SOME OBSERVATIONS ON THE STIMULATION OF ERYTHROPOIESIS BY HUMORAL FACTORS

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The regulatory mechanisms responsible for the normal erythroid steady state and the nature of the stimuli to erythropoiesis during periods of increased need have long attracted the attention of investigators. Until recently, however, little was known concerning the manner in which erythrocytic equilibrium was maintained or restored. The endocrine glands, the nervous system, the spleen, hypoxia of myeloid elements, and a humoral factor have all been implicated in the control of red blood cell production. Although the former three are capable of modifying erythropoiesis, they do not appear to constitute the primary erythropoietic stimulus, whereas the role of arterial oxygen tension as a basic determinant of erythropoietic activity has been widely confirmed by observations on experimental animals and human subjects. However, the original theory that this effect was due to hypoxia of myeloid erythrocytic precursors has been disproved.

Carnot and Deflandre[1, 2] first described a humoral erythropoietic stimulating factor in 1906 and named this hypothetical substance hemopoietine. In the course of research on the regeneration of organs, these investigators discovered that the serum of bled rabbits induced erythrocytosis when administered to normal rabbits. In subsequent years this intriguing concept of a humoral erythropoietic regulatory mechanism was responsible for a number of attempts to confirm the existence of such an erythrocytogenic agent. Some of these studies were in accord with those of Carnot and Deflandre, but many were in disagreement. The evident discrepancies in data that supported this hypothesis and the negative findings of other investigators were such that, prior to the past few years, the humoral control of erythropoietic activity was considered by most to be speculative and lacking in experimental support.

Observations such as those of Reissmann[3] in 1950 on the stimulation of erythropoiesis in parabiotic rats maintained in a normal atmosphere following exposure of their partners to reduced oxygen tension, the need for administering large quantities of anemic plasma or serum in order to elicit an erythropoietic response in recipient animals as pointed out by Erslev[4] in 1953, and the demonstration of erythropoietic activity in heat-denatured anemic plasma by Borsook and his co-workers[5] and Gordon and his associates[6] in 1954 greatly accelerated interest in the humoral control of erythropoiesis. On the basis of recent studies in many laboratories,[7] the existence of a humoral erythropoietic regulatory mechanism can no longer be denied. However, the nature, site of production, and mode of action of the substances responsible for this effect have not yet been defined clearly.

Our observations[8-10] indicate that there are both thermostable and relatively thermolabile plasma erythropoietic factors that control, respectively, the seemingly diverse physiological activities concerned with erythroblastic cellular division and the synthesis of hemoglobin. Extracts of active plasmas, which have been processed by boiling for 30 min., induce a singular type of erythropoietic response when administered to normal rats. This effect on erythropoiesis is characterized by erythrocytosis and reticulocytosis

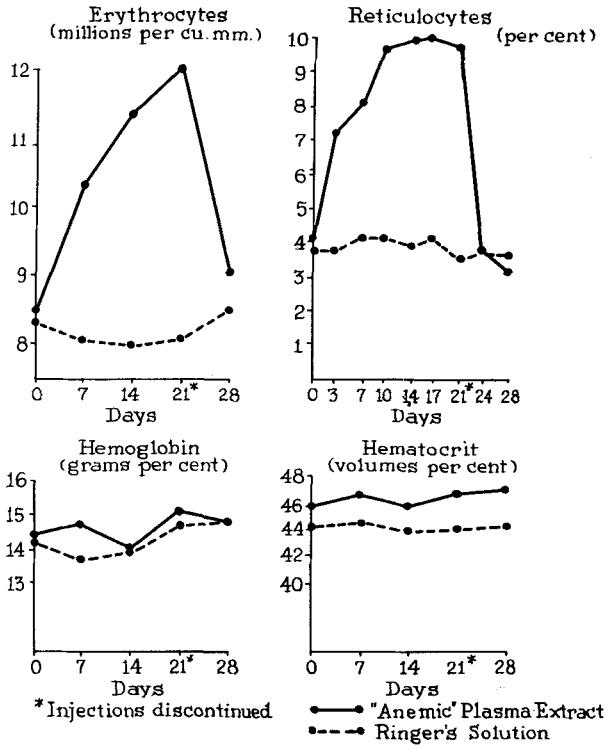

FIGURE 1. Erythrocytosis and reticulocytosis without increase in the hemoglobin or hematocrit values in normal rats given 18 daily injections (Sundays excepted) of boiled plasma extracts from rabbits made anemic by phenylhydrazine. Each injection was equivalent to 2 per cent of the recipient's body weight. Average determinations of 24 animals receiving the anemic plasma extract and a similar number of controls injected with Ringer's solution.

