Fluorescent Microscopic Studies on Various Exudate Cells in the Inflammatory Focus

II. Observations on the Immature Cells of Bone Marrow and Lymph Node

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Cytological studies of exudate cells have been carried out by numerous investigators, and recently remarkable advance has been made in this field of research with the aid of the electron-microscope. In order to clarify the cell functions, however, it is desirable to use not only cytophysical but also cytochemical techniques.[1] In this respect, the use of the fluorescent microscope has such a great advantage that it makes possible to investigate both physical and chemical phases of cytology simultaneously. It should be emphasized here that by the fluorescent microscope the cell components can easily be examined microchemically in vivo, without injuring the cells, by simple procedure using fluorescent dyes.

With respect to the physical and chemical aspects of the cellular changes in the inflammatory focus, *Menkin*[2] has given a fresh turn to the old concept of inflammation by considering many physico-chemical factors. As one of the chief sources of exudate cells, the peripheral blood cells have been investigated by the fluorescent microscope and the results were reported in the preceding paper.[3] This paper describes the observations on the immature cells of the blood-forming tissues.

The literature concerning fluorescent microscopic studies of the cell has been briefly reviewed in the preceding paper.[3]

MATERIAL AND METHODS

The immature cells in the normal bone marrow and lymph node of human, mouse and rat were observed.

Staining dyes, apparatus, microscope and filters are the same as used in the preceding study.[3][4][7]

FINDINGS

1. Erythrocytic series (Fig. 1.)[5][6]

Series A illustrates the changes both in cell size and cytoplasmic color by *Amano*'s staining[8] (azur II, methylenblue and eosin) at the same time. Series B shows the

color of the nucleus: Series C the condition of chromatin nets in the nucleus; and Series D schematic illustration of the summarized findings by neutral red and Janus green supravital staining and phase-contrast microscopic observations, and finally Series E the fluorescent picture.

As shown in Figure 1, the green fluorescence in the cytoplasm decreases in intensity as hemoglobin content increases. The fluorescence of the nucleus changes from yellowish tone to green gradually. And at the stage of the reticulocyte, its fluorescence is indistinct, and at more advanced stage of maturation, it becomes non-fluorescent, showing only cell outline. Generally the mature erythrocyte is non-fluorescent, but the fluorocyte which has been so called from the ancient time may occasionally be noticed as autofluorescent, but in blood only. The chromatin pattern in the nucleus is rough and massive, and as the cell develops, the nucleus becomes piknotic and homogenous. The nuclear membrane is indistinct, and finally disappears completely. Immature cells of the erythrocyte series, the erythroblasts, emits an intense green fluosrecence, but the cytoplasm of some macroblasts gives no clear fluorescence. In general, the erythroblast is characterized by the concentric localization of the nucleus.

2. *Granulocytic series* (Fig. 2.)[5)6)]

Generally, the discrimination of different types of the blast-cells appears to be difficult. The chromatin structure of the nucleus of the blast-cell of this series is fine and netlike, whereas the nuclear membrane of the lymphoblast is thick and the chromatin structure is beam-like. The monoblast is larger than the former two in size, and has a concave nucleus surrounded by wide cytoplasm which shows a irregular outline. The chromatin is fine, showing a irregular, rough net-like structure.

The discrimination of each blast must be made by the structure of the nucleus. The fluorescent color of each nucleus is green, but as regards the brilliancy of fluorescence, the cell of the lymphocyte series is the most intense, that of the monocyte series is feeble, and that of the myelocyte sereis is of the middle grade.

As the maturation of the cell goes on, the granule components characteristic of each cell type appears, and these granules give yellow-red or red fluorescence. At stage of mature myelocyte, the fluorescent picture shows considerable difference according to cell types. Namely, the neutrophil leukocyte emits yellowish red fluorescence from the perinuclear region, which is enlarged progressively as cell maturation proceeds. But, the periphery of the cytoplasm or the hyaloplasm reserves irregular green fluorescence yet. The chromatin of the nucleus becomes dense and block, and an increase in yellow-green tone of fluorescence is noticed. The ensinophil, even at the relative young stadium, has a little large granules which shows diffuse distribution; these granules, however, become aggregated densely as the maturation of the cell goes on. These so-called eosinophil granules emit red fluorescence. The periphery of the cytoplasm is similar to that of the neutrophil. The fluorescent color of the nucleus is of more intense yellow tone than in the cells of the other series. The chromatin structure shows no essential difference among different cell types of

the myeloid series. In the basophil the granules are also scattered diffusely and emit dark red fluorescence. The fluorescence of the nucleus is of yellow tone.

The chromatin structure of the myeloid blast-cells is generally fine and delicate, but becomes dense in the later stages of maturation. The nucleus emits yellow red fluorescence, but this fluorescence is easily influenced by pH as well as by the concentration of the staining solution.

As mentioned above, different types of immature cells of the granulocyte series can be discrimainted from each other, by observing the intensity of the luminescence, structure of the nucleus, the distribution of characteristic granules and the graduation of their differentiation with the fluorescent microscope.

3. Megakaryocytic series (Fig. 3.)[5)6)]

The cells of this seires are larger than those of the other series. The nucleus is irregularly shaped and the chromatin is rough, block and dense even at the relative immature stage. The fluorescent tone of the nucleus is yellow green, and that of the cytoplasm is light green at the immature stage, and begins to emit red fluorescence as the cell maturation proceeds. At the mature stage capable of producing the thrombocytes, it radiates an intense red fluorescence. After discharging thrombocyte, the mature megakaryocyte loses its cytoplasm and becames naked.

4. Monocytic series (Fig. 4.)[5)6)]

Generally, the cell of this series may be easily discriminated from the cells of the other series by the fact that the chromatin of the nucleus is delicate and irregularly netlike. The outline of the cell is irregular. The nucleus emits yellow green fluorescence, and the cytoplasm light green one. The promonocyte, on the other hand, gives reddish-yellow fluorescence because it contains more numerous in the cytoplasm granules than the other cells, which closely resembles the monocyte, but the latter cell can be discriminated from the monocytic cell by the fluorescent picture of the nucleus and the cytoplasm mentioned above. The cytoplasm of the mature monocyte, as described previously,[3)] emits yellowish red fluorescence. The digestive vacuoles, however, luminate dark red one. The rosette-formation in the cytoplasm is influenced by pH, concentration and staining time of staining solution. The rosette-formation in the monocyte from the abdominal cavity of mouse, however, is clearly noticed.[7)8)]

5. Lymphocytic series (Fig. 5.)[5)6)]

As mentioned above, the luminous intensity of fluorescence of the nucleus of the lymphoid cell is the most intense among different cell types. The green fluorescence of the nucleus is really very impressive. The nuclear membrane is very thick, and the chromatin structure is beam-like and dense. The cytoplasm emits feeble green fluorescence. As the cell maturation proceeds, a few round granules which seem to correspond azur granules radiate yellow-red fluorescence and these granules are seen scattered in the cytoplasm. The chromatin structure of the nucleus becomes

progressively dense and homogenous. In the mature lymphocyte, light red fluorescence is occasionally seen in the cytoplasm, this seems to be luminated from the azur.

