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Abstract

With an improved method of tissue culture of peripheral leucocytes of our own design, the authors carried out systematic observations on the peripheral leucocyte culture from the normal, various kinds of leukemias, leukemoid reactions, and hypoplastic anemia. As for the culture method we have devised a method of silicon oil coating on the blood containers, a method which will least affect the cell function as compared with the conventional culture method. As the results we have found that the tissue growth in the case of peripheral leucocyte culture of normal persons ceases after six-hour culture and also we have recognized a peculiar finding, a growth like a corona-shape, in which an empty space appears in the inner part of growth area along with the lapse of time. In every leukemic case, without presenting growth area like a corona, the cell density is high and the outer zone of growth area becomes sharply demarcated as in the case with bone-marrow culture and also the growth continues even after 12 hours. At this instance we have noticed many mitoses of immature cells. Moreover, the maturation of immature cells has been observed and it has also been possible to distinguish to what type these immature cells belonged. In the leukemoid reaction no growth pattern Characteristic to the leukemia can be recognized, and at a glance it can easily be differentiated from the leukemia. In the case of hypoplastic anemia although the growth area is like that of the normal presenting a corona-like shape, the cell density is lower and the function of leucccytes is lesser than the normal. From these results we believe that the peripheral leucocyte culture is clinically useful.

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FUNDAMENTAL STUDIES ON THE PERIPHERAL LEUCOCYTE CULTURE AND ITS CLINICAL APPLICATION*

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The culture of peripheral leucocytes has its beginning with the successful pure culture of large monocytes of chicken and the observations carried out on the motility of these cells by CARREL and EBELING[1] in 1922. On the heel of this, MAXIMOW[8] reported the transformation of lymphocytes to fibroblasts in his culture of chicken leucocytes loaded with visceral extracts, and FISCHER[2] studied the morphological changes of cells by culture in the medium loaded with various extracts. Later, LEWIS and LEWIS[7] in their droplet culture of blood cells obtained from various vertebrates observed the transformation of monocytes to histiocytes or Langhans' giant cells. In our country SUZUKI[9] described the life span and the morphology of peripheral leucocytes in the blood culture of leukemic patients in the medium loaded with sera of various animals, and UNO[10] reported on the change of blood corpuscles to fibroblast-like cells in his leucocyte culture of chicken. In addition, HARA[3] examined the microscopic structure of the cells by silver staining, especially the morphological changes of mononuclear leucocytes in his culture of chicken leucocytes. As is clear from these there are many reports on the culture of peripheral blood cells but all these belong to the fundamental research and none belong to the field of clinical application. In our laboratory we have already obtained many new findings[4-6] by the fundamental and the clinical studies of the bone marrow culture, and we have designed a research plan on the peripheral leucocytes by the tissue culture method, bearing in mind the relationship it will have to the study of bone marrow. First of all, after re examining the conventional methods and devising a method that will maintain the cell function to the highest degree, we conducted a series of systematic studies of the tissue growth and the cell

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function of peripheral blood obtained from normal persons and persons with various leukemias, leukemoid reaction, and hypoplastic anemia. The results of our studies are described in the following.

MATERIALS AND METHODS

Test tubes are used as containers of blood and syringe as blood drawing apparatus. These are sterilized, dried and coated with 2% petroleum ether solution of silicon oil produced by Shinetsu Chemical Mfg. Co. (KF 99). Ten ml. each of blood is drawn aseptically from the elbow vein of subjects, and centrifuging at 1, 500 rev/min. for ten minutes, leucocyte layer is separated and then serum is eliminated. In the silicon oil and paraffin coating methods this layer is placed in an incubator at 37°C for a few minutes to coagulate without any addition, while in the case using anticoagulant, extracts of chick embryo and 0.1% calcium chloride solution are added and then put in the incubator. Taking out the coagulated leucocyte layer, red cells are eliminated by washing it with Ringer's solution and cutting it into small slices of about 1 cu. mm., these small slices are used as the materials for the tissue culture.

