

The Effect of Irradiation on the Plasma Erythropoietic Stimulating Factor

By JAMES W. LINMAN AND FRANK H. BETHELL

With the technical assistance of HELENA K. TASCOTT

THE DEMONSTRATION of erythropoietic stimulatory activity in protein-free "anemic" plasma extracts by Borsook and his co-workers¹ and subsequently by Gordon et al.² has greatly facilitated investigation of the humoral factor or factors capable of influencing erythropoiesis. Studies in this laboratory³ have confirmed the presence of such a factor in protein-free extracts of plasma from rabbits made anemic either by phenylhydrazine or repeated bleedings. When administered subcutaneously to the normal rat, these extracts are capable of stimulating red blood cell production as evidenced by an erythrocytosis, reticulocytosis, and increased marrow erythropoietic activity. The erythrocytes produced in the normal rat as a result of this stimulus are of decreased size without change in mean corpuscular hemoglobin concentration or appreciable increases in the hemoglobin or hematocrit.

This factor is stable over a wide range of temperature, acid soluble, not destroyed by prolonged boiling, and is not precipitated by perchloric acid. Therefore it is almost certainly nonprotein and it is apparently not species specific and nonantigenic. The absence of stimulatory activity in plasma extracts that have been ashed is in favor of its organic nature.

Erslev and Lavietes⁴ have reported that the administration of nitrogen mustard to rabbits, prior to rendering them anemic by hemorrhage, does not impair the production of the erythropoietic stimulating factor. The studies to be described were undertaken to obtain similar information with respect to x-irradiation and to determine whether anemia due to irradiation alone is a stimulus to its production.

Materials and Methods

Adult New Zealand rabbits weighing 2.5 to 3.0 kilograms were given total body irradiation. The radiation factors included: total dose—750 r, filtration— $\frac{1}{2}$ mm. copper and 1 mm. aluminum, 245 Kv., and the dose rate was 17.6 r per minute. The rabbits were then divided into three groups. One group was exsanguinated on the first post-irradiation day, the plasma was pooled and saved, and the protein-free plasma extract (hereafter designated as PFPE) was prepared according to the method described by Borsook et al.¹ and was precipitated with perchloric acid. These animals had normal hemoglobin levels. The second group was started on daily subcutaneous injections of 1 ml. of a 2.5 per cent solution of phenylhydrazine 24 hours following irradiation. After three to four such injections the animals were anemic with hemoglobin values ranging from 2.2 to 6.6 and averaging 3.7 Gm. per cent. They were then exsanguinated and PFPE was prepared as described above. The

From the Atomic Energy Commission Biological Effects of Irradiation Laboratory, The Department of Internal Medicine, and the Simpson Memorial Institute, University of Michigan, Ann Arbor, Michigan.

This study was carried out under contract No. AT(11-1)-75 for the United States Atomic Energy Commission at the University of Michigan.

Submitted May 23, 1956; accepted for publication June 29, 1956.

third group was followed for 2 to 3 weeks after irradiation until their hemoglobin fell to levels below 7 Gm. per cent when they were exsanguinated and PFPE prepared. These animals had hemoglobin values averaging approximately 6 Gm. per cent at the time of killing. Another group of rabbits was not irradiated and was made anemic by the daily administration of phenylhydrazine in the manner described above. Six to seven daily injections were required before they were sufficiently anemic with hemoglobins between 3.2 and 6.6 and averaging 5.2 Gm. per cent. They were then exsanguinated and PFPE prepared.

Forty-six female Wistar strain rats weighing 140-160 Gm. were divided into five groups. Three groups of 8 rats each were given 18 daily subcutaneous injections over a three week period (Sundays excepted) in amounts equivalent to 2 per cent of their body weight of anemic (by phenylhydrazine) PFPE, anemic (by phenylhydrazine) post-irradiation PFPE and normal (post-irradiation) PFPE. Sixteen rats received Ringer's solution and the fifth group of 6 rats was injected with anemic (by irradiation) PFPE.

