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Abstract

Our methods of tissue culture of the bone marrow, lymphnodes, and peripheral blood were described. Furthermore, for the purpose of promoting wide clinical application of bone marrow tissue culture, our simple vital inspection method was also stated.

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THE METHOD OF TISSUE CULTURE (MAINLY OF THE BONE MARROW) AND A SIMPLE METHOD OF OBSERVING LIVING TISSUE

By

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For investigation of the movement of various living bone marrow cells, human and animal bone marrow tissues were cultivated and moving pictures were taken.

We brought this method into clinical practice and especially, the diagnosis of different blood diseases has been greatly facilitated in our department. The tissue culture was used mainly for the bone marrow, but also for the lymphnodes and peripheral blood.

In the following, we will chiefly explain the methods of bone marrow culture.

They are the coverslip, flask, fluid medium and rollertube methods. The coverslip method was used for morphologic and photographic study, the flask method for the study of the metabolism of the tissue, the fluid medium method for quantitative observation of cells and hemoglobin, and the rollertube method for cultures of a longer duration.

In order to make clinical use of the bone marrow culture easier and in order that it might be more widely propagated to general physicians, we simplified the coverslip culture method and made up a special method for observing living bone marrow.

A. Method of tissue culture of the bone marrow.

1. Materials :

Normal and pathological human and animal (rabbits, dogs, cats, mice, rats, frogs, and fowls) bone marrow. The human bone marrow was obtained mainly by sternal aspiration and partially from vertebral and iliac bones.

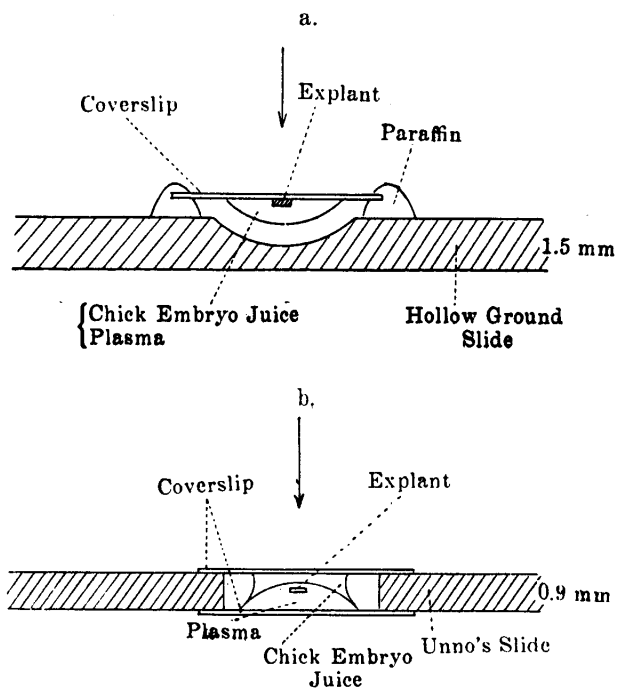
In animals, it was taken mainly from the femoral bones, and for fluid medium culture, from the femoral, tibial and brachial bones. It is, of course, understood that the following methods were completely aseptic.

2. *Methods of culture :*

a. Culture in coverslips (hanging drop-culture)..... (Fig. 1, a, b.)

Because the hollow ground slide could not be applied to the phase contrast microscope, "*Unno*'s slide" (perforated slide), Fig. 1, b. was used.

Fig. 1.



But "*Unno*'s slide" was inconvenient for thin layer cultures, therefore for bright field microscopy we used hollow ground slides (Fig. 1, a.).

As substrate, heparinized plasma of normal humans and rabbits was used, and, as growth promoting substance, the juice of chick embryos of 7—9 days old was used. To make the juice, several embryos were compressed by *Fischer*'s apparatus. The gruel was centrifuged for 15 minutes at 3000 r. p. m..

The supernatant fluid was diluted with Tyrode solution (supernatant.... 2 parts; Tyrode.... 1 part) in the case of rabbits, while in humans the supernatant fluid was directly used. Procedure of culture: (1) place a drop of heparinized plasma on a coverslip and spread it to a diameter of about 10—12 mm, (2) put a piece of the tissue on it, (3) add a drop of chick embryo juice, (4) after the coagulation of the plasma, cover the whole with a hollow ground slide and then seal with paraffin (or cover with a perforated slide touched with balsam, one side of which has been covered with a coverslip), (5) insert it in a 37°—38°C warm incubator. The microscopy was made in a warm box of the same temperature. Since "*Unno*'s slide" was only 0.9 mm thick, the fluid substance that was used as a medium filled the hole.

