A Comparison of Some Physical and Chemical Properties of Erythropoiesis-Stimulating Factors from Different Sources

By Wendell F. Rosse and Thomas A. Waldmann

Erythrocytosis has been noted in man in association with various tumors and cysts including renal cysts, hypernephromas, cerebellar hemangioblastomas, and pheochromocytomas. Patients with this association have an elevated red cell mass and usually have a normal white blood cell count, platelet count, and arterial oxygen saturation. In the majority of these patients, resection of the tumor or cyst results in a remission of the erythrocytosis. In certain cases, extracts of the tumor or cyst have been shown to contain erythropoiesis-stimulating activity.

Several substances are known to produce erythrocytosis in the experimental animal. These include erythropoietin, thyroid hormone, androgenic hormones, corticosteroids, dinitrophenol, and batyl alcohol. In the past decade, considerable evidence indicates that erythropoietin is the major factor in the physiologic stimulation of erythropoiesis. Although this substance has not been purified, it has been determined by indirect means that it is a mucoprotein with a molecular weight of 25,000-40,000, which requires sialic acid and the protein structure for biological activity.

The nature of the erythropoiesis-stimulating factors produced by tumors and cysts has not been characterized. We have previously shown by radiation inactivation that erythropoiesis-stimulating factors from renal cyst fluid and cerebellar hemangioblastoma cyst fluid are similar, if not identical in size (molecular weight 25,000-35,000) to erythropoietin derived from the urine of an anemic patient. In the present investigation, the erythropoiesis-stimulating factors present in anemic serum, anoxic serum, renal cyst fluid, and cerebellar hemangioblastoma cyst fluid were compared with reference to their electrophoretic mobility and the effect of treatment with trypsin, sialidase, and antibody to erythropoietin on their erythropoietic activity.

Materials and Methods

The four biological materials containing erythropoiesis stimulating activity used in these studies were: (a) Anemic serum—Blood was removed under sterile conditions from a patient with chronic lymphocytic leukemia and red cell aplasia at a time when her hemoglobin was 6.7 Gm. per cent. The blood was centrifuged and the serum removed and frozen. The whole serum from this patient was found to contain 9.7 units of erythropoietin/ml. as defined by Goldwasser and White. (b) Anoxic serum—Serum was likewise obtained from a patient with transposition of the great vessels. The patient had marked arterial oxygen desaturation (68 per cent) and polycythemia (hemoglobin 22.3 per cent). His whole serum was found to contain 0.52 units of erythropoiesis-stimulating activity per
ml. (c) Renal cyst fluid—Fluid was obtained from a single unilocular renal cyst of a patient with mild polycythemia. (Hct. 54). The fluid was yellow, clear, and contained 0.98 units of erythropoiesis-stimulating activity per ml. (d) Cerebellar hemangioblastoma cyst fluid—Fluid aspirated from a cerebellar cyst in a patient who had polycythemia (Hct. 63), and normal arterial oxygen saturation (95 per cent).* Using the present assay system, this cyst fluid was found to contain 14.9 units of erythropoiesis-stimulating activity per ml.

The erythropoiesis-stimulating activity of samples was determined in the polycythemic mouse assay previously described.23 Female NIH strain mice weighing 18–22 Gm. were injected intraperitoneally on days 1 and 2 of the assay with 0.7 ml. of an 80 per cent suspension of homologous red blood cells. On day 5, an additional 0.5 ml. of this suspension was given. On days 5 and 6, 0.5 ml. of test material was injected subcutaneously into each of 6–8 mice. Saline was injected into 8 mice as a negative control. On day 7, 0.5 μc. Fe59 citrate in 0.15 M NaCl was injected intraperitoneally. On day 9, the mice were bled by decapitation into tared bottles which were then reweighed. The radioactivity due to Fe59 was determined in a well-type gamma ray spectrometer. The per cent incorporation of the isotope in 1 ml. of peripheral blood was determined by comparison with an appropriate standard. A dose response curve performed with standard material obtained from the Hematology Study Section, National Institutes of Health, showed a linear relationship between dose of erythropoietin and the erythropoietic response between .24 and 2.4 units of the hormone. The minimum detectable dose tested was 0.03 units which caused an iron-59 incorporation of 0.41 per cent compared to an incorporation of 0.09 per cent per ml. in animals receiving saline. Samples containing more than 2.4 units per ml. were diluted with 0.15 M NaCl and reassayed.

