Title
Inhibition of the Biological Activity of Erythropoietin by Neuraminidase in Vivo

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INHIBITION OF THE BIOLOGICAL ACTIVITY OF ERYTHROPOIETIN BY NEURAMINIDASE *IN VIVO*

By

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ABSTRACT

The ability of exogenous erythropoietin to stimulate erythropoiesis in the plethoric mouse is abolished when neuraminidase is injected into the mouse immediately after the hormone. Similarly, the ability of plethoric mice to respond with increased erythropoietic activity to a brief hypoxic exposure is drastically decreased when neuraminidase is injected immediately before or after the hypoxic exposure. Injections of similar concentrations of neuraminidase 24 hours after the initiation of a wave of erythropoietic activity only moderately diminishes the erythropoietic response, suggesting that the enzyme acts primarily on erythropoietin itself rather than on nucleated erythroid cells. The destruction of the biological activity of circulating plasma erythropoietin in rats by the intravenous injection of neuraminidase provides additional evidence for this view.

The ability of erythropoietin to stimulate erythropoiesis is destroyed when the hormone is pretreated *in vitro* with neuraminidase,[1,2,3] a glycosidase which cleaves terminal sialic acid from heterosaccharides or glycoproteins.[4] Mild acid hydrolysis, which removes sialic acid, also inactivates the hormone.[5,6] It has been reported that, although neuraminidase-treated erythropoietin cannot initiate erythropoiesis *in vivo*, it does stimulate heme synthesis in bone marrow cultures.[7]

These experiments were undertaken to determine whether neuraminidase *in vivo* prevents the biological activity of endogenous and exogenous erythropoietin.

Materials and Methods

Female LAF_1/JAX mice weighing about 25 gm. were made plethoric by exposure to increasing amounts of carbon monoxide for 3 weeks as described by Fogh.[8] The mice were used 7 days after removal from the CO chamber when erythropoiesis was maximally suppressed. Erythropoiesis was stimulated either by intravenous injection of sheep plasma erythropoietin or by a 6-hour exposure to a simulated altitude of 22,000 ft. (321 torr.). The erythropoietin was a Step 1 preparation containing about 0.4 I.R.P. units per mg dry weight obtained from Connaught Medical Research Laboratories, Toronto, Canada. 0.5 μCi of ^{59}Fe as the citrate was injected intravenously 56 hours after erythropoietin injection or the beginning of the hypoxic exposure. The mice were bled 72 hours after the ^{59}Fe injection, and the radioactivity in 0.5 ml. blood was measured. The results are expressed as the per cent of the injected ^{59}Fe in the calculated blood volume which was assumed to be 7 per cent of the body weight. All hematocrits were above 60 per cent at the time of sacrifice. Each experimental group contained at least 6 mice.

Neuraminidase, derived from *Vibrio cholerae* strain Z-4 containing 500 units per ml., was obtained from General Biochemicals. A unity of activity

is equivalent to 1 μg. N-acetylneuraminic acid from a glycoprotein substrate in 15 minutes at 37° C. The enzyme was diluted with Gey's solution. Mice were injected intravenously with 20 or 40 units in a volume of 0.2 ml.

In one experiment 1 ml. aliquots containing 4 I.R.P. units erythropoietin were incubated with various concentrations of neuraminidase *in vitro* for 1 hour at 37° C. After incubation the solutions were placed in a boiling water bath for 5 minutes, cooled, diluted to 5 ml. with Gey's solution, and 0.5 ml. was given subcutaneously to plethoric mice to assay for erythropoietic activity.

Neuraminidase was iodinated with ^{125}I using chloramine T as described by Greenwood *et al.*[9] The labeled enzyme was injected intravenously into a group of normal mice, and blood samples were taken from the orbital plexus at various times after injection. The radioactivity in a known volume of plasma was measured.

A group of Sprague-Dawley rats weighing about 400 g were injected intravenously with 160 units of neuraminidase per 100 gm. body weight (250 units per ml. saline). Control rats were injected with an equivalent volume of saline. The rats were immediately placed in an altitude chamber at 22,000 ft. for 5 hours and then sacrificed. Two groups of rats were injected intravenously with 8 units of sheep plasma erythropoietin, and 10 minutes later one group was injected intravenously with neuraminidase, and the control group was injected with saline. One hour after the erythropoietin injection they were sacrificed.

