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Abstract

(1) The movement of the blood cells in the bone marrow was classified into 9 types. (2) The characteristics of moving types are so distinct according to the kinds of blood cells, that the differential diagnosis of the cells by moving types is easily and certainly made. In this way, (by the kind of blood cells), we have classified leukemia, as is described in our other articles. (3) The phagocytosis and vital staining of the blood cells in the bone marrow is different in degree and mode, according to the kind of blood cells, and thus becomes valuable ground for the differential diagnosis of the cells.

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OBSERVATIONS OF VARIOUS LIVING BLOOD CELLS BY TISSUE CULTURE OF THE BONE MARROW

By

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The studying methods of hematology by means of smear staining preparations such as Giemsa- or May-Giemsa-stain, which stained dead blood cells, were simple to be practiced and should not be neglected for the fundamental subjects of hematology. But in the respect of the classification of pathological immature blood cells, it could not be expected for its progress to exceed the limitation unless any other excellent staining methods were devised. In this time, we performed the tissue culture of the bone marrow of human beings and rabbits, and observed the movement, phagocytosis and vital staining of living marrow cells under the microscope.

At the same time, by microcinematography we studied the characteristics of various living blood cells on the screen.

Method: refer to the previous article

Observation;

A. Movement

1. Classification of the cycles in cellular movement

In our classification we have the following 6 stages (fig. 1):

a. Preparatory stage: This stage is immediately prior to the formation of the pseudopodium, that is, at the time of the acceleration of the molecular movement in the local granules when the stream of granules starts.

b. Stage of the pseudopodium formation: Varius pseudopodia start forming (explained in the following pages), and the granules follow after the pseudopodia, which enlarges gradually, until the movement of the nucleus starts.

c. Migratory stage of the main body (migratory stage of

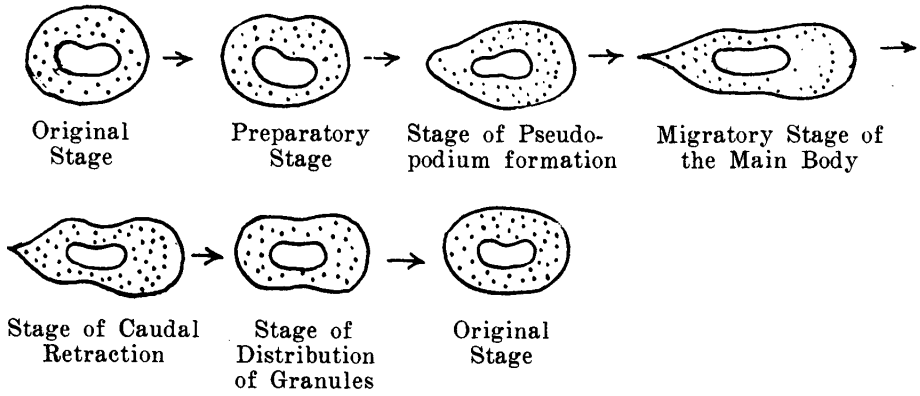

Fig. 1. Classification of Cycles in Cellular Movement.

the nucleus): When the enlargement of the pseudopodia reaches the maximum, the nucleus starts migration and the granules behind the nucleus start to move. This is the stage where the extension of the body reaches its maximum. The swelling of the caudal part appears and sometimes caudal thread (Pseudo-fragella) is observed

d. Stage of caudal retraction: When the progress of the pseudopodia stops, the anterior and then the posterior part of body also stops. Then the caudal part retracts, as if it is sucked into the body.

e. Stage of distribution of the granules: Distribution of the granules is observed to all parts of the immobile and round cell body.

f. Original stage (immobile stage): Pseudopodium completely disappears, the cell body becomes round-shaped, and no more granules flow.

All of these six stages were observed typically in type A and B (explained in the following pages), but in other types, some shortening of the cycle was seen at times.

2. *Classification of types in cellular movement*

Philipsborn[4, 5, 6] first classified the cellular movement of human leukocytes of the peripheral blood into 5 types. But no classification for the leukocytes of the bone marrow has been made. We have made the classification for the leukocytes of the bone marrow according to the tissue culture findings, i. e. the pseudopodia into 5 types and the types of cellular movement into nine— (A-I).

Classification of pseudopodia: (fig. 2)

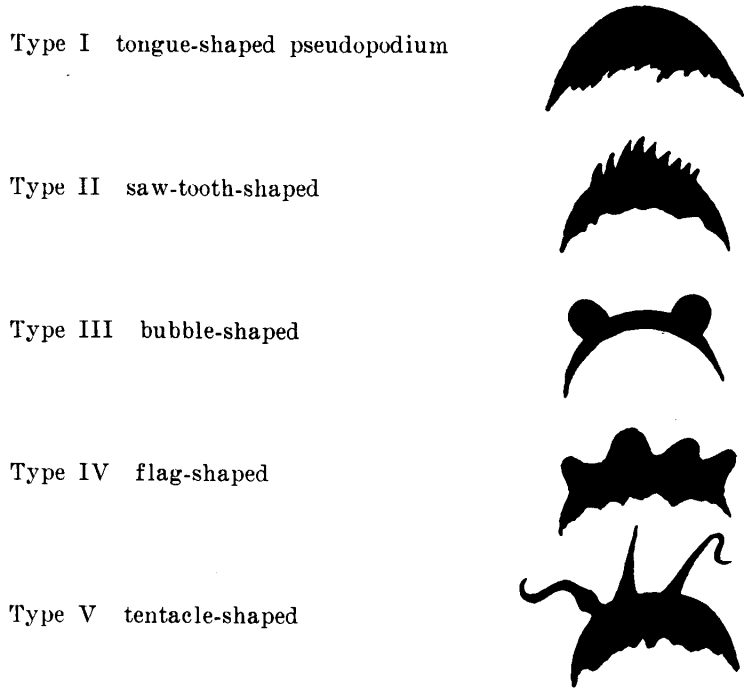

Fig. 2. Classification of Pseudopodium

Type I. The broad and thick tongue-shaped pseudopodium (tongue-shaped pseudopodium).

