The Selective Membrane Filtration of an ESF-generating Factor (EGF) in the Presence of Erythropoietin (ESF)[1] (35888)

J. P. Lewis, Emily T. Welch, W. A. Neal, R. R. Moores, W. G. Lewis, III, C.-S. Wright, C. M. DuBose, Jr., and Linda L. Smith

Department of Research, Veterans Administration Hospital and Department of Medicine and Biochemistry, Medical College of Georgia, Augusta, Georgia 30904

An EGF has been demonstrated (1–13). Erythrogenin, the renal erythropoietic factor (REF), seemed to be an enzyme that acted on a serum substrate to produce an erythropoiesis stimulating factor (ESF) (6). Although EGF and REF appeared to perform the same function, the exact identity of the two factors has not been established.

Recently the optimum pH for EGF activity was determined on a fraction isolated from the urine of a female patient with an anemia secondary to multiple myeloma (MM) (12). An unsuccessful attempt was made to demonstrate a pH activity dependence on a fraction isolated in the same way from the urine of a male patient with paroxysmal nocturnal hemoglobinuria (PNH). This report describes an additional step in the fractionation of the urine from the PNH patient, which was necessary to obtain a similar EGF.

Materials and Methods. The urine was from a male PNH and a female MM patient. The urinary ESF concentrate, fraction II + III, was prepared by column chromatography on DEAE-cellulose (14). An ESF was removed from fraction II + III by selective membrane permeability (9–13). The retentates, after exhaustive dialysis and lyophilization, were dissolved in 0.02 *M* phosphate buffer at pH 7.4 and 0.5 *M* NaCl and fractionated by selective membrane filtration with Amicon XM-50 centriflo membranes supported in 50-ml conical centrifuge tubes. These membranes were designed to retain molecules at a molecular weight of about 50,000 or greater. (The membranes were purchased from the Amicon Corporation, Lexington, Mass.). Centrifugation was continued at 1100*g* and 4° until all the solution passed through the membrane. The filtrate was collected, and the residue on the membrane was dissolved in the same phosphate buffer. The volumes of the solutions were adjusted so as to contain the extract from 0.05 or 0.20 mg of the fractions/ml, respectively, from the urines of the MM and PNH patients. ESF and generated ESF activity were determined on all fractions (9–12, 15). The posthypoxic–polycythemic mouse was used as an assay animal for ESF activity (15). The results of ESF and generated ESF activity are expressed as percentage RBC-^{59}FE incorporation and also as international standard[2] B units. The generation of ESF activity by incubation of ESF with rabbit and mouse serum, *in vitro* as well as *in vivo*, has been demonstrated (9–12). The EGF activity was pH dependent, whereas the ESF activity was not. The pH-ESF activity data were obtained on the fractions as reported (10–12). The EGF was inactivated with 10 μM of dithiothreitol (DTT), whereas the ESF was not (13). Nitrogen was determined by a micro-Kjeldahl method.

Results. After centrifugation of the retentate as described from the urine of the MM patient, the EGF activity (equivalent to 0.26 ± 0.01 IU of ESF/ml) was in the filtrate, and no ESF or EGF activity was found in the residue (Table I). After similar treatment of the comparable retentate from the urine of the PNH patient, the EGF activity (equiv to 0.19 ± 0.00 IU of ESF/ml) was in

[1] Supported in part by Grants HE-10591-07 (HEM), FR-0061, FR-5365, and HE-12958 from the National Institutes of Health.

[2] ESF standard B was obtained through the courtesy of Drs. D. R. Bangham and Mary Cotes, Medical Research Council, National Institute for Medical Research, London, England.

the filtrate; but ESF activity (0.20 ± 0.01 IU of ESF/ml after dissolving the residue as described in Methods) was only in the residue.

The optimum pH for the activity of the EGF in both filtrates was about 7.4. The EGF fractions from the urines of the MM and PNH patients were about 39.4 and 62.0% protein, respectively, whereas the residue (PNH) ESF fraction was 61.3% protein. The EGF solution, when stored frozen, retained its activity indefinitely.

