STUDIES ON BONE MARROW IN VITRO

III. THE EFFECT OF ANOXIA AND HYPEROXIA ON EXPLANTED BONE MARROW

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The effect of low oxygen tension on hemopoiesis is well known, since numerous observations have been made on men and animals living at high altitudes. It has been established that the increase in red cells and hemoglobin at high altitudes is not due to hemoconcentration or to an abnormal distribution of the blood, but to a real increase in production. The latter is manifested by a rise in reticulocytes in the peripheral blood and by erythroblastic hyperplasia of the bone marrow. These facts led to the widely accepted conception that even under normal circumstances bone marrow activity is largely regulated by the oxygen tension in this organ. Minot and Castle stated that "in the presence of the necessary chemical factors for normal maturation, the supply of red blood cells is regulated largely by the oxygen tension of the bone marrow." (p. 13) It is, however, unknown whether the bone marrow is directly influenced by the fluctuations of the oxygen tension, or whether the response of the bone marrow is provoked indirectly by other factors, initiated by the low oxygen tension, such as the appearance of incompletely oxidized metabolic products, or vasomotor phenomena. It seemed desirable, therefore, to study the direct effect of various oxygen tensions on bone marrow activity. Isolated bone marrow surviving in vitro offered a good opportunity for this purpose.

MATERIAL AND METHODS

The bone marrow from the tibia of 6-8 weeks old rabbits was used. The technic of explantation was that described in the first paper of this series. The glass tubes containing the bone marrow explants were placed open in a glass flask into which the desired gas mixture was introduced. The gas chamber (diameter 5.5 cm. and height 15 cm.) consisted of two parts, the flask and the cover, connected with a ground glass joint. The flask contained a rack for four culture tubes and a few cc. of water at the bottom to prevent desiccation. After placing the tubes with the cultures in the flask, this was tightly closed with the cover. The cover contained an inlet and an outlet tube of glass, through which the gas was circulated, the inlet tube; reaching the bottom of the flask, the opening of the outlet tube being only a few cm. below the top of the cover. In each experiment two liters of the desired gas mixture were driven through the apparatus and it was then closed by two glass stopcocks in the inlet and outlet tubes.

The gas mixtures were prepared in graduated bottles over water. For mixtures containing less than 20 per cent O₂, atmospheric air was diluted with nitrogen. For mixtures containing more than 20 per cent oxygen, pure oxygen and nitrogen...
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were mixed. Oxygen and nitrogen were taken from commercial cylinders, the nitrogen being passed before use over heated copper turnings. The in-going gas was always at atmospheric pressure. The gas chambers thus prepared as well as the control cultures were placed in the incubator at 37°C for 24 hours. Gas mixtures containing 1, 3, 5, 10, 12, 15 and 50 per cent oxygen were used. The control tubes containing atmospheric air were closed with corks as usual.

After incubation the bone marrow explants were fixed together with the plasma clot in Zenker's fluid. Serial sections 4 μ in thickness were cut from the material embedded in calloidin-paraffin. They were stained with hematoxylin-eosin and with Giemsa's stain.

Observations

Cultures of bone marrow incubated in an atmosphere containing 1 per cent oxygen.

The cultures maintained in an atmosphere containing 1 per cent oxygen were severely damaged. There were in all 7 cultures treated in this way and all of them presented a uniform picture of disintegration. The stroma appeared brownish in the hematoxylin-eosin preparation, and the stroma cells were mostly damaged. The hemic cells, erythroid as well as myeloid, showed marked signs of degeneration. The nuclei of the erythroblasts and normoblasts showed margination of chromatin or were fragmented, the promyelocytes and myelocytes showed karyolysis. Nuclear debris was scattered throughout the whole preparation. The only cells well preserved were the megakaryocytes. On the periphery of some explants single well preserved polymorphonuclear leucocytes and their precursors could be observed, and very exceptionally a myelocyte was found in mitotic division.

The surrounding plasma contained undamaged polymorphonuclear leucocytes.

