STUDIES ON THE MECHANISM OF ERYTHROPOIETIC STIMULATION IN PARABIOTIC RATS DURING HYPOXIA

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ALTHOUGH it is well established that polycythemia induced by exposure to low oxygen pressures is due to an increased erythropoietic activity, the mechanism by which the bone marrow is stimulated under these conditions is open to speculation. The widely accepted theory that the low pO_2, i.e., the partial pressure of oxygen, in the blood supplying the bone marrow represents the primary stimulus of erythropoiesis is based on the simultaneous finding of a low oxygen saturation in the arterial blood and an increased red cell production during hypoxia. But, so far, no experimental proof has been obtained of any direct influence on the erythropoiesis by the fluctuation of the pO_2 in the bone marrow blood. Besides, some evidence has recently been presented against that mechanism. Rosin and Rachmilewitz,[1] who discussed older objections against the pO_2 theory, exposed tissue cultures of bone marrow in plasma clots to various pO_2 by using as gas phase oxygen-nitrogen mixtures containing 1, 3, 5, 10, 12, 15, 21 and 50 volumes per cent O_2. They found the greatest erythropoietic activity in cultures exposed to 50 vol. per cent O_2 and an injurious effect of the gas mixtures containing from 1 to 12 vol. per cent O_2. They concluded that under these experimental conditions the decreased pO_2 did not stimulate erythropoiesis. With regard to stimulation of erythropoiesis in vivo, however, the results of these experiments must be interpreted with caution, especially because neither the O_2 consumption of the cultures, nor the O_2 diffusion constant of the particular medium, nor the pH were known. Consequently, the actual O_2 tension in the immediate vicinity of the explanted tissue was unknown and, judging from the results of the metabolic studies on tissue cultures, reviewed by Fischer,[2] was possibly extremely low with most of the gas mixtures used. Magnussen[3] used Osgood's bone marrow culture technic to study the production of erythrocytes in vitro. In a liquid medium, bone marrow of rabbits was kept in Warburg vessels in equilibrium with 100, 80, 50, 40, 30, 20.95, 10, 5 and 2.5 per cent oxygen in nitrogen. No remarkable difference was observed in cell formation at 30, 20 and 10 per cent of oxygen. Both 40 and 5 per cent gave a slight inhibition; at 50 and at 2.5 per cent of oxygen the production of erythrocytes was completely arrested. Magnussen concluded that in these experiments the direct action of a low oxygen tension on the erythroblasts was not capable of stimulating the production of erythrocytes in vitro.

Grant and Root[4] and Grant[5] measured the O_2 content in the blood drawn directly from the bone marrow of dogs both under normal conditions and after inducing anemic hypoxemia by either single large or repeated small hemorrhages. They found that a striking and prolonged stimulation of the erythropoiesis occurred even though the O_2 saturation of the bone marrow blood remained normal.

In the light of these observations the theory gains in interest, relating the

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stimulation of the erythropoiesis to humoral factors, the so-called erythropoietins. According to this theory, advanced by Carnot and Deflandre[6] and by Mueller,[7] it is believed that somewhere in the organism hypoxia produces specific factors which reach the bone marrow via the blood and stimulate erythropoiesis. A number of authors have tried to prove the existence of the erythropoietins by injecting plasma or serum, drawn from animals with induced hypoxia, into test animals and by observing its effects on erythropoiesis. The results of these studies, reviewed by Westphal[8] and recently by Bonsdorff and Jalavista,[9] are rather contradictory. However, it must be kept in mind that with this method only relatively small amounts of serum can be injected to avoid hemodilution or nonspecific protein effects. Besides, the weak response of the bone marrow to intermittent exposure to low pO_2 suggests that the erythropoietin—if it exists—is formed in small amounts only or destroyed rapidly. Therefore, this procedure offers a poor chance to demonstrate either the existence or nonexistence of the erythropoietins. For this reason, we used parabiotic rats, i.e., artificial Siamese twins, as a more adequate approach to this problem.

