The Role of the Kidney in the Erythropoietic Response to Hypoxia in Parabiotic Rats

By Wendell F. Rosse and Thomas A. Waldmann

The existence of a specific humoral substance stimulating erythropoiesis (erythropoietin, erythropoiesis stimulating factor, ESF) was first suspected by Carnot and Deflandre in 1906 and has since been amply confirmed. The site of production of this substance is the subject of considerable controversy. The occurrence of erythrocytosis with renal tumors and cysts and with hydronephrosis and the presence of anemia in chronic renal disease suggest that the kidney may be an important site. Some investigators (Jacobson et al., Naets, Reissmann et al.,) have not been able to demonstrate ESF in the serum of nephrectomized anemic, anoxic or cobalt-treated animals, whereas others (Mirand et al., Piliero et al., Gallagher et al.) have found increased serum ESF in response to some stimuli. The differences in the results may reflect the relative insensitivity of the assay systems used, which depend upon the injection of serum from the stimulated animals into recipient animals. Measurement of erythropoiesis directly in the animal stimulated by hypoxia, anemia or cobalt is a more sensitive system for measuring the presence of ESF than the indirect measurement which these workers used. Unfortunately, nephrectomized animals develop significant uremia in spite of peritoneal lavage before adequate stimulus can be given and response measured. Lack of erythropoietic response to anemia in such nephrectomized animals has been interpreted as being due to either the absence of the site of production or to the metabolic effects of uremia.

Parabiosis provides a preparation in which nephrectomy can be performed in one partner with survival of that partner and without uremic suppression of erythropoiesis of the other partner. Reissmann has shown that ESF passes from one animal to the other in such a preparation. Therefore, we chose to use parabiotic rats in an effort to estimate the role of the kidney and of extrarenal sites in the erythropoietic response to hypoxia.

Methods

Female litter mate Fischer strain rats weighing 50–65 Gm. were matched for size and parabiosed by the modified Bunster Meyer separate coelom technic. Parabiosis intoxication was not seen as determined by hematocrit determinations, weight gain and appearance of the rats. When the pairs weighed 220–240 Gm. (6–10 weeks after parabiosis), they were starved and divided into four groups. In three groups, one partner in each pair was nephrectomized bilaterally through flank incisions; in the fourth group, the ureters of one partner were ligated and cut.

Lucite chambers, modeled after those used by Reissmann, were used to provide an hypoxic environment to one partner only. Each chamber was divided into two compartments, each about the size of one partner, by a partition which was attached to the lid (fig. 1). This partition had a hole of such size and shape to admit the anastomosis be-
tween the partners. At the front of each compartment was an inlet through which humidified air from a wall jet with 20 volumes per cent O₂ or an hypoxic gas mixture could be admitted. Hypoxic gas mixtures were made by combining air from a wall jet in proper proportion with water pumped pure nitrogen to the desired oxygen saturation as monitored by an electromagnetic oxygen analyzer.* In an early group of experiments, 10–12 volumes per cent O₂ was used as the stimulus; later, 9 volumes per cent O₂ was used as it was found to be a better and yet well tolerated stimulus. The oxygen saturation within each compartment could be measured through two small holes near the front of the compartment using the oxygen analyzer. The flow of air or gas mixture was maintained at two liters per minute into each compartment as measured by a flowmeter;* the open rear of the compartment permitted flow rates of this magnitude.

When the animals were to be placed in the cage, their anastomosis was wrapped with vaseline petrolatum gauze. One partner was placed in each compartment and the lid and attached partition was screwed in place. Wooden blocks were inserted laterally as needed to restrain the animals lightly. The tails of the partners were loosely taped together with adhesive tape to prevent the animals from turning in the compartment. When these precautions were taken, it was easy to maintain the desired O₂ concentrations in each of the compartments.

The animals remained in the chambers for two 16-hour periods, the first the evening after operation, the second the following evening. The four groups were treated as follows: Group I (11 pairs): one partner nephrectomized, neither partner hypoxic; Group II (10 pairs): hypoxic air to nephrectomized partner; Group III (10 pairs): hypoxic air to the unnephrectomized partner; Group IV (8 pairs): hypoxic air to ureter-tied partner, room air to normal partner.

*Beckman Model D₂, obtained from Arnold O. Beckman, Inc., South Pasadena, Calif.
*Hoke model 2113 Flowmeter, obtained from the Hoke Co., Englewood, N. J.
KIDNEY IN ERYTHROPOIETIC RESPONSE TO HYPOXIA

Erythropoiesis was measured by a modification of the starved rat assay of Fried et al. Eight hours after removing the pair from the chamber following the second period of confinement, one μc of Fe⁵⁹ citrate in one ml. of saline was injected intraperitoneally into each partner. Sixteen hours later, blood was removed by cardiac puncture from each partner and the radioactivity in one ml. was determined in a well type scintillation counter having an activated NaI crystal. The per cent incorporation of Fe⁵⁹ into the circulating red blood cells was estimated using the following formula:

$$\text{counts per minute ml. x 0.05 x wt. of pair} \div \text{total counts injected per partner}$$

where blood volume is assumed to be 5 per cent of body weight and the total counts injected are estimated using a diluted standard. As estimated in this way, the differences in iron incorporation between partners of a pair were small, averaging 1.4 per cent, since the two had a common intravascular system. Thus, in final calculations, the average of the incorporations for the two partners was considered the incorporation of the pair.

