The Isolation of Erythropoiesis Regulatory Factors by an Electrofractionation Technique Combined with Selective Membrane Permeability[1] (37008)

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Selective membrane permeability to a relatively small erythropoiesis stimulatory factor (ESF) was demonstrated (1–3). A mixture of a relatively large ESF and an ESF-generating factor (EGF) was separated by selective membrane filtration (4). This report describes the isolation of four erythropoiesis regulatory factors (ERF), including an erythropoiesis inhibitory factor (EIF), by combining an electrofractionation technique with selective membrane permeability.

Materials and Methods. A urine concentrate of ESF, fraction II + III from a patient with paroxysmal nocturnal hemoglobinuria, was the starting material (5). The posthypoxic-polycythemic mouse was used as an assay animal (6). Results were calculated as the mean and standard error of the mean of the ESF activity of five or more mice and reported as percentage ^{59}Fe incorporated during 48 hr and as International Units of standard B (7); assays with high activities were repeated at levels commensurate with linear dose–response data. Nitrogen was determined by a micro-Kjeldahl method.

Fraction II + III was placed in the center section of an electrofractionation apparatus (Fig. 1) designed and constructed for the purpose of isolating ERF. Since the buffers became progressively acidic at the anode and alkaline at the cathode during operation, the buffers chosen were 0.13 *M* phosphate and pH 7.9 at the anode and 5.0 at the cathode. The entire system was 0.7 *M* NaCl. The solutions in the anode and cathode buffer compartments were replaced every 3.5 days to reestablish the initial pH gradient between the compartments; during the period of 3.5 days the pH of the solution in the center section decreased from 7.4 to 7.0. Although the experiments were done at 3 ± 2° and high ionic strength, enough phenol was added to the buffers to make the entire system 0.1% phenol as recommended by Lowy and Keighley (8). The chlorine and hydrogen gases evolved during electrolysis were aspirated. The solutions in the sections nearest the buffer compartments were stirred continuously. The fractions in the first anode and cathode sections were collected and replaced with fresh buffer after every 2 wk of electrophoresis through 8 wk. During this period the pH of the solution in the center section decreased to 5.9.

During preliminary experiments various combinations of membranes[2] were tried. Ultimately an appropriate selection of membranes was made as follows: The buffer compartments (capacity 1,500 ml each) were separated from the fraction collecting sections (capacity 250 ml each) by membranes with a molecular weight cutoff at 10,000; the first anionic fraction collecting section was separated from the second section by a membrane with a molecular weight cutoff at 20,000; the first anionic fraction collecting section was separated from the center section by a membrane with a cutoff at 30,000; the center section (capacity 500 ml) was separated from the first cationic fraction collecting section by a membrane with a cutoff at 50,000; the first cationic fraction collecting section was separated from the second

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[2] Purchased from the Amicon Corp., Lexington, MA.

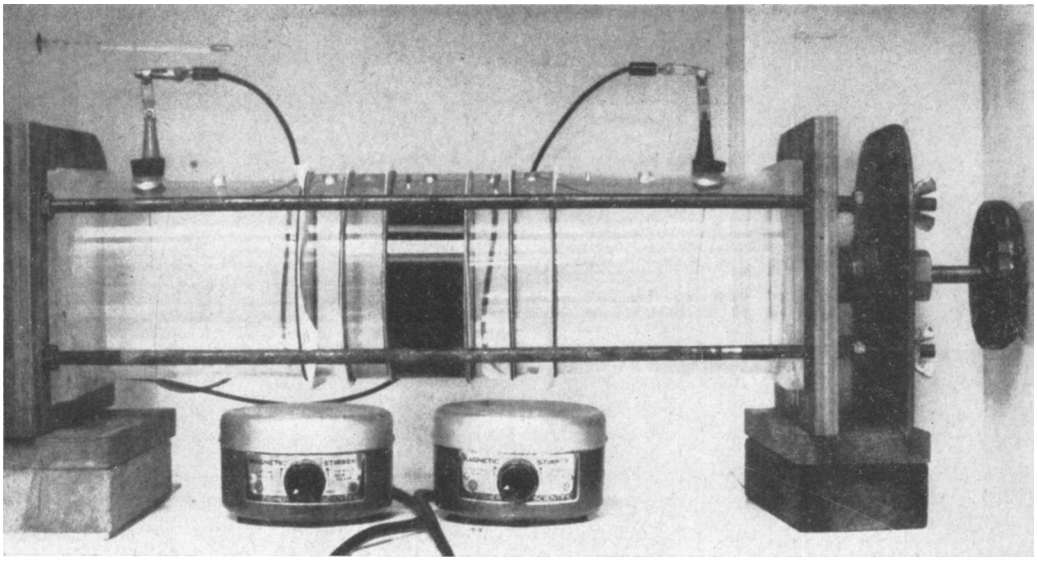

FIG. 1. Electrofractionation apparatus (see text for details).

by a membrane with a cutoff at 30,000. EGF, ESF and EIF activities were measured on the fractions.