without associated increase in the hemoglobin or hematocrit levels (FIGURE 1). Myeloid erythrocytic hyperplasia involving a roughly proportional increase in all recognizable nucleated red cell precursors is also evident in the recipients of these anemic plasma extracts (FIGURE 2), and it provides, together with the erythrocytosis and reticulocytosis, conclusive evidence of increased erythropoietic activity. The newly formed cells responsible for the erythrocytosis are microcytic. Their small size is apparent on stained films and demonstrable graphically by Price-Jones measurements.[11, 12] In our opinion,

this type of response is the result of accelerated erythroblastic cellular division without augmentation in hemoglobin synthesis.

Following discontinuation of the plasma extract injections, restoration of normal erythrocytic equilibrium is prompt. Recent studies[12] have shown that this phenomenon apparently is due to impaired viability of the microcytes produced in response to this particular stimulus. Quantitative photocolorimetric measurements of erythrocyte osmotic fragility in normal rats injected daily for two weeks with a boiled anemic plasma extract were not found to differ from the controls. However, simultaneous determinations

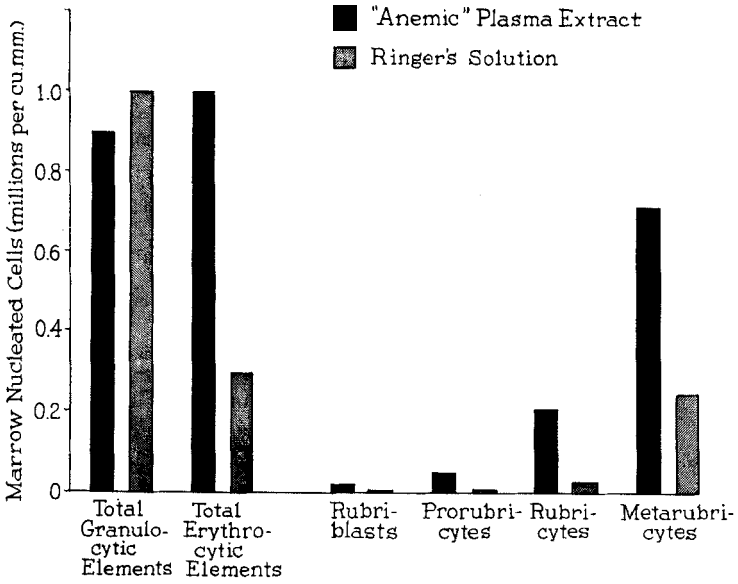

FIGURE 2. Myeloid erythrocytic hyperplasia demonstrable at the end of the injection period in the normal rat recipients of anemic rabbit plasma extracts (see FIGURE 1). There was a roughly proportional increase in all recognizable nucleated red cell precursors. Mean cell counts of 12 animals in each group.

by a direct cell enumeration technique employing red cell pipettes and hypotonic salt solutions as diluents demonstrated decreased osmotic resistance of the microcytes in the stimulated animals. Reversion to normal osmotic behavior accompanied the re-establishment of pretreatment erythrocyte counts and Price-Jones curves when the injections of plasma extract were stopped. Since this fragility abnormality is not evident in situations involving increased activity of both humoral factors, it cannot be assumed to be due to a specific action of the thermostable factor and most probably results from alterations in cell size and/or shape, secondary to accelerated erythroblastic cellular division in the absence of a comparable stimulus for increased hemoglobin synthesis.

Extracts of active plasmas prepared by boiling for 30 min. or more, which induce erythrocytosis and reticulocytosis but fail to augment circulating

hemoglobin or red cell mass, are also ineffective in enhancing the uptake of Fe^{59} in erythrocytes of recipient animals. However, the response to the same source materials, when tested in the unmodified state or after boiling for only short periods of time, is characterized by an increase in both hemoglobin and erythrocyte values and in iron-59 incorporation in hemoglobin. The effect of boiling on the activity of anemic plasma, as measured by the incorporation of Fe^{59} in the hemoglobin of nitrogen mustard-treated rats, is shown in TABLE 1. Although activity was demonstrable by this technique in recipients of the whole anemic plasma and of material boiled for 5 min., the responses to filtrates processed by boiling for longer periods were comparable to that of normal plasma. In addition to these findings, the thermostable erythrocytogenic agent that affects the rate of cellular division is soluble in ether,[10] whereas the relatively thermolabile substance that stimulates hemoglobin formation is not.