Plan of Investigation

In a parabiotically united pair of rats the vascular union (mainly capillaries) provides a constant transfer of blood from one partner to the other. There is, however, no connection of nerves or larger blood vessels (Schmidt,[10] Møller-Christensen[11]). Van Dyke and co-workers[12] found that of the total red cell mass approximately 0.64 per cent per minute flowed from one animal to the other, and that this rate is fairly independent of the duration of the parabiotic union as well as of the type of anastomosis used (muscle-skin connection or celiac anastomosis). The exchange of blood-borne substances has been demonstrated for hormones, antibodies and metabolic products (cf. Møller-Christensen,[11] Hill[13], Biddulph,[14] Furth,[15] Grollman,[16] Granger[17]).

On the other hand, the exchange of blood in a parabiotic pair is small enough to permit the experimental production and maintenance of different levels of O_2 saturation in the blood of the two animals. To this end, the parabiotic pair of rats is exposed to different O_2 tensions by means of a special breathing chamber. In this chamber the left partner breathes an oxygen-deficient gas mixture (e. g., 10 per cent O_2) and the right partner normal air. Whereas the O_2 saturation in the blood of the right partner remains normal, a chronic hypoxemia and, with it, the well known stimulation of the red bone marrow is produced in the left partner. It now remains to investigate whether or not the right partner, having normal oxygen saturation of the blood, shows an influence on its erythropoiesis.

Because of the exchange of blood cells between the two parabiotic partners, the erythropoietic response cannot be ascertained in the usual way from the peripheral blood values. Therefore, the percentage of nucleated red cells in marrow smears was used as criterion for the erythropoietic activity.

Methods

Rats (littermates weighing 70 to 100 Gm. from an inbred strain) were parabiotically united by an anastomosis of skin and muscles from the ears to the roots of the tails in ether anesthesia. The modifica-

tion of Bunster and Meyer,[15] suturing the shoulder blades of the two animals together, proved very helpful. It was essential to observe the animals for at least 3 months after the operation in order to exclude pairs with disharmonic parabiosis.

For five weeks the parabiotic pair was exposed in a special breathing chamber (fig. 1) to defined gas mixtures in such a way that the left partner was breathing an oxygen-nitrogen mixture whose oxygen content was gradually reduced from 12 to 8 vol. per cent C_2, whereas the right partner was breathing normal air. The breathing chamber was divided by a partition into two compartments, each of which accommodated one partner of the pair. The upper part of the partition was removable and, when adjusted, left an oval opening in which the parabiotic anastomosis was inserted. Before the animals were placed into this chamber the anastomosis was packed in greased cotton. The chamber was closed by a Plexiglas lid which was screwed on. The tails, protruding from the chamber, were tied with adhesive tape to prevent the animals from turning lengthwise. An airtight separation of the two compartments was not feasible because of the risk of restricting the blood flow in the anastomosis. Yet, to maintain the desired difference in the oxygen levels within both compartments, a constant flow of gas at a rate of approximately 1.5 liters per minute entered each compartment of the chamber through the funnel-shaped front opening and left it through the rear opening. Since dry gas was badly tolerated, the gas mixtures were saturated to a humidity of 80 per cent. During the first week of the experiment the animals were

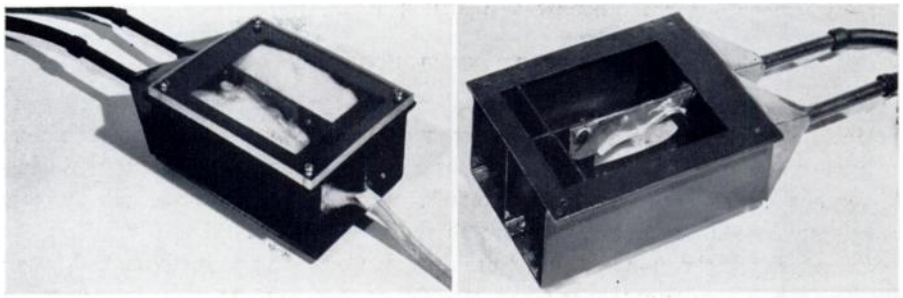

FIG. 1.—Breathing chamber for exposing parabiotic rats to different oxygen levels.

kept in this chamber for twelve hours and, later, as long as twenty-two hours a day. It was essential for a successful course of the experiment that the animals learned from the very beginning to eat the food (Purina laboratory chow and carrots) placed in the funnel part of the compartments. If the loss of weight exceeded 15 per cent of the original weight the experiment was stopped, likewise when the site of anastomosis showed inflammation or necrosis.