6. Plasmocytic series (Fig. 6.)[5)6)]

These cells are mainly recognized in the bone morrow, lymph node, spleen and perivascular tissue, etc. Roughly speaking, these cells are similar to the lymphocyte. The fluorescence of the nucleus closely resemble to that of the lymphocyte. The cytoplasm radiates green fluorescence. However, the plasmocyte differ from the lymphocyte in the point that the former has a relative wide Golgi complex in comparison with that of the other cells. Even in younger cells, the non-fluorescent part—Golgi complex—is noticed as a part of perinuclear cytoplasm. With the advance of the degree of cell maturity, the Golgi area becomes larger and the fluorescence of the nucleus turns to yellow. In mature plasmocytes the chromatin structure of the nucleus does not show wheellike structure and perinuclear halo also is not clear. During the process of cell maturation, the cytoplasm luminates light red yellow fluorescence and the Russel's body intense red one. The changes are similar to those in the lymphocyte.

7. Other cell types.

Reticulum cell in the lymph node and bone marrow: The cytoplasm is not clear, and only nucleus of delicate and net-like structure emits feeble green fluorescence. These features of the nucleus are similar to those of the young monocyte.

Endothelial cell: Only the nucleus radiates moderate intense green fluorescence, which is especially intense in the nuclear membrane. The cytoplasm is not clear.

Histiocyte: The nucleus radiates yellow green fluorescence, this is similar to the that of the myelocyte, the nucleus of which has relative dense chromatin structure. The cytoplasm, on the other hand, radiates red fluorescence. This is similar to that of the monocyte.

Fibroblast: The nucleus radiates intense green fluorescence. This is similar to that of the lymphocyte. The cytoplasm is not clear.

As mentioned above, the fluorescent picture of normal hematogenic cells shows a series of characteristics. Furthermore, with the advent of cell maturation, the fluorescent intensity of all these cells except for the erythrocyte becomes progressively intense and appearently exhibits some changes in the fluorescent color.

A comparison of the fluorescent picture of each of the cell types in which discrimination is difficult from each other are illustrated in Figures 7 and 8.[5)6)] It can be seen that the different cell types can be easily discriminated from each other by the author's method.

DISCUSSION

Fluorescence of each of the cell components

On the basis of the fluorescent findings described above, the author wishes to discuss the relation between the fluorescent picture and the pattern of cytochemical staining of the nucleus and cytoplasm.

Nucleus.[8)-21)]

The chief constituents of the nucleus are nuclear-substance, nuclear-lymph, and nuclear membrane. Chemically, the nuclear component is composed mainly of nucleoprotein; namely, basophilic (histon) and acidophilic (nonhiston) protein, DNA and RNA, etc.[9)13)15)] *Caspersson*[10)] pointed out that the amount of nucleoprotein may be measured by the DNA content. It was found that the fluorescence of the nucleus was green yellow color, and that other color tone had not been recognized as yet. From this fact, the binding between the fluorescent dye and the nucleoprotein may easily be presumed. It is generally accepted that the DNA content in sorts of the cell is almost costant,[12)17)18)] but the author believes that there are differences in the density of nucleoprotein among different cell types, leading to the differences in the color tone and the intensity of fluorescence.

It is considered that the greater intensity of brilliancy in the mature cells may be explained to be a relation between dye and its combining protein molecule. Namely, the action of Van der Waals' force may be easily imagined. In view of the fact that this force is determined with the size of the protein molecule, the nucleoproteins of the mature cells may be of large molecular size than those of the immature ones. It is well known, on the other hand, that the protein of the nucleolus is RNA, the fluorescent color of which is red-yellow.

If the fluorescent phenomenon is assumed to be interpreted as a photochemical reaction between the constituting protein and the changes in radiation energy of ultraviolet in the field of the cell, the fluorescent radiation itself should be accepted as one of the molecular alterations of the protein. In fact, it has been demonstrated by *Caspersson* and *Amano* and *Koga*[8)9)10)11)12)] that nucleic acid absorbs very intensely ultraviolet-rays. The relation between nucleic acid and nucleo-protein has also be shown by these authors.

The chromatin (chromosome mass) combines with dye and radiates green fluorescence, but the nuclear fluid (lymph) does not. The chromosomes of the myeloid series are seen as stick form, and those of the immature cells of the lymphocytic series as fleck-like. These findings are especially clear in the blood cells of the newt because of their very large size. The fluorescent substances in the nucleus are very distinct and situated near the nuclear membrane or at the center of the nucleus. A similar condition is also seen in the phagocytes.

The nucleolus is not always observable depending upon the changes in pH and

concentration of the dye solution. It should be stressed, however, that the nucleus is cytochemically quite different from the other elements.

Protoplasm.[8)22)-32)]

It is a well known fact that the cytoplasm is in a condition of gel and sol fluidity and contains different kinds of granules characteristic of each cell series. However, the earliest forms of immature cells are almost devoid of granules.

Mitochondria.[29)]

The mitochondria emits red-yellow fluorescence, which, however, gradually disappear after a long period of irradiation by ultraviolet-rays. Nevertheless, this does not mean disintegration of the mitochondria, because the phase-contrast microscopic observations demonstrated that they had not been destroyed by the ultraviolet rays.

These consist chiefly of lipoid with a lesser amount of nucleoprotein cytochemically. It is easily imagined that the nucleoprotein may conjugate with the dye.

Granules in the granulocyte.[23)24)28)]

Generally, these radiate intense red-yellow fluorescence which also diminishes gradually in intensity. It seems that the affinity of the dye to the granules, the eosinophil and basophil ones in particular, is very strong and emit an intense fluorescence. This is attributable to the shape, the size and the number of the granules. The eosinophil granules are said to consist of lipid, chiefly phospholipid and protein. The basophile granules, on the other hand, are believed to be composed of heparin, vitamin B12 and protein (histamin), according to the recent studies. It can be said that both types of the granules contain protein and consequently the protein component may be correlated with the fluorescence, as already mentioned. The neutrophil granules are smaller in size than the other two, and may be subdivided into A, B, and C types by the electron microscope. These granules as a whole emit feeble red yellow fluorescence, but it is not clear which types of the granules radiates the fluorescence. However, it is probable that one type which shows intense basophilia is fluorescent. It is said that this types of the granules are rich in glycogen. Nevertheless, as shown clearly by the observations of the liver cells, the fluorescent radiation cannot be caused by the combination of glycogen with the dye.