TABLE 1

EFFECT OF BOILING ON THE ERYTHROPOIETIC STIMULATORY ACTIVITY OF PHENYLHYDRAZINE-INDUCED ANEMIC RABBIT PLASMA AS MEASURED BY THE INCORPORATION OF Fe^{59} IN HEMOGLOBIN OF NITROGEN MUSTARD-TREATED RATS

Mean of 4 Rats in Each Group ± 1 Standard Deviation

Materials tested	Percentage Fe^{59} RBC uptake		
	18 hours	24 hours	42 hours
Unmodified anemic plasma	16 ± 2	19 ± 4	34 ± 5
Anemic plasma boiled 5 min	13 ± 2	20 ± 3	35 ± 2
Anemic plasma boiled 10 min	7 ± 3	10 ± 3	23 ± 4
Anemic plasma boiled 30 min	7 ± 2	9 ± 2	23 ± 4
Anemic plasma boiled 45 min	5 ± 2	6 ± 2	25 ± 3
Normal rabbit plasma	6 ± 2	9 ± 2	27 ± 5

We believe that these separable erythropoietic effects exerted by active plasmas are due to the presence of two factors that differ in nature and mode of action. One is characterized by an accelerated rate of erythroblastic cellular division and is related to a thermostable, ether-soluble fraction of plasma. The other, which involves hemoglobin synthesis, appears to be due to a relatively thermolabile, ether-insoluble factor.

The possibility that processing procedures might alter the chemical and physiological characteristics of a single factor must be considered, but appears quite unlikely. Ether extracts of unmodified anemic plasma evoke responses in recipient rats identical to those induced by boiled filtrates of such plasmas or their ether-soluble fraction. Furthermore, the demonstration of microcytes with decreased osmotic resistance in the blood of patients with polycythemia vera (FIGURE 3), a disorder associated with increased plasma erythropoietic activity,[9, 13, 14] and the retention in ether-insoluble fractions

of active plasmas of an enhancing effect on the incorporation of Fe^{59} in hemoglobin provide additional evidence against such an occurrence.

We have yet to study an active plasma, regardless of the experimental or clinical conditions under which it was obtained, that did not possess activity attributable to both humoral factors. Although their combined effects in normal recipients on hemoglobin synthesis and cellular division result in the production of increased numbers of normal cells, their properties are such that

