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Publication Date
2023-09-06

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DISSOCIATION OF ERYTHROPOIETIN FROM ERYTHROPOIETIN-ANTI-ERYTHROPOIETIN COMPLEX

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Running Title: Erythropoietin dissociated from its antibody

This work was done under the auspices of the United States Atomic Energy Commission.

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In 1962 Schooley and Garcia (1) first showed that serum from rabbits, immunized with human urinary erythropoietin, will neutralize the erythropoietic activity of human urinary erythropoietin. This observation was later confirmed and extended to include erythropoietins from other species and sources (2,3,4,5). Extensive studies supporting the specificity of such antisera for erythropoietin were performed showing that the erythropoietin neutralizing ability was not absorbed by a variety of other hormones and proteins, and that it existed in the gamma globulin fraction of the immune serum (6). All the evidence obtained to-date supports the hypothesis that the neutralization of the biological activity of erythropoietin by the immune serum is the result of an antigen-antibody reaction.

During continuing studies directed to the development of a radioimmunoassay for erythropoietin, it was observed that erythropoietin labelled with ^{125}I was recoverable from its combination with anti-erythropoietin (7) by the application of a simple plasma erythropoietin extraction technique first demonstrated by Borsook et al. (8). The present investigation was initiated in an effort to obtain biological confirmation of this observation.

Materials and Methods. Human urinary erythropoietin was extracted from the urine of severely anemic patients by pressure filtration through a collodion membrane (9). Such an erythropoietin extract, with a specific activity between 20 and 30 units per mg, was used for this study. Using 5% human serum albumin as the diluent, halving dilutions of erythropoietin were made such that 4 concentrations were obtained equivalent to 32, 16, 8, and 4 units of the I.R.P. standard per ml. A 1 ml aliquot of each of these concentrations was pipetted into each of 4 tubes. One tube at each of the erythropoietin concentrations was left without further treatment. Three ml of saline were added to the second tubes, which were then acidified

to pH 5.0 with 0.1 N HCl and placed in a boiling water bath for 5 min. They were then cooled, centrifuged for 10 min, and the supernatants decanted. The precipitates were washed once with 4 ml saline, and the supernatants combined for each dose of erythropoietin. Finally, the pH was neutralized to approximately 7.5 with 0.1 N NaOH. To the third tube of each erythropoietin concentration was added 100 λ of anti-erythropoietin antiserum which was then allowed to incubate overnight at $4^{\circ}C$. The final tube of each erythropoietin concentration received the same volume of anti-erythropoietin, and was also incubated overnight at $4^{\circ}C$, but the following day was given the same acidification-heat treatment as outlined above. The final volume of all tubes was brought to 10 ml with a final human serum albumin concentration of 5%.

Rabbits were immunized with human urinary erythropoietin combined with complete Freund's adjuvant as previously described (6). The best anti-erythropoietin producing rabbits were selected and were given a booster immunization with a highly purified human urinary erythropoietin.[1] The particular antiserum used in this study was obtained 9 days after such an immunization. This anti-erythropoietin antiserum was of the highest potency we have as yet produced. Each ml of this antiserum could neutralize greater than 300 units of human erythropoietin.

Erythropoietin was assayed in female LAF_1 mice made polycythemic by exposure to increasing amounts of carbon monoxide for 3 weeks as described by Fogh (10). One week after removal from the carbon monoxide chamber the mice were injected subcutaneously with 1 ml of the test solutions. Fifty-six hours later they were given an intraperitoneal injection of 0.5 µCi of ^{59}Fe citrate. The mice were bled by cardiac puncture 72 hr later, and the radioactivity in 0.5 ml blood was measured. The results are expressed

as the per cent of ^{59}Fe in the blood volume, calculated at 7% of the body weight. The results on mice with hematocrits of less than 55% were discarded. The mean hematocrit for all of the animals at the end of the assay was 67.6% with a standard error of the mean of 0.23%.

Results. The results are summarized in Table I. Although human urinary erythropoietin receiving the acidification-heat treatment appears to give a somewhat lower iron incorporation into red blood cells than untreated erythropoietin, the difference is not significant except for the 2 lowest doses. The p value (Fisher's T test) for the 0.4 unit dose was < 0.001, and for the 0.8 unit dose was < 0.05.

Combination with anti-erythropoietin serum completely neutralized the biological activity of all the erythropoietin concentrations. Even the 32 units of erythropoietin used to make up the highest dose were completely neutralized by 100 λ of the antiserum used. In this study then, this antiserum showed the ability to neutralize at least 320 units of erythropoietin per ml.