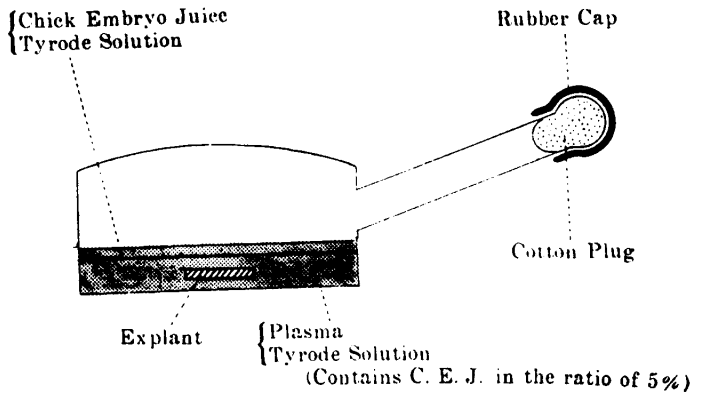
Thus the top of the plasmaclot viz. the specimen was brought into proximity with the opposite coverslip.

Therefore the migrating cells attached themselves to the surface of the glass. At this point the observation was performed from the side of the coverslip with the phase contrast microscope and also by photography.

As the hollow ground slide was too thick at the place of concavement to be observed by the phase contrast microscope, bright field microscopy was used.

In this case, therefore, the filming with the phase contrast microscope was impossible, but filming of the tissue growth by bright field microscopy was excellent. This was by far the best method for filming phagocytosis of carbon particles.

Fig. 2,

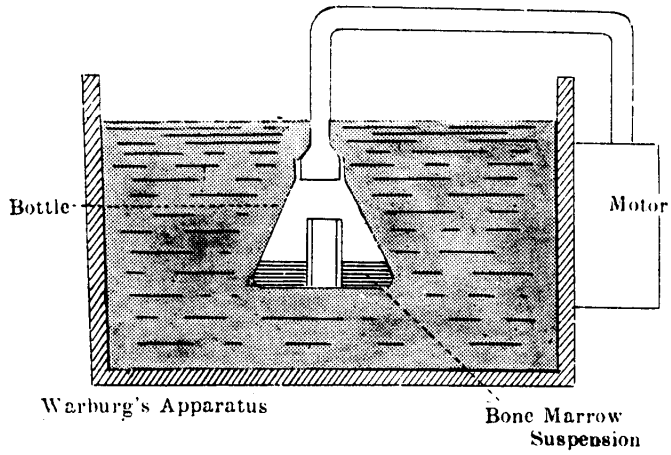


b. Culture in flasks (Fig. 2):

The medium for this method consisted of a solid medium and the supernatant fluid. The procedure of culture: (1) introduce 0.13—0.5 cc of plasma through the neck into the flask, (2) put the specimen in it, (3) add 0.07—0.15 cc of Tyrode solution with 10% chick embryo juice, (4) add the same fluid again after the plasma coagulates, (5) sterilize the opening with fire and insert a cotton plug and rubber stopper, (6) place in a 37°C incubator.

c. Culture in fluid medium (Fig. 3):

Fig. 3.



Osgood's[7,8,9] original method and *Norris*[6] and *Hay*'s[5] modification were further modified in our department. (1) The bone marrow as obtained from the femoral, tibial and brachial bone of young rabbits, was put in the *Gey*'s first fluid, (2) homogenized for 1 minute at a slow rate of rotation, (3) after centrifuging for 15 minutes at 3000 rotations per minute, its supernatant fluid was taken off, and the sediment was put into the Tyrode solution without glucose.

Thus the cell suspension was made. (4) 2 cc of this suspension were poured into each bottle of *Warburg*'s apparatus (38°C) and cultivated by shaking.

For human bone marrow, 0.4 cc aspirated fluid from the sternum was used and cultivated in the same way as for rabbits, but no homogenizing was necessary.

d. Culture in rollertubes :

The tissue fragment was cultivated on three places of a 60 mm long, 12 mm wide and 0.13—0.17 mm thick glass, in the same way as the cover slip method.

Then it was placed in a 15 mm caliber and 150 mm length tube, and the medium of 2 cc, consisting of *Hanks*' solution, E. E. I. and serum, was added.