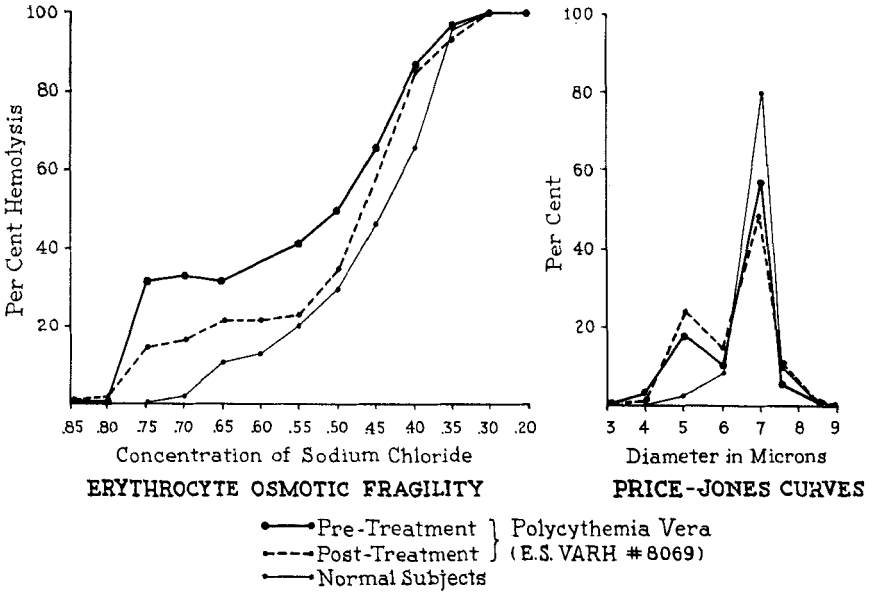

FIGURE 3. Erythrocyte osmotic fragilities determined by a direct cell enumeration technique and red cell diameter distributions (Price-Jones measurements) of a patient with polycythemia vera demonstrating the presence of microcytes with decreased osmotic resistance similar to those observed in normal rats injected with the thermostable, ether-soluble erythropoietic factor. These findings persisted, as did enhanced plasma erythropoietic activity, after normal erythroid values had been attained following treatment with P^{32}. Prior to therapy the hematocrit, hemoglobin, and erythrocyte count in this patient were 63.5 volumes per cent, 18.2 gm./100 ml., and 8,500,000 per cu.mm., respectively, with similar determinations of 45.0, 15.2, and 5,555,000 at the time the posttreatment plasma was obtained. Control values represent the composite curves of 8 normal subjects.

the methods used to demonstrate stimulatory activity and prepare materials for testing assume paramount importance in the interpretation of experimental results. Unmodified active plasmas or their extracts boiled for only short periods of time will yield evidence in recipient animals of altered erythropoietic activity irrespective of the assay methods employed. The response to these materials in normal rats given 10 to 14 daily injections is characterized by augmentation in all erythroid determinations, together with myeloid erythrocytic hyperplasia. The presence of the relatively thermolabile factor can also be detected by the short-term method that utilizes

the incorporation of iron-59 in hemoglobin as a means of demonstrating increased hemoglobin synthesis. Ether extracts of these same plasmas or their filtrates processed by prolonged boiling, however, contain only the factor that affects erythroblastic cellular division, and they do not enhance the formation of hemoglobin. Therefore, the differences in chemical and physiological attributes of the humoral erythrocytogenic agents would appear in many instances to explain the apparent disparity in experimental observations that have been described by various investigators.

The majority of our studies in regard to the chemical identities of the plasma erythropoietic factors have dealt with the thermostable, ether-soluble factor, and current information indicates that it is most probably a lipid. Similarities in the chemical and physical characteristics of batyl alcohol, the monoglycerol ether of *n*-octadecyl alcohol, and the ether-soluble plasma erythropoietic factor suggested a possible relationship between these two substances. Batyl alcohol was isolated from bovine yellow bone marrow by Holmes and his associates[15] in 1941, and a few reports have indicated that it possesses erythropoietic[16] and leukopoietic[17-19] activity. Our observations[20] have confirmed the erythrocytic stimulatory effect of batyl alcohol, which was manifested in normal rats given daily injections of batyl alcohol in peanut oil over a period of four weeks by erythrocytosis due to microcytes with decreased osmotic resistance, reticulocytosis, and myeloid erythrocytic hyperplasia without increase in their hemoglobins or hematocrits. Batyl alcohol is also ineffective in enhancing the incorporation of Fe^{59} in hemoglobin of recipient animals.[21] On the basis of this erythrocytic response, which was identical in all respects demonstrable by the methods used to that observed following the administration of the thermostable, ether-soluble plasma erythropoietic factor, together with their common chemical and physical properties, it is suggested that batyl alcohol, originating in yellow bone marrow, may be identical with or closely related to this factor, perhaps as precursor material.

An increase in circulating thrombocytes has been a consistent finding in all animals to which we have given batyl alcohol with a prompt return to base line and control values when the injections are stopped.[20] Although daily doses of 25 mg. or less of batyl alcohol used in the above experiment did not alter the recipients' leukocyte counts, other studies[21] indicate that it also possesses granulopoietic stimulatory activity. However, greater amounts apparently are necessary to evoke this response in normal rats than are needed to elicit erythrocyte and thrombocyte increases.

Since certain lipid substances induce hemolysis which may be associated with thrombocytosis and leukocytosis, this possibility must be excluded before true myelopoietic stimulatory properties can be assigned to batyl alcohol. All of the hematological phenomena ascribed to batyl alcohol are compatible with a compensated hemolytic state with the exception of the erythrocytosis, and this could conceivably represent red cell cytoclasis with impaired viability of the resultant erythrocytic fragments. However, it would then be necessary to postulate definite augmentation in hemoglobin synthesis as a prerequisite for the maintenance of normal hemoglobin and

hematocrit values, and enhanced iron-59 incorporation in hemoglobin should be evident. The failure of batyl alcohol to increase erythrocyte iron-59 uptake in recipient rats[21] would appear to exclude hemolysis and supports the conclusion that this compound does exert a primary stimulatory effect on hematopoiesis.

