

The Origin of Erythropoietin in Rats with Transplants of an Adrenal Cortical Carcinoma¹ (34280)

HOWARD A. MEINEKE²
(Introduced by Roger C. Crafts)

Department of Anatomy, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45219

A polycythemia or erythrocytosis has been reported to accompany the presence of various types of tumors in experimental animals and man, and the pertinent literature has been reviewed (1-6). In many of these cases erythropoietin was demonstrated in the serum or in extracts of the tumors. The present study involved a transplantable adrenal cortical carcinoma derived from one first described by Snell and Steward (7) in the Osborne Mendel strain of rats. While this tumor was accompanied by a true polycythemia directly related to the presence of the tumor, Swarm *et al.* (8) and Waldmann *et al.* (3) were unable to demonstrate erythropoietin in the serum or in tumor extracts. Furthermore, the erythrocytosis was not reduced by blood transfusion but did cease after surgical removal of the tumor. These results suggested the presence of an erythropoietic factor other than erythropoietin. Testing the possibility that this agent was a combination of adrenal corticoids and androgens, Waldman *et al.* (3) were unable to duplicate the degree of polycythemia induced by the tumor with prolonged combined injections of testosterone enanthate and ACTH in normal rats. In contrast to these findings, Meineke and Feleppa (9) demonstrated that erythropoietin could be detected in the serum of rats bearing such tumors when the serum was obtained within approximately 3 weeks after transplantation.

This discrepancy in findings prompted the present study. Experiments were designed (i)

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to see whether these differences can be resolved, (ii) to verify that the erythropoietic factor is actually erythropoietin, and (iii) to determine its source.

Materials and Methods. Rats of the Osborne Mendel strain were used; these were inbred within a colony maintained in this laboratory. The stock animals and original tumor were obtained through the courtesy of Dr. Richard Swarm. Rats were given food and water (containing oxytetracycline) *ad libitum*, supplemented once a week with lettuce. Tumors were transplanted subcutaneously in all cases. A modification of the method described by Contrera *et al.* (10) was used for erythropoietin assays. Mice, CFI females, were made polycythemic by interrupted exposure to a simulated altitude of 23,000 feet for a total of 200 hr over a period of 10 days. They were removed from the chamber on day 0, and injected with 0.5 ml of test material on days 4 and 5; 0.5 μ Ci of ⁵⁹Fe was injected on day 6 and the rate of ⁵⁹Fe uptake was measured 48 hr later, on day 8. In the calculation for the percentage of uptake, total blood volumes were estimated to be 7.3% of body weight. In the studies where tissue or tumor extracts were used, material was obtained as follows: the tissue, or encapsulated tumor mass, was excised and wet weighed; this material was homogenized mechanically with saline and the resultant sludge was centrifuged at 2000 rpm for approximately 1 hr; the supernatant was removed and diluted with saline so that 1 ml of final solution represented 2 g of original tissue. In the experiments where enzymatic digestion was employed, neuraminidase (*Vibrio cholerae*)³ was used. Phosphate buffer,

³ Calbiochem, Grade B.

TABLE I. The Effects of Serum and Saline Tumor Extracts of Nephrectomized and Sham Operated Tumor Bearing Male and Female Rats on the ^{59}Fe Uptake of Polycythemic Mice.^a

	Serum				Tumor extract			
	Female		Male		Female		Male	
Normal (6)	Sham (10)	Neph. (7)	Sham (10)	Neph. (13)	Sham (8)	Neph. (8)	Sham (7)	Sham (9)
0.34 ± 0.05	13.27 ± 2.37	10.10 ± 1.58	2.53 ± 1.40	3.80 ± 0.76	11.38 ± 2.21	8.84 ± 2.13	10.00 ± 2.20	8.18 ± 1.10

^a All values are % \pm SE.

pH 5.6, was used as a diluent and incubation was carried out for 60 min at 37°. After incubation the material was divided into aliquots for each day of injection and frozen until used.

Procedure and Results. The experiments were divided into essentially three separate studies as follows: (I) those studies designed to determine whether the level of erythropoietic factor in the serum of animals having transplanted tumors for short periods of time differed from that of rats bearing tumors for a long interval of time; (II) experiments directed at determining the source of the erythropoietic factor; and (III) those studies designed to provide evidence that the erythropoietic factor in these animals was actually erythropoietin.

I. In the first series of studies male rats were used. Serum was pooled from each of 6 rats (hematocrits all above 52) having had tumor transplants 24 days prior to autopsy and from each of 4 rats (hematocrits all above 60) bearing tumor transplants for 60 days. The serum was assayed by measuring the ^{59}Fe uptake of polycythemic mice following injection of the sera as described previously. The results showed that there was no significant activity in the serum of the older rats compared to normal rat serum, $0.47 \pm 0.02\%$ ⁴ vs. $0.38 \pm 0.02\%$, whereas significant activity, $2.44 \pm 0.39\%$, was found with the serum from the rats bearing tumors for a short term.