Vacuole.

This part is noticed as a punched hole of non-fluorescence. The so-called digestives vacuoles in a condition of pinocytosis show red fluorescence. For example, Russel's body of the plasmocyte emits an intense red fluorescence. This also provides evidence to indicate that protein plays an important part in the fluorescence.

Enzyme and Vitamin.

The autofluorescence of some vitamins is noticed at present, but it is not clear in the enzymes. These will be confirmed with other various dyes in the near future.

Basophilia.

This is dependent upon RNA content, namely the amount of microsomes—endoplasmic reticulum, etc. It is considered that the fluorescent brightness of the protoplasm may be influenced by the combining force between RNA protein and dye.

Azur granules.

Their fluorescence is not clear. In view of the fact that some of the lymphocytes radiated red fluorescence, it seems probable that the granules may be the seats of combining the dye with some protein.

Golgi apparatus.[38]

This is non-fluorescent. This is not so well developed in most cell types and appears not so distinct, being completely covered by many granules in the cytoplasm. In contrast this apparatus of the plasmocyte is larger and clearly discernible, but its outline is not so clear as that of the vacuoles. It is said that this consists mainly of lipoid.

Hemoglobin.

This is a sort of heme-pigment protein. It shows no fluorescence. It is noteworthy, however, that the fluorocytes are occasionally found among the erythrocytes. The nature of the fluorocytes are unknown, but they may be considered to be the malformed erythocytes caused by the abnormality of synthetic mechanism of hemoglobin.

As mentioned above, the fluorescence of the cell becomes intensified with the advance of cell maturation. This can be interpreted to be due to the fact that in the mature cell the functional activities are greater than in the immature form, so that combining force between the component protein and dye strengthened. The relationship between the stractures, and their cytochemistry has also been demonstrated with the aid of the fluorescent microscope, on the basis of the characterized fluorescent pictures of the different types of cells. This type of investigation must be extended to cells other than exudate cells.

Mechanism of fluorescent staining

As regards the immediate effects of the ultraviolet-rays on the living cell, it seems evident that, so far as the wood's light is used, no pronounced injury of the cell was observed until 30 minutes after making preparations. Hereafter, if the observation was continued, color change in the fluorescence would definitely take place and the cell would show homogenous feeble green fluorescence, regardless of cell types. Inability to discriminate the nucleus from the cytoplasm signifies death of the cell (homogenous yellow brown fluorescence).

The light source and filters were not the same as used in the previous studies by other workers reported in the literature. The light source of "Ortholux" (Leitz) may be stronger than that of the apparatus used here, but the dyes used were the same. The dye solution in a consentration of 1:10,000 at pH 7.1—7.6 (weak alkali) was used. Under these conditions, the obtained findings were similar to those of

Kosenow et al.[33]

With respect to the mechanism of the fluorescence, however, *Bethe, Schümerfeder* and *Strugger*[35][36] et al. postulats that it is due to the glycolytic activity of the cell as well as to the lipoid solubility and the degree of accumulation of the dye. *Schümerfeder* emphasizes that the dye-storing activity of the cell is in close relation to its high glycolytic potential. *Bethe* notices, on the other hand, the importance of the alkaline medium to enhance the fluorescent phenomenen. As already stated, the author is of the opinion that the significance of the relation between the dye and the histoprotein should be stressed for the development of fluorescent radiation. *Contier* reports[34] that fluorescent color has a close relation to the "Speicherungsgrade" of the dye in the cell; namely, weak "Speicherung" of the dye emits green fluorescence, whereas intense one copper-red. Of course there is the gradation of color tones between these extremes. In fact, various color scale between green and red was observed. However, the author cannot agree with the view of *Contier*, because an intense red color is also emited from the dead cells or from the degenerating cells that have been kept in the dye solution for a long period. *Schümerfeder* also stated that in the young living cell it is difficult to store the fluorescent dye and shows the weak fluorescence. By measuring the concentration of intracellular fluoretcent dye, *Strugger* reported that the living cell contains dye in a concentration of 1:10,000, whereas the dead cell which emits homogenous red fluorescence contains dye in a much greater concentration, 1:100.

This appears to give further support to the author's view that the fluorescent phenomenon depends greatly upon the relation between the dye and the histoprotein.

SUMMARY

The fluorescent findings of the immature blood cells in the bone marrow and lymph node of human, mouse and rat were described. Each cell type emits characteristic fluorescence. The fluorescence of the cells of the lymphocytic series is most intense, that of the monocytic series is very feeble, and that of the myeloid series is of the middle grade. The cytoplasm of the immature cells generally emits green fluorescence, whereas that of mature cells gives red fluorescence. It should be noticed, however, that the cytoplasm of the mature lymphocyte emits green fluorescence except for the azur granules, which sometimes show red fluorescence. The plasma cell is easily discriminated from the cell of the other series by the presence of non-fluorescent large *Golgi* complex.

As regards the differences in the fluorescent color of the cell, the action of the *Van der Waals'* force between the size of the protein molecule and the dye seems to play an important role in producing such differences.

The dead cells show homogenous red fluorescence, so that the discrimination of the living and dead cells can easily be made by the fluorescent method. Besides,

labelling of the cell can easily be made without injurying of the living cells by the use of fluorescent dyes.