These observations on the erythropoietic, thrombopoietic, and probable granulopoietic activity of batyl alcohol are in accord with the theory that all aspects of myelopoiesis may be under the influence of humoral regulatory mechanisms. Since erythrocytes, thrombocytes, and granulocytes are all derived from the multipotential myeloid reticulum cells, a single substance or activator-inhibitor complex may control the formation of all these hemic elements. If this concept is confirmed by further investigation, the pathogenetic mechanisms responsible for a number of heretofore poorly understood hematological findings involving hyperactivity of myeloid elements en masse may be clarified. These include, among others, the granulocytosis and thrombocytosis that accompany acute blood loss and certain types of intravascular hemolysis and the increase in these hemic cells in patients with polycythemia vera.

The precise physiological and pathophysiological significance of the humoral erythropoietic factors is not known, but available data support the hypothesis that they constitute the primary erythropoietic stimulus. Enhanced plasma activity has been found in a number of clinical and experimental situations which, although of diverse etiologies, all with the exception of polycythemia vera have hypoxia as a common denominator. Therefore, it may be inferred that elaboration of the plasma erythropoietic factors is probably dependent on the relationship between oxygen supply and tissue requirements at the as yet undetermined site or sites of their formation. Since some activity is demonstrable in the plasma of normal subjects, especially after concentration,[9, 22] it may be concluded that the humoral factors contribute to the maintenance of the normal erythroid steady state and probably constitute the primary erythropoietic stimulus.

A well-balanced equilibrium appears to exist that ensures, under normal conditions, an oxygen-carrying capacity of the blood commensurate with cellular needs. Apparently this humoral regulatory mechanism is capable of responding to even minor changes in the dynamic relationship between oxygen supply and metabolic requirements. Erythropoietic activity thereby is altered accordingly, and equilibrium is re-established at the same or a different level, as the situation warrants.

The plasma factors are probably involved to some extent in all anemias irrespective of their basic cause, but since erythropoiesis is dependent on a number of things, certain deficiencies or abnormalities of myeloid elements may alter the production of red cells regardless of the integrity of the humoral regulatory mechanism. Enhanced plasma erythropoietic activity has been demonstrated in experimental animals and human subjects with both hemorrhagic and hemolytic anemias and undoubtedly is responsible for the myeloid erythrocytic hyperplasia that accompanies increased removal of red cells from the circulation. Since there is no associated defect of the myeloid

reticulum, the marrow responds to this humoral stimulus by increasing the production of erythrocytes. Anemia occurs only when erythrocytogenesis fails to keep pace with the peripheral loss. Therefore, it is apparent that the anemias due to increased removal of red cells from the peripheral circulation are intimately related to plasma erythropoietic stimulatory activity. Although the humoral agents per se are not of pathogenetic significance in these anemic states, their increased elaboration would appear vital for the restoration of normal values and in the case of persistent hemorrhage or hemolysis equally indispensable to temper the severity of the anemic hypoxia. It is possible that some failure or defect in the humoral regulatory control of erythropoiesis may be responsible, at least in part, for the "aplastic crises" that have been described in some types of hemolytic disease; this subject needs further study.

The pathophysiological activity of the plasma factors in the anemias due to defective erythrogenesis is more speculative, but experimental observations support certain conclusions. The stimulatory effect of erythropoietic factors presupposes adequate amounts of the various erythrocytic constituents together with an intact myeloid reticulum. It is evident that a normal marrow response is otherwise precluded. Studies on the effect of total body X irradiation on plasma erythropoietic activity indicate that hematopoietic elements are not involved in the elaboration of the factors and that a regenerative marrow is not a prerequisite for their formation.[23] Plasma extracts from normal and irradiated rabbits rendered anemic by phenylhydrazine and from those with anemia secondary to irradiation alone were each effective in stimulating erythropoiesis in normal rats. It follows that enhanced plasma activity secondary to anemic hypoxia should be demonstrable in the anemias that result from specific deficiency states or injury to myeloid elements of known cause. These conditions would include deficiencies of substances such as vitamin B_{12} or iron; a reduction in myeloid erythrocytic precursors by physical or chemical means, displacement by foreign cells, or diversion of growth potential by leukemic transformation; and the hereditary hemoglobinopathies. Although the number of patients studied is as yet small, increased activity has been found in each of these disorders. However, the existent myeloid deficiency or defect prevents a normal marrow response to the humoral stimulus.