II. The second series of studies, designed to determine the source of the erythropoietic factor, was divided into two parts. In the first, tumors were removed from the animals

used in the above study, plus those from 3 additional older male rats, and extracts were prepared as described in "Materials." Extracts of normal liver and of livers from tumor-bearing rats served as controls. The ^{59}Fe uptakes were $19.03 \pm 1.37\%$ for the extracts of the tumors from the younger rats and 16.34 ± 1.49 for the older tumors. These are highly significant responses when compared to similarly prepared extracts of normal liver, $0.25 \pm 0.05\%$, and liver tissue from the tumor-bearing rats, $0.37 \pm 0.05\%$. The results of the first part of this study suggested that the tumor itself is the source of the erythropoietic factor. These values also show, in contrast to the findings for the serum of these animals, that the tumor extract from the older animals is just as active as that from the younger rats.

The second part of this series utilized both male and female rats. This part of the experiment was directed at determining whether the circulating erythropoietic factor might be the result of some stimulatory action of the tumor on the kidney so that erythropoietin production was in this manner indirectly related to the presence of the tumor. Tumors were transplanted and 17 days later rats were placed into 2 groups, nephrectomized (4 female and 6 male) and sham operated (4 female and 4 male). Forty-eight hr later, serum and tumor extracts were obtained and pooled for each group. The results were shown in Table I.

The failure to see significant differences in any of the groups supports the view that the erythropoietic factor in the serum of tumor-bearing rats is due to its production by the tumor itself and not through a secondary

⁴ Standard Error of the Mean.

effect on the kidney. It should also be noted that there was a difference in the erythropoietic activity of the serum in the male and female rats, a finding that has not been consistently seen in all other studies. In contrast, the activity of the tumor extracts was essentially the same in the two sexes.

III. In view of these and other findings, it seemed advisable to present more direct evidence that the active material was indeed erythropoietin and not some combination of steroids. Accordingly, a large pool of tumor extract was obtained from 15 male and 11 female rats 16–20 days after transplantation. After the extract was prepared as described previously, one alteration in the procedure was instituted; *i.e.*, the extract was ultracentrifuged for 2 hr at 144,000g; the supernatant from this was divided into several aliquots and served as the source of material for most of the following studies.

Initial studies were done to see if the responses in ^{59}Fe uptake seen in the previous assays were reflecting maximum activity of the extract. One aliquot was taken and divided so that dilutions of 2, 4, and 8 were made. When these were assayed, the ^{59}Fe uptakes were 50, 37, and 17% of the value for the undiluted extract, suggesting that the responses seen with the undiluted material were indicative of the total erythropoietic potential of the extract.

Another aliquot was divided into two portions, acidified to pH 5.6 and immersed in boiling water for either 5 or 20 min. The resultant material was centrifuged, the supernatant was removed and diluted to the original volume with saltine and assayed. The unheated extract resulted in a ^{59}Fe uptake of $14.27 \pm 1.49\%$ compared to $6.80 \pm 0.85\%$ after 5 min of boiling and $0.52 \pm 0.10\%$ after 20 min. Saline treated assay mice showed a $0.30 \pm 0.03\%$ uptake. The material was thus somewhat heat stable, 52% reduction in activity occurring after 5 min, but could be heat inactivated as indicated by the result for the 20-min treatment. Another extract, from another group of rats, was tested for activity following treatment with an organic solvent. The material was mixed vigorously with 4 vol of petroleum ether and

the solvent was removed by drawing off the upper layer and then placing the remainder in a vacuum to remove any remaining trace of the solvent. Assay mice injected with the untreated material showed a ^{59}Fe uptake of $9.31 \pm 0.76\%$; following lipid extraction the activity of the remaining aqueous phase was practically unchanged, $8.90 \pm 0.84\%$. These results indicate that the material is protein rather than steroid in nature.

As a further test of the nature of the material, portions were treated with neuraminidase, an enzyme known to inactivate erythropoietin. Preliminary analyses⁵ of the extract were done to determine total protein (Lowery method), total sialic acid, free sialic acid, and the sialic acid released from 1 ml of the extract by 500 units of neuraminidase. The amount hydrolyzed by the neuraminidase was determined to be 61.9% of the total. Because a large amount of extraneous protein was found, 4 different concentrations (125, 250, 500, and 1000 units) of neuraminidase were used on the extracts. However, the final results indicated that there was no difference in the response of assay mice in the various groups, so the results of all 38 assay mice were pooled to represent the enzyme-treated group. The original extract was diluted 1:2, plus an additional 2 ml of buffer to equal the maximal volume of enzyme added. Appropriate adjustments were made for the various volumes of enzyme added in the other groups. All samples were incubated together. The diluted control material produced a ^{59}Fe uptake of $4.89 \pm 0.79\%$. Following neuraminidase treatment, this was reduced to $0.55 \pm 0.05\%$ as compared to $0.36 \pm 0.03\%$ for control assay mice treated with buffer to which 1000 units of enzyme had been added. These results indicate that the active material is a glycoprotein and substantiates our previous assumption that the material is erythropoietin.