**Electrophoresis**

Zonal electrophoresis was performed according to the method of Müller-Eberhart24 as modified by Fahey and McLaughlin.25 Geon (polyvinyl chloride particles) and Pevikon (polyvinyl chloride, polyvinyl acetate copolymer particles) in equal amounts were used in supporting matrix. Four hundred and eighty Gm. of the mixture was equilibrated with 300 ml. of sodium barbital buffer, pH 8.6, ionic strength of 0.075, and was placed in a Lucite electrophoresis chamber 36.5 cm. long which was divided longitudinally into 4 subunits, each of which is 5.5 cm. wide. Two to four ml. of the sample were applied in a narrow groove cut in the matrix 12 cm. from the cathodal end of the unit. A current of 50 ma. was applied for 18 hours. The Geon-Pevikon blocks were then divided into 1–4 cm. segments cut perpendicular to the direction of the electromotive force. The protein was eluted with 0.15 M NaCl, using 5 ml. per cm. segment of the block. The relative protein concentration of the eluate from each segment was found by determining the optical density at 280 μm in a Beckman DU spectrometer.

Undiluted aliquots of these eluates were tested for erythropoiesis stimulating activity in the assay described above. The electrophoretic mobility of the erythropoiesis-stimulating activity was compared with the mobility of proteins whose electrophoretic characteristics were known. To do this, albumin, haptoglobin (an α2-β globulin) and transferrin (a β globulin) were labeled prior to electrophoresis with Bromphenol Blue, hemoglobin and Fe59 citrate (0.5 μc.) respectively. The position of albumin was identified after electrophoresis by the position of the blue band near the anodal end as determined either by inspection or by determining the peak optical density of the eluates read at 600 μm (optimum for bromphenol blue). The position of the maximum optical density due to bromphenol blue corresponds to a peak of protein concentration (fig. 1, top panel). The position of the haptoglobin-hemoglobin complex following electrophoresis was similarly identified by the red band(s) as noted on inspection or determined by the peak optical density at 412 μm.

*The clinical course of this patient has been previously reported in full.10
1 Obtained from Goodrich Chemical Company, Akron, Ohio.
2 Obtained from Stockholm’s Superfosfat Fabriks A.—B., Stockholm, Sweden.
Fig. 1.—A comparison of the electrophoretic mobility of erythropoiesis-stimulating activity in anemic serum, hypoxic serum, renal cyst fluid, and cerebellar hemangioblastoma cyst fluid. Migration from the point of application (origin) is shown on the abscissa. The relative protein concentration of segments taken at centimeter intervals from the origin are shown for a typical run by the solid line in the top panel. The position of the erythropoiesis stimulating activity is shown in black, of albumin in cross-hatched areas (determined by spectrophotometry in the top panel and by inspection in the lower panels), of hemoglobin-heptoglobin complex in the dotted areas (spectrophotometrically in the top panel, by inspection in the lower panels), and of transferrin labeled with Fe59 citrate by the broken line in the top panel.

\( m\mu \) (optimum for hemoglobin). The position of transferrin was determined in one case by determining the peak of radioactivity in the eluate due to Fe59 as monitored in a well-type single channel \( \gamma \)-ray spectrometer.

In order to ascertain that the relative mobilities of proteins on Geon-Pevikon were the same as those commonly seen in other technics of zonal electrophoresis, agar gel electro-
The electrophoretic mobility on agar gel of proteins eluted from a Geon-Pevikon block after electrophoresis compared to the electrophoresis pattern of whole serum (WS). The distance in centimeters from the origin at which the segment was taken is shown by the numbers; positive numbers indicate that the segment was in the anodal direction from the origin, negative numbers, in the cathodal direction. In the agar gel electrophoresis shown here, the anode was at the top of the plate.

Enzymic Reactions

Sialidase* of clostridial origin, free of non-specific proteolytic activity, was incubated with each of the materials in order to determine whether the removal of sialic acid inactivated the erythropoiesis-stimulating activity. The reaction was carried on according to the method described by Blumberg and Warren, using 1–6 ml. of test material, depending

*Kindly supplied by Dr. Leonard Warren, National Institute for Arthritis and Metabolic Diseases, National Institutes of Health and from Behringwerke, Marburg-Lahn, Germany.
upon the erythropoiesis-stimulating activity titer. The reaction was continued until no further sialic acid was produced (6–10 hours at 37 C.) was measured by the thioarbiturate method of Warren. Controls for each incubation were treated identically except that saline instead of sialidase was added to the mixture. Both control and test mixtures were assayed for erythropoiesis-stimulating activity in polycythemic mice.