The O_2 contents of the gas mixtures in both compartments were measured every three hours with the Beckman electromagnetic oxygen analyzer and checked occasionally by gas analysis with the Haldane method.

O_2 content and O_2 capacity were determined in 0.5 cu. cm. of blood, drawn by puncture from the left ventricle, with the manometric method of Peters and Van Slyke. The heart puncture was performed quickly and carefully to insure unimpaired respiration. For the determination of the O_2 capacity the relatively small amount of blood was saturated in the syringe.

Tail blood was used for blood counts and hemoglobin determination after a ten minute immersion of the tail in water at a temperature of 40 C. Hgb was measured as oxyhemoglobin in the Evelyn photoelectric colorimeter. The reticulocytes were stained with brilliant cresyl blue in wet preparations. The bone marrow biopsies were performed on the femur after a skin incision 1 cm. long had been made under local anesthesia. After exposure of the bone a hole was drilled into the femur with an electric drill and a small amount of marrow was aspirated. The marrow was smeared and stained in the usual way. By using alternately the left and right femur and various parts of the bone, weekly marrow specimens could be obtained for seven weeks without difficulties and without impairment of the animals. For the differential count of the marrow only larger groups of cells were considered and 1000 cells were counted in each of two smears of the same marrow.

Results and Discussion

The maintenance of the different levels of O_2 saturation in the blood of the two partners was controlled: (a) by determination of the pO_2 in the two compartments of the breathing chamber and (b) by blood gas analyses of both partners. The gas mixtures inside the breathing chamber always differed by less than 1 vol. per cent O_2 from the expected values, i. e., if a gas mixture with 8 vol. per cent O_2 was administered to the left side and normal air to the right, the values found in the chamber were 8 to 9 per cent in the left and 20 to 21 vol. per cent O_2 in the right compartment. Repeated blood gas analyses could not be performed during the experiment because it was not possible, under these conditions, to draw arterial blood repeatedly without injurious effects. Attempts were made to determine the oxygen saturation in the heated tail by the oximeter method, but the results proved unreliable because the arterialization of the tail blood could not be achieved in every attempt. Separate experiments were, therefore, performed in several pairs to determine whether in parabiotic rats the arterial saturation corresponds to the inhaled gas mixtures, in particular whether one partner while breathing a gas mixture with low oxygen content influences the arterial saturation of the other twin breathing normal air, or vice versa. The results of one experiment are listed below:

Left Partner	*Right Partner*
Breathing Normal Air	Breathing Normal Air
Arter. Saturat. 97.6%	Arter. Saturat. 96.4%
Breathing 7.6% O_2	Breathing Normal Air
Arter. Saturat. 63.0%	Arter. Saturat. 97%

As was expected, on account of the minimal exchange of blood, no mutual influence was found on the arterial saturation of the two partners while breathing gas mixtures with different O_2 contents. Therefore, the analysis of the gas mixtures in the two compartments of one breathing chamber was considered to be an adequate control.

After observation for more than three months, 36 of the 139 operated pairs were found to be in harmonic parabiosis and were eligible for use in the experiments. Most of the other pairs showed unequal growth, or the so-called parabiosis poisoning (disharmonic parabiosis), in which, along with other disturbances, severe anemia develops in one partner, the mechanism of which is not understood so far. In 21 pairs, the experiment had to be discontinued because of weight loss of more than 15 per cent of the original weight, or because of necrosis on the site of anastomosis, or because of intercurrent diseases. In 9 pairs, the experiment was performed over a period of five weeks, as scheduled. In the left partner of these pairs, hypoxemia was induced as described, while the right partner was breathing normal air. Six more pairs were used as controls over the same period. Both partners of 3 control pairs were exposed to gas mixtures with low oxygen content, and both partners of the other 3 pairs to normal air in the breathing chamber.

