The Selective Inactivation of an ESF-Generating Factor (EGF) in the Presence of Erythropoietin (ESF)[1] (35666)

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An ESF-generating factor (EGF) has been demonstrated (1–12). EGF appeared to be an enzyme that acted on a serum substrate to produce ESF, which is analogous to the suggested role of the renal erythropoietic factor (REF or erythrogenin) described by Gordon *et al.* (6). Some anemic patients could be deficient in the EGF-substrate concurrent with a sufficiency of EGF. Since both factors bring about the eventual incorporation of ^{59}Fe into the heme group of hemoglobin in posthypoxic-polycythemic mice, as indicated with the ESF bioassay, it becomes of importance to be able to distinguish between the two activities.

Cleland (13) described a protective reagent for sulfhydryl (SH) groups, 2, 3-dihydroxy-1,4-dithiolbutane (dithiothreitol or DTT), which maintains monothiols completely in the reduced state and reduces disulfides (S–S) quantitatively. The present report describes the application of DTT to the problem of differentiating between the activities of EGF and ESF. Heretofore, it was impossible to distinguish between the activities of EGF and ESF in a mixture of both. However, McDonald *et al.* (14) demonstrated an immunochemical difference between REF and ESF. It is not known whether the EGF described herein is the same as REF. Some differences in the two factors have been observed, which possibly were due to a relative degree of purity. (11, 12).

Materials and Methods. Fraction II + III, the urinary ESF concentrate used in this work, was prepared by column chromatography on DEAE-cellulose (15). About 86% of the proteins were removed from the fraction, and most of the ESF activity was retained. The urine was obtained from one patient with paroxysmal nocturnal hemoglobinuria (PNH) and from a preterminal patient with an anemia secondary to multiple myeloma (MM). Fraction II + III was fractionated further by selective membrane permeability (9–12, 16). The dialysates and retentates were then dialyzed in 24/32-in. (i.d.) tubing to remove the salts, and were dried by lyophilization. The dried powder was weighed and mixed either with a 0.02 *M* phosphate buffer (1 ml) at pH 7.4 as a control or with the buffer plus DTT and incubated for 30 min at 37° prior to ip injections into posthypoxic-polycythemic assay mice (17). The generation of ESF activity by incubation of EGF with normal serum *in vitro*, as well as *in vivo*, has been demonstrated (10–12). The EGF activity was pH dependent, whereas the ESF activity was not. The results of ESF activity and generated ESF activity are expressed as percentage RBC-^{59}Fe incorporation and also as international standard[2] B units.

Results. Incubation of the equivalent of 0.28 ± 0.01 (20 mice) IU ESF/mouse of the EGF (obtained from the retentate of the urine from the MM patient) with 0.1, 5.0, or 10.0 μM of DTT inactivated the EGF (Table I).

Incubation of 0.17 ± 0.02 (18 mice)

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[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.

TABLE I. The Selective Inactivation of an ESF-Generating Factor (EGF) with Dithiothreitol (DTT).

	(A) ESF activity (mean ± SEM)				(B) ESF activity (mean ± SEM)		
Wt-fraction (mg/mouse)	RBC-^{59}Fe (%)	(IU)	(No. of mice)	DTT added (μM)	RBC-^{59}Fe (%)	(IU)	(No. of mice)
0.05-EGF[a]	12.9 ± 0.6	0.28 ± 0.01	(20)	0.1	0.9 ± 0.9	0.02 ± 0.02	(10)
	12.9 ± 0.6	0.28 ±0 .01	(20)	5.0	0.6 ± 0.6	0.01 ± 0.01	(5)
	12.9 ± 0.6	0.28 ± 0.01	(20)	10.0	2.8 ± 0.9	0.06 ± 0.02	(10)
0.50-ESF[b]	8.0 ± 0.9	0.17 ± 0.02	(18)	10.0	7.7 ± 0.9	0.16 ± 0.02	(18)
0.50-II+III[c]	20.8 ± 0.6	0.46 ± 0.01	(15)	0.5	8.8 ± 0.6	0.18 ± 0.01	(5)
	20.8 ± 0.6	0.46 ± 0.01	(15)	10.0	8.8 ± 0.6	0.18 ± 0.01	(5)
0.30-II+III	18.7 ± 1.4	0.41 ± 0.03	(10)	10.0	7.0 ± 0.6	0.14 ± 0.01	(15)
	19.0 ± 0.6	0.43 ± 0.01	(10)	10.0	8.8 ± 0.6	0.18 ± 0.01	(15)

[a] ESF-generating factor from the urine of a preterminal patient with an anemia secondary to multiple myeloma. The phosphate buffer solvent for all fractions was 0.02 *M* at pH 7.4.