Baseline studies consisted of hemoglobin and hematocrit determinations and erythrocyte and reticulocyte counts. The former three were repeated at 7 day intervals and the latter twice weekly. Hemoglobin was determined by an oxyhemoglobin photocolormeter technic, hematocrits were done by a microhematocrit method, and reticulocytes were counted per 1,000 erythrocytes on dried brilliant cresyl blue coverslip films counterstained with Leishman's stain. The erythrocytes were enumerated by hemacytometer. At the conclusion of the injection period 10 rats receiving Ringer's solution and 4 rats in each of the other groups were killed and femoral marrow specimens were obtained and examined by the method previously described.³ All recipient animals were weighed twice weekly and the dosage of PFPE adjusted accordingly.

RESULTS

Plasma extracts from rabbits made anemic by the administration of phenylhydrazine beginning 24 hours following total body irradiation were capable of stimulating erythropoiesis in the normal rat as manifested by the erythrocytosis and reticulocytosis depicted in figures 1 and 2. The onset of increased erythro-

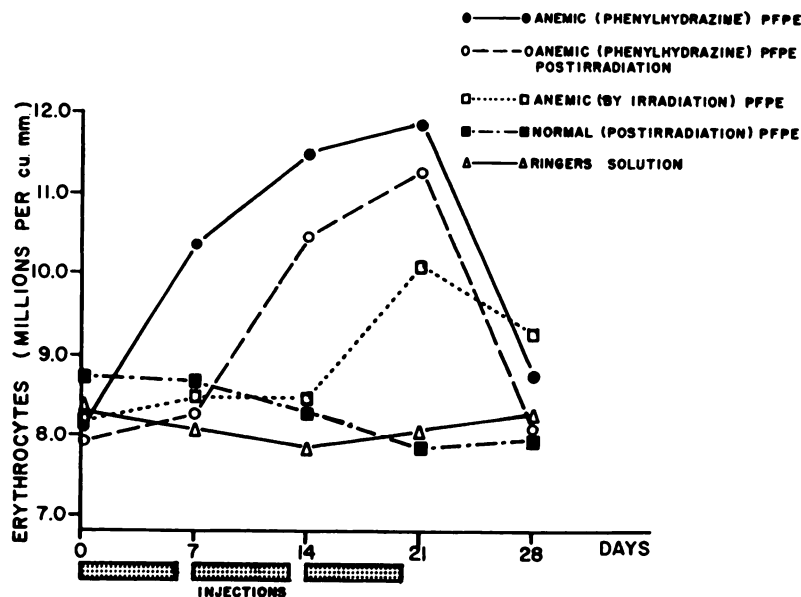


FIG. 1.—The average weekly erythrocyte counts showing an increase in the rats given plasma extracts from nonirradiated and irradiated rabbits made anemic by phenylhydrazine and from rabbits rendered anemic by total body irradiation alone.

poietic activity was, however, not evident in the rats receiving this material until one week later than in rats injected with PFPE from nonirradiated rabbits made anemic with phenylhydrazine. Rabbits rendered anemic by total body irradiation alone were also able to produce the plasma erythropoietic stimulating factor but the evidence for accelerated erythropoiesis was even further delayed in the rats which received extracts of plasma from animals treated in this manner. At the conclusion of the three week period of injections, the average increases over their baseline values in the erythrocytes per cu. mm. were 3,700,000; 3,320,000; and 1,890,000 respectively in the rats receiving PFPE-anemic by phenylhydrazine, PFPE-anemic by phenylhydrazine immediately after irradiation, and PFPE-anemic by irradiation. The reticulocytosis became evident 3, 10, and 17 days after beginning injections in these 3 groups in the order listed above, with erythrocytosis first noted 3 to 4 days later. There was no evidence of erythropoietic stimulation in the rats injected with normal one day post-irradiation PFPE.