The slide-glass method devised by UNNO as well as the simple coverslip method of our own device were used for the tissue culture, and observations were carried on in the warm box under a phasecontrast microscope. The tissue growth and cell function were determined by the bone-marrow culture in coverslip, the regular method[4] employed in our laboratory.

RESULTS

An improvement on the culture method ; In the culture of peripheral leucocytes, it is important to avoid the coagulation at the time of blood drawing and to obtain the coagulated layer of leucocytes after centrifugation. By the conventional method heparin solution diluted 10^{-3} or 3.8% sodium citrate solution are used as anticoagulants or test tubes are paraffin coated to avoid coagulation. However, in all such cases, it is unavoidable to diminish the cell function and in the latter it also requires a highly skilled manipulation in coating. Furthermore, it gives rise to many difficulties in the manipulation as human blood is much more apt to coagulate than the blood of animals. Taking the advantage of water repellent action of silicon oil and relative ease with which it can be smeared, we have used the silicon oil for coating the containers of blood as aforementioned and compared ours with conventional methods.

As the coagulating agent for the leucocyte layer the extracts of chicken embryo has long been in use, but we have used 0.1% calcium chloride solution to take place of the chick-embryo extracts.

Using five sets of methods, combining various blood drawing methods with coagulants, namely, with the use of (1) silicon oil, (2) paraffin, (3) heparin with extracts of chick embryo, (4) heparin with calcium chloride, and (5) sodium citrate with calcium chloride solution, the culture of peripheral blood from normal persons was performed with the purpose to compare the growth area, cell density and the wandering velocity of neutrophils. As the result the growth area was largest in the case with silicon method, followed by the heparin and the paraffin coating methods, and it was smallest in the case with the sodium citrate method as shown in Fig. 1. The cell density was likewise greatest in the silicon method.

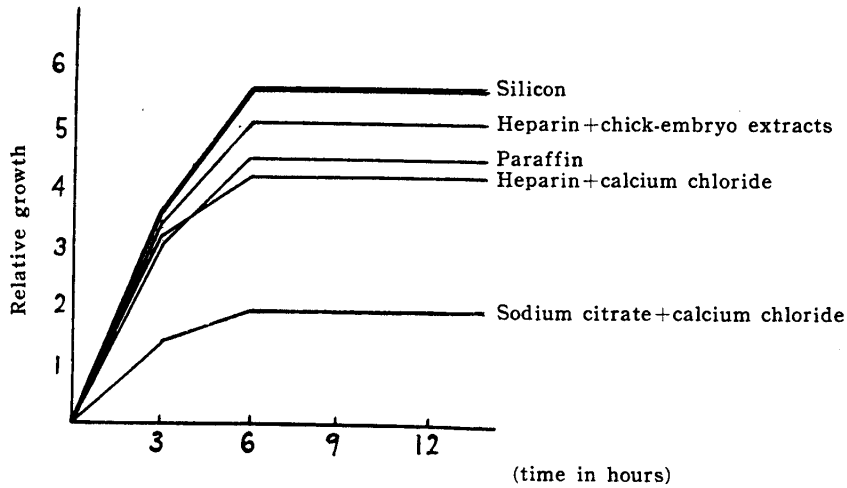

Fig. 1. The growth area of the In-Vitro peripheral blood culture of normal persons

The wandering velocity of neutrophils was, as shown in Fig. 2, greatest in the case of the silicon method followed by the paraffin, the heparin and the sodium citrate methods, in that order, and in general it has been found that the silicon method is most effective and that by the paraffin method the wandering velocity is about the same as that in the case of the silicon method but its growth area is smaller, and by the sodium citrate method both of these values decrease, indicating a considerable adverse influence upon the cell function. Therefore, all the culture findings described below are the results obtained with the use of the silicon method.

