# The Destruction by Neuraminidase of the Biological Activity of Erythropoietin When Complexed with Antierythropoietin[1] (35833)

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The sialic acid moiety of erythropoietin has an important physiological role. The ability of exogenous erythropoietin to stimulate erythropoiesis in the plethoric mouse is completely abolished if the hormone is incubated *in vitro* with neuraminidase (1–3). Recent evidence indicates that the injection of neuraminidase into plethoric mice abolishes their ability to respond to erythropoietin (4). Mild acid hydrolysis with conditions known to remove sialic acids from glycoproteins (0.01 *N* $H_2SO_4$, 80°, 1 hr) also inactivates erythropoietin (5, 6). The removal of sialic acid from erythropoietin enzymatically or by acid hydrolysis does not, however, alter the ability of the hormone to stimulate hemoglobin synthesis (7) or stromal protein synthesis (8) in marrow cultures *in vitro*. Goldwasser (9) has suggested that sialic acid may play a protective role *in vivo* preventing the excretion or inactivation of the hormone.

The observation that the antigenicity of erythropoietin is considerably reduced after mild acid hydrolysis (6) suggested that sialic acid might be an antigenic determinant in the reaction of the hormone with antierythropoietin antibody. This suggestion has been reinvestigated utilizing the recent finding that erythropoietin can be quantitatively recovered from the erythropoietin–antierythropoietin complex by heating the complex in acidic conditions (10).

*Materials and Methods.* A series of tubes containing about 4.0 IRP units of human urinary erythropoietin in 1 ml of saline were prepared. The treatment of each tube was different, but all tubes were exposed to the same periods of incubation, etc. Antierythropoietin and neuraminidase, either alone or in combination, were added to some tubes. All tubes remained at room temperature for 2 hr after the addition of antierythropoietin. Some tubes were acidified with 0.1 *N* HCl to pH 5.0 and then placed in a boiling water bath for 5 min. The solutions were then cooled, neutralized, centrifuged for 10 min, and the precipitate was washed with 2 ml of saline. The supernatants were combined, diluted to 10 ml, and human serum albumin was added to obtain a 5% solution. The order of addition of erythropoietin, antierythropoietin, and enzyme, as well as the time of acidification and heat treatment, are important, and are indicated in the Results.

Each tube, treated with neuraminidase, received a total of 25 units or 6.5 units of enzyme/unit of erythropoietin. The neuraminidase was purchased from General Biochemical, *Vibrio cholerae* strain Z4, containing 500 units/ml. The enzyme was allowed to act for 1 hr.

The antierythropoietin used in these experiments had a high potency. One milliliter of immune sera could neutralize more than 300 units of human urinary erythropoietin. The antibody was prepared in rabbits by a schedule previously described (6); but, 9 days before serum sampling, the rabbit received a booster immunization with highly purified human urinary erythropoietin.[2] Each test tube received 50 μl of the immune sera or 12.5 μl/unit of erythropoietin.

The erythropoietic activity of the various

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[2] 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/mg.

TABLE I. Effect of Neuraminidase on the Biological Activity of Erythropoietin When Combined with Antierythropoietin.

| Group | | 72-hr $^{59}$Fe incorporation |
|---|---|---|
| 1 | Erythropoietin | 12.4 ± 1.6[a] |
| 2 | Erythropoietin + antierythropoietin | 0.28 ± 0.01 |
| 3 | Erythropoietin + heat, pH 5.0 | 9.6 ± 1.5 |
| 4 | Erythropoietin + antierythropoietin + heat, pH 5.0 | 11.9 ± 0.94 |
| 5 | Erythropoietin + neuraminidase + heat, pH 5.0 | 0.44 ± 0.05 |
| 6 | Erythropoietin + antierythropoietin + neuraminidase + heat, pH 5.0 | 0.53 ± 0.05 |
| 7 | Erythropoietin + antierythropoietin + heat, pH 5.0 + neuraminidase | 0.42 ± 0.03 |
| 8 | Neuraminidase + heat, pH 5.0 + erythropoietin | 14.8 ± 1.2 |
| 9 | Erythropoietin + antierythropoietin + pH 5.0 + neuraminidase + heat | 11.7 ± 0.73 |
| 10 | Uninjected | 0.56 ± 0.04 |