Type II. The broad and saw-toothed pseudopodia at the anterior margin (saw-tooth-shaped pseudopodia).

Type III. One to several round-shaped projection-like pseudopodia appear simultaneously (bubble-shaped pseudopodia).

Type IV. Thin membraneous pseudopodium with a slow waving movement like a flag (flag-shaped pseudopodium).

Type V. Tentacle with a sharp tip (tentacle-shaped pseudopodium).

Types I and II could be observed by bright field microscopy. Types II, IV and V, however, could be more easily observed by phase contrast microscopy. Sometimes they could not be seen by bright field microscopy.

The activity of the wandering was the best in type I and gradually decreased in the order of II, III, IV and V. Types I and II were seen in the most active stage of the neutrophils,

pseudoeosinophils, eosinophils and lymphocytes, and types III and V in a little weaker stage of activity than they. Type IV was mostly seen in the monocytes and basophils.

Type of cellular movement (fig. 3):

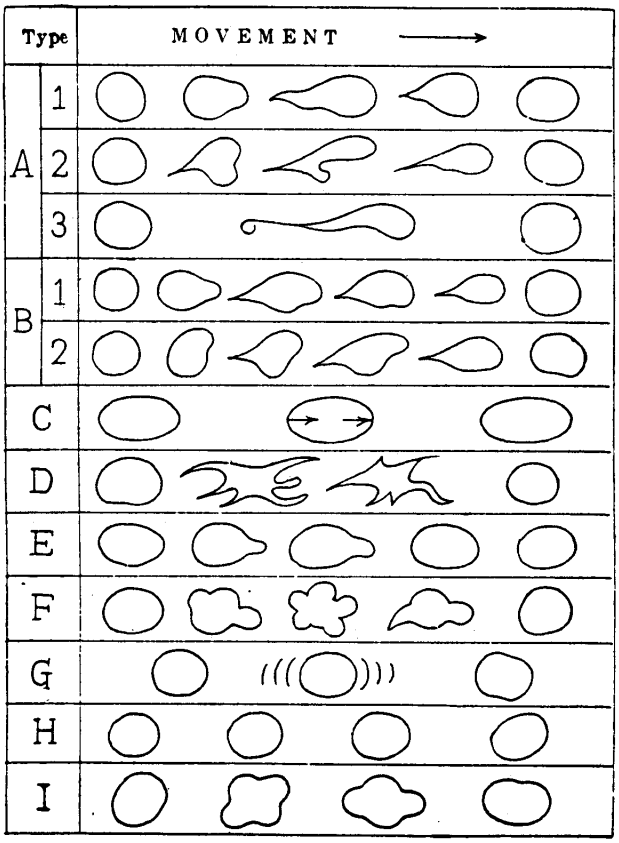

Fig. 3. Classification of Type of Cellular Movement

Type A.

During the migratory stage, when a tongue-shaped or saw-tooth-shaped pseudopodia stretched out very rapidly, the long diameter becomes more than three times the length of the short diameter. The characteristics were; 1. Distinct stages of movement; 2. A stream of granules and protoplasma, and the easily seen migration of the nucleus; 3. Easily distinguishable caudal part; 4. A great wandering distance of the cell body during one cycle; 5. The highest wandering velocity.

This type was observed in the initial stage, when the move-

ment was most active, especially in the neutrophils, pseudoeosinophils, eosinophils and lymphocytes.

Among these, the movement which only has a singular pseudopodium is called type A-I. The movement that has more than two pseudopodia at the same time or that has branched pseudopodium is called type A-II.

In type A-II the cell body proceeds in the direction of the main pseudopodium, and then the protoplasm and granules of the accessory pseudopodium stop moving and return to the cell body.

Even if there are no obstacles such as erythrocytes or foreign bodies, the formation of the branches still occurs. When the adhesion of the caudal part is so strong that it is extremely extended and reaches to a distance of more than two times the diameter of the cell, we call it type A-III. In type A-III the wandering velocity decreases because of its adhesion. The flow of the granules can be seen in the extended caudal part, which is rapidly withdrawn into the cell body when the adhesion is released.

Type B.

This type has a similar, but slightly smaller pseudopodium than type A and the duration of the migratory stage of the main body is shorter than that of type A, so that the long diameter is less than three times the length of the short diameter. This type is most commonly seen in the initial stage and in the neutrophils, pseudoeosinophils, eosinophils and lymphocytes, and is classified into types B-I and B-II. The wandering velocity is ranked next to type A.

Type C.

The migratory stage of the main body, and the stage of caudal retraction come at the same time and never return to the original stage. Therefore, each stage of the cycle is not clearly distinguishable, and is very peculiar, because neither extension nor retraction of the body is observed during the wandering. This type is frequently seen in the lymphocytes and basophils, and sometimes in the neutrophils, etc.