Discussion. In considering tumors associated with a polycythemia they had been divided into two broad categories, those ac-

⁵ The author wishes to thank Dr. Curt Bubel, Department of Microbiology, for these studies and advice.

accompanied by an increase in erythropoietin and those without (1). It is of special interest to note the findings of Mann *et al.* (5) in which they describe, in humans, a tumor of the adrenal gland associated with increased erythropoietin production. The present experiments support our previous assumption (9) that the active erythropoietic agent in the rat bearing a transplanted adrenal cortical carcinoma is erythropoietin. Further, they demonstrate that the erythropoietin is produced by the tumor itself.

In addition, the experiments were designed to resolve the discrepancy between a previous report that erythropoietin could be detected in the serum of the tumor-bearing rat (9), and one stating that no increase could be found (8). This was accomplished in part; however, the experiments introduced other complicating observations. The present study did show elevated levels of serum erythropoietin in the relatively early stage following transplantation that were not demonstrable after a longer period of time elapsed after transplantation. This time element seemed, at first, to resolve the differences in the two reports. However, in the present study extracts from tumor tissue were found to contain erythropoietin, in contrast to the negative findings of Swarm *et al.* (8). In discussing these differences in results with Dr. Swarm, the possibility that the activity of the tumor changed during the serial transplantation in the two separate laboratories seemed a feasible explanation for the discrepancy.

The complicating feature in the present study is the observation of undetectable levels of erythropoietin in the serum of the older animals in the face of high concentrations in the tumor itself. Several possibilities exist as an explanation of this observation. It is possible that as the tumor produces the hormone, the animal increases in size and the blood volume increases so that the erythropoietin is diluted beyond the sensitivity of the assay. It is also possible that there is a gradual reduction in production and release from the tumor so the tumor level, per milligram of tissue, remains fairly constant, but the amount in the serum goes down. However, these explanations do not seem likely

since there is a continuing reticulocytosis in these animals. Another possible explanation is that utilization by the hyperactive erythropoietic system accounts for the low serum level during prolonged stimulation. This thesis has been proposed by some authors (11, 12) but has been questioned by others (13, 14). Another interpretation was recently proposed (15) in which it was shown that high levels of erythropoietin are necessary to initiate erythropoiesis but low levels could maintain increased erythropoietic activity once it had been established. Either of these two explanations could fit the present situation. It should also be noted in the present study that there was a difference in the level of activity in the serum for the males as compared to the females. This difference has not always been seen and may have some bearing on interpretation of the results. That there is erythropoietically active material in the serum and tumors of our animals seems undisputable; however, the reason for the several variations described needs further study.

It is of interest to note the studies of Leaders *et al.* (6) in which they found erythropoietin would stimulate tumor growth in rats and mice and that plasma from persons with a variety of cancers would stimulate ^{59}Fe incorporation in rats. Plasma from rats bearing Novikoff tumors did not stimulate ^{59}Fe uptake. In contrast, they found that liver extracts of the hepatoma and of normal liver decreased ^{59}Fe uptake in their assay rats. This is in contrast to our observation that liver extracts did not influence ^{59}Fe uptakes in the polycythemic mouse. This may be related to the severe depression of ^{59}Fe uptake in the polycythemic mouse compared to their assay animal, the fasted rat, so that a depressive influence could not be seen.

In recent years it has become increasingly evident that there is an extrarenal source of erythropoietin in a variety of animal species. The literature pertaining to this fact is well reviewed in a recent study of Mirand *et al.* (16) in which they observed an extrarenal source of the hormone in the baboon. The origin of this fraction of erythropoietin is unknown. In addition, the studies of Gordon and co-workers (17) might be mentioned.

They have described a system of erythropoietin production in which a factor (REF) produced by the kidney, which in itself is erythropoietically inactive, when incubated with normal plasma produces the active erythropoietin. What relationship this system has to that operating in the extrarenal sources is unknown. It seems possible that further experimentation on abnormal systems, such as that described in the present study, might provide information relating the system of erythropoietin production in the kidney to that which exists for its production from extrarenal sources.

Summary. Rats bearing a transplantable adrenal cortical carcinoma showed the following features: elevated levels of an erythropoietic factor in the serum 2-4 weeks after tumor transplantation; no increase in erythropoietic factor in the serum 2 months after transplantation; significant levels of an erythropoietic factor in saline extracts made of both the young and the old tumor tissue; and no significant reduction of erythropoietic factor in the serum or tumor 48 hr after nephrectomy of the tumor bearing rats. This evidence indicates that the site of production of the erythropoietic factor is the tumor itself. Several features of the active tumor extracts, primarily its inactivation by neuraminidase, supports the contention that the erythropoietically active material in these rats is

erythropoietin.

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