with Fe 32, are about twice as much diluted with blood. Besides this fact, we are necessarily confronted that fixed cells such as RES is to be torn off from tissue. Comparing the percentages of phagocyting cells of sectioned specimens in 1 000 nuclei containing cells with those in smear specimens of extracted bone marrow, we obtained the following results.

Table 2 has already shown that phagocyting cells in smear specimens are in the ratio of 3 to 1 of sectioned specimen. Even when we prepared smear specimens of extracted bone marrow, so much difficulty of separating from tissue was proved. Therefore, much more difficulty is to be expected when we observe smear specimens aspirated from vital bone marrow.

TABLE 2. Appearance Frequency of Phagocyting Cells in Bone Marrow

Laboratory animals	Experimental conditions	Sectioned specimen (%)	Smear specimen (%)
Albino rats	Venous injection of 2 ccm of I.i., examined 30 m. after injection	4.1	1.2
Rabbits	3 ccm of I.i. for 3 days, succeedly 5 ccm of 2.5% l.c. for 5 days	I.i. and l.c. 3.0 only I.i. 1.5	0.9 0.5
Rabbits	3 ccm of I.i. for 3 days, succeedly 5 ccm of 2.5% l.c. for 13 days	4.2	1.7
Rabbits	10 ccm of 1.0% trypan blue for 4 days	3.6	1.1

I.i.: India ink, l.c.: lithium carmine.

DISCUSSIONS AND CONCLUSIONS

In order to identify the RES cells appearing in smear specimen of bone marrow, we utilized their phagocytic activity, which, since Aschoff, has been considered to be one of their characteristic functions. But it is doubtful if this method can throw a light on all the RES cells, without exception. It has for long been discussed from various points of view, if complete blockade of RES is possible or not. Hesse,[23] performing the experiments under various conditions, described that vital staining progresses continuously if favorably furnished with factors such as quantity of vital staining dyes injected, duration of injection and regeneration of RES cells. Recently, Onishi,[21] using lithium carmine, has made experiments on this problem, too. A far as our experiments could show, RES cells proved progressive in phagocyting the vital staining dyes we have identified, and so it is probable that we have identified only some, not all, the RES cells. In brief, it may conveniently be concluded that we have made a morphological study of what are called "macrophages" of Rohr in his phenomenalistic classification.

It is duly conceivable that RES cells are liable to be stimulated or degenerated to some degree by phagocyting foreign substances; therefore it may safely be assumed that RES cells were observed at various functional phases in our experiments. For example, most of these cell which phagocyted India ink particles in a large quantity, contain pycnotic nuclei, and there appear a few, or none, with Janus green granules. Therefore, by observing the morphological aspect of RES cells which phagocyted India ink particles in small quantity on the one hand, and studying the morphology of RES cells showing no visible phagocytic activity, on the other hand,—they are considered identical with Rohr's

"lymphoid reticulum cells"—we attempted to give them unity by throwing light on the cytological characteristics applicable to all the RES cells with or without phagocyting activity. It is, however, naturally conceivable that even the lymphoid reticulum cells phagocyte various substances, to various degrees, though invisible at this stage of optical devise. Various cytological studies on lymphoid reticulum cells have been carried out though individually, by many in our clinic. Ito[20] described that the Golgi apparatus of this cell group showed a complex form, as a large mesh work or "Golgi-rest" from in the case of smear specimens of human bone marrow. Uetani,[18] employing the same materials, stained mitochondria by Kunii's method, and described that there were seen beside the nuclei of these cells, Altmann's granules which were rod like or granular. According to Haga[24] who employed supravital staining with 0.5%–1.0% brilliant cresyl blue and Heidenhein's iron hematoxylin staining, the nucleoli of this cell group, around which dense pigmentation were perceived, are of middle size, and sometimes contain, something like partitions. Hence, they present a different aspect from other cell elements of in hematopoietic organs.

These cytological characteristics were rarely missed in the case of strongly phagocyted RES cells, but, "macrophages" also generally showed the above-mentioned cytological signs as far as the materials examined by us were concerned. The following are relatively marked features of RES cells so far obtained in our cytological and cytochemical studies, setting apart the views represented by Giemsa staining and Janus green-neutral red supravital staining etc., which have been described by our seniors. Namely, the peculiar nuclear structure and nucleolar feature, observed under the phase microscope, showed dark green cytoplasm by Papanicolaou's staining and polysaccharides masses in cytoplasm by PAS staining. We believe that these features are considerably effective in differentiating RES cells from other cell elements, as far as the bone marrow is concerned.