Type D.

One to several flag- and tentacle-shaped pseudopodia and projections (when the cell body does not move in its direction, it

is called a projection) are stretched out and withdrawn in all directions simultaneously. The cell remains in the original place and the pseudopodia show the flag- and tentacle-like movement. Later, one pseudopodium becomes hypertrophic, and when cytoplasm flows into it and moves forward, the other pseudopodia retract. Thus the wandering velocity of the cell body is very slow compared with it's marked deformity, and therefore, the efficiency of the movement is quite low. This type is characteristically seen in the monocytes, neutrophils and others with decreased activity of movement. The flag-shaped pseudopodium, however, is seen only in the monocytes.

Type E.

Smaller tongue- and saw-tooth-shaped pseudopodia than those of type B are stretched out slowly, and the wandering of the main body and the caudal retraction is also slow. In comparison with other stages, it is characterized by the fact that the time in which it is in the original form is longer, and interrupted pseudopodium formation is also more frequent. This type is seen in some of the myelocytes (neutrophilic, pseudoeosinophilic, and eosinophilic) and in the metamyelocytes—also with diminished activity in the neutrophils, pseudoeosinophils, eosinophils and lymphocytes.

Type F.

One to several bubble-shaped pseudopodia and projections are stretched out and retracted from all parts of the cell body, which moves in the direction of one of them. At that time, the other pseudopodia retracts. This type is seen in diminished activity in the neutrophils, pseudoneutrophils and eosinophils. It is also frequently seen in the monoblasts, which only stretch out and withdraw bubble-shaped projections reciprocally from any side of the body, but do not move. Furthermore, the bubble-shaped pseudopodia sometimes stretch out a bubble-shaped daughter pseudopodium and the cell body moves in its drection.

Type G.

The cell bodies movement is a sort of trembling movement, like Brownian one. No formation of pseudopodium can be observed. The movement is visible under the microscope, but better observed by moving picture photography. This type is observed mostly in monocytes.

Type H.

A rotating movement around the center of the cell body is observed without any formation of pseudopodium. There are two types of movement. One is with a rotation of 360° and the other is with a more limited rotation (90°—180°) to the right or left. This type is seen in lymphoblasts, myeloblasts, promyelocytes and partially in the myelocytes.

Type I.

The deformation of the cell body usually takes about a minute, but sometimes lasts for several minutes. There is no pseudopodium formation. Even though the deformation is too slight to be observed by bright field microscopy, it can be observed very well by photography. This type appears in lymphoblasts, myeloblasts, promyelocytes and myelocytes, and even in other mature cells with extremely diminished activity.

In the various types of movement mentioned above, is repeated for a while, but after a long time, it is changed into another type. Especially, in the stage of accelerated movement, types A, B and C are most frequently seen. They are, however, changed into type D, etc. in case of decreased movement. Type A or B, in the stages of accelerated movement, may also be changed alternately into the other.

The migration velocity decreased in the order of A, B, C, D, E and F.

3. Motility of various kinds of bone marrow cells

a. Myelogenous cells

(1) Myeloblasts: Concerning the motility of these cells, *Grossmann*[2] observed a little deformaton in the bone marrow culture of guinea pigs, but only Rich et al. by moving pictures of the tissue cultures of human and rabbit bone marrow and leukemic peripheral blood, described a movement of the pseudopodium in torsion-like movement. In the tissue culture we never found the formation of a pseudopodium, but a slight deformation (Type I) and rotating movement (Type H).

(2) Promyelocytes: Concerning the motility of these cells, there is a little deformation, as is generally known. We observed occasionally the movement of H and I types of these cells and sometimes intracellular dislocation of the nuclei, but no distinct migration. In the protoplasm, a few moderate sized granules,

looking like weak, brilliant, black spots (characteristic of the neutrophils) were seen in molecular movement.

(3) Myelocytes: The motility of these cells is as yet not generally accepted. In guinea pig bone marrow culture, *Grossmann*[2] has observed an ameboid movement. We observed not only the movement of H and I types, but of E type (location was changed by means of small pseudopodia), however, without any migration. The protoplasm was filled with peculiar granules, which had active molecular movement and some of them were observed to flow. The protoplasm looked solid. Their nuclei were distinct and were moving in the cytoplasm, but did not show any deformation. The nuclei remained at the posterior part of the cytoplasm in the movement of type E.

(4) Metamyelocytes: The migration of these cells is commonly known. We found a slight pseudopodic movement in the myelocytes, but the movement of these cells was distinctly B and E types. The protoplasm looked soft and the flowing of the granules was distinct. The molecular movement of the granules seemed to have less activity than that of the myelocytes. The nucleus was kidney-shaped, rather indistinct, and during migration it appeared in the center or in the posterior part of the body. The deformation of the nucleus was scarcely noticeable.