It can be assumed that the plasma erythropoietic factors are not involved in the actual production of the depressed erythroid values in these clearly defined anemias. Nevertheless, their presence is probably essential for the continued attempt on the part of the marrow to produce some erythrocytes, and they probably represent the mechanism by which normal erthyrocytic equilibrium is restored if the basic deficiency or defect can be corrected. The possibility exists, however, that some abnormality in the humoral erythropoietic regulatory mechanism involving impaired formation of the plasma factors, defective utilization, or both, may be of pathogenetic importance in certain other anemias of questionable or unknown cause. Progress in this regard has been slow because of the difficulties associated with measuring relative degrees of activity.

Polycythemia vera and secondary polycythemia are the hematological disorders that, except for time factors, most closely simulate the imbalance in erythrocytic equilibrium induced in normal rats by the administration of the plasma factors. Enhanced plasma erythropoietic stimulatory activity is demonstrable in patients with polycythemia vera and secondary polycythemia, and has been a consistent finding in all of the patients with these disorders whom we have studied. The overproduction of the plasma factors in patients with erythrocytosis due to decreased arterial oxygen saturation is not surprising in view of the well-documented relationship between hypoxia and erythropoietic activity. Under these conditions, the hypoxic hypoxia is undoubtedly the stimulus for the increased elaboration of the humoral factors which in turn transmit the need for augmentation of the oxygen-carrying capacity of the blood to the myeloid reticulum. However, depending upon the severity of the arterial hypoxemia, the resultant increase in erythropoiesis may be insufficient to restore the normal relationship between oxygen supply and tissue requirements. As a consequence, the physiological regulatory mechanism produces an unphysiological secondary polycythemia.

Increased amounts of the humoral erythropoietic factors in patients with polycythemia vera occur in the absence of demonstrable hypoxia. Since the factors are apparently not a product of hyperactive myeloid elements, the augmented plasma erythropoietic activity in this disorder supports the view that polycythemia vera may be caused by a metabolic imbalance resulting in the production of excessive amounts of the plasma factors or, conversely, in the failure to inactivate such materials at rates sufficient to maintain normal erythrocytic equilibrium. Enhanced plasma activity should then persist in these patients irrespective of the institution of specific myelosuppressive therapy. To date, we have studied the plasma of six patients with polycythemia vera after normal erythroid values had been achieved following treatment with radioactive phosphorus. Myeloid erythrocytic hyperplasia (TABLE 2) was evident in the normal rats injected with ether extracts of the plasma from each of these patients and was accompanied by erythrocytosis and reticulocytosis.

Augmented amounts of both the thermostable and relatively thermolabile plasma factors have been demonstrated in polycythemia vera, thus explaining the increase in all erythroid values that these patients manifest. However, microcytes with decreased resistance to lysis in hypotonic media similar to those observed in normal rats given the thermostable plasma erythropoietic factor are evident in the peripheral blood of patients with polycythemia vera, an example of which is shown in FIGURE 3. Simultaneous osmotic fragility measurements by a quantitative photocolorimetric method in these patients have not been found to be significantly at variance with those of normal control subjects. The small cells with abnormal osmotic behavior are still discernible during therapeutically induced remissions (FIGURE 3), a not unexpected finding in view of the persistence after treatment of enhanced erythropoietic activity as determined by *in vitro* plasma assays. Therefore, in contradistinction to the evenly balanced stimulatory effect of each plasma factor in normal and many anemic states, the humoral agent that controls erythroblastic cellular division appears to predominate in polycythemia vera.

These observations add further support to a causal relationship between the erythropoietic factors and polycythemia vera and are in agreement with the findings of Berlin and his co-workers[24] of a bimodal red cell population in patients with polycythemia vera consisting of erythrocytes with a normal life span and a second group with a survival time of only a few days.