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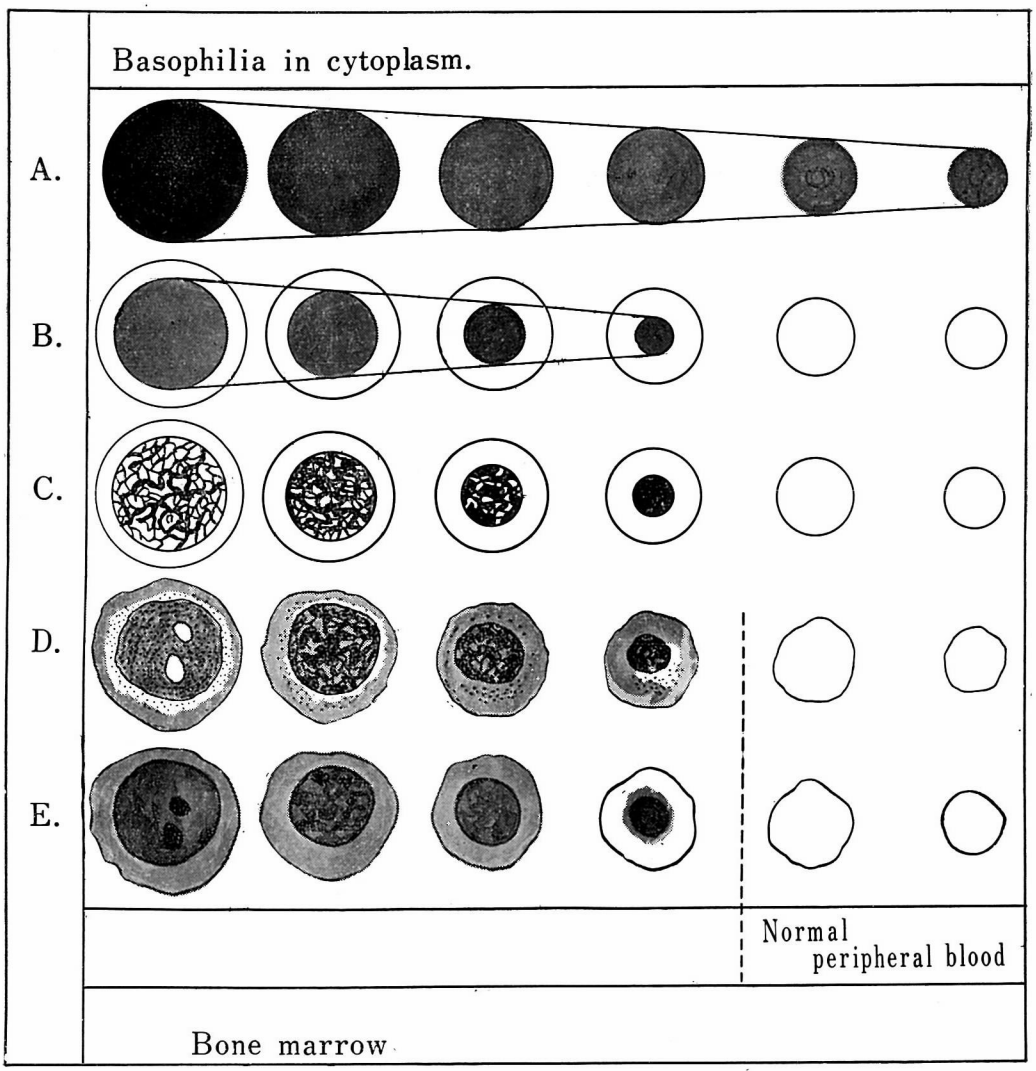


Fig. 1. Diagram of the fluorescent picture of the erythrocytic series.
A. Cell size and cytoplasm color.
B. Nuclear size and color.
C. Nuclear chromation structure.

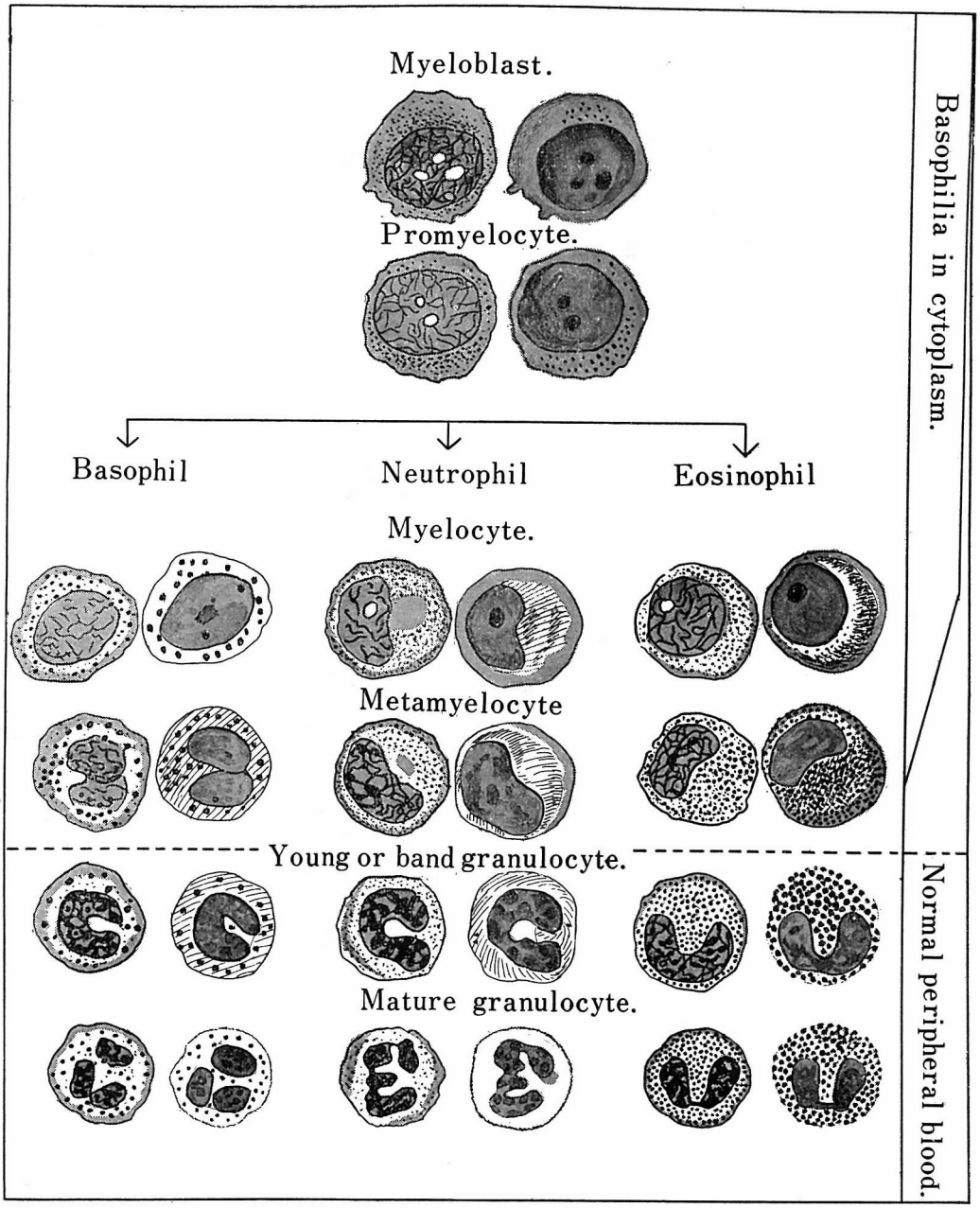


Fig. 2. Diagram of the fluorescent picture of the glanulocyte series.