Tryptic digestion of each of the materials was carried out using twice crystallized trypsin of bovine pancreatic origin.* The course of the reaction was monitored by precipitating the protein of aliquots of the reaction mixture with 5 per cent perchloric acid and noting the optical density of the supernatant at 280 nm in the Beckman DU spectrometer. The reactions were continued until there was no further increase in the optical density at this wave length. Controls for each incubation were treated identically except that saline instead of trypsin was added to the mixture. Both control and test mixtures were assayed for erythropoiesis-stimulating activity in the polycythemic mouse assay as described above.

Immunologic Reactions

An antibody to human urinary erythropoietin was prepared by a modification of the method described by Schooley and Garcia. An extract of urine from the aplastic patient described above was made according to the method of Gordon. The lyophilized powder was reconstituted to a concentration of 1.5 mg./ml. At the onset of immunization, 2 ml. of this mixture was injected with 2 ml. of Freund’s complete adjuvant. Two weeks later a further 2 ml. was injected with 2 ml. of Freund’s incomplete adjuvant. Two weeks later, a progressive intravenous immunization with increasing amounts of alum-precipitated urine extract was begun according to the plan of Kabat and Meyer. Five days after the completion of a three-week course, serum was harvested. Two of six rabbits so treated, developed a factor in the serum which destroyed erythropoietin activity in serum of anemic patients. This factor was found to migrate as a gamma globulin on zonal electrophoresis performed as described above, was destroyed by heating to 65 C. for two hours, but was stable for one year when kept frozen. The serum was thus considered to contain antibody to erythropoietin.

The ability of this antiserum to neutralize the erythropoiesis-stimulating activity of the test material in vitro was determined by mixing equal portions of the immune rabbit serum and the material to be tested, incubating the mixture for 1/2 hour at 37 C. and then overnight at 0 C. Any precipitate formed was removed by centrifugation. Controls were performed in which the antiserum in the reaction mixture was replaced by saline or serum from rabbits immunized with an extract of urine that did not contain erythropoietin. The erythropoiesis-stimulating activity of the reacted mixture was assayed as described above.

RESULTS

The electrophoretic mobility of Geon-Pevikon block of the erythropoiesis stimulating factors from anemic serum, anoxic serum, renal cyst fluid, and cerebellar hemangioblastoma cyst fluid is shown in figure 1. The mobility of the biologically active factor in each of the tested materials is in the a2-globulin region. In each case, the Fe59 incorporation of the saline controls has been subtracted from the total. Although the incorporation of Fe59 into the blood of the assay animals injected with the eluates of the a2-globulin sections of the renal cyst fluid and the anoxic serum is low, in each case, it was significantly above that of saline controls (p < 0.01).

The effect of reaction with trypsin, sialidase and rabbit anti-erythropoietin antiserum on the biological activity is shown in table 1, 2 and 3 respectively. In each case the procedure completely destroyed the biological activity of

*Obtained from Worthington Biochemical Corporation, Freehold, New Jersey.
Table 1.—Inhibition of Erythropoiesis-Stimulating Activity by Reaction with Sialidase

<table>
<thead>
<tr>
<th>Source of Erythropoiesis-Stimulating Activity</th>
<th>Assay Results [% incorporation Fe(^{59}) in 1 ml. of blood (transfused mouse)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without enzyme</td>
</tr>
<tr>
<td>Anemic serum</td>
<td>4.55 (0.34)*</td>
</tr>
<tr>
<td>Anoxic serum</td>
<td>1.00 (0.27)</td>
</tr>
<tr>
<td>Cerebellar hemangiendo-blastoma cyst fluid</td>
<td>5.39 (1.11)</td>
</tr>
<tr>
<td>Renal cyst fluid</td>
<td>1.03 (0.56)</td>
</tr>
<tr>
<td>Saline control</td>
<td>0.15 (0.01)</td>
</tr>
</tbody>
</table>

*Standard error of the mean shown in parentheses.

Table 2.—Inhibition of Erythropoiesis-Stimulating Activity by Trypsin Digestion

<table>
<thead>
<tr>
<th>Source of Erythropoiesis-Stimulating Activity</th>
<th>Assay Results [% incorporation Fe(^{59}) in 1 ml. of blood (transfused mouse)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without enzyme</td>
</tr>
<tr>
<td>Anemic serum</td>
<td>5.62 (0.55)*</td>
</tr>
<tr>
<td>Anoxic serum</td>
<td>2.05 (0.66)</td>
</tr>
<tr>
<td>Cerebellar hemangiendo-blastoma cyst fluid</td>
<td>7.46 (1.15)</td>
</tr>
<tr>
<td>Renal cyst fluid</td>
<td>1.87 (0.25)</td>
</tr>
<tr>
<td>Saline control</td>
<td>0.05 (0.01)</td>
</tr>
</tbody>
</table>

*Standard error of the mean shown in parentheses.

the tested materials. Reaction of the materials with a rabbit antiserum to an extract of normal urine did not affect the biological activity.