[a] Standard error of the mean; 7 plethoric post-CO female $LAF_1$ mice/group.

solutions was assayed in female $LAF_1$/JAX mice made plethoric by exposure to increasing amounts of carbon monoxide for 3 weeks as described by Fogh (11). The mice were used 1 week after removal from the CO chambers, when erythropoiesis was almost completely suppressed. Fifty-six hr after the sc injection of 1 ml of the test solutions, the mice were injected iv with 0.5 $\mu$Ci of $^{59}$Fe citrate. All mice were bled 72 hr after the $^{59}$Fe injection, and the radioactivity in 0.5 ml of blood was measured. The results are expressed as the percentage of the injected $^{59}$Fe in the calculated blood volume, which was assumed to be 7% of the body weight. The hematocrits averaged 69 ± 0.23% at the end of the assay.

*Results.* The results are shown in Table I. The order of addition of the reagents reads from left to right. The addition of antierythropoietin to erythropoietin completely neutralized the biological activity of erythropoietin (Group 2 compared to Group 1). The biological activity of the erythropoietin–antierythropoietin complex (Group 2) was actually slightly less ($p<0.001$) than the $^{59}$Fe uptake observed in the uninjected control (Group 10). This suggests that an excess of antierythropoietin was available which neutralized the biological activity of a small amount of endogenous erythropoietin always present in plethoric mice. We consistently found that the injection of antierythropoietin into plethoric mice, produced either by transfusion or by posthypoxic exposure, depressed the $^{59}$Fe uptake below the control value.

The acidification and heating of erythropoietin did not significantly decrease its biological activity (Group 3 compared to Group 1). Acidification and heating of the erythropoietin–antierythropoietin complex released the erythropoietin from the complex, and the biological activity (Group 4) returned to that observed with erythropoietin alone (Group 1).

Incubation of erythropoietin alone (Group 5) or the erythropoietin–antierythropoietin complex (Group 6) with neuraminidase completely destroyed the biological activity, as did the addition of the enzyme to the erythropoietin–antierythropoietin complex after the acidification and heat treatment (Group 7). The acidification and heat treatment not only dissociates the erythropoietin–antierythropoietin complex but completely destroys the activity of neuraminidase so that it cannot act on erythropoietin (Group 8). If neuraminidase was added to the antigen–antibody complex after acidification, and the tube was rapidly mixed and immediately placed in a boiling water bath, the enzyme did not destroy erythropoietin released from the antigen–antibody complex (Group 9).

This result suggests that the inactivation of erythropoietin seen in Group 6 occurs while the hormone is complexed with antierythropoietin and not during the few moments when the erythropoietin–antierythropoietin complex is dissociated by the heat treatment.

*Discussion.* Neuraminidase destroys the *in vivo* biological activity of erythropoietin by specifically removing the sialic acid moiety. It is unlikely that the carbohydrate portion of γ-globulin plays any role in the antibody-combining site (12). We assume that neuraminidase does not alter the combination of antierythropoietin with erythropoietin. We conclude that sialic acid is not an important antigenic determinant in the reaction of the hormone with its antibodies, since the sialic acid can still be easily removed from the hormone after combination with antierythropoietin. The decreased antigenicity of acid hydrolyzed erythropoietin, which we previously observed (6), was apparently not related to removal of the sialic acid; possibly slight changes in the tertiary structure of erythropoietin produced by the less specific acid hydrolysis were involved.

*Summary.* The enzyme neuraminidase destroys the biological activity of erythropoietin even when the hormone is combined with antierythropoietin antibody. It is concluded that the sialic acid moiety is not an important antigenic determinant in the combination of the hormone and its antibodies.

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