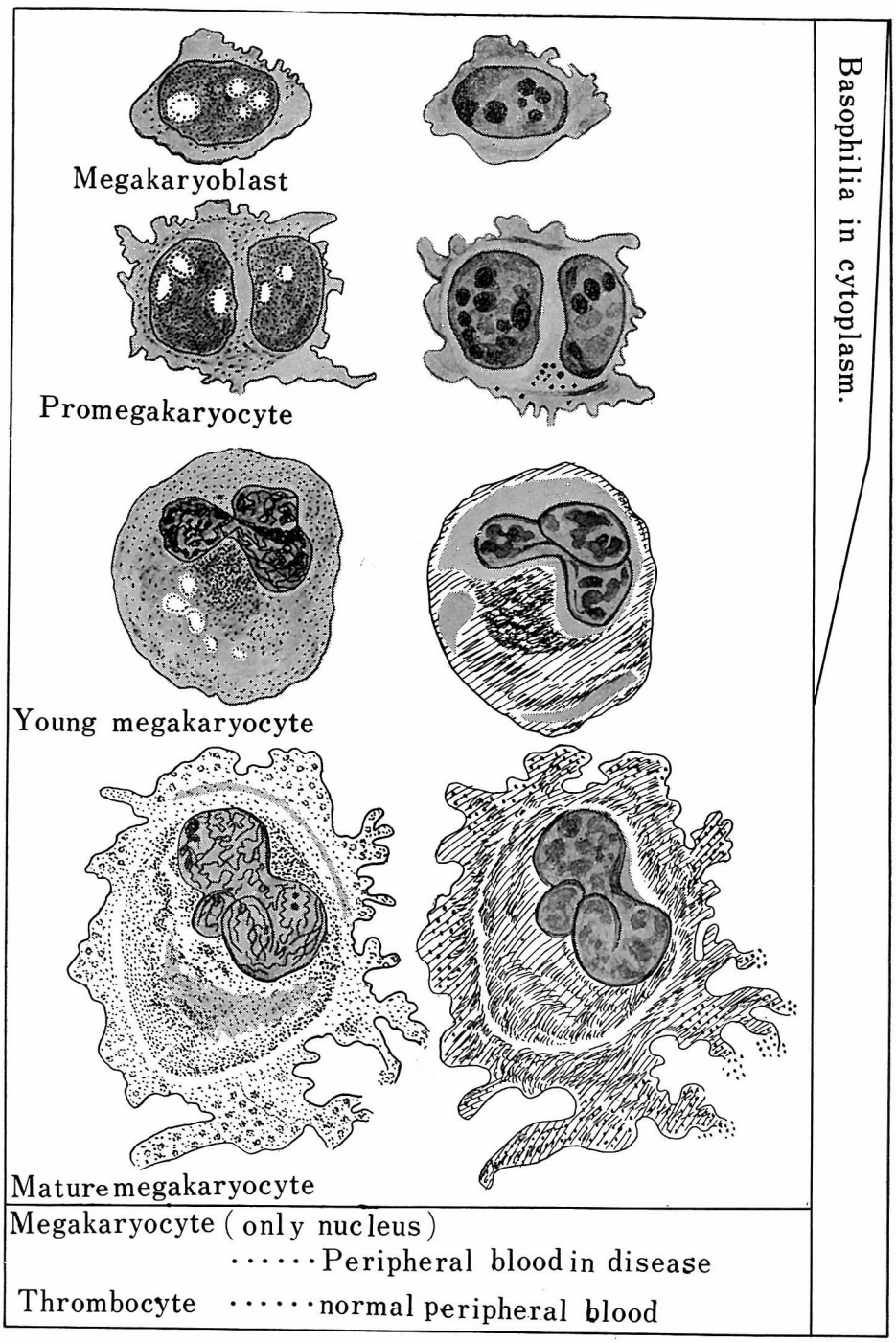


Fig. 3. Diagram of the fluorescent picture of the megakaryocytic series.

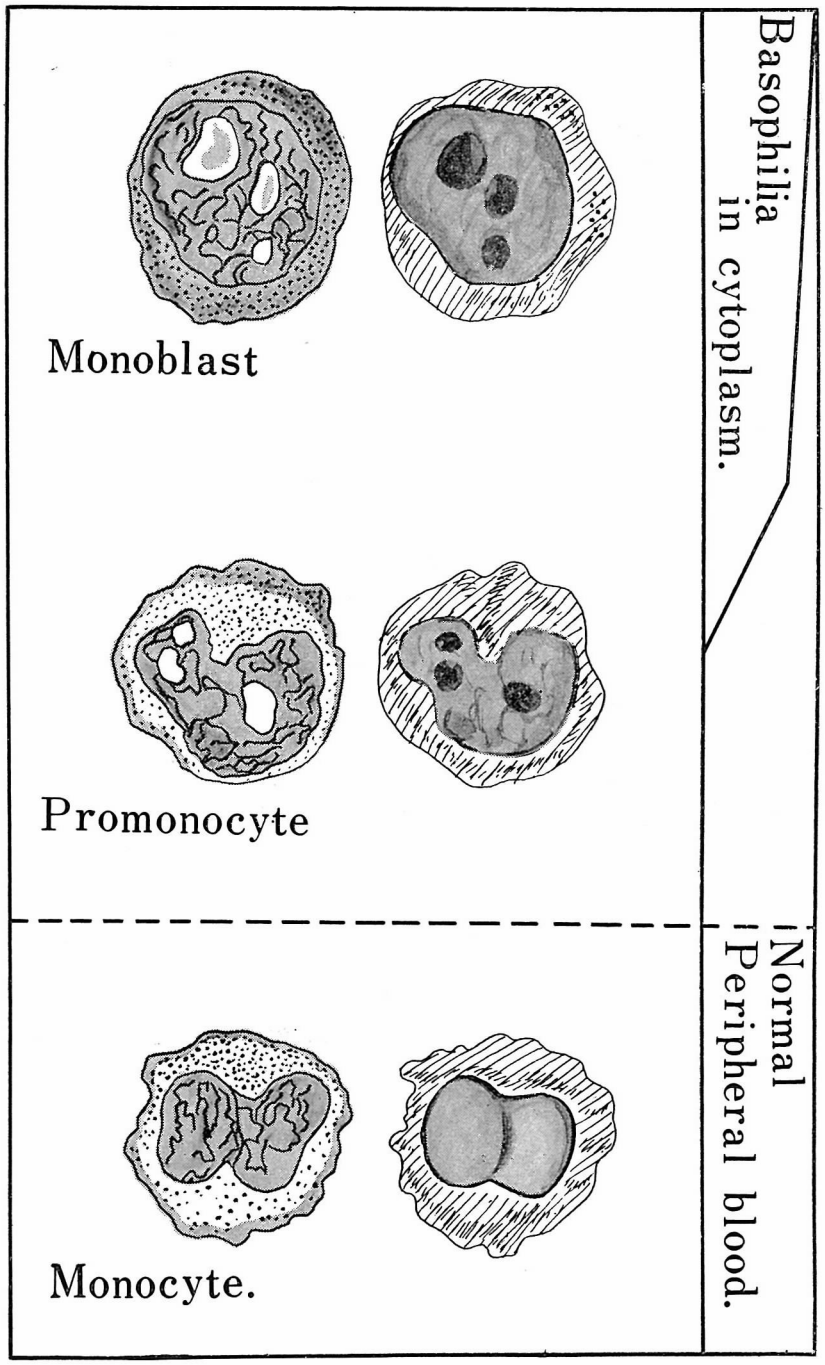


Fig. 4 Diagram of the fluorescent picture of monocytic series.