DISCUSSION

Although the hormone, erythropoietin, has not been purified, concentrates of great biological activity have been prepared from the serum of anemic rabbits\(^{31,33,34}\) and sheep\(^{19,32}\) and from the urine of anemic patients.\(^{29}\) Some estimates of the molecular characteristics of erythropoietin have been made by analysis of these partially purified preparations. The assumption is made that the chemical and physical characteristics of the majority of the protein of the preparation reflect the characteristics of the biologically active molecule. Analyses of this sort have indicated that erythropoietin is a mucoprotein with a high sialic acid, carbohydrate, and hexoseamine content.\(^{31,32}\) The electrophoretic mobility of these partially purified preparations has been found to be in the \(\alpha_1\)-globulin\(^{32,34}\) region by some groups and in the \(\alpha_2\)-globulin region by others.\(^{31}\) In the present studies, the electrophoretic mobility of the erythropoiesis stimulating factor in each of the materials tested was found to be in the \(\alpha_2\)-globulin region by determining the mobility of the biological activity directly. Since whole serum or cyst fluid could be used, the possibility of alterations in the electrophoretic mobility by purification procedures was eliminated.

Information about the nature of erythropoietin molecule has also been ob-
Table 3.—Inhibition of Erythropoiesis-Stimulating Activity with Anti-Erythropoietin Neutralizing Antibody

<table>
<thead>
<tr>
<th>Source of Erythropoiesis-Stimulating Activity</th>
<th>Assay Results (% incorporation of Fe²⁺ in 1 ml. of blood (transfused mouse))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemic serum</td>
<td>Without antibody: 1.61 (0.60) * 0.13 (0.01)</td>
</tr>
<tr>
<td>Anoxic serum</td>
<td>With antibody: 2.83 (0.42) 0.04 (0.01)</td>
</tr>
<tr>
<td>Cerebellar hemangioblastoma cyst fluid</td>
<td>Without antibody: 5.67 (0.81) 0.07 (0.01)</td>
</tr>
<tr>
<td>Renal cyst fluid</td>
<td>With antibody: 2.66 (0.57) 0.03 (0.01)</td>
</tr>
<tr>
<td>Saline control</td>
<td>0.09 (0.01)</td>
</tr>
</tbody>
</table>

*Standard error of the mean shown in parentheses.

By noting the effect of physical or chemical treatments upon the biological activity. Using this approach, we have previously demonstrated that the size of biologically active portions of the molecule is equal to a molecular weight of 25,000–35,000 and that this size is the same for erythropoiesis-stimulating factors from renal cyst fluid and cerebellar cyst fluid. Likewise, this approach has been used by others to show that the biological activity of erythropoietin from anemic sources is dependent upon the protein structure and the presence of sialic acid on the molecule. The present studies demonstrate that erythropoiesis-stimulating factor(s) from human anemic serum, anoxic human serum, renal cyst fluid and cerebellar cyst fluid have the same requirements for biological activity.

The destruction of biological activity by a single specific antiserum may be used to demonstrate the immunologic similarity of proteins. Thus, Garcia and Schooley have found that antiserum to human urinary erythropoietin made in rabbits neutralizes the erythropoietic activity of serum from anoxic rats or anemic mice, rabbits, sheep and man. Likewise, in the present studies, the antiserum to human urinary erythropoietin neutralized the erythropoietic activity of human anemic and anoxic serum and cyst fluid from renal and cerebellar hemangioblastoma cysts as well as that of anoxic rabbit and anemic rat serum. However, this antiserum does not destroy the erythropoietic activity present in the serum of anoxic Japanese quail. This indicates that the erythropoiesis-stimulating factors from both “normal” and pathologic sources in man and from other mammals share common antigenic determinants which are not present in the erythropoiesis stimulating factor of birds and frogs.