This was then placed in a roller and cultivated at 12 rotations an hour.

After the chick embryo juice had been extracted, *Hanks*' solution was then added to the remaining substances, (ratio.... 1 : 1). These were then mixed together and centrifuged again.

The resultant supernatant fluid is called E. E. I.

3. *Method of observation :*

a. Measurement of the tissue growth (coverslip method) :

(1) Measurement of growth area :

In the coverslip method, the new growth of tissue was traced with Abbe's projectoscope, and its area was planimetrized. The difference between the areas before and after the culture (the absolute increase was measured), the ratio of this difference to the original area was estimated as the rate of relative increase of the growth area, and the ratio of this rate to the normal rate of relative increase was described as the growth index.

In the case of the flask method, because it could not be used, we applied a reflecting apparatus which we devised.

(2) Measurement of the cell density (Fig. 4) :

A microscope with the magnification of 100 × objective and 5 × eyepiece was used, and the peripheral, intermediate and central areas of the growth zone were investigated.

The number of cells in one field was counted and the total number of cells in all 3 fields was called the density rate.

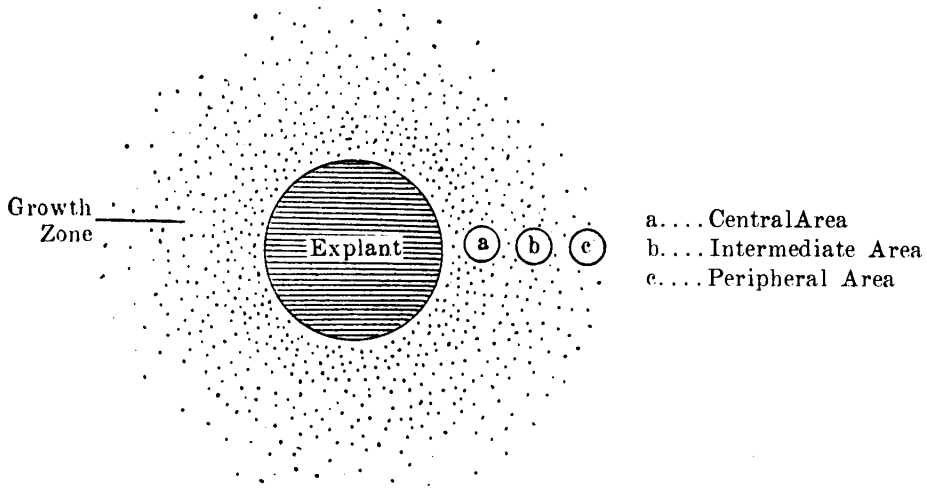
The ratio of this density rate to the normal density rate was described as the density index.

b. Observation of the function of cells (coverslip method) :

(1) Measurement of the wandering velocity : The wandering distance of the cell center was measured with a curve meter.

(2) Observation of the migration types : The migration was observed under the phase contrast microscope and bright field microscope.

Fig. 4.



$a+b+c$ = Density Rate

$$\text{Density Index} = \frac{\text{Density Rate}}{\text{Normal Density Rate}}$$

We then took moving pictures (process will be explained later) and studied them on the screen.

(3) Investigation of the phagocytosis of carbon particles: India ink was previously mixed with an equal quantity of chick embryo juice and then added to the medium. The phagocytosis was classified into 0—4 degrees, and the mean degree of phagocytosis for one cell was calculated.

(4) Investigation of vital staining: 0.01% of Neutral Red (medium concentration) was used as a basic dye, and 0.03% of Lithium Carmine (medium concentration) was used as an acid dye.

Each of them was previously mixed with the juice, half and half, and 1.5 droplets of the mixture were put into the medium.