The results of the bone marrow biopsies of these three groups are presented in table 1.

For interpretation of the results two fundamental sources of error had to be ruled out. The erythropoietic activity was evaluated by determining the percentage of nucleated red cells in bone marrow smears, i. e., the relative number of these cells as compared with other cells. The validity of this procedure, therefore, requires that the number of these other cell types, especially the granulocytes, remain constant within certain limits. To control this, the number of the peripheral

TABLE 1.—*Percentage of Nucleated Red Cells in the Bone Marrow of Parabiotic Rats before and during Exposure to Different pO_2*

Pair No.	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
	Before Exposure		During Exposure				
Group I: Left Partner Exposed 14–21 Hours Daily to 8–12% O_2 Right Partner Exposed to Normal Air							
37							
Left	30	34	40	54	52	62	66
Right	33	30	33	37	48	50	58
49							
Left	21	29	34	42	56	54	60
Right	28	28	37	41	49	53	55
67							
Left	41	27	29	51	56	71	64
Right	36	37	41	43	62	64	66
85							
Left	28	—	42	47	52	49	61
Right	31	—	24	32	47	61	58
117							
Left	21	39	32	47	64	63	67
Right	28	24	41	43	50	58	57
121							
Left	36	32	—	61	63	—	71
Right	32	28	—	51	52	—	64
128							
Left	—	29	42	49	57	71	66
Right	—	34	31	49	57	54	61
132							
Left	31	27	26	48	—	61	62
Right	24	26	34	43	—	58	60
Group II: Both Partners Exposed 14–21 Hours Daily to 8–12% O_2							
38							
Left	42	28	38	56	68	74	76
Right	28	34	41	59	71	68	73
42							
Left	22	34	—	68	58	72	68
Right	31	38	—	65	74	78	72
138							
Left	35	37	40	60	68	67	71
Right	42	37	44	64	61	70	70

TABLE 1.—*Concluded—Group III:* Both Partners Exposed to Normal Air

27							
Left	27	34	36	29	31	24	29
Right	31	32	26	31	35	25	—
68							
Left	35	35	26	31	—	32	29
Right	28	33	22	31	—	28	31
104							
Left	38	29	—	32	35	28	26
Right	26	31	—	31	27	29	35