From these considerations, it is clear that the substance stimulating erythropoiesis found in renal cyst fluid, cerebellar hemangioblastoma cyst fluid, and serum from an anoxic patient is similar, if not identical to the erythropoietin found in the serum of an anemic patient. Thus, the pathogenesis of the polycythemia seen in association with renal cysts and cerebellar hemangioblastoma is probably due to the secretion of erythropoietin by these lesions.

It is not surprising that erythropoietin is found in renal cyst fluid since it has been shown in experimental animals that the kidney is a major source
of erythropoietin produced in response to anemia or anoxia.\textsuperscript{38,39,40} Considerable evidence indicated that extra-renal production of erythropoietin occurs in response to anemia and anoxia.\textsuperscript{39,40} The sites, other than the kidney, where the hormone is produced are unknown. It is of interest that the four tumors associated with polycythemia in which erythropoietin assays of tumor tissues or cyst fluid have been positive (i.e., hypernephroma, renal cysts, pheochromocytoma, and cerebellar hemangioblastomas) may all be components of the von Hippel-Lindau syndrome.\textsuperscript{41} This suggests that logical places to seek the extra-renal sites of production of erythropoietin in anemic or anoxic animals might be the adrenal and the posterior fossa.

Hepatomas\textsuperscript{5,42,44} uterine fibromas\textsuperscript{43} and virilizing adrenocortical tumors\textsuperscript{44} and ovarian tumors\textsuperscript{12} have been associated with polycythemia. The assay for erythropoietin in tumor tissue or serum from these patients, except in one instance,\textsuperscript{5} has been negative when done.\textsuperscript{42,44} It is entirely possible that these tumors secrete substances which stimulate erythropoiesis which are not erythropoietin and are not detected by the assay systems used. Androgenic hormones, adrenocortical steroid hormones, and thyroid hormone, all of which are known to stimulate erythropoiesis in the experimental animal, give in fact negative results in the starved rat or polycythemic mouse assay for erythropoietin.\textsuperscript{31,34} Thus, although the present studies show that the factor stimulating erythropoiesis elaborated by some tumors is the same, or nearly the same, as erythropoietin produced in response to anemia or anoxia, the mechanism by which certain other tumors produce polycythemia requires further study.

**SUMMARY**

In the present study we have compared some molecular characteristics of factors stimulating erythropoiesis found in the serum from patients with \(a\) aplastic anemia and \(b\) with erythremia due to anoxia, and in cyst fluid from patients with erythremia secondary to \(c\) a renal cyst and \(d\) a cerebellar hemangioblastoma. The biological activity of each of the four materials was found to migrate as an \(a_2\)-globulin on zonal electrophoresis and was destroyed by treatment with sialidase and trypsin; this implies that these materials are mucoproteins with similar electrophoretic mobility. The erythropoietin activity was also completely neutralized by an antiserum made in rabbits to an extract of urine of anemic patients. This implies that these materials share common antigenic determinants. Thus, the erythropoiesis-stimulating factors in human anemic and anoxic serum, cerebellar hemangioblastoma cyst fluid, and renal cyst fluid appear to be similar, if not identical. Therefore, the pathogenesis of the erythrocytosis seen in association with renal cysts and cerebellar hemangioblastomas appears to be due to the secretion of erythropoietin by these lesions.

**SUMMARIO IN INTERLINGUA**

In le presente studio nos ha comparate certe characteristicas molecular de factores de stimulation erythropoietic le quales se trova in le seros de patientes con \(a\) anemia aplastic, \(b\) erythremia cause per anoxia e in
PROPERTIES OF ERYTHROPOIESIS-STIMULATING FACTORS

le liquido de cystes de patientes con erythremia secundari a (c) un cyste renal, (d) un hemangioblastoma cerebrellar. Esseva trovate que le activitate biologic in cata-un del quatro materiales migra in electrophorese zonal como un globulina o_2 e es destruite per le tractamento con sialidase e trypsina. Isto indica que le materiales es mucoproteinas con simile mobilitate electrophoretic. Le activitate erythropoietinic esseva etiam neutralisate completamente per un antiserio, facite in conilios, anti un extracto de urina ab patientes anemic. Isto signifia que le materiales ha in commun determinantes antigenic. Assi il pare que le factores stimulante le erythropoiese le quales es trovate in sero human ab patientes anemic e anoxic, in le liquido de cyste in hemangioblastoma cerebrellar, e in le liquido de cystes renal es simile, si non identic. Per consequente, le pathogenese del erythrocytose que es vidite in association con cystes renal e hemangioblastomas cerebrellar pare esser causate per le secretion de erythropoietina per ille lesions.

ACKNOWLEDGMENTS

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