The hemoglobin and hematocrit levels were not significantly changed in any group (tables 1 and 2) whereas the mean corpuscular volume and the mean

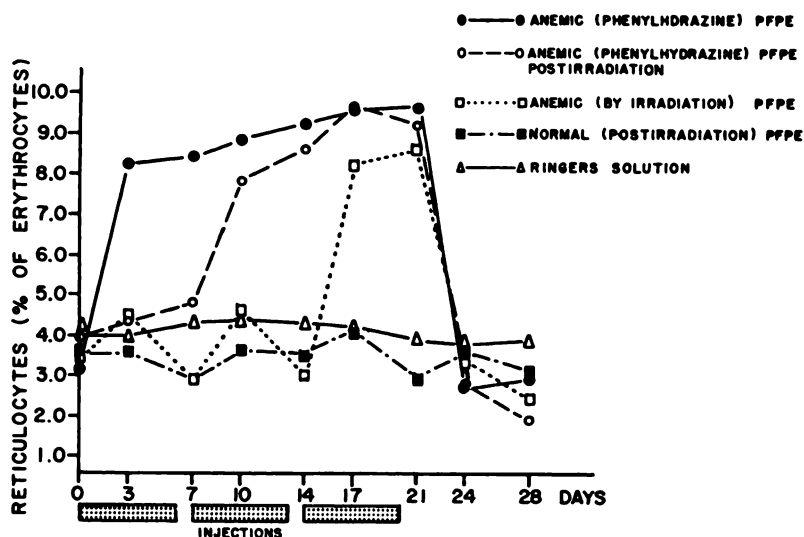


FIG. 2.—The average reticulocyte percentages in all groups demonstrating reticulocytosis in the rats with the erythrocytosis as depicted in figure 1.

TABLE 1.—Average Hemoglobin Values (Gm. per 100 ml.)

Groups	Baseline	1 Week	2 Weeks	3 Weeks*	4 Weeks
Anemic (Phenylhydrazine) PFPE	14.0	15.3	15.2	15.9	14.8
Anemic (Phenylhydrazine) PFPE Postirradiation	13.4	13.7	14.1	15.6	14.5
Anemic (By Irradiation) PFPE	14.5	14.1	13.7	14.4	14.2
Normal (Postirradiation) PFPE	14.0	14.4	13.9	14.6	14.5
Ringer's Solution	14.3	14.0	13.9	14.6	14.9

* Injections discontinued.

TABLE 2.—Average Hematocrit Determinations (Volumes per cent)

Groups	Baseline	1 Week	2 Weeks	3 Weeks*	4 Weeks
Anemic (Phenylhydrazine) PFPE.....	45.5	47.8	47.4	47.2	48.1
Anemic (Phenylhydrazine) PFPE Postirradiation.....	43.0	42.7	45.6	46.3	45.1
Anemic (By Irradiation) PFPE.....	46.7	43.3	43.1	43.9	42.4
Normal (Postirradiation) PFPE.....	45.2	44.3	43.8	43.2	44.2
Ringer's Solution.....	44.6	44.5	44.7	44.1	43.4

* Injections discontinued.

