THE EFFECT OF REDUCED OXYGEN TENSION ON PLATELET FORMATION IN VITRO

BY

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It is now generally accepted that platelets are formed in the bone marrow from megakaryocytes. This suggestion was first clearly made by Wright (1906), who based his case on embryological and microscopical arguments as well as on considerations of comparative biology. The question was, however, settled when Thiery and Bessis in 1956 produced their beautiful film showing conclusively that megakaryocytes throw out long processes which become fragmented to form platelets. This was confirmed by Izak, Nelken, and Gurevitch in 1957.

Examination in this laboratory of some hundreds of bone-marrow cultures from human beings and animals by tissue culture methods did not, however, in any case disclose activity in megakaryocytes (Pulvertaft and Humble, 1956). On two occasions only they were seen to rotate; on one spidery processes were seen reminiscent of the film by Thiery and Bessis; in all other cases they remained spherical until, after a lapse of a few days, they disintegrated.

Nearly all the specimens examined in this series were, however, human; Thiery and Bessis worked with the rat. More important is the fact that they worked with isolated megakaryocytes seeded between coverslip and microscope slide, whereas the specimens personally examined were mounted on agar in a special chamber.

It was found that when bone-marrow cultures were submitted to low oxygen tensions great activity was shown by megakaryocytes, including platelet formation.

Technique

The culture chamber used was similar to a blood-counting chamber, having a central pillar recessed by 2 mm., surrounded by a ditch which was normally half-filled with culture medium. The central pillar was coated with 2% agar, and the medium used was 10% human serum, 0.1% "difo" yeast extract, and 90% Hanks' balanced salt solution. Streptomycin and penicillin were added, 100 units per ml.

The marrow was collected into heparinized human serum, usually from the same patient. When this is poured into a dish and allowed to settle, small fragments are usually seen. These consist of fat and blood vessels with bone marrow, and the smallest possible fragments are placed on the agar. A coverslip is applied and sealed with beeswax. After half-filling the ditch with medium through a lateral aperture, later sealed, the preparation is incubated, coverslip downwards.

Agar repels cells, and glass attracts them; the marrow cells are therefore flattened on to the glass in a manner not seen unless agar is used. Motile cells move peripherally, and in 18 hours megakaryocytes are clearly seen as dense spheres. They congregate along blood vessels and are usually not obvious until migration is established (Figs. 1, 2, 3).

After 18 hours the culture medium is withdrawn, and the slide is placed in a desiccator with the lateral aperture unsealed. The desiccator is then evacuated with a mercury pump, and the air is replaced by a mixture of 95% nitrogen and 5% CO₂. Meanwhile culture medium is placed in shallow dishes, and all dissolved gas is removed by a mercury pump in a desiccator. When bubbling has ceased, 95% nitrogen and 5% CO₂ is introduced, and the desiccator is sealed for 18 hours, during which time the medium is saturated with this gas mixture. Five per cent. CO₂ is of course essential in order to maintain pH at 7.4. The chamber is then filled with this fluid, making sure that no bubble of air remains. It is sealed with beeswax.

The chamber is mounted on a phase-contrast microscope in an incubator box. Cinematographic records are taken by time-lapse.

Results

During the first five hours the cells normally motile continue to move, but the megakaryocytes remain spherical. After five hours motility of megakaryocytes is observed.

It must be appreciated that these cells are in various stages of maturity, and that only fully mature cells can form platelets. These can be recognized from their peculiar mottled texture, identical with the appearance of coherent platelets collected from the buffy coat of heparinized blood.
FIG. 1.—(a, b, c) Specimen of mouse bone marrow (normal) between glass surfaces, to show blood vessels and megakaryocytes.
(d) Peculiar capillary end body.
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around, so that they cover a wide circular area, extending over many microscopical fields of a 1/12 lens. Such cells never develop further (Figs. 4 and 5).

They may also become sausage-shaped, and then become motile, progressing and elongating simultaneously (Fig. 6). Since they are at first spheres they make only point contact with the coverslip, and when this serpentine development sets in they are not adherent to the glass or agar, but progress in the fluid. Serpentine development may proceed to platelet formation.

Finally, true platelet formation develops by the extrusion of thick processes which elongate progressively and which eventually become fragmented. This process alone represents platelet formation, and only megakaryocytes which have the peculiar mottled appearance characteristic of maturity develop in this way (Figs. 7 and 8). Such cells in the early stages of platelet formation, while still spherical, are seen to be studded with small projections which make contact with the coverslip.

The very tenuous threads which are developed frequently are seen to expand and to contain nuclear fragments. The threads are readily broken up by motile cells, and it must be remembered that these "in vitro" experiments are unlike conditions in living marrow in that there is no circulation of blood; in life doubtless these threads are broken up by currents very quickly. In the slide preparations the threads often persist for 24 hours.

Those who do not examine living cells on a warm stage are apt to forget that the spherical forms seen in sections and stained films are not representative of living and active cells. The sphere is a defence mechanism, a form adopted when cells are chilled or otherwise insulted, and

Four types of motility are observed. The first is rotation, in either direction, at a rate of one full rotation in three to four minutes. This continues for many hours, and rotating cells have not been observed to develop in any way. Although the nuclei of cells often rotate, I have never seen any other human cell rotating in nitrogen, in air, or in oxygen.

Secondly, megakaryocytes are seen to spread over the surface of the coverslip. This they do by sending out pseudopodia symmetrically all
Fig. 5.—Spreading megakaryocyte, in nitrogenated medium. Sequence from film. $\times$ 840.
FIG. 6.—Elongating and progressing megakaryocyte, in nitrogenated medium. Sequence from film. x 390.
FIG. 7.—Platelet formation in nitrogenated medium. Sequence from film. × 840.
Fig. 8.—Platelet formation in nitrogenated medium. Sequence from film. × 390.
this is perhaps true of megakaryocytes as well as of polymorphs. We do not know from observation what the shape of a living megakaryocyte may be. They tend to collect around blood vessels, and therefore probably do not wander far. However, the number of controls in the present experiments is exceptionally large; about 2,500 bone-marrow preparations have been examined, in fluid saturated with air and 5% CO₂, without noting motility or any other activity in those cells, except the rotation twice observed. Almost invariably activity is seen in nitrogen after a lapse of approximately five hours.

It is not possible to say precisely how much oxygen is present in the preparations showing activity. The nitrogen used contained a maximum residuum of 0.1% oxygen, and not all the oxygen could have been removed from the agar cap by the vacuum.

The stimulus to megakaryocyte activity, including platelet formation, does, however, appear to be a sudden fall in oxygen tension, and this fits in with the known fact that platelets are increased following haemorrhage and in persons living at high altitudes. It is plausible to suggest that the reaction is a defence mechanism, considering the many ways in which platelets control haemorrhage.

The other forms of activity may be related to speed of megakaryocyte maturation. The larger the surface area of a cell the greater is its metabolic activity; oxygen deficiency may thus accelerate the development of these cells to the peculiar form which Bessis (1954) describes as thrombocytogenic. However, such maturation was not observed in vitro.

Summary

In the course of the examination of 2,500 slide cell cultures of human bone marrow, megakaryocytes remained spherical in all cases, and the only activity noted was rotation in two cases. When the oxygenated culture medium was replaced by one saturated with nitrogen and 5% CO₂, they showed activity after the lapse of five hours. The activity noted was (1) rotation, (2) elongation and progression, (3) wide spreading over a plane surface, (4) platelet formation.

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References