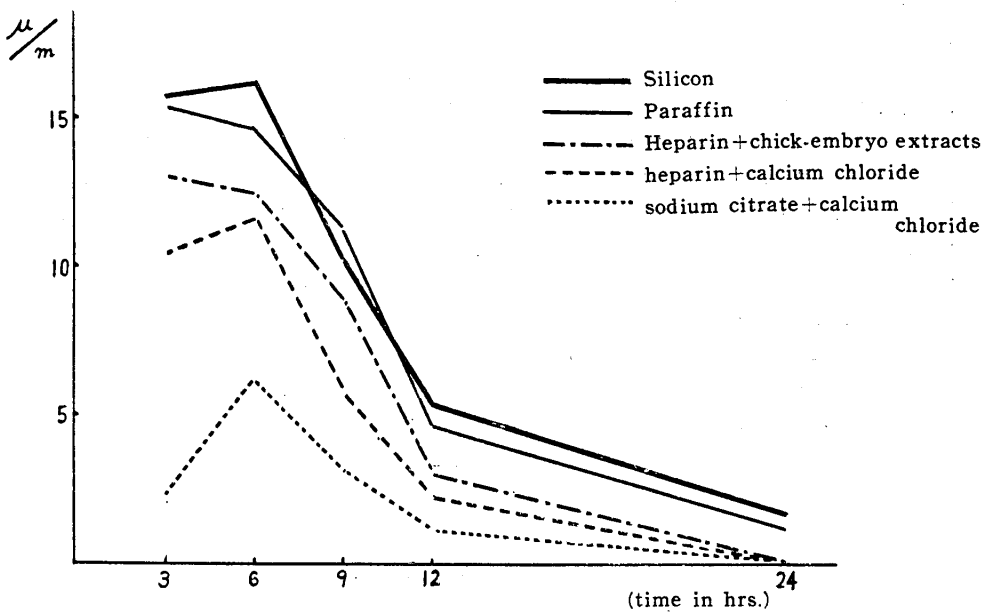

Fig. 2. The wandering velocity of neutrophils of normal persons in In-Vitro peripheral blood culture

Findings of peripheral leucocyte culture of normal persons : As for the growth area relative growth rate is, as shown in Fig. 1, 5.72 after six-hour culture, but unlike the gradual and continuous growth in the case of bone marrow culture the growth ceases thereafter. This seems to be due to the fact that those freely movable cells around the explant all wander out in very short time because the peripheral leucocytes of normal persons are all consisted of mature cells and possess no mitotic faculty, at the same time their wandering velocity is greater than immature cells. In his in vitro culture of chicken leucocytes, HARA[3] observed at each stage of the culture, the majority of cells in each zone of the growth area are consisted of polynuclear leucocytes which are especially more numerous in the outer zone. Generally mononuclear leucocytes are chiefly located in the intermediate zone, while lymphocytes are mainly located near the explant, but also being extensively distributed in the entire growth area. FISCHER[2] in his culture of human peripheral blood found after 24-hour culture leucocytes wandering markedly around the explant forming a shape somewhat like a big corona and observed neutrophils and eosinophils occupying the outer zone, and large monocytes occupying mostly the intermediate zone, and lymphocytes principally the innermost zone. He further stated that after two-day culture only wander-

ing cells surviving were large monocytes and all other leucocytes were found dead. In our observations carried out further in detail up to 24 hours of culture, most cells observed in the early stages in the growth area were neutrophils but after six-hour culture number of lymphocytes and eosinophils gradually increased ; and these cells began to migrate to the peripheral zone after nine hours, making the central zone more sparsely populated by degree, and an empty space appeared between the explant and the wandering cell zone as shown in Fig. 3; and 12 hours afterward monocytes wandered out into this space.