Discussion. It was of interest to find that the optimum pH for activity of the EGF fraction obtained from the urine of the patient with PNH was about 7.4, the same as that previously found for the EGF from the urine of the patient with MM (10–12). It was not possible to demonstrate the pH effect on the EGF from either urine without first separating ESF from EGF; the response to ESF flattened out the pH activity curve of EGF as previously demonstrated.

It was also of interest that there was an ESF in the urine of the PNH (but not the MM) patient that appeared to be larger than the EGF, or attached to a carrier protein larger than the EGF. An ESF smaller than EGF, or also attached to a carrier protein smaller than EGF, was demonstrated previously in the urines of the PNH and MM patients, and the fraction was about 11% protein (9–12). The difference in the percentage protein of the two EGF fractions was possibly due to a relative degree of purity. These observations help to explain the variable results reported for the molecular weight of ESF (16–21).

In a previous report EGF was indicated to be stable for more than 3 years, after lyophilization and during storage at —12° in a desiccator over calcium chloride and under nitrogen (12). Upon dissolving the dried fraction in 0.02 *M* phosphate buffer at pH 7.4 and freezing, however, the activity disappeared entirely during 3 weeks. In the current work, the buffer solutions were also 0.5 *M* NaCl, which improved the stability of EGF indefinitely, possibly by maintaining EGF in its natural conformation.

Summary. A mixture of an erythropoiesis

TABLE I. The Selective Membrane Filtration of an ESF-Generating Factor (EGF).

Disease[a]	Retentate[b] ESF activity (mean ± SEM)			Residue[c] ESF activity (mean ± SEM)		Filtrate[c] ESF activity (mean ± SEM)	
	RBC-^{59}Fe (%)	IU	Wt (mg/mouse)	RBC-^{59}Fe (%)	IU/ml	RBC-^{59}Fe (%)	IU/ml
MM	12.9 ± 0.6	0.28 ± 0.01	0.05 (20)[d]	0.1 ± 0.1	0.00 ± 0.00 (10)	12.3 ± 0.6	0.26 ± 0.01 (10)
PNH	15.0 ± 0.6	0.33 ± 0.01	0.20 (20)	9.9 ± 0.5	0.20 ± 0.01 (15)	9.1 ± 0.3	0.19 ± 0.00 (25)

[a] MM, female preterminal patient with an anemia secondary to multiple myeloma; PNH, male patient with paroxysmal nocturnal hemoglobinuria.

[b] The retentate was obtained after exhaustive dialysis (using selective membrane permeability) of fraction II + III, a concentrate of human urinary erythropoietin.

[c] The volumes of the residue and filtrate solutions were adjusted so as to contain the extract from 0.05 or 0.20 mg of the retentate fractions/ml, respectively, from the urines of the MM and PNH patients. The pH dependent EGF activity was demonstrated only with the filtrate in each case, and ESF activity was found only in the PNH residue. DTT inactivated the EGF in both retentates, but not the ESF in the PNH retentate, as previously described (13). DTT inactivated 47.2% of the activity in the retentate (PNH), whereas the EGF activity in the filtrate (PNH) was 48.6% of the total activity recovered. The Amicon XM-50 centriflo membranes were supported in 50-ml conical centrifuge tubes.

[d] Number of mice is given in parentheses.

stimulating factor (ESF) and an ESF-generating factor (EGF), isolated from the urine of a patient with paroxysmal nocturnal hemoglobinuria (PNH), was separated by selective membrane filtration. The optimum pH for activity of the EGF fraction from the urine of the PNH patient was about 7.4, the same as that previously found for the EGF from the urine of a patient with an anemia secondary to multiple myeloma (MM). The urine from the PNH patient contained an ESF that appeared larger than EGF, whereas the urine from the MM patient did not; the urine from both patients, however, contained an ESF that appeared smaller than EGF as previously noted.

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Received April 19, 1971. P.S.E.B.M., 1971, Vol. 138.