When the erythropoietin-antibody complex was acidified to pH 5.0 and heated in a boiling water bath for 5 min, essentially all of the biological activity of the erythropoietin was recovered. The results of this treatment were not significantly different from the results obtained with the various doses of erythropoietin which received no treatment. However, the 0.4 unit and 0.8 unit dose of erythropoietin, when complexed with anti-erythropoietin and then acidified and heat-treated, resulted in significantly greater iron incorporation than the same 2 doses receiving only the acidification-heat treatment without the presence of anti-erythropoietin. The p values for the 0.4 and 0.8 unit doses were < 0.01 and < 0.05 respectively.

Discussion. Borsook et al. (8) demonstrated that acidified, boiled extracts of plasma from rabbits with phenylhydrazine anemia were capable of

stimulating erythropoiesis in normal rats. Their procedure was to acidify the plasma to pH 5.5 and then boil for 15 min. Goldwasser (11) extracted the plasma from normal rabbits by a similar procedure and arrived at a normal rabbit plasma erythropoietin level of approximately 0.02 units per ml. By the addition of a known amount of rat erythropoietin, he estimated the recovery as a result of this procedure to be approximately 85%. Thus, plasma erythropoietin appears to be quite stable to temperatures of 100°C for short times, even in low concentrations.

In the present study, at low concentrations, human urinary erythropoietin appears to be appreciably destroyed by application of an acidification-heat treatment. Comparison with a dose-response curve would indicate that on the order of 75% of the 0.4 unit dose and 50% of the 0.8 unit dose were destroyed by this treatment as compared to untreated erythropoietin. At the higher urinary erythropoietin concentrations, no significant destruction occurred as a result of the acidification-heat treatment. However, at all dose levels used, no significant reduction in erythropoietin was observed when the erythropoietin was incubated with a small, but neutralizing, amount of anti-erythropoietin antiserum before the acidification-heat treatment. At low concentration, complexing with its antibody appears to offer some protection to urinary erythropoietin from this form of treatment. Further studies in progress indicate that gamma globulin fractions separated from the combination of erythropoietin with anti-erythropoietin will also yield biologically active erythropoietin after such acidification-heat treatment.

We feel that these studies indicate that the erythropoietin molecule is not irreversibly altered as a result of the neutralization of its biological activity when combined with serum from rabbits immunized with

erythropoietin. This provides further support for the immunologic nature of the reaction involved.

Summary. The biological activity of human urinary erythropoietin is completely neutralized when combined with serum from rabbits immunized with erythropoietin. Acidification to pH 5.0, followed by heating in a boiling water bath for 5 min, recovers essentially all the erythropoietic biological activity from such a combination. These data further support the immunologic nature of the reaction between erythropoietin and the neutralizing serum.

Acknowledgments

The technical assistance of Mrs. V. Havens, Miss L. Mahlmann, Mrs. P. Smith and Mrs. S. Walker, and the secretarial assistance of Mrs. G. Walpole are gratefully acknowledged.

Footnote

[1] A gift, kindly supplied by Dr. Joaquin Espada, Catedra de Bioquimica, Facultad de Medicina, U.N.N.E., Corrientes, Argentina. The specific activity of this erythropoietin was stated to be about 8000 units per mg.

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Table I

Effect of acidification-heat treatment on erythropoietin and erythropoietin-anti-erythropoietin complex

Treatment of erythropoietin	Erythropoietin dose per mouse units I.R.P.	72-hr red cell ^{59}Fe %	S.E.
None	0.4	13.3	± 1.4
	0.8	19.9	± 1.9
	1.6	28.5	± 1.8
	3.2	33.9	± 1.8
Acidified pH 5.0, heated 5 min in boiling H_2O	0.4	5.28	± 0.73
	0.8	14.2	± 1.0
	1.6	25.2	± 0.84
	3.2	31.9	± 1.4
Combined with anti-erythropoietin	0.4	0.43	± 0.09
	0.8	0.46	± 0.03
	1.6	0.52	± 0.06
	3.2	0.58	± 0.09
Combined with anti-erythropoietin, acidified pH 5.0, heated 5 min in boiling H_2O	0.4	10.4	± 0.92
	0.8	18.0	± 1.0
	1.6	26.7	± 0.34
	3.2	35.4	± 1.5

6-8 mice per group.