The problem of monocytes, which, like RES cells, exhibit phagocytic activity is most puzzling when we try to differentiate them from RES cells. Kojima[25,26] studied this subject in detail. From strictly morphological points of view, we discerned the difference between RES cells and blood monocytes, as is shown in table 1. Besides the pictures presented by Giemsa staining, oxidase or peroxidase reaction and Janus green-neutral red supravital staining, we wish to reckon the following as features of blood monocytes, namely, negativity of polysaccharide in cytoplasm demonstrated by PAS staining, small nucleolus and Demel's granules finer than those of RES cells.

Secondly we will refer to RES cells in bone marrow from the standpoint of their location. Since Ashoff and Kiyono, the concept of "RES" has oftener been as a functional unit than a cell group of morphological similarity, Siegmund,[27] who assumed that sinus endothelium cells were nothing but reticulum cells flattened and brought into contact with the blood stream, demonstrated their identity, the former being called "Uferzellen" by him. In recent years, however, Akazaki[6] and his pupils,[21,25,28,29] attaching importance to argyrophile fibers, advanced a new theory that reticulum cells are, morphologically as well as genetically, different from sinus endothelium cells in type.

From this standpoint, the question arises—are "macrophages" derived from reticulum cells or from sinus endothelium cells? Tischendorff and Franck,[5] who conducted animal experiment with India ink, described that endothelium cells comprised a large percentage of India ink/phagocyting cells, and histiocytic cells comprised the rest. Amano[30] also presumed that so-called Rohr's "macrophages" were derived from sinus endothelium cells.

According to our experiment, for instance, when rabbits or albino rats recieved injections of a small amount of India ink into the blood circulation, sinus endothelium cells predominate in the India ink phagocyting cells in bone marrow. However, in the course of time, reticulum cells or histiocytes in parenchyma come to show considerable phagocytic activity. Furthermore, when injections of longer duration are carried out, reticulum cells or histiocytes, as well as sinus endothelium cells, are observed to phagocyte India ink particles. This fact is applicable to the case of vital staining with lithium carmine. As Onishi[21] has described, in the case of rabbits daily injected with 5 ccm of 2.5% lithium carmine continuously for about 5 days, sinus endothelium cells predominantly show phagocytic activity. As the injection is continued, however, reticulum cells or histiocytes increasingly show phagocytic activity, finally dominating the sinus endothelium cells. Thus, it may justly be argued that sinus endothelium cells, as well as reticulum cells or histiocytes, exhibit phagocytic activity and it depends on the experimental conditions whether the former or the latter exhibit superiority in phagocytic activity.

Furthermore, employing lithium carmine and India ink, we were able to differentiate sinus endothelium cells from reticulum cells by the substances phagocyted by them. Our success, however, should be ascribed to a model experiment. That is to say, we could only demonstrate the possibility of determining the origin of "macrophages" as a phenomenen, and that, under a certain experimental condition.

Now, let us observe those cells which phagocyted pigments. We obtained them from the pathological bone marrow of a human autopsy. Hagino[29] observed, in the case of sepsis, malaria and Gaucher's disease, that sinus endothelium cells scarcely phagocyted vital pigments, while reticulum cells in medullary cord remarkably phagocyte the pigments. He toiled at how to interpret these phenomena. We could observe with sectioned specimens of bone marrow in the case of leucomoid reaction, that reticulum cells remarkably phagocyted the pigments, while, in the case of myeloma accompanied by hemosiderosis, that sinus ondothelium cells predoninantly phagocyted the iron-reaction-positive substances. According to these facts, we can not hastily determine from what are derived "macrophages" appearing in smear specimens of bone marrow, reticulum cells or sinus endothelium cells.

In this connection, we felt difficulty in drawing a distinct line between sinus endothelium cells and reticulum cells in their functional aspects which were represented by morphologically visible phagocytic activity. If that be the case, we must proceed to the next problem as to whether or not these cell elements can be differentiated from each other from a purely morphological standpoint.

Rohr, stating the difficulty in differentiating them, described only that the

cytoplasm of sinus endothelium cell is usually longer than that of reticulum cell. He, consequently, did not reckon "sinus endothelium cells (or reticuloendothelium cells)" when he classified RES cells in his myelogram. As mentioned above, Akazaki's pupils—Kojima,[25] Omori[28] and others[29]—brought forward the new doctrine that these two cell elements have respectively different characteristics in their genetical and morphological aspects, concluding their differences in origin. According to them the following are the factors in differentitaing those two cell groups—namely, picture obtained by Giemsa staining, quantity and size of neutral red granules, scattering modes of Janus green granules and some features of Colgi apparatus etc.

By means of Giemsa staining and single staining with hematoxylin, we investigated the morphological aspect of these two cell elements appearing in smear specimens of bone marrow; and we could, as a rule, differentiate both cell elements according to their phagocyted substances, employing lithium carmine and India ink. The following features were obtained as results, that is, most of the sinus endothelium cells have long-oval cytoplasm and nucleus, their nuclear structure is usually dense and their nucleoli are rather small in size.