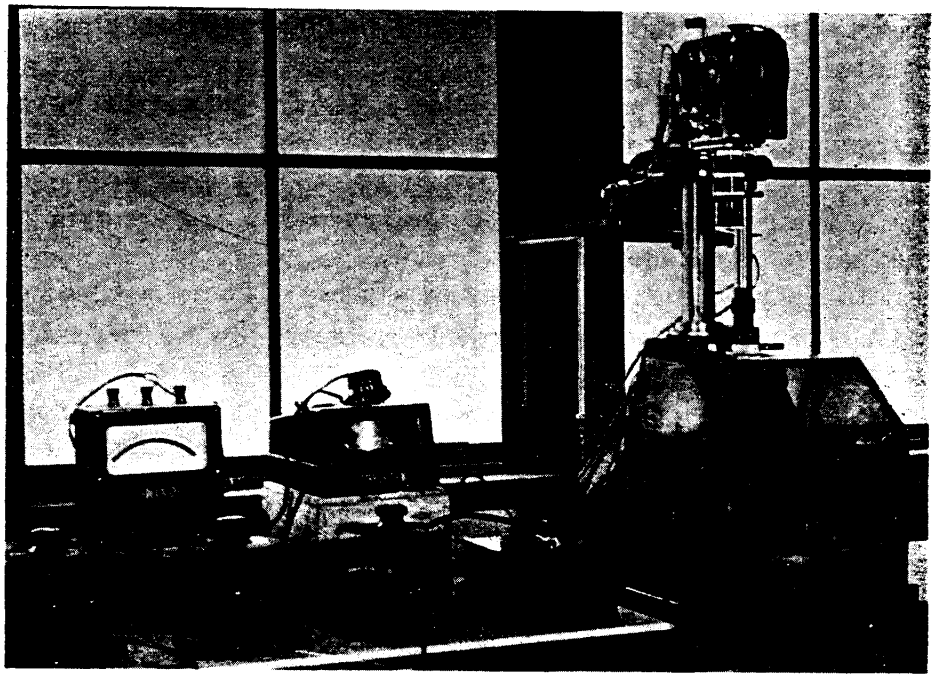
The mean degree of staining was calculated in the same way as that for phagocytosis.

c. Addition of the various substances into the medium. (coverslip method):

In the case of addition of serum, remedies, etc., a suitable quantity should be added before mixing the juice and the medium.

d. Photography (Fig. 5), (coverslip method)

Fig. 5. Microcinematographic apparatus.



For taking moving pictures, we used the *Bell* and *Howell*'s 16 mm cinecamera and *Paillard Bolex* 16 mm cinecamera and filmed each frame at intervals of 1 second and at intervals of 1 minute (according to materials).

e. Fixation and staining of the cultivated tissue (coverslip method):

(1) Fixation and staining of the growth zone; *Zenker*'s solution, *Bouin*'s solution and *Carnoy*'s solution were used for fixation. Staining was performed in *Giemsa*'s solution.

(2) Staining of the tissue. *Helly*'s solution was used for fixation. The tissue fragment was embedded in fresh paraffin and stained in *Giemsa*'s solution.

f. Quantitative observation of blood cells and haemoglobin (fiuid medium culture):

(1) Erythrocytes: The suspended cells that were being shaken in *Warburg*'s apparatus were sucked up with a sterillized melangeur and mixed appropriately with *Hayem*'s solution, and then counted.

(2) Nucleated cell count: *Tuerk*'s solution was used and observation was made in the same way.

(3) Reticulocytes: They were stained with 1% brilliant-cresyl blue and the number found in 2000 erythrocytes was counted.

(4) Quantity of haemoglobin: It was measured by the ferricyanide method using *Beckmann*'s electric spectrophotometer.

g. Quantitative analysis of the glucose and protein in the medium: (flask method)

(1) Quantitative analysis of glucose: The glucose of the supernatant fluid in the flask was measured by the *Hagedorn-Jensen* method.

(2) Quantitative analysis of protein: The total amount of protein was measured by *Biuret*'s method using *Beckmann*'s spectrophotometer. *Saito*'s method was used for the protein fraction and each fraction was measured by the *Biuret*'s method using a spectrophotometer. Fibrinogen was separated by calcium chloride and dissolved by caustic soda and then measured by the *Biuret*'s method using a spectrophotometer.

4. Condition of culture (coverslip method):

The tissue in the culture was influenced by various physical and chemical conditions of the medium. Therefore we investigated the influence of pH, temperature, and osmotic pressure, on the tissue culture of the bone marrow.

The results were as follows:

a. pH of about 7.63 was the optimum for growth.

b. Because the temperature of 35°—37°C showed the highest growth index, cell functions did not decline. Therefore, this temperature was best for cytological study. But for keeping the cells alive longer, a lower temperature was suitable.

c. Osmotic pressure: Growth was promoted somewhat by using in some hypotonic solution between (ΔT 0.45 ~ ΔT0.33) but generally we found the change to be negligible.