Cultures of bone marrow incubated in an atmosphere containing 3 per cent oxygen.

Eleven explants were incubated in an atmosphere containing 3 per cent oxygen. Practically all of them showed signs of severe injury of the erythroid and the myeloid cells, similar to those seen in the cultures in 1 per cent oxygen. Here also the megakaryocytes were well preserved. The only difference worth mentioning between the cultures in 1 and 3 per cent oxygen was the presence in some explants maintained in 3 per cent oxygen of a peripheral zone containing a greater number of undamaged cells of all varieties and in all stages of maturation. In this zone mitoses were not rare. In the plasma numerous polymorphonuclear leucocytes were present.

Cultures of bone marrow incubated in an atmosphere containing 5 per cent oxygen.

In all 24 cultures were maintained in 5 per cent oxygen. Of these 19 were damaged and the remaining 5 were similar to the controls. The degree of damage was not the same in all injured cultures. Some offered a picture of complete disintegration, others were less uniformly damaged and showed well preserved cells in some peripheral areas of the fragment. In these areas usually the cells of the myeloid series prevailed. The mature leucocytes and erythrocytes were always well preserved.
Mitoses were present in the less damaged cultures, but their number was always less than in the controls.
Fig. 3, Experiment R32. Hematoxylin-Eosin. (X 450.)

a) Explant of bone marrow in atmospheric air after 24 hours of incubation.
b) Explant of bone marrow in an atmosphere containing 30% oxygen after 24 hours of incubation.

Cultures of bone marrow incubated in an atmosphere containing 10 and 12 per cent oxygen.

There is no essential difference between the behavior of the bone marrow cultures maintained in an atmosphere of 10 and 12 per cent oxygen, and that of cul-
tures maintained in an atmosphere of 5 per cent oxygen. Out of 28 cultures kept in an atmosphere containing 10 and 12 per cent oxygen, 22 were severely damaged. The remaining 6 were fairly well or well preserved. Here, too, cultures consisting predominantly of erythroid cells were more damaged than those in which the myeloid elements prevailed. The well preserved cultures contained cells in mitotic division, their numbers being, however, less than in the controls.

Cultures of bone marrow incubated in an atmosphere containing 15 per cent oxygen.

The cultures maintained in an atmosphere containing 15 per cent oxygen presented quite a different picture as compared with the cultures grown in atmospheres with lower oxygen content. Out of 11 cultures 8 were in good condition and similar to the controls. 3 cultures were somewhat damaged, the normoblasts being here also the most affected. Mitoses in the well preserved cultures were nearly as numerous as in the controls.

**Table 1.—Effect of Hyperoxia (50% Oxygen) on the Mitotic Activity of Bone Marrow in Vitro**

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>24 hours old explants in atmospheric air</th>
<th>24 hours old explants in an atmosphere containing 50% oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>R92</td>
<td>3.8</td>
<td>7.8</td>
</tr>
<tr>
<td>R93</td>
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</tr>
<tr>
<td>R94</td>
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<td>2.4</td>
</tr>
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</tr>
<tr>
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<td>4.8</td>
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<td>4.2</td>
</tr>
<tr>
<td>R99</td>
<td>0.6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Cultures of bone marrow incubated in an atmosphere containing 50 per cent oxygen.

The cultures maintained in an excess of oxygen presented a picture of definitely stimulated bone marrow. They were characterized by their rich cellularity and excellent condition of the individual cells (figs. 1 and 2). In all, 32 cultures were maintained in an atmosphere containing 50 per cent oxygen. Close examination of the cultures maintained at 50 per cent oxygen revealed a distinct feature in their cellular composition, namely the abundance of mature cells, mainly segmented leucocytes. They contained also a varying amount of precursors, predominantly myelocytes, often in mitotic division. The mitotic activity of the cultures was determined by counting the number of mitoses present in 500 cells capable of undergoing mitotic division. The counts made on the experimental cultures and on the controls revealed that the cultures maintained in 50 per cent oxygen contained a higher number of mitoses than the controls (table 1). It thus seems that the higher O₂-tension had a stimulating effect on multiplication and maturation.