The cells considered as reticulum cells or histiocytes showed cytoplasm of irregular form, the nucleoli being rounder, the chromatin net bright and nucleoli usually larger. These morphological differences, however, are not so distinct, and we felt considerable difficulty in differntiating these two cell elements in the case of practical observation of bone marrow smear specimen, because of the morphological affinity between them. It is presumably ascribed to the fact that fixed cells such as RES cells tend to grow round by a mechanical treatment such as preparing of smear specimen.

Thirdly, we will refer to the endothelium cells proper. According to Hagino,[29] the sinus endothelium cells are, genetically, closely related to endothelium cells proper; while according to Bargmann's[31] and Hashimoto's[32] description, the sinus endothelium cells are located contiguous to endothelium cells proper and distinguishable merely by their vigorous phagocytic activity. Therefore, it is of some significance to describe cytological characteristics of endothelium cells proper in correlation with those of sinus endothelium cells. It is interesting to discern some cytological similarity between the cytological features of the former, indicated by us, and those of the latter indicated by Akazaki's pupils.

SUMMARY

I) On experimental basis, we, described the cytological and histochemical features of RES cells in bone marrow, identified by their phagocytic activity, and by so doing, we made some contributions to the differentiation of this cell group from other cell elements in smear specimens of bone marrow.

2) We, simultaneously, demonstrated the cytological features of blood monocytes and endothelium cells proper, which one might confuse with those of RES cells.

3) In the case of bone marrow of rabbits, injected with India ink and lithium carmine, we demonstrated a way to differentiate sinus endothelium cells from reticulum cells by their phagocyting modes, and referred to the cytological

features of these two cell groups by means of smear specimen in this case.

4) The appearance frequency of phagocytic cells in bone marrow decreased in smear specimens when compared with sectioned specimens. This fact enables us to presume the difficulty of separating this cell group from its tissue.

REFERENCES

1) ASCHOFF, L. *Erg. inn. Med. Kinderh.* **26**: 1, 1924.
2) KIYONO, K. *A Study on vital Staining.* Tokyo: Nankodo, 1920 (Japanese).
3) JAFFE, R. H. *Downey's Hb. of Hematology,* **II**: 973. Hoeber: New york, 1938.
4) ROHR, K. *Das menschlich Knochenmark.* Leipzig: Thieme, 1940; Stuttgart: Thieme, 1949.
5) TISCHENDORFF, W., U. A. FRANK. *Dtsch. Arch. klin. Med.* **186**: 272, 1940.
6) AKAZAKI, K. *Nippon Rinsho* **4**: 340, 1944. (Japanese); *Saishin Igaku* **7**: 406, 1952 (Japanese).
7) TANABE, T. *Acta haemat. jap.* **2**: 309, 1938 (Japanese).
8) MORITA, H. *Saishin Igaku* **3**: 24, 1948 (Japanese).
9) FORKNER, C. E. *J. exp. Med.* **50**: 121, 1929.
10) BRACHET, J. *Ann. N. Y. Acad. Sci.* **50**: 815, 1950.
11) HOTCHKISS, R. O. *Arch. Biochem.* **16**: 131, 1948.
12) HIBINO, S., A. YASUMA AND S. ONO. *Saishin Igaku* **5**: 44, 1950 (Japanese).
13) YASUMA, A., S. ONO AND K. YAIDA. *Nagoya J. med. Sci.* **14**: 1, 1951.
14) YASUMA, A. AND T. ICHIKAWA. *Medicine and Biology* **19**: 197, 1951 (Japanese) and *J. Lab. clin. Med.* **41**: 296, 1953.
15) GOMORI, G. *Proc. Soc. exp. Med. Biol.* **42**: 23, 1939.
16) HIRAMATSU, K. *Acta haemat. jap.* **14**: 355, 1951 (Japanese).
17) ONO, S. AND A. ERI. *Gendai Igaku* **1**: 38, 1951 (Japanese).
18) UETANI, T. *Nagoya J. med. Sci.* **12**: 117, 1938.
19) KUNII, S. *Rinshobyori Ketsuekigaku Z.* **4**: 1063, 1935 (Japanese).
20) ITO, S. *Acta haemat jap.* **6**: 351, 1942 (Japanese).
21) ONISHI, N. *Acta haemat. jap.* **15**: 365, 1952 (Japanese).
22) FADEM, R. S. AND I. BERLIN. *Blood* **6**: 760, 1951.
23) HESSE, I. *Ziegler's Beitr.* **93**: 36, 1934.
24) HAGA, K. *Symposia of hematology,* **II**: 95. Osaka: Nagai, 1949 (Japanese).
25) KOJIMA, Z. *Acta haemat. jap.* **13**: 10, 1950; **14**: 153, 1951 (Japanese).
26) KOJIMA, Z. AND N. ONISHI. *Acta haemat. jap.* **15**: 399, 1952 (Japanese).
27) SIEGMUND, H. *München med. Wshr.* **70**: 5, 1923.
28) OMORI, Y. *Soc. Pathol. Jap.* **38**: 34, 1949; *Acta haemat. jap.* **15**: 127, 1952 (Japanese).
29) HGINO, Y. *Niigata Igakukai Z.* **63**: 287, 1949 (Japanese).
30) AMANO, S. *The foundation of hematology,* **1**: 282. Tokyo: Maruzen, 1948 (Japanese).
31) BARGMANN, W. *Z. Zellforsch.* **11**: 1, 1930.
32) HASHIMOTO, M. *Fukuoka Igakukai Z.* **29**: 2450 1936 (Japanese).