After the 12-hour culture the cell density yielded the value which was about the same as in the case of bone marrow culture. The wandering velocity of neutrophils after 3-hour culture was, as shown in Fig. 2, 15.7 μ/m. which proved to be inferior to that of the bone marrow culture, while after six hours it reached the maximum of 16.1μ/m. and thereafter it dropped rapidly. The average rate of carbon particle phagocytosis by neutrophils showed its maximum of 0.9 after six hours as shown in Fig. 4, which proved to be far lower than maximum of 1.95 yielded in the case of bone marrow after three-hour culture ; and 0.69 after 12 hours, 0.53 after twenty-four hours, and on the whole it gradually fell making a rather slow curve.

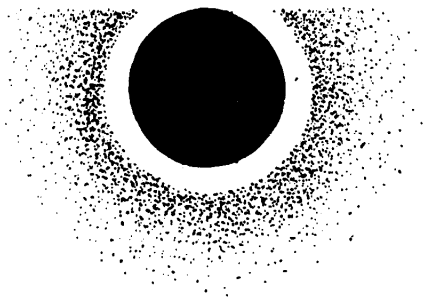

Fig. 3. Peripheral blood culture of normal person (model)

Stainability of neutrophils to neutral red vital staining proved to be quite high in the early stages of culture as shown in Fig. 5, and the average stainability reached the maximun value of 1.62 after three hours; but falling to 1.21 six hours afterward, thereafter they gradually lost color.

Findings of peripheral leucocyte culture of various leukemias : As has previously been reported about the bone marrow culture conducted in our laboratory[6], because immature cells in leukemia proliferate excessively by mitosis and the majority of them are consisted of immature cells either with a little wandering capacity or none at all and because even mature cells have a poor wandering faculty due to the decrease in their function, the cell density in the growth area becomes extremely high and the boundary around it becomes so clear-cut that it can be readily distinguished as leukemia at a glance. However, the authors have

Fig. 4. Carbon-particle phagocytosis of neutrophils of normal persons in In-Vitro peripheral blood culture

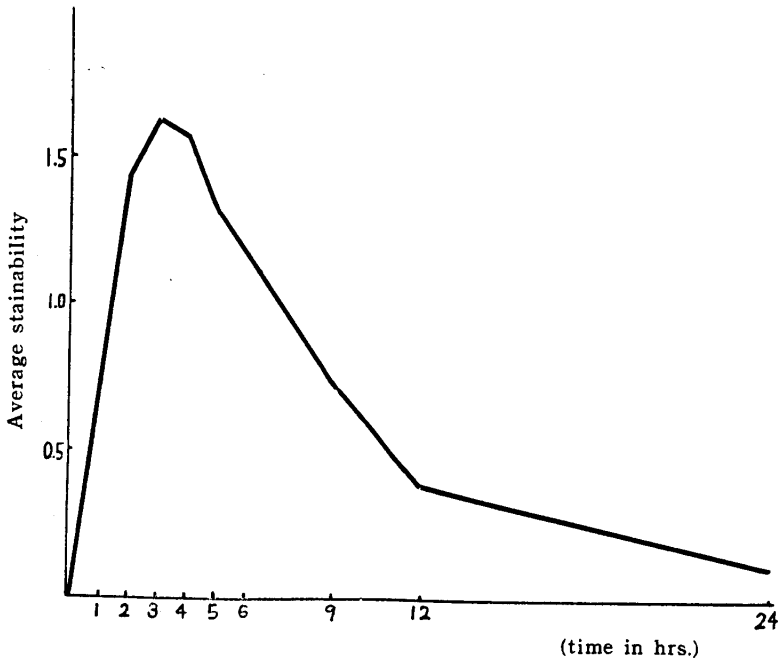

Fig. 5. Stainability of neutrophils to neutral red staining

likewise recognized in peripheral blood a peculiar leukemic type of growth; namely, in the central zone of this growth area no empty space

appears as in the case of normal persons aforementioned but immature cells occupy it densely all through the course of culture, and the tissue growth can be recognizable even after twelve-hour culture. This is due to the quite active mitotic proliferation of immature cells of leukemia and at this stage many nuclear divisions can be recognized.