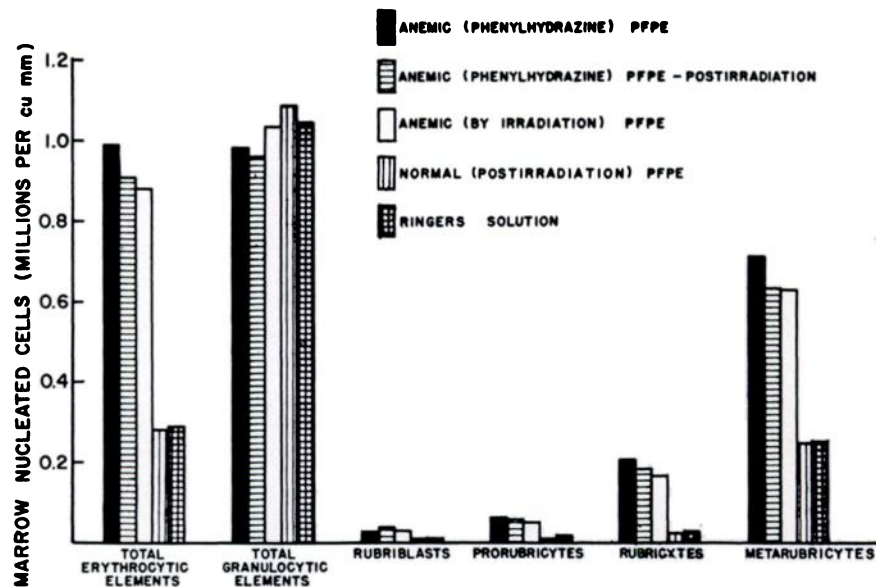


FIG. 3.—Average marrow nucleated erythrocytic and granulocytic cell counts from 4 rats in each group receiving the different plasma extracts compared to similar values for 10 rats injected with Ringer's solution. The increase in total marrow cellularity in the animals showing reticulocytosis and erythrocytosis was due to erythrocytic elements.

corpuseular hemoglobin fell in the rats demonstrating erythrocytosis and reticulocytosis. The mean corpuseular hemoglobin concentration remained normal in all groups.

There was increased marrow cellularity in rats with peripheral evidence of accelerated erythropoiesis (fig. 3) involving a roughly proportional increase in all erythrocytic precursors. As previously noted, the increase in total marrow cellularity was due solely to nucleated cells of the erythrocytic series, the granulocytic elements being essentially the same in all groups.

All rats gained weight in amounts comparable to the animals in the control group and demonstrated no adverse local or generalized effects from the injections.

DISCUSSION

Our observation of the production of the plasma erythropoietic stimulating factor in rabbits made anemic by phenylhydrazine following total body irradiation

tion is in accord with the findings of Erslev and Lavietaes⁴ regarding the effects of the administration of nitrogen mustard prior to bleeding on the formation of the factor. Therefore, it would appear that the erythropoietic stimulating factor is not produced by hemopoietic or other radiosensitive tissues and, in view of its presence in plasma from rabbits made anemic by total body irradiation alone, a regenerative marrow is apparently not a requirement for its formation.

The variation in the time of onset of the peripheral evidence of increased erythropoietic activity (i.e., reticulocytosis and erythrocytosis) is, we believe, dependent on the concentration of the erythropoietic stimulating factor present in the various plasma extracts assayed. It has been previously shown in this laboratory in experiments involving varying doses, that the response to the factor is determined not only by the dosage but by the length of the injection period with the time of maximal response varying inversely with the amount of the factor administered. Small amounts given over long periods of time are capable of evoking almost the same degree of response as larger doses given over shorter periods. This would explain, at least in part, the delayed response in the rats injected with plasma extracts from rabbits made anemic by total body irradiation alone. These rabbits were not as anemic as the others and the anemia developed slowly over a much longer period of time. Although the reticulocytosis and erythrocytosis in the rats receiving such material were not as great as in the other groups, there was definite increased marrow erythropoietic activity comparable in degree to that present in the rats given plasma extracts from both irradiated and nonirradiated rabbits made anemic by phenylhydrazine.

The reason for the slight delay in the onset of reticulocytosis and erythrocytosis in the rats injected with plasma extracts from irradiated rabbits with phenylhydrazine induced anemia as compared to the nonirradiated phenylhydrazine treated donors is not readily apparent. The former were more anemic than the latter and their plasma might have been expected to contain a higher concentration of the factor. A possible explanation may be that the duration of their anemia was less as they required fewer daily injections of phenylhydrazine, to attain comparable hemoglobin levels, or the slight difference may merely reflect individual variations in response to the erythropoietic stimulus.

The possibility that the more delayed and less marked erythrocyte and reticulocyte response in the rats given material from irradiated rabbits does actually reflect some degree of irradiation induced depression of the factor's formation, must be considered. The previously demonstrated absence by the assay method used, of an erythropoietic stimulatory factor in normal "nonanemic" plasma indicates that the failure to find accelerated erythropoiesis in the recipient would not require as a prerequisite total destruction of the factor's formative tissue in the donor. If one assumes that the factor is produced by hemopoietic elements, it would then appear essential to postulate in the irradiated rabbits a rate of hemopoiesis greater than that present in the normal state. In view of the known sensitivity of hemopoietic tissues to irradiation, the small amount of phenylhydrazine required to render the irradiated rabbits anemic, and the absence of reticulocytosis in both the irradiated rabbits made anemic by phenylhydrazine and by irradiation alone, it would be difficult to envision hemopoietic activity in these animals greater than that present normally. This is especially true in the rabbits with anemia secondary only to irradiation. It would, seem therefore,

that considerably less or no activity should have been found in the plasma from irradiated rabbits in order to implicate hemopoietic tissue as the site of its production.