(5) Neutrophils (fig. 4-a, b & c): They moved most actively in A and B types, but a weaker movement was seen in D, E and F types and a much weaker movement in H and I types. The granules in the protoplasm were black spots of moderate size, having the above mentioned peculiar weak brilliancy. The flowing was the most active of all, and we also found molecular movement. The protoplasm looked softer than that of the metamyelocytes and was deformed in various ways. The streaming was active. The nuclei which were not distinctly visible (particularly in movement), easily changed their location. They were located in the center of the posterior part during the movement of the protoplasm. The deformation of the nuclei was not as distinct as that of the lymphocytes and monocytes. The pseudoeosinophilic promyelocytes, myelocytes and metamyelocytes were different from those of the neutrophilic cells in that, that they had very brilliant and rather large granules, but in other respects they were quite similar to the latter. We found not

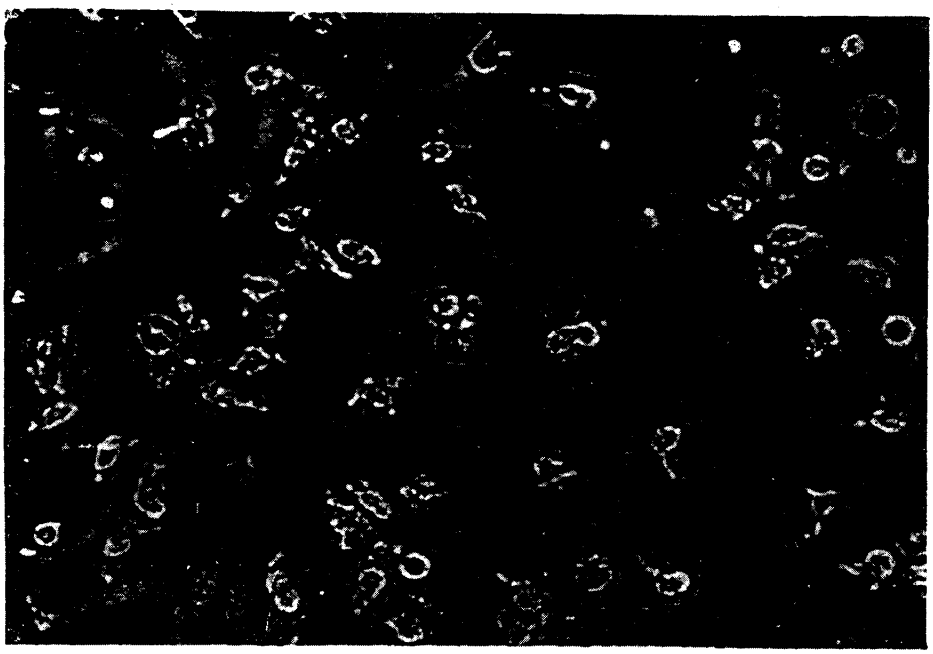

Fig. 4 — a. Neutrophils. Bright field. 600×.

Fig. 4 — b. Neutrophils. Bright field. 1000×.

only a similar difference between the mature pseudoeosinophils and neutrophils, but also a little more rapid wandering velocity in the former which was the greatest in all the cells of the bone marrow.

(6) Eosinophils (fig. 5-a & b): Next to the neutrophils,

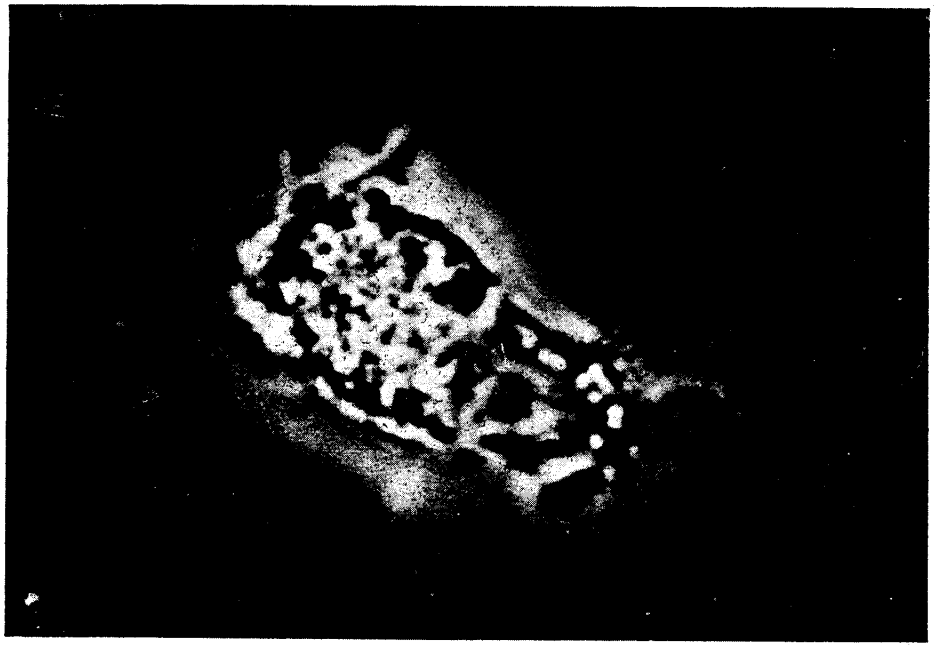

Fig. 4 — c. Neutrophil. Phase contrast. 1500×.