**Discussion**

No data are available in the literature on the effect of various oxygen tensions on bone marrow cultivated in vitro. The behavior of cultures of other tissues under different oxygen tensions has been the subject of study by a number of investigators.
Burrows (1926-12) working with cultures of heart fibroblasts derived from chicken embryos of 4 to 5 days of incubation, found that they could survive and grow temporarily in the absence of oxygen. Fibroblasts of 10 to 15 day old embryos, however, grew only in an atmosphere containing 1.8 and 5.4 per cent oxygen respectively.

Wind (1926) examined the behavior of Rous sarcoma cultures under entirely anaerobic conditions. He found that in anaerobic conditions the cultures survived and the cells migrated and divided normally for 48 hours. If they were transplanted and cultivated for a further 48 hours under anaerobic conditions, migration occurred, but not as extensively as in the aerobic control cultures. During the third passage the cells in the anaerobic cultures died.

Mottram (1927) studied the effect of reduced oxygen tension on cultures of rat fibroblasts, kidney and Jensen sarcoma. He found that no cell migration took place in the cultures of fibroblasts and kidney at 40 mm. Hg. (3.2 per cent O₂) or lower tensions of oxygen. The cultures of Jensen sarcoma were less sensitive to oxygen want, since cell migration could be observed in these cultures even at 20 mm. Hg. (3.6 per cent O₂). No observations were made as to the occurrence and frequency of cell division in these cultures.

Wright (1928) determined the critical tension required for cell division in cultures of various tissues. He found that the lowest tension at which cell division takes place was: for chicken heart fibroblasts about 12 mm. Hg. (1.6 per cent O₂), for Jensen rat sarcoma 6 mm. Hg. (0.8 per cent O₂) and for mouse carcinoma 12.46 about 3 mm. Hg. (0.4 per cent O₂). The condition of the cells was good under tensions of 14.7 mm. Hg. (1.9 per cent O₂) and above.

Ephrussi, Chevillard, Mayer and Plantefol (1929), working with cultures of heart fibroblasts of chicken embryos, found that migration took place even in the complete absence of oxygen. However, cell division was inhibited under oxygen tensions of 7 mm. Hg. (0.9 per cent O₂).

Lipmann (1933) observed that outgrowth was possible, but at a very reduced rate, in fibroblast cultures under entirely anaerobic conditions. If respiration was reduced to 1/3, increase of area was diminished to 1/4.

Julius (1933) examined cell cultures in gas mixtures containing oxygen from 0 to 96 per cent. He found increase in area even at 1 per cent oxygen. The optimal conditions for growth were seen in an oxygen concentration corresponding to that of atmospheric air. Mitoses were scarce in pure nitrogen.

Gomirat (1934) found inhibition of growth, but no death of cells, in cultures of heart fibroblasts from chicken embryos maintained in pure nitrogen.

Knake (1934) is the only author who claimed a growth stimulating effect of reduced oxygen tensions (3 per cent) on fibroblast cultures. In very low oxygen tensions, such as 0.2 per cent, as well as in very high oxygen concentrations, i.e. 100 per cent, the cultures were damaged. Oxygen concentrations of 33 and 66 per cent did not affect the growth of fibroblasts.

The investigations cited above indicate that growth of different tissues in vitro is possible under very low oxygen tensions, and even under anaerobic conditions, but that the growth rate is considerably reduced. The few observations on mitoses in tissue cultures under low oxygen tensions always registered inhibition of mitotic activity. According to Wright mitoses in fibroblasts may be present in oxygen concentrations as low as 1.6 per cent, according to Ephrussi et al. even in concentrations of 0.9 per cent. Growth stimulation of mesenchymal cells, measured by the the area of outgrowth, caused by low oxygen tensions, was found only by Knake.