Microhematocrit determinations were performed on each sample of blood. Blood urea nitrogen determinations were done on the blood from each partner by the urease method.

RESULTS

The results of the iron incorporation into peripheral red blood cells are tabulated in Table 1. Groups designated IIA, IIIA, and IVA are from preliminary experiments wherein the hypoxic stimulus was 10–12 per cent O₂. This degree of hypoxia was felt to be inadequate because of insufficient differences between unstimulated pairs (Group I) and pairs in which the hypoxic partner was not nephrectomized (Group IIIA). Therefore, the oxygen saturation was reduced to 9 per cent ± 0.5 per cent O₂. Pairs receiving this stimulus are designated Group II, Group III and Group IV.

At this level of hypoxia, there is a very significant difference in red cell radioiron incorporation between unstimulated pairs (Group I) and pairs in which the non-nephrectomized partner was hypoxic (Group III) (t = ±5.45, P < 0.01), indicating that the stimulus was adequate. There is no difference between Group III and Group IV (ureter-ligated partner hypoxic), indicating that mild uremia of the stimulated partner does not alter the ability of the pair to respond. There is significantly less red cell radioiron incorporation in Group II (nephrectomized partner hypoxic) than in Group III (non-nephrectomized partner hypoxic) and/or stimulated with hypoxia.

Table 1.—The Per Cent Fe⁵⁹ Incorporation into Peripheral Blood Cells in Parabiotic Rats, One Partner of Which Was Nephrectomised or Ureter Ligated and/or Stimulated with Hypoxia

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of pairs</th>
<th>Partner nephrectomised</th>
<th>Partner ureter tied</th>
<th>Per cent O₂ used as stimulus</th>
<th>Per cent Fe⁵⁹ incorp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I IIA</td>
<td>5</td>
<td>left</td>
<td>0</td>
<td>10–12%</td>
<td>7.2 (1.72)</td>
</tr>
<tr>
<td>IIIa</td>
<td>5</td>
<td>left</td>
<td>0</td>
<td>10–12%</td>
<td>11.8</td>
</tr>
<tr>
<td>IVa</td>
<td>4</td>
<td>0</td>
<td>left</td>
<td>10–12%</td>
<td>13.9</td>
</tr>
</tbody>
</table>

*Standard error of the mean.
tomized partner hypoxic) \( (t = \pm 2.55, p = 0.02) \) or Group IV (ureter-ligated partner hypoxic) \( (t = \pm 2.24, 0.05 > p > 0.02) \), suggesting that the kidney plays an important role in stimulation of erythropoiesis by hypoxia. But most important is the highly significant difference between Group I (unstimulated) and Group II (nephrectomized partner hypoxic) \( (t = \pm 2.96, p < 0.01) \), suggesting that stimulation of erythropoiesis can take place in the absence of the kidneys.

The results of blood urea nitrogen determination are tabulated in table 2. The values in the left partners are elevated in all groups (normal BUN for the rat = 12 mg. per cent). The BUN of the right partners is slightly elevated, possibly a reflection of increased urea load from the operated partner. However, there are no significant differences in the average BUN of the right partners of groups I-IV or of the left partners of these groups.

**Discussion**

The significant differences in the Fe\(^{59}\) incorporation into peripheral red blood cells between those pairs in which the nephrectomized partner was stimulated (Group II) and those in which the non-nephrectomized or ureter-ligated partner was stimulated (Groups III and IV) suggest that hypoxia of the kidney is a potent stimulus for erythropoiesis. Since the blood urea nitrogen level is the same in the ureter-ligated and nephrectomized groups, suppression of either production of ESF or of the responsiveness of the marrow of the stimulated partner cannot explain this difference in iron incorporation. This work appears to confirm the findings of others which indicate that the kidney is an important site of production and/or activation of ESF.\(^7\)

More important, the significant difference in Fe\(^{59}\) incorporation between the animals of Group I (nephrectomized and unstimulated) and Group II (hypoxia to the nephrectomized partner) suggests that the kidney is not necessary for an erythropoietic response to hypoxia and infers that there exists an extrarenal site or sites of production or activation of ESF. It is possible that the erythropoietic effect seen in Group II is not mediated by a humoral agent, but a considerable body of evidence has shown that hypoxia has no stimulating but rather a suppressive effect directly on erythropoiesis in the bone marrow.\(^19\)