B. Culture of lymphnodes and pheripheral blood.

1. Culture of lymphnodes (coverslip method):

The same substrate and growth promoting substance as for

the bone marrow culture were used. Coagulated lymph, as a substrate, extract of spleen, *Lock*'s solution, extract of lymph-nodes and lymph as a growth promoting substance were useful substitutes.

2. *Culture of peripheral blood (culture of leucocytes)*:

a. Coverslip method: The heparinized peripheral blood was centrifuged for 10 minutes at 2000 r. p. m.. Its plasma was removed and 1—2 droplets of chick embryo juice were added to the upper leucocytic layer and then kept in an incubator for 5 minutes.

After the leucocytic layer coagulated, it was taken up with *Graefe*'s knife, cut into small pieces (after washing with *Riger* solution) and cultivated in the same way as the bone marrow.

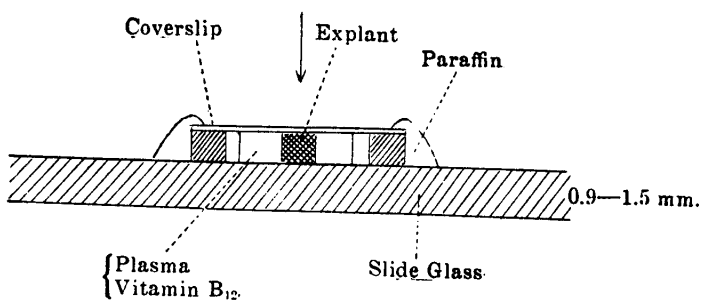
b. Fluid medium culture: The leucocytic layer was put into *Gey*'s first solution and centrifuged. The sediment was put in *Warburg*'s apparatus with 2 cc of serum of the same kind.

The same method as was used for the bone marrow was applied in the following procedure:

C. Simple method of observation of the bone marrow tissue and cells. (fig. 6).

As mentioned in the preceding part, the tissue culture of the bone marrow of various blood diseases by sternal aspiration was performed and a lot of new knowledge was obtained. This method was generally difficult for routine clinical use, because the materials (plasma, chick embryo juice, etc.) were not easily acquired, and skilled techniques were required.

Fig. 6.



Therefore, we made efforts to simplify this method in order

to make it more widely available for clinical use.

Our efforts proved successful. Therefore we were able by the following method to get results similar to that of the above-described culture method, and the phase contrast microscopy was also easy.

1. Materials :

We used serum and V. B_{12} instead of the plasma and chick embryo juice.

(a) Incubator, (b) microscope, (c) sternal aspirator, (d) knife and tweezers, (e) V. B_{12} (sold in market), (f) paraffin, (g) glass slide (as is shown in fig. 1, it has 2 ridges to the height of 100μ), and a coverslip.

2. Procedure :

a. The tissue fragment obtained by sternal aspiration was washed in *Ringer* solution.

b. A droplet of serum was placed on a glass slide and spread into a circle with a diameter of 1.5 cm.

c. The tissue fragment was placed in the center of the circle and a drop of V. B_{12} (100γ) was added.

d. A coverslip was laid on the bank so that it covered the medium.

e. After sealing with paraffin, it was placed in an incubator (37 C) with the side of the coverslip turned downward.

In this method, the growth area, cell density, and the functions of neutrophils (wandering velocity, phagocytosis of the carbon particles, and vital staining) were estimated in the same way as in the above-described tissue culture.

Comparing this with the tissue culture method, the tissue growth area in this method was somewhat larger. but its density index was slightly smaller.

As to the functions of the neutrophils, the wandering velocity was almost equal. but the degree of phagocytosis of the carbon particles was lower.

However the vital staining showed a higher degree.

When either the serum or V. B_{12} were used separately, both the tissue growth and the functions of the neutrophils were markedly depressed.

That is, until the serum and V. B_{12} were used together. it was impossible to maintain the functions of the cells.

Therefore. by combining serum and V. B_{12}, the functions of the cells could be satisfactorily maintained.

As mentioned above, for comparatively short periods of observation (24 hr.), this simple method showed almost the same results as the tissue culture method, and its procedure was so simple, that it could be widely applied to clinical use. It is especially recommended that this simple method be used by clinics, for the diagnosis of leukemia and aplastic anemia. This method is also the most convenient for phase contrast microscopy and we are now using it to study fine structures of cells.

Summary.

Our methods of tissue culture of the bone marrow, lymph-nodes, and peripheral blood were described. Furthermore, for the purpose of promoting wide clinical application of bone marrow tissue culture, our simple vital inspection method was also stated.

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