Another possibility is that the irradiation may have curtailed elaboration of the factor to a greater degree than was apparent with an accumulation in the plasma due to an inability of the target tissue to utilize it in blood formation. Although the mode of action of the humoral erythropoietic factor is unknown, it does not seem likely that hemopoietic injury to such an extent that utilization of the factor was seriously impaired could occur without serious interference with its formation, if the site of the latter were the blood-forming or other equally radiosensitive tissue. For these reasons we interpret the data to indicate that the plasma erythropoietic stimulating factor is not produced by highly radiosensitive tissues.

The role of the plasma erythropoietic factor in the maintenance of the steady state or in recovery from anemia is unknown. The evidence presented above for its production in sites other than hemopoietic tissues and for its formation in the absence of a regenerative marrow suggests strongly that the factor has an important physiologic role in the regulation of erythropoiesis. The failure to demonstrate erythropoietic activity in the plasma from normal animals by the assay methods herein employed may be due to the small amount of the factor present rather than to its absence.

It has been postulated⁸ that, in the normal intact rat over a relatively short experimental period, the plasma erythropoietic stimulating factor acts chiefly by causing increased cellular division of already existent erythrocyte precursors without significant increases in red cell mass. Its possible mode of action in anemic states, however, remains to be defined.

SUMMARY

1) Protein-free plasma extracts from rabbits made anemic by the administration of phenylhydrazine immediately following total body x-irradiation are capable of stimulating erythropoiesis in the normal rat as demonstrated by erythrocytosis, reticulocytosis, and increased marrow erythropoietic activity.

2) Plasma extracts from rabbits made anemic by total body x-irradiation alone contain an erythropoietic stimulating factor.

3) These data would indicate that the stimulating factor is not produced by hemopoietic or other radiosensitive tissue and its formation is not dependent upon a regenerative marrow.

SUMMARIO IN INTERLINGUA

1. Extractos non-proteinic de plasma, obtenite ab conilios que habeva devenite anemic in consequentia del administration de phenylhydrazine immediate mente post le roentgeno-irradiation del corpore total, es capace a stimular le erythropoiese in rattos normal. Iste effecto se manifesta in le erythrocytose, le reticulo-cytose, e un augmento del activitate erythropoietic in le medulla.

2. Extractos de plasma ab conilios rendite anemic per roentgeno-irradiation del corpore total sin administration de phenylhydrazina contine un factor de stimulation erythropoietic.

3. Iste datos pare indicar que le factor stimulatori non es producite per textos hemopoietic o altere textos de character radiosensibile e que su formation non depende de un medulla regenerative.

REFERENCES

- ¹ BORSOOK, H., GRAYBIEL, A., KEIGHLEY, G., AND WINDSOR, E.: Polycythemic response in normal adult rats to a nonprotein plasma extract from anemic rabbits. *Blood* 9: 734, 1954.
- ² GORDON, A. S., PILIERO, S. J., KLEINBERG, W., AND FREEDMAN, H. H.: A plasma extract with erythropoietic activity. *Proc. Soc. Exper. Biol. & Med.* 86: 255, 1954.
- ³ LINMAN, J. W. AND BETHELL, F. H.: The plasma erythropoietic stimulating factor. Observations on circulating erythrocytes and bone marrow of rats receiving protein-free extracts of rabbit plasma. *Blood* 11: 310, 1956.
- ⁴ ERSLEV, A. J. AND LAVIETES, P. H.: Observations on the nature of the erythropoietic serum factor. *Blood* 9: 1055, 1954.