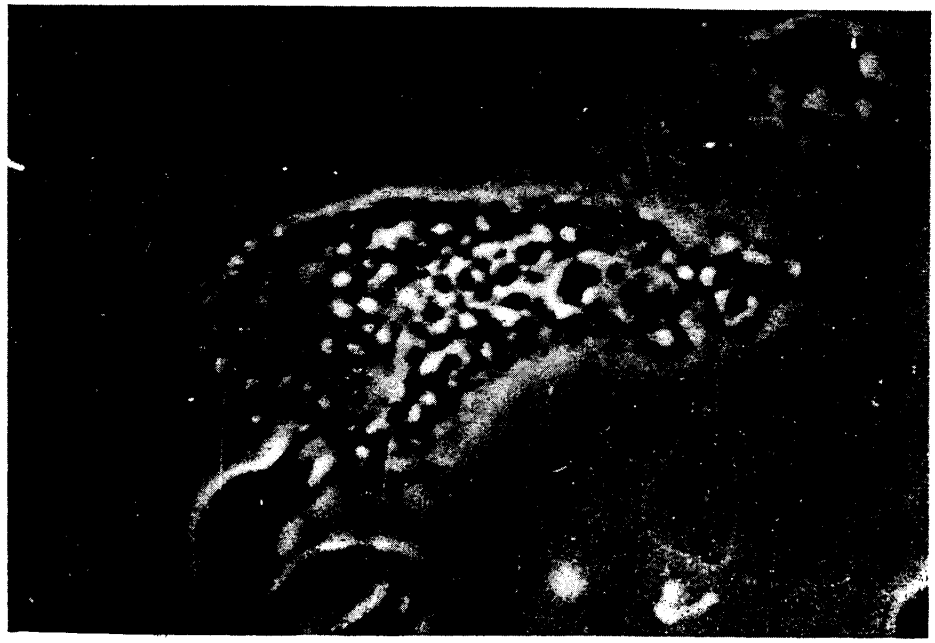

Fig. 5 — a. Eosinophil. Phase contrast. 1500×.

Fig. 5 — b. Eosinophil. Phase contrast. 1500×.

these migrated most actively. The movement belonged to types A and B, but showed D, E and F types during decreased activity. In the movement of types A and B, the deformation of the protoplasm was slight, compared with the neutrophils. These granules were larger and more brilliant than the granules of other bone marrow cells. The granules were round, ellipsoidal and oval, but during active migration, they showed mostly an ellipsoidal form. The molecular movement of the granules was also seen. The nuclei were more easily visible than those of the neutrophils and were located in the center or posterior part during movement. They were not easily deformed.

(7) Basophils (fig. 6., and fig. 7): They moved mainly in types B and C, and stretched out the characteristic tentacle-shaped and flag-shaped pseudopodia like the monocytes. Different from the monocytes that stretched out pseudopodia from any side of the cell body, these cells only stretched them out from the anterior part. The wandering velocity was next to that of the eosinophils. Their protoplasm looked thin and wide and contained many fine granules, but without any brilliancy. The peculiar granules in these cells, at staining, were only visible by a phase contrast microscope. In May-Giemsa staining, these cells resembled the neutrophils more than they did the monocytes, but in cultivation, the above mentioned motility, as well as the

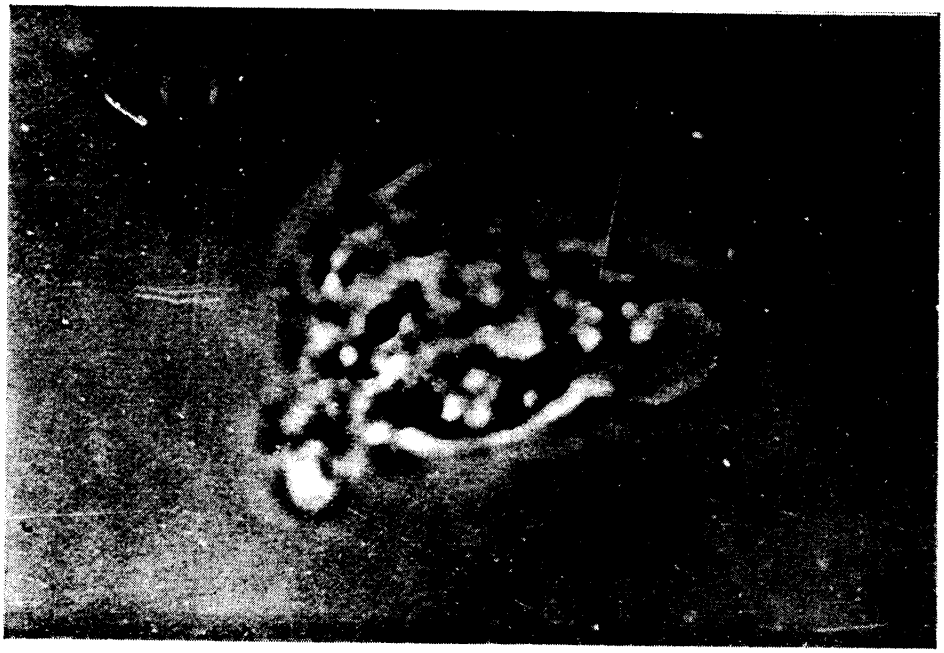

Fig. 6. Basophil. Phase contrast. 1500×.