TABLE 2
AVERAGE MARROW NUCLEATED CELLS PER CUBIC MILLIMETER OF RATS RECEIVING ETHER EXTRACTS OF PLASMAS FROM PATIENTS WITH POLYCYTHEMIA VERA IN THERAPEUTIC REMISSION

Source of plasma	Number of rats	Granulocytic elements	Erythrocytic elements
VARH—6738	3	1,059,125	1,280,000
VARH—8069	3	1,137,526	1,156,198
UMH—852687	3	1,008,570	1,096,517
UMH—772026	3	1,053,988	1,447,074
UMH—791448	3	862,802	1,065,074
UMH—811765	3	1,036,858	1,037,635
Normal subject	6	1,016,577	291,851

TABLE 3
AVERAGE THROMBOCYTE COUNTS (MILLIONS PER CUBIC MILLIMETER) OF RATS RECEIVING ETHER EXTRACTS OF PLASMAS FROM PATIENTS WITH POLYCYTHEMIA VERA

Source of plasma	Number of rats	Base line	1 week	2 weeks
Active disease				
UMH—880933	6	0.714	0.984	0.898
UMH—711011	6	0.676	0.900	0.832
UMH—876961	2	0.676	0.847	0.950
Therapeutic remission				
UMH—811765	6	0.759	0.938	0.956
UMH—852687	5	0.751	1.015	0.982
UMH—772026	6	0.755	1.188	0.940
UMH—791448	3	0.699	0.874	0.973
Normal subject	18	0.680	0.838	0.824
Ringer's solution	12	0.632	0.600	0.646

In view of the evidence that implicates the erythropoietic factors in the pathogenesis of polycythemia vera, experiments were designed to study the possible existence of humoral factors that might be responsible for the thrombocytosis and leukocytosis so commonly associated with this disease. These studies are still in progress, but preliminary observations indicate that ether extracts of plasma from both treated and untreated patients with polycythemia vera do exert a thrombocytosis-promoting effect in normal rats (TABLE 3) in addition to accelerating erythroblastic cellular division. A

slight but definite increase in platelets has been a consistent finding in all recipients given such extracts daily for two weeks, with a prompt return to base line values after the injections were stopped. Although the normal unconcentrated human plasma extract was erythropoietically inactive, it would appear to contain minimal thrombocytic activity when the platelet counts of animals given this material are compared to those of rats injected with equivalent amounts of Ringer's solution. Definitive conclusions in regard to these observations must await additional study; however, they assume increased significance in the light of the apparent myelopoietic stimulatory effect of batyl alcohol, the evidence linking batyl alcohol with the thermostable, ether-soluble erythropoietic factor, and the findings that suggest that activity attributable to the latter factor may predominate in patients with polycythemia vera. To date, all attempts to detect granulopoietic activity in polycythemic plasma extracts have been unsuccessful, but the possibility of varying degrees of sensitivity of myeloid elements to such a stimulus cannot be excluded.

Summary

Our studies indicate that at least two humoral factors exert regulatory control over erythropoiesis. A thermostable, ether-soluble agent stimulates erythroblastic cellular division, but does not augment hemoglobin synthesis, which appears to be governed by a relatively thermolabile, ether-insoluble factor.

It is suggested, on the basis of similarities in their chemical and physiological properties, that the thermostable plasma erythropoietic factor and batyl alcohol may be the same or related compounds.

The thrombopoietic and probable granulopoietic activity of batyl alcohol, in addition to its effect on erythropoiesis, support the hypothesis that all aspects of myelopoiesis may be under the influence of humoral regulatory mechanisms. Since erythrocytes, thrombocytes, and granulocytes are all derived from the multipotential myeloid reticulum cells, a single agent or activator-inhibitor complex may control the formation of all of these hemic elements. Batyl alcohol, originating in yellow bone marrow, may be of primary importance in such a system.

Although the precise physiological and pathophysiological significance of the plasma erythropoietic factors is not yet known, they most probably constitute the primary erythrocytogenic stimulus. These humoral agents appear to contribute to the maintenance of the normal erythroid steady state and to be responsible for the increased erythropoietic activity in hypoxic and some types of anemic hypoxia.

Observations on the erythropoietic and probable thrombopoietic activity of plasma from patients with polycythemia vera indicate that the plasma factors are of etiological importance in this disorder. The stimulus to this derangement in the humoral regulatory mechanism remains unknown.

Further study of the humoral control of myelopoiesis should aid in clarifying the pathogenesis of certain obscure anemic states and hematological responses.

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