a) *Chronic myelogenous leukemia :* In the growth area of this disease the cell density is high as shown in Fig. 6 and it is consisted of two zones, namely, a young cell inner zone with clear-cut boundary and a mature cell outer zone, as observable in the bone marrow culture of this disease. Soon after the beginning of culture the area of the young cell inner zone gradually starts to grow larger, reaching the maximum after 6-hour culture; but it again becomes by degree smaller with lapse of time. This seems to be due to the fact that the young cells on the periphery of the inner zone, maturing and acquiring the motility, have migrated to the outer zone. The relative growth rate after 12 hours was 26.2, and the cell density likewise proved to be quite high. As for the function of neutrophils, the wandering velocity after 6-hour culture proved to be 8.7μ/m. and the rate of phagocytosis of carbon particles was highest after six hours, namely, 0.75 and decreased slowly thereafter. The stainability to neutral red vital staining after three or four hours showed 1.71 and the color faded rapidly thereafter. Namely, the function of neutrophils on the whole was slightly lower than that of the normal persons.

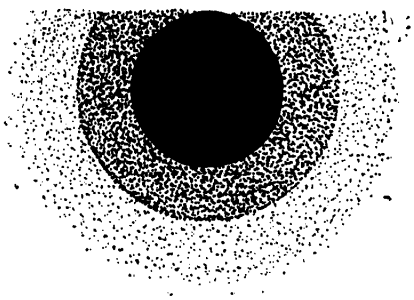

Fig. 6. Growth pattern in chronic myelogenous leukemia (model)

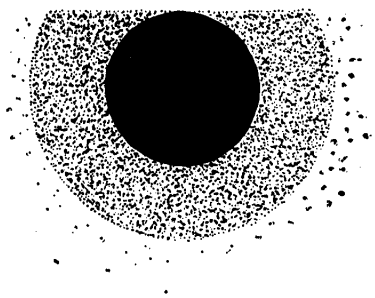

Fig. 7. Growth pattern of peri pheral blood culture in acute leukemia (model)

b) *Acute leukemia :* As shown in Fig. 7, in every type of this discase the periphery of the growth area, consisted of immature cells, is distinctly demarcated, presenting the findings characteristic of leukemia. Differing from that of chronic type, in the case of acute leukemia during the culture no mature cell outer zone can be seen as observed in chronic

leukemia. In addition, even in this instance the maturation of some immature cells can be observed and it is also possible to determine to what specific type of leukemia these cells belonged. The relative growth rate in the case of myelogenous leukemia was 9.15, which proved to be higher than that of the normal persons (5.72); while both that of monocytic and lymphocytic ones were below those of normal persons. As for the cell density, lymphocytic one gave the highest value, then myelogenous one; and monocytic one the lowest. These are, of course, much higher than that of the normal persons. The function of neutrophils decreased far greater than in the chronic type; and as for the wandering velocity after six-hour culture, myelogenous one gave 6.4 μ/m.; lymphocytic 4.8 μ/m.; and the rate of carbon particle phagocytosis at that time was 0.53 in myelogenous one, 0.52 in lymphocytic one; which decreased slowly thereafter. The stainability to neutral red vital staining is high in the early stages of the culture, namely, myelogenous one showed 1.82 and lymphocytic one 1.90 after three hours, fading rapidly thereafter. From these findings, in the peripheral leucocyte culture of acute leukemia the function of neutrophils was proved to have decreased. According to the literature, in his myelogenous peripheral blood culture of human leukemia of both chronic and acute types in the media of sera from various animals, SUZUKI[9] failed to touch upon the peculiar leukemic type of growth in the blood culture of leukemic patients such as mentioned so far in this paper.