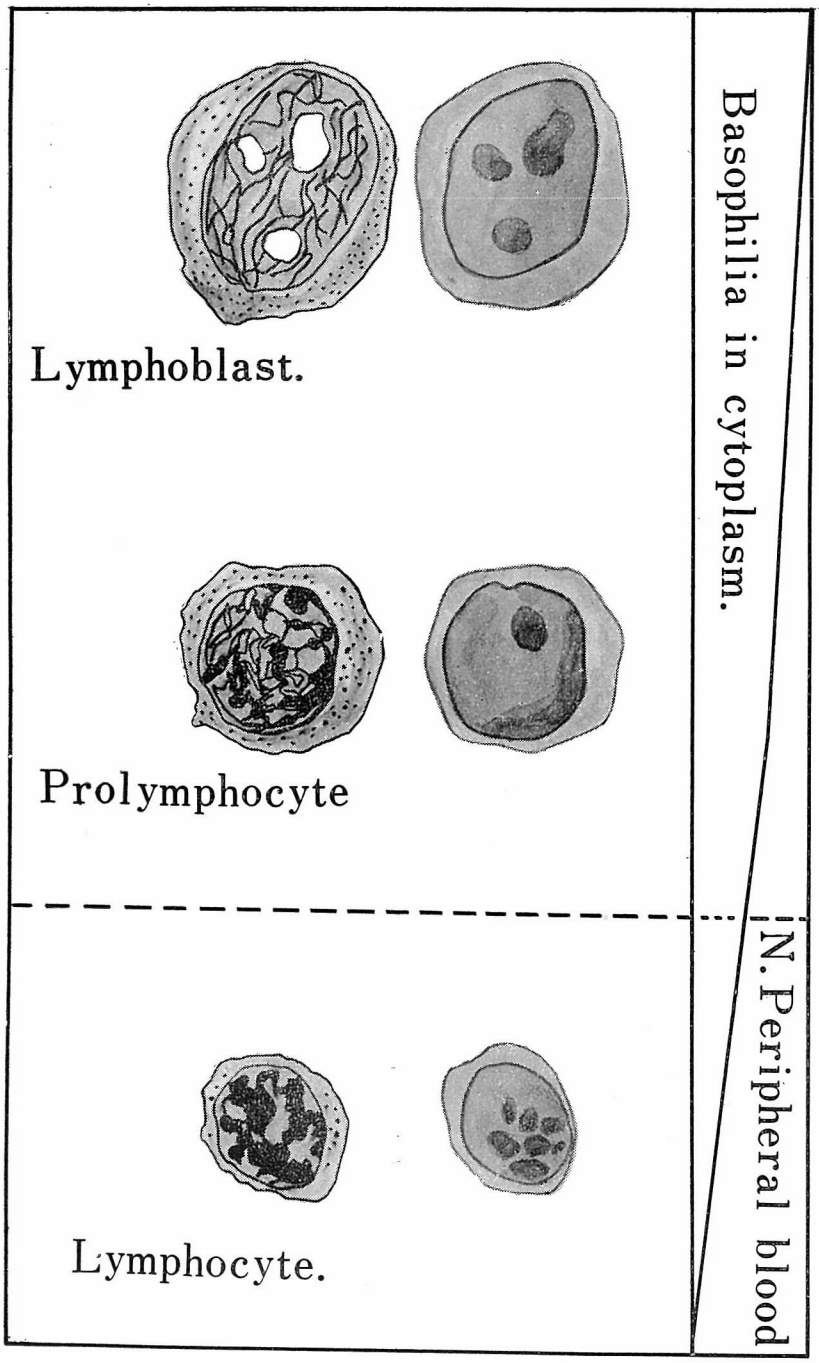


Fig. 5. Diagram of the fluorescent picture of the lypmhocytic series.