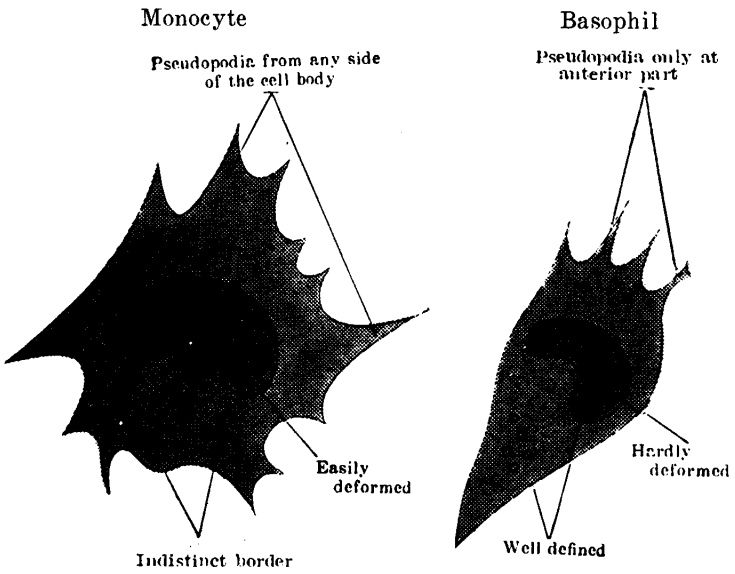

Fig. 7. Bone Marrow Culture

findings concerning the protoplasm and granules, resembled the monocytes more. This was very significant. The nuclei were a little more easily found, and had mostly two lobes like those in

the eosinophils. They proceeded to the anterior part of the body during the movement. The deformation of the nucleus was rarely seen. The basophils were different from the monocytes in that the nucleus of the former looked more solid, better defined, and was harder to be deformed than the latter, which stretched out tentacle- and flag-shaped pseudopodia from any side of the cell body.

b. Lymphogenous cells

(1) Lymphoblasts: As to the motility of these cells, only Rich et al. observed this in his moving picture study of the cultivation of rabbit lymphnodes and of the peripheral blood of lymphatic leukemia. He described it thus: These cells stretched out small pseudopodia from their anterior edge and moved in the form of a hand mirror, having a small pseudopodium from the anterior margin and a cauda from the posterior margin. From our observation, they sometimes moved in H and I types, but pseudopodic movement was never seen. These cells were a little smaller than the monoblasts and myeloblasts. The protoplasm was narrow and transparent, and many of them contained no granules. Others, however, showed a few fine non-brilliant granules around the nucleus. The nuclei looked almost round, sometimes irregularly formed, but the deformation was slight and slow. Though the nucleus could be easily seen, the nucleic substance was thinner than that of the myeloblast.

(2) Lymphocytes (fig. 8): They moved in A, B, C and D types, and their velocity was next to that of the basophils.

As they contained no specific granules, their movement was very gentle and soft. Therefore, the flowing of the protoplasm was indistinct. The protoplasm contained no specific granules, so it was transparent. Sometimes, however, we found fine nonbrilliant granules around the nucleus. The protoplasm was generally narrow and its contour was more distinct than that of the monocyte.

The nuclei were round, ellipsoidal or kidney-shaped and quite visible. They proceeded to the anterior part and were easily deformed during movement.

C. Monocytic cells:

(1) Monoblasts. Their movement was in F, G, H and I

Fig. 8. Lymphocyte. Phase contrast. 1500×.

types. The movement was more active than that of myeloblasts and lymphoblasts and showed the characteristic F or G type. The protoplasm contained no specific granules and was a little wider than those of other blastcells. The nucleus had a tendency to show an indention, looked soft, and its contour was a little more indistinct than that of the myeloblast. There were several nucleoli, but less than that of myeloblast. The size, however, was larger.

(2) Promonocytes: Migration was seen. They moved in E type and sometimes also in F type. The protoplasm contained non-brilliant fine granules and was cloudy, but its contour was indistinct. The nuclei were rather hard to be seen and indented, and looked softer than those of the promyelocytes.

(3) Monocytes (fig. 9-a-b): Their movement was characteristically type D, and different from the basophils, they stretched out flag- and tentacle-shaped pseudopodia from any side of the cell body. This movement was like that of a wiggling octopus, so we called it an octopus-like movement. It's efficiency was therfore very low. These cells were rather large; the protoplasm was thin and broad; and its contour was indistinct.

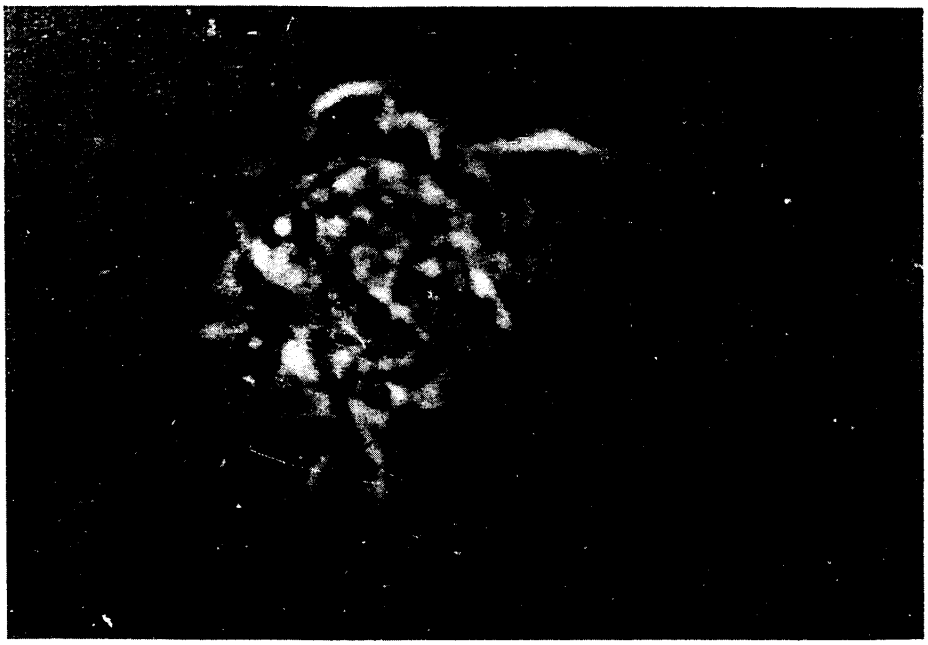

Fig. 9 — a. Monocyte. Phase contrast. 1500×.