The EIF fraction was purified further by preparatory column electrophoresis (9), and 5.0 mg were injected intraperitoneally into groups of four or more normal AKR mice (6–8 wk old) each day for 1–9 and then 30 days; one group received 1 day of EIF treatment, a second group 2 days, other groups as much as 3–9 days, and the last group 30 days of EIF treatment. Control mice were injected with saline or 0.1 ml of normal human serum, which contained 5 mg of protein. Twenty-four hours after the final injection of EIF or the control solutions and 48 hr before killing the mice for blood sample collections the mice were injected with 0.5 μCi of ^{59}Fe. Hematologic data were obtained on all mice 3 days after the final EIF injections. Hemoglobin concentrations were measured by the cyanmethemoglobin technique (10). The percent ^{59}Fe incorporation was calculated the same way as for the bioassay for erythropoietin (ESF) activity. Inhibition was also demonstrated *in vitro* as previously reported (9).

Results. Table I is a compilation of data obtained during the electrofractionation of three ESF concentrates. The results are expressed as the mean and SEM of the ESF activity as measured with 15–20 mice for each mean during three electrofractionation experiments. The EGF was in the first cathode section, and it generated ESF activity during incubation with normal rabbit serum or assay mouse serum and was pH dependent and inactivated by dithiothreitol, whereas ESF was not (11). An ESF was collected from the first anode section, and an ESF remained in the center section. No activity passed to the anode or cathode sections after eight weeks; however, about 110 IU of an ESF remained in the center section. An EIF was in the second cathode fraction collecting section.

Figure 2 illustrates the depression of erythropoiesis as measured by the incorporation of ^{59}Fe in peripheral blood, after injection of the EIF, which continued through the sixth sample collected after killing the mice for the daily data and was followed by a steady climb back to the control level by the ninth postmortem blood sample. The Day 3 collection corresponded to the Day 1 EIF injection, which was not different from the saline control collected on the same day. No change was observed until after 2 daily injections, which corresponded to the Day 4 collection. The hematocrits decreased maximally about 2% and the concentrations of the hemoglobin about 1% during the EIF injections, which

TABLE I. The Selective Membrane Electrofractionation of Erythropoiesis Regulatory Factors.

Fraction	Time (wk)	ESF activity[a] (mean ± SEM) RBC ^{59}Fe (%)	ESF activity[a] (mean ± SEM) IU/ml	(No. of mice)	Sp act IU/mg N
II + III[b]	0	33.1 ± 0.9	0.72 ± 0.02	20	0.91
Anode[b] (ESF)	2	22.6 ± 1.3	0.50 ± 0.03	15	3.95
	4	19.6 ± 0.6	0.43 ± 0.01	15	5.58
	6	32.0 ± 1.3	0.68 ± 0.03	20	8.64
	8	17.0 ± 0.8	0.36 ± 0.02	15	5.80
	10	0.0 ± 0.2	0.00 ± 0.00	15	—
Cathode[b] (EGF)	2	21.1 ± 0.7	0.47 ± 0.01	15	4.47
	4	21.8 ± 1.7	0.49 ± 0.04	15	6.44
	6	18.0 ± 0.8	0.40 ± 0.02	15	5.32
	8	13.0 ± 0.6	0.27 ± 0.01	15	3.13
	10	0.2 ± 0.6	0.00 ± 0.01	15	—
Center section (ESF)	10	10.6 ± 0.9	0.22 ± 0.02	15	0.34
reclaimed activity			3.82 ± 0.02	15	

[a] Data from three electrofractionation experiments.

[b] The volume in the center section was twice the volumes in the fraction collecting sections. The recovery of ESF activity was calculated to be 281%.

was not considered significant. The EIF also inactivated ESF *in vitro* as previously demonstrated (9).

The EIF fraction was about 7.5% protein and was soluble in ethanol–acetone (9/1, v/v). The yield of the EIF fraction at the end of the first 2 wk of the electrofractionation was 6.4 ± 0.2 mg (mean ± SEM for 28 experiments); this was the amount recovered after dialysis, lyophilization and further separation by preparatory column electrophoresis (9) of the fraction in the second cathode section obtained during the electrofractionation of 500 ml of the ESF fraction II + III concentrate. The fraction II + III was concentrated 5× from whole urine (5).

Discussion. Several results have been reported for the molecular weight of ESF (12–16). Amicon membranes have been described by Michaels (17) and have been standardized with different proteins and other relatively small molecules. The anionic ESF that passed through the membrane with the permeability limited to less than a mol wt of 30,000 was retained by the membrane with a cutoff at 20,000; Rosse, Berry and Weldman (12) reported a value of 27,000. The ESF that remained in the center section did not pass toward either electrode through a membrane with a cutoff at 50,000, and so the state of charge on that ESF was not ascertained; the suggested values for the molecular weight of ESF by Hansen (13), Goldwasser and Kung (14) and Olsen and Fogh (15) were also relatively large. The cationic EGF that passed through the membrane with the permeability limited to less than a mol wt of 50,000 was retained by the membrane with a cutoff at 30,000; O'Sullivan *et al.* (16) reported a value of 32,600 for a nondialyzable ESF, but no attempt was made to demonstrate EGF activity with their factor. The EGF was comparable to the nondialyzable factor reported with a sedimentation coefficient between 2.7 and 4.5 S (2). The cationic EIF that passed through the membrane with the permeability limited to less than a mol wt of 30,000 was retained by the membrane with the cutoff at 10,000 and was comparable to the basic EIF fraction with a sedimentation coefficient of 1.99 $S_{20,w}$ (9).