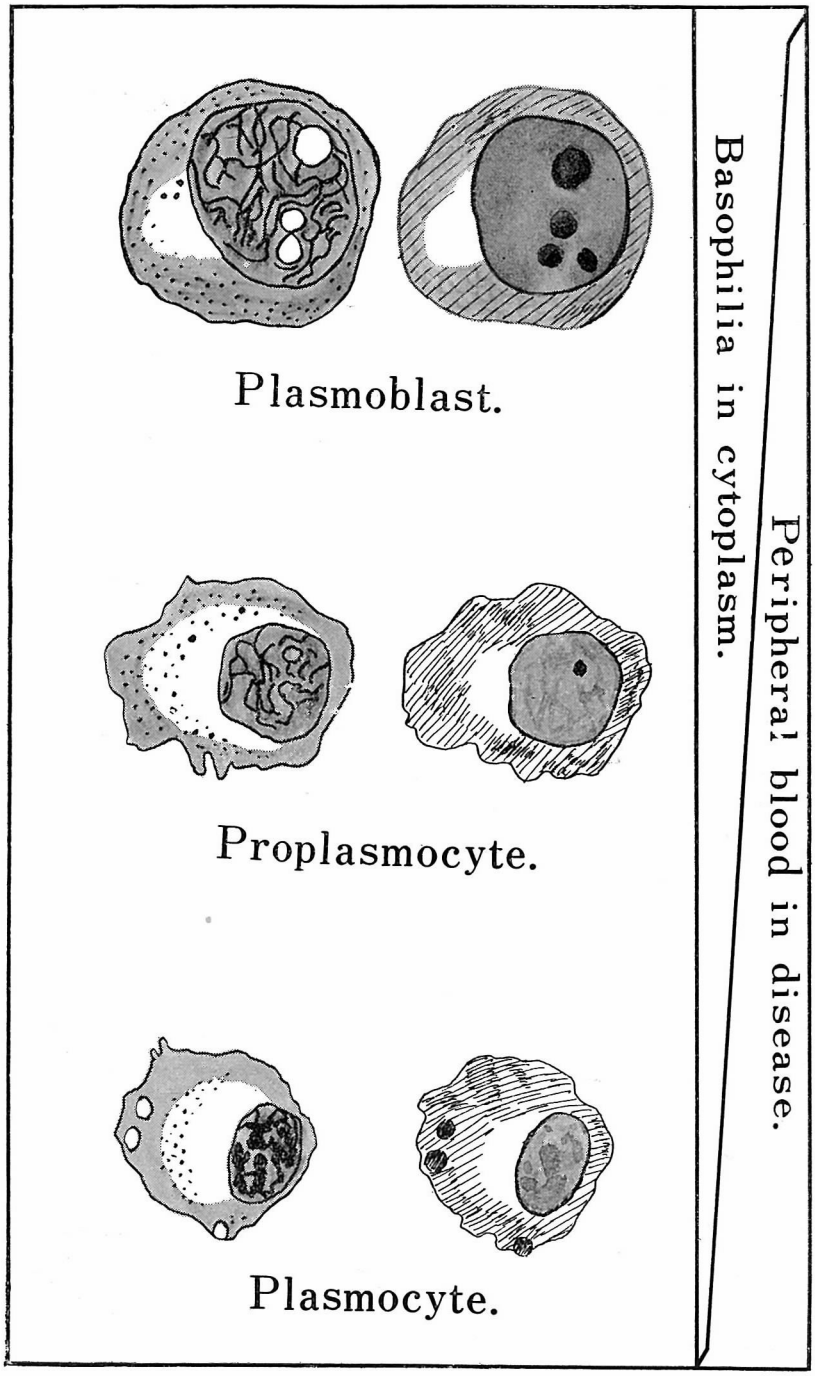


Fig. 6. Diagram of the fluorescent picture of the plasmocytic series.

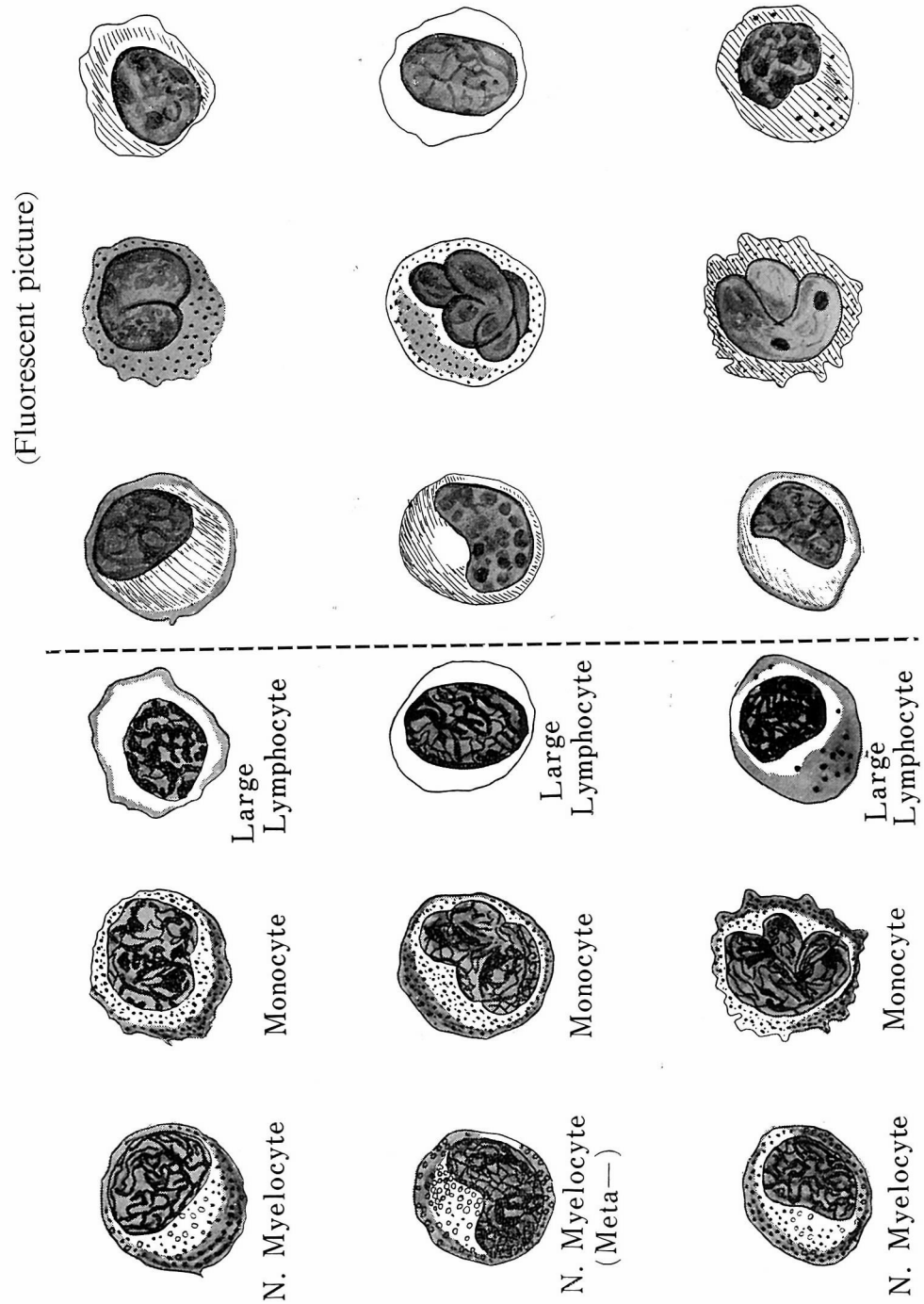


Fig. 7. Comparative mordholgy, granulocyte, monocyte and lymphocyte (fluorescent picture).