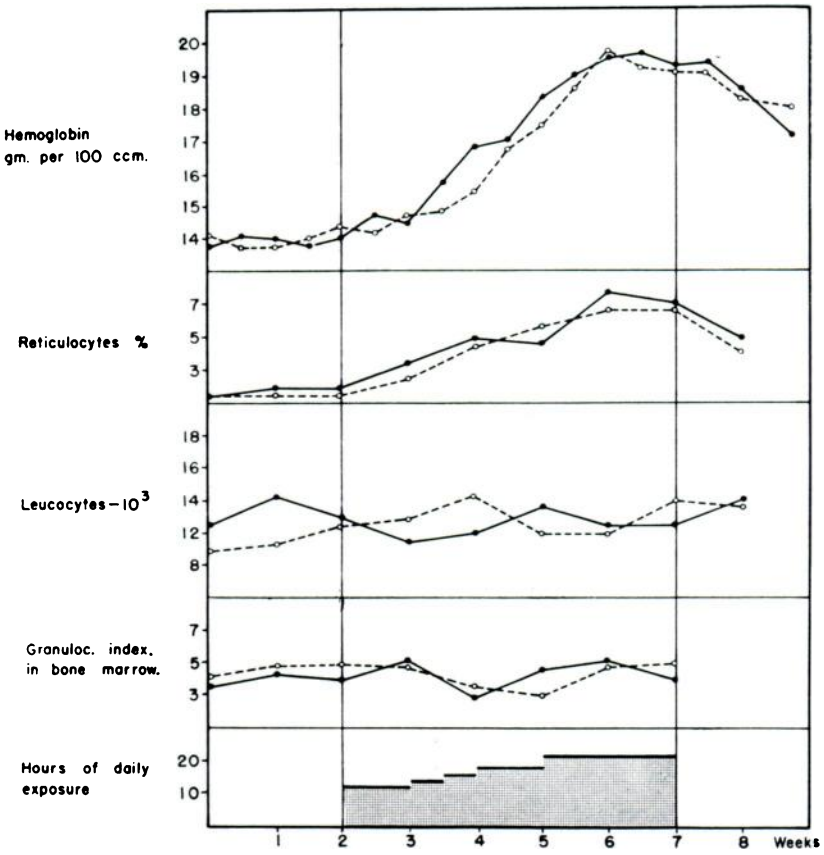

FIG. 2.—Blood values and granulocyte maturation index in one pair of parabiotic rats. Left partner (solid line) exposed to low pO_2, right partner (broken line) to normal air.

leukocytes and the maturation index of the granulocytes (promyelocyte-myelocytes: juvenile-segmented) in the marrow were determined regularly. Animals showing a leukocytosis or a shift of the granulocyte maturation index during the experiment were discarded.

Another source of error would arise if the mutual exchange of blood cells in both

partners were not in equilibrium. If, for instance, the partner breathing normal air supplied the hypoxemic partner with more cells than he received, the former would develop an anemia with a concomitant stimulation of the erythropoiesis. However, determinations of hemoglobin and erythrocytes in three-day intervals throughout the experiment lent no support to the existence of such a mechanism. To give an example, figure 2 shows the data of one pair during the experiment.

For purpose of the statistical analysis the results presented in table 1 were classified as follows:

Group	Exposure	Measurements of Table 1 Used
A	Both partners normal air	All of Group III, all before exposure in Group I and II
B	Both partners low pO_2	All of Group II during exposure (last three weeks)
C	Left partner, low pO_2. Right partner normal air	Left partners of Group I during exposure (last three weeks)
D	As in Group C	Right partners of Group I during exposure (last three weeks)

For these groups the observed means are:

Group	Number of Observations	Mean Percentage of Nucleated Red Cells
A	77	30.78
B	18	69.94
C	22	61.27
D	22	56.45

In an evaluation of the differences among groups as just defined, tests of significance using student's t and the pooled variance of 26.415 were employed. The results are summarized below:

Tests of Significance

Between Group Means	t	$d.f.$	$P.$
$\overline{A}$ and $\overline{B}$	29.10	93	0.0001
$\overline{A}$ and $\overline{C}$	24.54	97	0.0001
$\overline{A}$ and $\overline{D}$	20.66	97	0.0001
$\overline{C}$ and $\overline{D}$	3.11	42	0.001

On the basis of this statistical significance it seems reasonable to conclude that: (1) the increase in percentage of nucleated red cells is greatest when both partners are exposed to low pO_2; (2) when only one partner is exposed to low pO_2 and the other to normal air, both partners show substantial increases of the erythropoiesis, although not as substantial as when both partners are exposed; (3) when only one partner is exposed to low pO_2, the exposed partner shows an increase slightly above that of the partner breathing normal air.

The results suggest that in those parabiotic pairs in which one partner was exposed to low pO_2 and the other to normal air, an erythropoiesis-stimulating factor was produced in the organism of the hypoxemic animal. By way of the parabiotic anastomosis this factor was humorally transmitted to the other partner, in which it effected, although to a lesser degree, a stimulation of the erythropoiesis. The relatively lower erythropoietic stimulation in these pairs, as compared with a pair of which both partners were exposed to hypoxia, may be explained as due to either a larger distribution of the factor over two animals or to countermeasures taken by the animal living in normal pO_2.

Summary

Chronic hypoxemia was induced in one partner of a pair of parabiotic rats by exposure to defined gas mixtures with low oxygen content. The other partner of the pair was kept in normal atmosphere throughout the experiment and showed normal values of the oxygen saturation of the blood. The erythropoiesis, estimated by the percentage of nucleated red cells in the bone marrow, showed a statistically significant stimulation in both animals. It is, therefore, concluded that under these experimental conditions, the stimulus is not the partial pressure of oxygen in the bone marrow directly but a humoral factor elicited by the hypoxemia in the one partner and transferred to the other one.

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