Several authors observed an injurious effect of low oxygen tensions on the cells. Wright found injury of fibroblasts in cultures kept in an atmosphere containing only 1.9 per cent oxygen, and Knake in an atmosphere containing 5 per cent oxygen. It must, however, be kept in mind that these authors studied the outgrowing cells and not the cells in the explant itself, as we did. Our experiments on bone marrow growing in vitro show that the reduction of oxygen concentration below 15 per cent has an unfavorable effect on the marrow parenchyma. This is manifested by
the poor preservation of the explants maintained under low oxygen tensions, and by inhibition of multiplication, whereas cell migration took place under all O$_2$-tensions tested. The lower the oxygen tensions under which the cultures were kept the more pronounced was the cell damaging effect and the reduction of mitotic activity. Single cells in mitotic division could be found even in gas mixtures containing 1 per cent oxygen, but the mitotic frequency was greatly decreased under reduced oxygen tensions as compared with the control cultures in air. Our observations indicate that erythroid cells are more sensitive to oxygen want than myeloid cells, hence the particularly poor preservation of cultures derived from predominantly erythropoietic bone marrow.

It is generally accepted that the oxygen physically dissolved in the plasma represents the immediate source of supply to the tissues. The quantity of the physically dissolved oxygen depends on the partial pressure of the oxygen in the atmosphere. Thus plasma saturated with alveolar air, containing 14 per cent oxygen, takes up 0.3 per cent oxygen, plasma saturated with pure oxygen takes up 2.2 per cent oxygen. The oxygen consumed by the tissues is constantly replaced in vivo by the oxygen dissociated from oxyhemoglobin. In our in vitro experiments the only source of oxygen supply is the respective gas mixture in which the cultures are kept. The volume of the gas mixture is comparatively large in relation to the size of the cultures, so that an ample supply of oxygen is secured during the relatively short period of incubation. It must, however, be kept in mind that the oxygen supply may be different in various layers of the culture, the gas penetrating more readily into the superficial than into the deep layers. This reservation holds true for all the cultures, including the controls in air, and our conclusions are based on the comparison of the conditions of the cultures kept in various oxygen tensions with those of the controls.

The lack of bone marrow stimulation by low oxygen pressure cannot be explained by mere technical shortcomings of our method, especially if a comparison is made between the cultures kept under low oxygen tensions and those under high oxygen tensions, i.e. 50 per cent oxygen, when a distinct stimulation could be seen. The stimulation was shown by an increased rate of multiplication as evidenced by the mitotic count, and by accelerated maturation, especially of the myeloid cells. Thus the cultures maintained at 50 per cent oxygen could be easily recognized by the rich cellularity and by the excellent state of the cells. The stimulated maturation, particularly of the granulocytes, caused by an excess of oxygen, is interesting in view of the frequent finding of leucocytosis in the peripheral blood in animals and men under high oxygen tensions. It is not, however, yet established whether this leucocytosis is a real one, or whether it is due to inflammatory changes in the lungs caused by inspiration of air rich in oxygen.

It is obvious that observations made on tissue surviving in vitro have only a limited value for the interpretation of processes taking place in the living body. This is also true in the case of anoxia and its effects on blood regeneration. However our findings justify a critical analysis of the widely accepted view that deficient oxygen supply to the bone marrow is the main stimulus for the increased production of red blood cells and hemoglobin.
Paul Bert and Viault were the first to observe increased hemoglobin and red cell formation in men and animals living at high altitudes. Miescher then advocated the view that the physiological regeneration of red cells and hemoglobin was governed by a relative oxygen want existing under normal conditions in the bone marrow. In anoxia bone marrow activity is stimulated to increased blood formation. Dallwig, Kolls and Loevenhart observed in animals subjected to low oxygen tensions an increase in hemoglobin and a marked extension of the red bone marrow. In accordance with the view of Miescher they came to the conclusion that the bone marrow was stimulated by the low partial pressure of oxygen. The same view was adopted by Schaumann and Rosenquist and by Loewy.