Findings of leukemoid reactions in the peripheral leucocyte culture: By the peripheral blood cultures performed of the patients revealing the appearance of myeloblasts in the peripheral blood due to the metastasis of stomach cancer to the bone marrow thus presenting myelogenous leukemoid reaction as well as of the patients suffering from generalized tuberculous lymphadenitis complicated with tuberculous meningitis and presenting lymphocytic leukemoid reaction, it has been possible to distinguish these cases clearly from true leukemia. Namely, the margin of the growth area does not become clearly demarcated as in the case of the leukemia, but only the cell density yields a slightly higher value than that of normal persons and no decrease in the function of neutrophils as observable in the leukemia can be recognized.

Findings of the peripheral leucocyte culture of hypoplastic anemia; Although the number of leucocytes in blood of hypoplastic anemia is small, it is possible to perform the peripheral leucocyte culture by taking a larger quantity of blood. As shown in Fig. 8, it was very poor although the tissue growth shows a shape like a corona as in the case of the normal persons. Relative growth rate after three-hour culture was 2.85; after

six hours 3.83; and thereafter the growth ceased. Namely, the tissue growth rate of this disease is markedly lower than that of normal persons. In comparison with the normal persons the cell density also decreased. The wandering velocity of neutrophils yields the maximum value of 9.24 μ/m. after six hours, and decreasing rapidly down to 7.2 μ/m. after 9 hours and to 2.6 μ/m. after 12 hours; and after 24 hours almost no movement could be observed. The rate of carbon-particle phagocytosis gave 0.52 after six hours and it slowly decreased to 0.34, 12 hours later. The stainability to neutral red vital staining gave the maximum value of 1.68 after three hours; 0.84 after six hours; 0.11 after 12 hours; and thereafter it gradually faded. From these findings, in the peripheral leucocyte culture of hypoplastic anemia, the function of neutrophils was proven to have decreased.

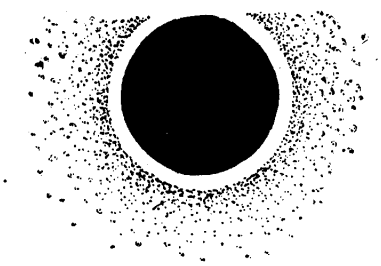

Fig. 8. Hypoplastic anemia in the peripheral blood culture (model)

CONCLUSIONS

With an improved method of tissue culture of peripheral leucocytes of our own design, the authors carried out systematic observations on the peripheral leucocyte culture from the normal, various kinds of leukemias, leukemoid reactions, and hypoplastic anemia. As for the culture method we have devised a method of silicon oil coating on the blood containers, a method which will least affect the cell function as compared with the conventional culture method. As the results we have found that the tissue growth in the case of peripheral leucocyte culture of normal persons ceases after six-hour culture and also we have recognized a peculiar finding, a growth like a corona-shape, in which an empty space appears in the inner part of growth area along with the lapse of time. In every leukemic case, without presenting growth area like a corona, the cell density is high and the outer zone of growth area becomes sharply demarcated as in the case with bone-marrow culture and also the growth continues even after 12 hours. At this instance we have noticed many mitoses of immature cells. Moreover, the maturation of immature cells has been observed and it has also been possible to distinguish to what type these immature cells belonged. In the leukemoid reaction no growth pattern characteristic to the leukemia can be recognized, and at a glance it can easily be differentiated from the leukemia. In the case of hypo-

plastic anemia although the growth area is like that of the normal presenting a corona-like shape, the cell density is lower and the function of leucocytes is lesser than the normal. From these results we believe that the peripheral leucocyte culture is clinically useful.

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EXPLANATION FOR PLATES

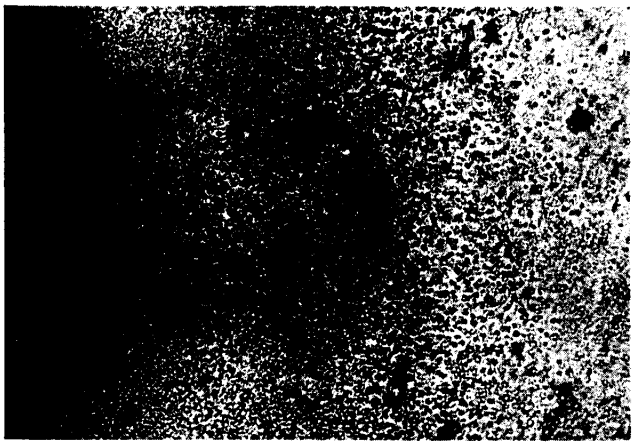
Plate 1.