EXPLANATION OF FIGURES

FIG. 1. Bone marrow of the rabbit, injected with India ink and lithium carmine. Hematoxylin staining.
FIG. 2. Various types of phagocytic cells, obtained by smear specimen of fig. 1. Hematoxylin staining.
FIG. 3. Phagocytic cell ingesting India ink particles. Giemsa staining.
FIG. 4. PAS-positive masses in phagocytic cell. Hotchkiss's PAS staining.
FIG. 5. Endothelium cells proper. Giemsa staining.
FIG. 6. Fat cell. Giemsa staining.

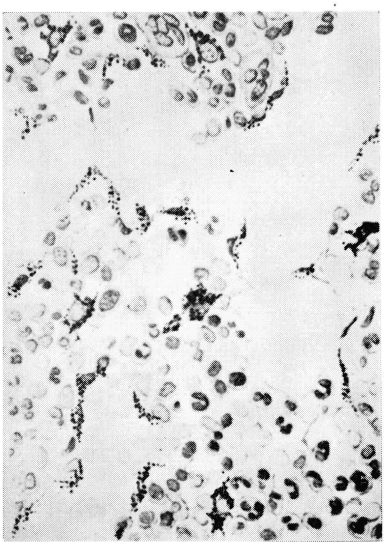

FIG. 1

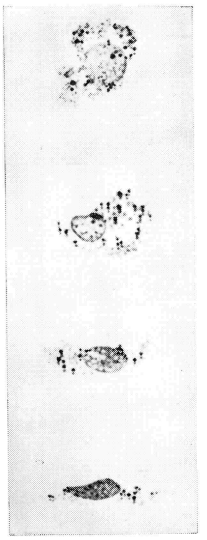

FIG. 2

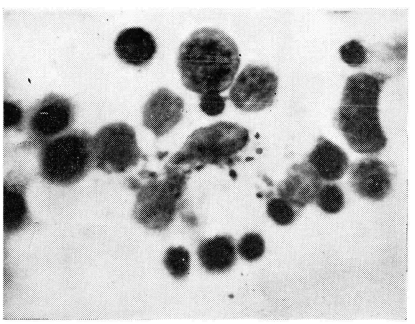

FIG. 3

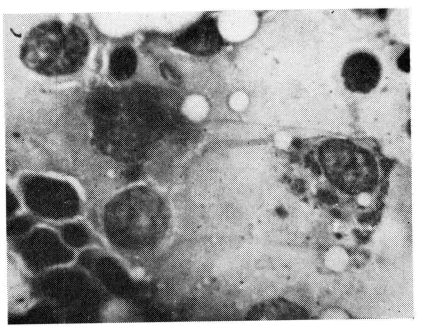

FIG. 4

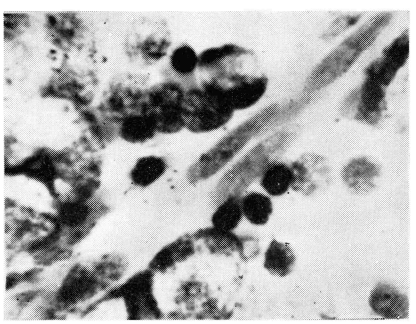

FIG. 5

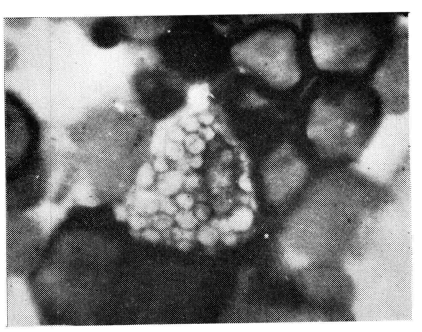

FIG. 6