There are certain facts, however, already pointed out by Miescher, Morawitz and by Loewy which are difficult to fit in with the theory that reduced oxygen tension in the bone marrow itself is responsible for the physiological and the accelerated blood regeneration. If oxygen want is directly responsible for the increased hemopoiesis at high altitudes, one might expect a certain relation between the diminution of the oxygen tension and the degree of blood regeneration. This is, however, not always the case; definite bone marrow stimulation is already found at altitudes of 600-700 meters where a diminished oxygen tension in the tissues can hardly be expected. The increased blood production at altitudes of 2000 to 5000 meters can easily be explained by the reduced oxygen tension, but this increase does not continue at higher altitudes, such as 6000 meters and more, where the proportion of reduced to oxygenated hemoglobin increases rapidly (Hurtado).

According to the theory of the stimulating effect of oxygen want, one should assume that strenuous exercise which increases the anoxia, should also augment the blood regeneration. This, however, is not in agreement with the observations of Cohnheim and Kreglinger and Gross and Kestner who found that strenuous exercise at an altitude of 10,000 feet produced a fall in hemoglobin.

Furthermore, if oxygen want is the factor responsible for bone marrow stimulation, one should expect that atmospheres rich in oxygen should depress bone marrow activity. Experiments to this effect made on animals reveal contradictory results. Some authors found a decrease, others an increase in red cells. According to Karsner, prolonged exposure to high oxygen concentrations produced no changes in the erythrocyte count. In this connection the experiments of Boykott and Oakley are significant. These authors failed to find in rats an inhibiting effect of high oxygen concentrations on the regeneration of red blood cells after hemorrhages. The reticulocyte response was rather greater in the rats exposed to 65 per cent oxygen after bleeding and the color index was regularly lower than in the controls.

The definite stimulation of bone marrow activity at high altitudes may be due not to oxygen want in the marrow, but to compensatory factors initiated by the low oxygen tension of the atmosphere. The factors which come into play under low oxygen tension and which may compensate for the oxygen want are: increased respiration, increased arterial pressure, increased cardiac rate, and at very high altitudes, increased minute cardiac output. Sands and de Graaf found in dogs subjected to a reduced oxygen tension in the inspired air an increased systolic discharge, an increased heart rate and a reduced peripheral resistance, all factors leading to an increase of the minute flow of blood through the body. Thus it may
be assumed that in moderate degrees of anoxemia the blood flow may also increase in the bone marrow so that actually there may be no oxygen want, but even a compensating hyperoxemia in this organ.

According to Gesell32 a greater utilization of oxygen by the tissues and an actual increase in oxidative energy occurs even during the period of oxygen want, when a normal increase in ventilation is allowed to combat the shortage of oxygen in conjunction with circulatory adjustments. Thus one could assume that a state of "anoxic hyperoxia" might arise in analogy to the "hyperoxic anoxia" postulated by Bean33 in oxygen poisoning.

The effect on the bone marrow of compensatory processes taking place in anoxic conditions might explain the increased blood regeneration already found in low altitudes, and the fact that at very high altitudes, where the circulatory and respiratory adjustments may become insufficient, no further increase in blood formation occurs.

SUMMARY

The effect of various oxygen tensions on explanted bone marrow fragments was studied. It was found that gas mixtures containing 1, 3, 5, 10 and 12 per cent oxygen have an injurious effect on hemic cells. Bone marrow maintained in these gas mixtures showed various degrees of degeneration, which was the more pronounced the lower the oxygen tension. Mitotic activity was also found to be reduced under the influence of low oxygen tension.

Bone marrow cultures maintained in a gas mixture containing 15 per cent oxygen did not show appreciable changes and were similar to the controls.

Increased rate of maturation and multiplication occurred in bone marrow cultures maintained in an excess of oxygen, i.e. 50 per cent.

The significance of these findings in the light of observations on the effect of anoxia in vivo has been discussed, and reported findings on the effect of low oxygen tensions on other tissues in vitro have been briefly reviewed.

REFERENCES

STUDIES ON BONE MARROW IN VITRO


STUDIES ON BONE MARROW IN VITRO: III. THE EFFECT OF ANOXIA AND HYPOXIA ON EXPLANTED BONE MARROW

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