[b] Erythropoiesis stimulating factor from the same source as the EGF.

[c] Fraction II+III contains EGF and ESF from the urine of a patient with paroxysmal nocturnal hemoglobinuria collected during two different periods. All fractions were incubated for 30 min at 37°, without (A) and with (B) the indicated amount of DTT, prior to ip injections into posthypoxic-polycythemic assay mice.

IU/mouse of ESF (obtained from the dialysate of urine from the MM patient) with 10 μM of DTT did not inactivate the ESF (Table I).

Incubation of the equivalent of 0.44 ± 0.02 IU (35 mice) ESF/mouse of fraction II + III, which has been demonstrated to contain EGF and ESF (from the urine of the PNH patient), with 0.5 or 10 μM DTT inactivated about 62% of the mixture (Table I).

Discussion. Since DTT maintains SH groups completely in the reduced state and reduces S–S groups quantitatively, it is evident that the nonoxidizable SH group or the reduction of S–S groups does eliminate EGF (but not ESF) activity. The work by Goldwasser and Kung (18) indicated that SH groups are not required for the biological activity of ESF. The present data do not indicate whether the DTT effect is exerted directly on EGF or indirectly by an effect on the EGF-substrate. The more highly purified that EGF and its substrate become the more likely the possibility that DTT can be used as a tool in the elucidation of the mechanism of ESF production.

In a previous report (19), the parallelism of the ESF dose–response with the oxidation of glutathione (GSH) was observed, and it was suggested that a common substance may be responsible for both responses. Glutathione is believed to maintain many enzymes in their active conformation. The current observation of the inactivation of EGF by DTT would further suggest a possible correlation between the GSH oxidation–reduction system and the production of ESF. Glutathione could be considered a cofactor with EGF providing that the two systems are proximate.

Fraction II + III has been demonstrated to contain EGF, ESF, and several contaminating proteins which could have SH groups that would be maintained in the reduced state by DTT. However, the fact that ESF was not inactivated, although the DTT was increased 100-fold, would suggest that ESF was not susceptible to the action of DTT under the prescribed conditions. It would appear that about 38% of the ESF activity in these II + III fractions was the result of the human ESF in the fraction as measured in the assay mouse, and 62% of the ESF activity was the result of EGF producing ESF from the serum substrate in the assay mouse. The

DTT (0.1–10.0 μM) prevented the production of 0.26 ± 0.01 IU of ESF (65 mice) when incubated with EGF or a mixture of EGF and ESF; the consistency of these data possibly could be explained by the relatively small variations in the amounts of serum substrate in the bioassay mice.

Hammond *et al.* (20) demonstrated the need of normal plasma by certain anemic children unresponsive to their own (apparent) high level of ESF (20). An anemic patient with a relatively high level of EGF but no serum EGF-substrate (or a deficit) would appear to have a high level of ESF, because EGF would produce ESF while circulating in the blood of the assay mice; such a patient would benefit from the infusion of normal plasma. DTT would prevent the production in the assay mice of the (apparent) ESF in the patient lacking the serum EGF-substrate, if applied to the urinary EGF as described in this report. If only part of fraction II + III is inactivated, it would seem that the patient would not be entirely deficient in the necessary serum substrate for EGF. This observation should give some indication as to whether an anemic patient would benefit from an infusion of normal plasma.

Summary. An ESF-generating factor (EGF), erythropoietin (ESF) and a mixture of both were incubated with dithiothreitol (DTT, a protective reagent for sulfhydryl groups). Ten μM DTT completely prevented the production of 0.26 ± 0.01 IU of ESF by EGF but did not inactivate ESF. DTT was used to indicate the relative amounts of EGF and ESF activity in a mixture of both.

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