a. Periphery of original tissue growing irregular (in the peripheral blood culture of normal person, 12 hr. culture, 7×10)
b. Double growth zone in the peripheral blood culture of chronic myeloid leukemia (6 hr. culture, 7×10)
c. Clear-cut margin in monocytic leukemia (12 hr. culture, 7×10)

Plate 2.

d. Mitosis in chronic myelogenous leukemia (peripheral blood culture, 12 hr. culture, slice specimen, Giemsa stain, 15×100)
e. Peripheral blood culture in hypoplastic anemia (12 hr. culture, 7×10)

Plate 1.



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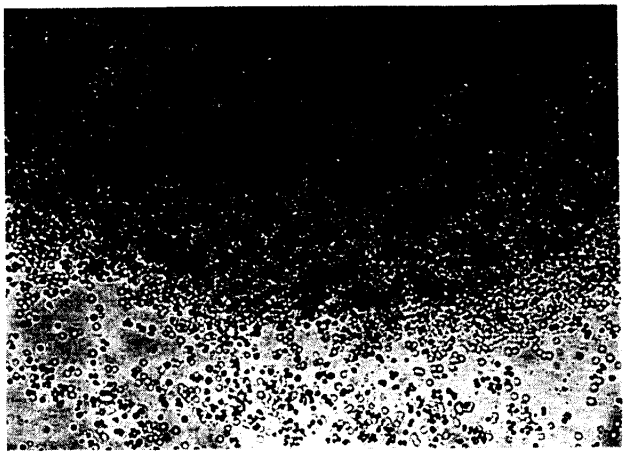

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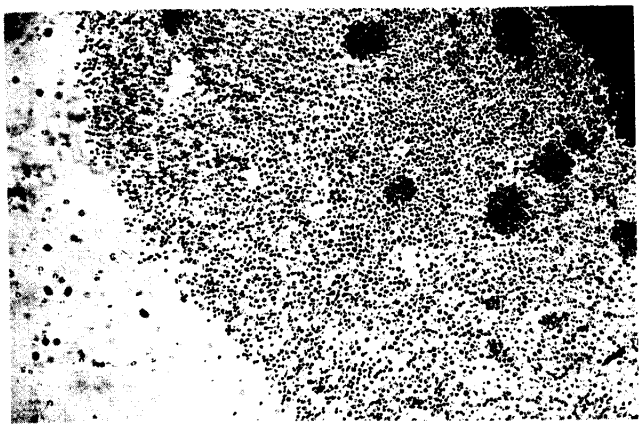

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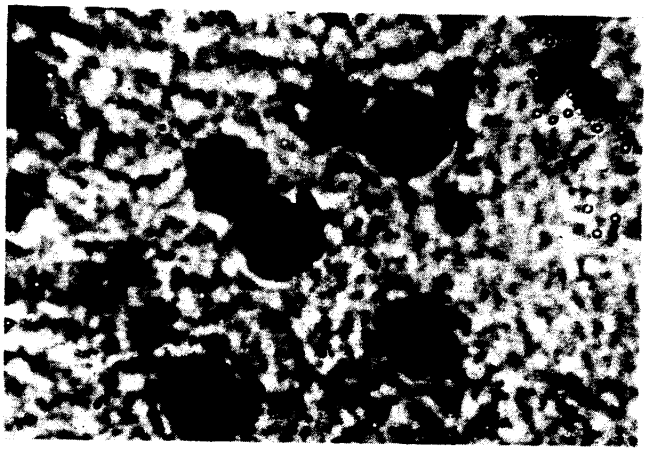
Plate 2.



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