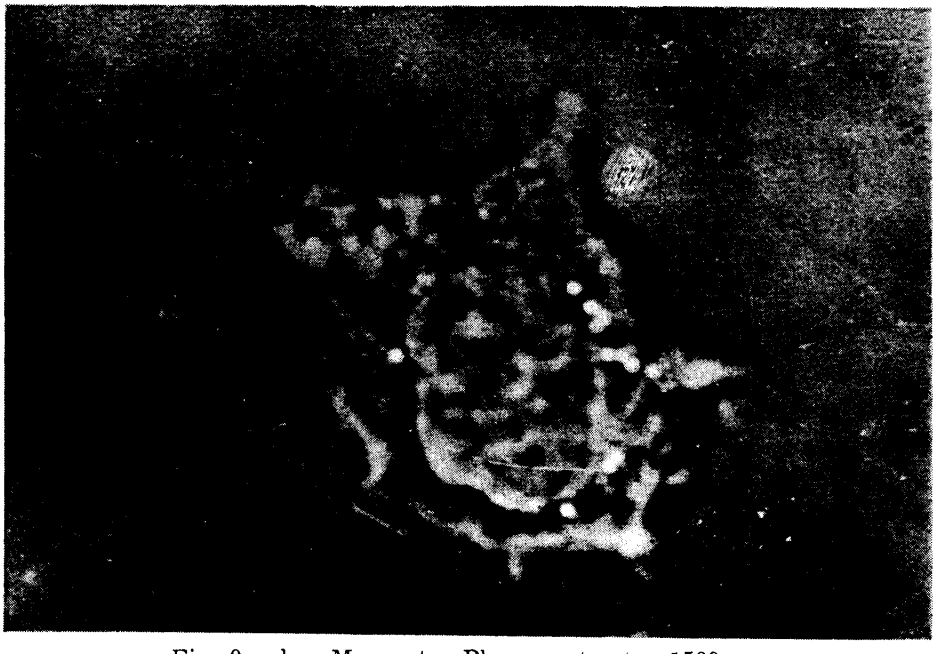

Fig. 9 — b. Monocyte. Phase contrast. 1500×.

In the protoplasm, many fine nonbrilliant granules appeared, and at a glance looked cloudy. After 4—5 hours

culture, a few very brilliant large granules appeared. These granules flowed through the same route, but sometimes in the reverse direction of the movement of the cells. The nuclei were mostly kidney-shaped or polymorphous, rather hard to be seen, and looked thin. They occupied mostly the center and sometimes the proceeding part during movement. Characteristically, they were continuously deformed.

d. Megakaryocytes

We observed a complicated pseudopodic movement in these cells, but as these details have been described in other articles, they are omitted here.

4. The wandering velocity of various myelogenous cells

There are many works, including *Philipsborn*'s[4,5,6], on the velocity of the peripheral leukocytes, but no one has as yet measured the wandering velocity of the leucocytes in normal bone marrow.

a. Comparison of the wandering velocity of various cells (at maximum velocity): The order was as follows: pseudoeosinophils 27.55 μ/m, neutrophils 26.31 μ/m, eosinophils 22.36 μ/m, basophils 18.45 μ/m, lymphocytes 17.63 μ/m, monocytes 15.78 μ/m and megakaryocytes 3.30 μ/m. In comparing these with the velocity rate of the cells in the peripheral blood, the pseudoeosinophils, neutrophils and eosinophils were found to be approximately similar, but the lymphocytes and monocytes had a far faster rate—particularly the monocytes (four times faster). The above mentioned difference seemed to be due more to the differnce in the method of observation (supravital observation and observation by culture) than to any real difference between the peripheral blood cells and the bone marrow cells.

b. The maximum migratory stage of each cell: In pseudoeosinophils and neutrophils, was 1—5 hours after the cultivation, in eosinophils, 4—12 hours, in lymphocytes, 5—13 hours, and in monocytes, 6—24 hours.