We feel that the increased erythropoiesis seen in Group II could not be due to arterial oxygen desaturation of the non-nephrectomized partner. According to Huff et al.,\(^21\) 0.66 per cent of the total blood volume is transferred across the anastomosis between parabiotic rats per minute. This amount of desaturated blood

| Table 2.—Blood Urea Nitrogen Determinations in Parabiotic Rats—The Left Partner of Each Pair Has Been Nephrectomized or Ureter-Ligated |
|---|---|---|---|
| Group | Operation to left partner | Blood urea nitrogen in mg. % |
|     |     | Left partner | Right partner |
| I   | nephrectomy | 48.9 (4.5)* | 23.6 (2.1) |
| II  | nephrectomy | 55.2 (6.8) | 28.7 (5.0) |
| III | nephrectomy | 65.8 (3.7) | 20.4 (2.1) |
| IV  | ureter ligation | 52.6 (2.4) | 21.2 (2.2) |

*Standard error of the mean.
blood from the hypoxic partner passing through capillary connections into the venous system of the other cannot possibly significantly lower the $O_2$ saturation of the arterial blood of the non-hypoxic partner. Also, in a very similar experimental situation, Reissmann\(^{16}\) measured the cardiac blood from each of the partners while one was receiving hypoxic air and found that the partner breathing room air had normal arterial oxygen saturation.

An extrarenal site of ESF production has been suggested by others. In more recent experiments, Jacobson et al.\(^{22}\) were able to demonstrate low and inconstant reticulocytosis in polycythemic mice injected with serum of nephrectomized mice subjected to anemia by phenylhydrazine or of nephrectomized rats stimulated by hypoxia. He considered this evidence that about 10 per cent of ESF may come from an extrarenal site of production.\(^{23}\) Gallagher et al.\(^{13}\) were able to demonstrate ESF in the concentrate of plasma of nephrectomized rabbits stimulated by hypoxia or anemia. Also, Erslev showed\(^{15}\) that the erythropoietic tissue in nephrectomized rabbits exposed to the stimulating effect of severe anemic anoxia maintains some functional activity despite the complete absence of renal tissue.

Tumors of organs other than the kidney have been associated with erythrocytosis. Hemangioblastoma of the cerebellum with or without other manifestations of von Hippel-Lindau syndrome have been associated with marked erythrocytosis and, in one case, Waldmann et al.\(^{24}\) demonstrated erythropoiesis stimulating material in the cyst fluid from such a tumor. Erythropoiesis stimulating activity has been demonstrated in tissue extracts of a pheochromocytoma associated with erythrocytosis which remitted on resection of the tumor.\(^{25}\) Tumors of other organs have also been associated with erythrocytosis but no evidence of ESF has been presented.

The extrarenal tissues which are sensitive to hypoxia in causing erythropoiesis in the present experimental situation are not known. Further, it is not possible to state whether the extrarenal tissues respond to stimuli other than hypoxia. These and other questions about extrarenal tissues which may play a part in the control of erythropoiesis remain to be answered.

**Conclusions**

1. Parabiotic rats, in which one partner was either nephrectomized or ureter ligated, have been used to determine the role of the kidney in the stimulation of erythropoiesis by hypoxia as measured by $^{59}$Fe incorporation into the peripheral RBC.

2. The radioiron incorporation into peripheral red blood cells was less in pairs in which the nephrectomized partner was hypoxic than in the pairs in which the unoperated or ureter ligated partner was hypoxic, suggesting that the kidney is important in the stimulation of erythropoiesis in response to hypoxia.

3. The iron incorporation in pairs in which the nephrectomized partner was hypoxic was greater than in those pairs in which one partner was nephrectomized but neither was hypoxic, suggesting that tissues other than the kidney contribute to the erythropoietic response to hypoxia.
SUMMARIO IN INTERLINGUA

1. Rattos parabiotic—con un del partenarios subjicite a nephrectomia o a ligation del ureter—esseva usate pro determinar le rolo del ren in le stimulation del erythropoiese per hypoxia, mesurate per le incorporation de ferro⁹ in le erythrocytos peripheric.

2. Le incorporation de radio-ferro in le erythrocytos peripheric esseva minus in pares con hypoxia in le nephrectomisate partenario que in pares con hypoxia in le non-operate partenario o in le partenario con ligation del ureter. Iste constatation pare indicar que le ren es importante in le stimulation del erythropoiese in responsa a hypoxia.

3. Le incorporation de ferro in pares con hypoxia in le nephrectomisate partenario esseva plus grande que in le pares in que un partenario esseva hypoxic durante que hypoxia esseva absente in ambes. Iste constatation pare indicar que tissu altere que le ren contribue al responsa erythropoietic a hypoxia.

REFERENCES

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