Previous reports suggested an essential role for a basic protein associated with ESF activity (18, 19), which was comparable to the cationic EGF described herein. We have demonstrated an ESF that appeared smaller than EGF (1–3), which was comparable to the anionic ESF described herein. We have also demonstrated an ESF that appeared larger than EGF (4), which was comparable to the

FIG. 2. The depression of erythropoiesis by an erythropoiesis inhibitory factor (EIF) as followed in the peripheral blood of normal AKR mice. The bars indicate the standard error of the mean (4 or more mice) of the percentage of ^{59}Fe incorporated. The days of sample collection correspond to 3 days after the last injection of EIF (5 mg/day) or control solution and 2 days after the injection of ^{59}Fe.

ESF that remained in the center section.

The relatively large recovery (281% for three experiments) of ESF activity was probably due to the removal of the EIF from the urinary concentrate. The inhibitor described herein was comparable to the one isolated by Erslev, Kazal and Miller (20); 0.01 mg inactivated 1 IU of ESF during 3 hr of incubation. The removal of 6.5 mg of such an EIF would account for the increased recovery of ESF activity (Table I), and we recovered 6.4 ± 0.2 mg (28 experiments) after lyophilization of the electrophoretic EIF fraction collected at the end of the second week, and further purified as described. The amount of EIF was calculated to be about 2.6 μg/ml of whole urine compared to 1.5 μg by a previous technique (9). The percentage recovery of ESF activity after removing the fractions once every 2 wk was about the same as when the fractions were removed once each week, which suggested that most of the inhibitor passed to the second cathode section during 1 wk.

The relatively large amount of nonprotein fraction in the EIF, the ESF-neutralizing capacity, and the fact that the EIF was soluble in organic solvents suggested a similarity with the renal lipid inhibitor reported by Erslev, Kazal and Miller (20). Whitcomb (21) noted that a serum EIF has been considered by some investigators as an anti-ESF. Since the EIF described herein passed through a membrane with the permeability limited to molecules with a molecular weight less than 30, 000, it would seem relatively small for an antibody.

Our results compare well with those obtained by Schooley and Garcia (22) who observed maximal erythroid cell depression in bone marrow 6 days after the first injection of anti-ESF and a return to normal by Day 9. Those authors presumed the increase that followed the decrease in erythroid cells to be due to the increased production of ESF by the mice and/or a decreased level of the injected anti-ESF as a result of the elimination of heterologous rabbit serum. Furthermore, Erslev, Kazal and Miller (20) indicated that normal serum inactivated some inhibitory activity. Similar possibilities could explain our data, and it is also possible that a neutralizing anti-EIF was formed. The erythropoiesis depression data appeared to follow a schedule that possibly was due to the time required to form an antibody; a similar rate for antibody formation was observed by Schoenheimer *et al.* (23). Schooley, Zanjani and Gordon have produced neutralizing anti-anti-ESF by inoculating goats with rabbit gamma globulins (24), which was analogous to the production of neutralizing anti-EIF sera.

There were two separatory processes in the fractionation technique, electrofocusing as described by Svensson (25) and selective membrane permeability. Electrofocusing separated proteins on the basis of isoelectric behavior, and the limiting factor was the permeability of the membrane. The extended duration for separation was due to the high ionic strength with NaCl, which allowed relatively little current for the ESF but was necessary to maintain the integrity of the ESF; without the salt all of the activity was lost. Sodium chloride was demonstrated previously to improve the stability of ESF (26). If the membranes remained intact within the apparatus and in the NaCl solution, the same membranes could be used for several months. Several sets of membranes were used during the course of this work. A recent set appeared thicker and required an extended period of exposure to the conditions of the experiments before the described factors could be isolated. A similar change in these membranes has been observed by others (27).

Summary. A urine concentrate from a patient with paroxysmal nocturnal hemoglobinuria contained four regulators of erythropoiesis that could be isolated by an electrofractionation technique combined with selective membrane permeability. An erythropoiesis-stimulating factor (ESF) passed toward the anode through a membrane with a cutoff at a molecular weight of 30,000, but was retained by a membrane with a cutoff at 20, 000, while another ESF was retained by a membrane with a cutoff at 50,000. An ESF-generating factor passed toward the cathode through a membrane with a cutoff at 50,000 but was retained by a membrane with a cutoff at 30,000.

An inhibitor of erythropoiesis (EIF) passed toward the cathode through a membrane with a cutoff at a mol wt of 30,000 but was retained by a membrane with a cutoff at 10,000. The EIF brought about maximal depression of erythropoiesis 6 days after the first of four daily injections, but erythropoiesis gradually returned to normal 3 days later, though the EIF injections were continued. The EIF appeared to be mostly lipid and about 7.5% protein.

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