B. Phagocytosis of carbon particles

1. Human bone marrow cells

No phagocytosis was found in myeloblasts and promyelocytes. In the myelocytes, it appeared rarely (±), in the meta-

myelocytes (±)~(+), and in the mature neutrophils, there was marked phagocytosis. Thus a close relationship was found between phagocytosis and maturity. In the eosinophilic series, no phagocytosis was seen. Only in the case of pathologic eosinophilia — for instance, ankylostomiasis and leukemia etc., did we find marked phagocytosis. In the monocytes, it was somewhat different from the results hitherto reported by *Yamashita*[9], *Tani*[8], and others. According to them, the phagocytosis was weaker than that of the neutrophils in the peripheral blood. Until 3 hours after the addition of Indian ink, the phagocytosis was weaker than that of the neutrophils, but later it became stronger and after 9—12 hours, the entire cell was completely filled with carbon particles. These cells showed no decrease of phagocytosis with the lapse of time, as the neutrophils did. Phagocytosis was never observed in lymphocytes. As *Amano*[1] reported, platelets showed phagocytosis and adhesion remarkably. Phagocytosis of the megakaryocytes is still disputable, and up to this date, we have found no phagocytosis in normal megakaryocytes. Concerning the variation of phagocytosis of the neutrophils by time: When the average degree of phagocytosis in the whole growth zone was examined along the line of radial direction from the marrow fragment, they exhibited the highest value (1.94), and the phagocytic rate also reached 90.8% after 3 hours of cultivation. Then immediately after, it fell rapidly, and gradually decreased further until it had declined to 0.48 after 24 hours. This initial drop of phagocytosis was due to new born cells, and the later declination was due to the discharge of carbon particles.

2. *Rabbit bone marrow cells*

As *Tani*[8] observed in the peripheral blood, and *Itoi*[3] observed in the supravital investigation of the bone marrow, phagocytosis of rabbit pseudocosinophils is weaker than that of human neutrophil. In the bone marrow culture, we found much weaker phagocytosis of the pseudoeosinophils. Therefore, when the chick embryo juice, diluted 2 times with normal rabbit serum was used, a remarkably good phagocytosis was obtained. In this case, the phagocytosis of each cell was similar to that of a human.

C. Vital staining

1. Basic dye (neutral red)

a. Findings in rabbits: The young cells have such a strong affinity for neutral red that they are stained heavily and diffusely from the very early stages. Cells that were older than the pseudoeosinophilic metamyelocytes only showed several stained granules (5—10 minutes after the administration of the dye). The stained granules were at first fine, small and of the same size, but they gradually increased their number, and then became confluent and swollen. Thus, they lacked uniformity. The staining reached the maximum about 5 hours later, and began to decline 7—8 hours later. About 24 hours later, they were almost completely discoloured. The eosinophils began to show a stain a little later, but when they were stained, 80% of all the granules were simultaneously stained and never became confluent or swollen until the very end. The colouring of these cells was weaker than that of the pseudoeosinophils and basophils, so that we could differentiate at a glance. They discoloured completely 17—18 hours later. In the basophils, all granules were simultaneously stained a deep red. These stained granules did not become confluent aud swollen and even after the discolouring of the pseudoeosinophils and eosinophils, they still retained their color. In the monocytes, the confluence and swelling of the stained granules was so marked that the number of granules decreased, while the colouring slightly increased. Thus the arrangement of the granules was peculiar and presented a beautiful rosette arrangement. In the lymphocytes, they were fine and heavily stained, but later, they became confluent and swollen like those of the monocytes, and occasionally formed a partial rosette arrangement, though never completely. In the megakaryocytes, they were stained to a moderate degree and confluenced slightly, but after 8—9 hours, they were completely discoloured. In the platelets, we also found confluenced neutral red granules.

(1) Variation of the colouring of the pseudoeosinophils by time: Immediately after the addition of the staining fluid, the colouring started. They showed the maximum mean colouring (1.31) and rate (96%) after 5 hours, then began to discolour

and after 24—30 hours they discoloured almost completely. As to the reason for discolouring, we think that it may be due to the destruction of the dye granules by dye toxity.

(2) Variation of the degree of colouring of pseudoeosinophils and neutrophils in the pathological stage: In the cultivation of the bone marrow of aplastic anemia and so-called *Banti*'s disease, we found poor growth of the bone marrow cells and depressed function of the leucocytes. In the cultivaron of the bone marrow of normal rabbits, the same result was found, if a restraining substance was added — for instance, serum from the above-mentioned patients, or p^{32} or benzene etc.. At the same time, they were stained more rapidly and heavily by basophilic dye and discoloured earlier than the normal ones. The fact that they were heavily stained, is easily explained by the cellular vital power. The early discolouring may be due to the fact that the chromatophilic granules are destroyed earlier than usual by dye toxity, and probably also by the depressed function of the cells.

b. Findings in humans: They are approximately equal to those of rabbits, but quite contrary to those of phagocytosis.

2. *Acid dye* (*Lithioncarmine*)

Different from that of a basic dye, we found that the granules were very weakly stained until several hours after the addition of the dye. In rabbits, about 9 hours later, several weakly stained granules appeared in 1—2 % of the pseudoeosinophils. About 12 hours later, they appeared in the monocytes and formed a rosette arrangement, but the stained cells occupied only a small part of it. 18 hours later, the histiocytes appeared, and their stained granules were relatively abundant. After that the majority of the histiocytes became stained. The staining of the pseudoeosinophils did not progress beyond about 9 hours. Thus although, the vital staining, using Lithioncarmine, in the bone marrow of rabbits was generally weaker than supravital staining, it was still much more weak in human bone marrow.

Summary

(1) The movement of the blood cells in the bone marrow was classified into 9 types.

(2) The characteristics of moving types are so distinct according to the kinds of blood cells, that the differential diagnosis of the cells by moving types is easily and certainly made. In this way, (by the kind of blood cells), we have classified leukemia, as is described in our other articles.

(3) The phagocytosis and vital staining of the blood cells in the bone marrow is different in degree and mode, according to the kind of blood cells, and thus becomes valuable ground for the differential diagnosis of the cells.

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