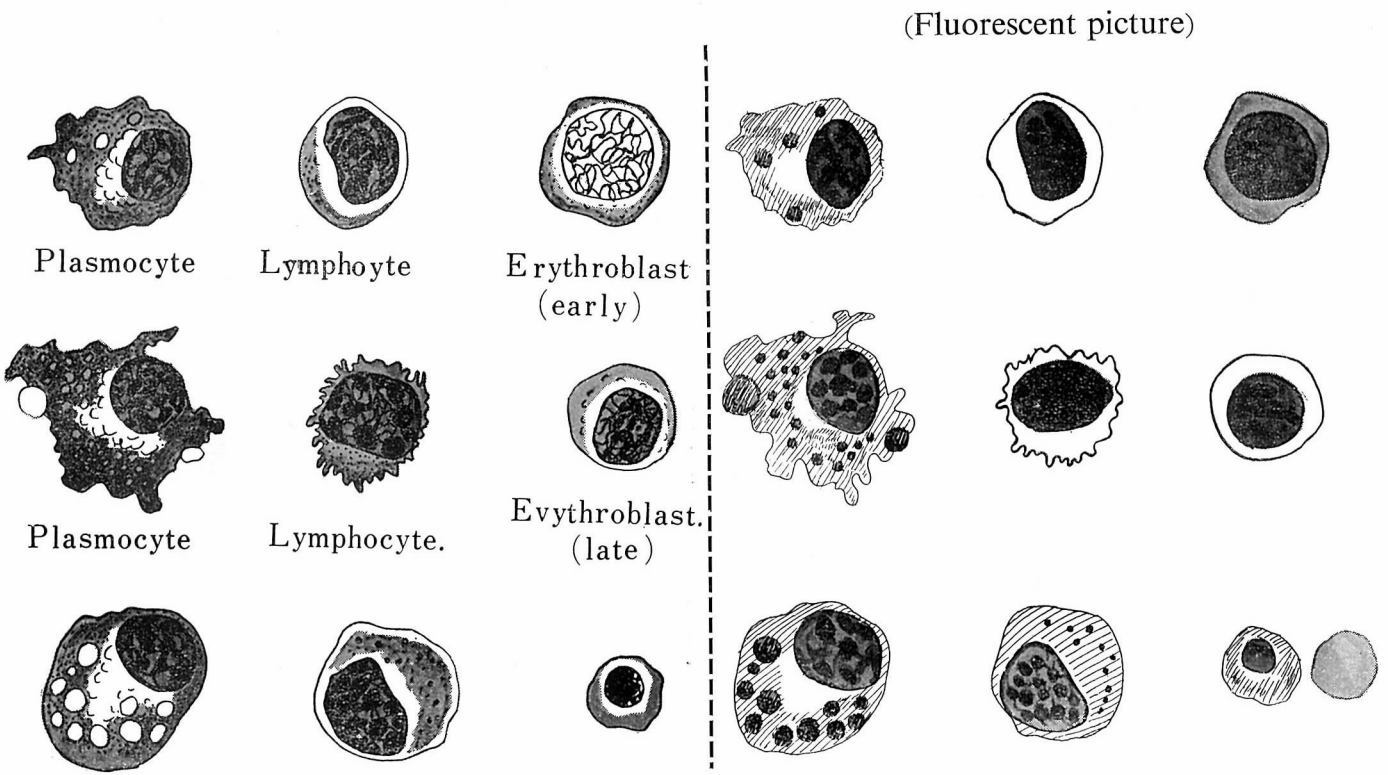
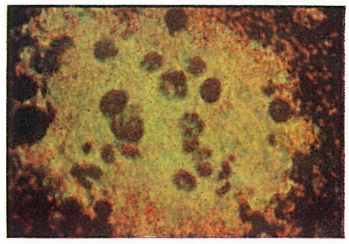


fig. 8. Comparative morpholgy, plasmocyte, lymphocyte, and immatured nucləated red cell (fluorescent picture).

EXPLANATION OF PHOTOMICROGRAPHS

1. Human bone marrow. Round dark specks represent fat globules. Intense luminous cells showing green fluorescence are the cells of the erythrocyte series, and those showing red-yellow fluorescence are the cells of the granulocyte series.
2. Fluorescence of the cells of the human bone marrow at different stage of maturation.
3. Mature granulocytes, metamyelocytes and lymphocytes in the human bone marrow.
4. Myelocyte and mature granulocytes in the human bone marrow of chronic myeloid leukemia.
5. Mass of immature cells of the granulocyte series (myeloblasts) in the human bone marrow from a case of chronic myeloid leukemia.
6. Lymphocyte, myeloblast, and mature granulocyte in the rabbit bone marrow.
7. Mouse bone marrow (matured granulocytes with ring nucleus).
8. Myeloblast, lymphocyte, and mature granulocyte in the mouse bone marrow.
9. Binucleated promyelocyte in the mouse bone marrow.
10. Megakaryocyte in the mouse bone marrow.
11. Lymphocytes without red fluorescence granules in their cytoplasm in the mouse lymph node.
12. Mouse peritoneal cells. Notice red fluorescent radiation of the cytoplasm. These cells are of the monocytic and granulocytic series.



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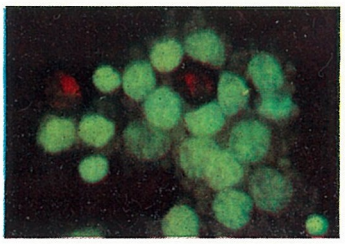

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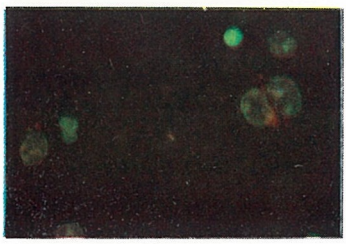

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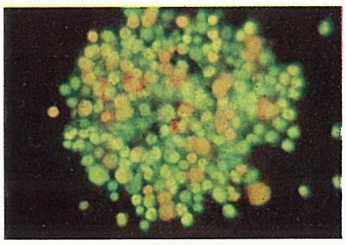

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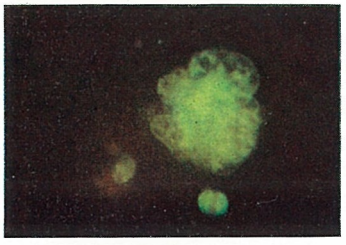

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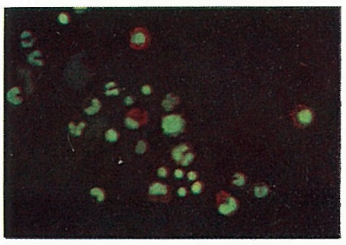

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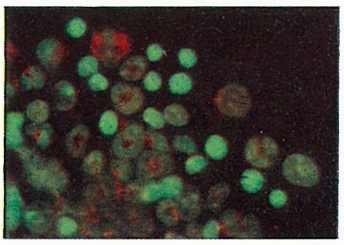

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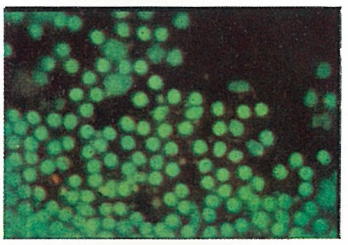

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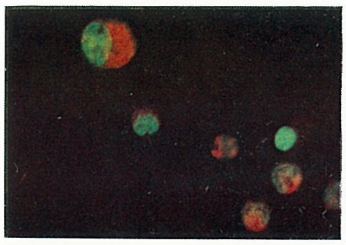

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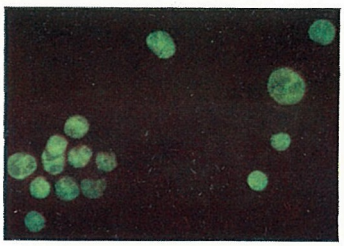

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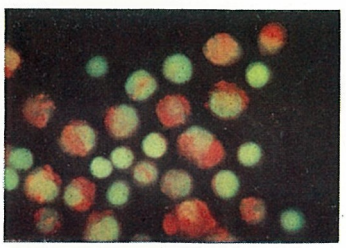

12.