All rats were bled from the dorsal aorta under ether anesthesia. The blood was chilled, centrifuged immediately, and the plasma removed. Ten ml. of the plasma were placed in 30 ml. of distilled water and adjusted to pH 5.0 with 1 N HCl. The solutions were placed in a boiling water bath for 5 minutes, cooled, centrifuged for 10 minutes at 5° C. The supernatant was

collected, lyophylized, and reconstituted to 10 ml. The final solution contained 5 per cent human serum albumin. The erythropoietic activity of the supernatant was assayed in plethoric mice after the subcutaneous injection of 1 ml.

Results

The biological activity of 1 I.R.P. unit of sheep erythropoietin was completely destroyed after incubation in vitro with 25, 12.5, 6.25 units of neuraminidase, whereas neuraminidase concentrations of 0.125 units or less had no effect. Neuraminidase concentrations of 2.5 and 1.25 units destroyed 88 per cent and 36 per cent of the biological activity.

The data in Table I show that by selectively modifying the variables of the dose of the hormone or enzyme and the time interval between injections, the biological activity of erythropoietin can be unaffected, reduced, or abolished. An injection of 0.4 unit erythropoietic immediately followed by an injection of 20 units neuraminidase dractically reduced the erythropoietic response (Groups 1 and 2); whereas the same amount of neuraminidase injected 24 hours later had little effect (Group 3). Similar results were observed when 40 units of neuraminidase were given (Groups 4 and 5). When 40 units of neuraminidase were given 24 hours after erythropoietin (Groups 1 and 5), a slight decrease, of doubtful significance ($P > 0.1$), in ^{59}Fe uptake occurred. In contrast, the injection of 40 units of neuraminidase 24 hours after the injection of 0.8 unit of erythropoietin significantly reduced ($P < 0.005$) the 72-hour ^{59}Fe uptake (Group 6 compared to Group 8). When 40 units of neuraminidase were injected immediately after this increased erythropoietin dose, the erythropoietic response was

almost completely abolished (Groups 6 and 7).

The data in Table II illustrate that the erythropoietic response of plethoric mice to a 6-hour exposure to a simulated altitude of 22,000 ft. can be intentionally altered by varying the time of injection and dose of neuraminidase. There was a significant depression in erythropoietic response ($P < 0.02$) when 20 units of neuraminidase were injected immediately before or immediately after the hypoxic exposure (Groups 2 and 3). There was no significant decrease in erythropoietic response when the same amount was injected 18 hours after the end of the hypoxic exposure (Group 4). Injection of 40 units of neuraminidase immediately before or immediately after the hypoxic exposure resulted in an erythropoietic response indistinguishable from that seen in the saline-injected controls (Groups 5 and 6). Injection of 40 units of neuraminidase 18 hours after the end of hypoxic exposure significantly ($P < 0.01$) decreased the erythropoietic response (Groups 6 and 1).

Following the injection of ^{125}I neuraminidase into normal mice, a multiexponential curve of several components was observed during the disappearance of ^{125}I radioactivity from the plasma. Only an approximation of the T-1/2 for neuraminidase can be made, since no measurements of changes in concentration of free neuraminidase in the plasma were taken, and it is quite likely that significant amounts of the enzyme may combine with receptor sites available on plasma proteins and red blood cells. It was estimated (with these limitations that the maximum T-1/2 for the plasma disappearance of neuraminidase was about 3 hours in normal mice.

The data presented in Table III indicate that circulating erythropoietin, produced in rats either by a hypoxic exposure or by the injection of exogenous erythropoietin, is inactivated <u>in vivo</u> by the injection of neuraminidase. Other experiments indicate that the acidification and heat treatment of the

rat plasma completely inactivates neuraminidase, but such treatment has little effect on plasma erythropoietin.[7]

Discussion

It is evident from these experiments that neuraminidase not only destroys the biological activity of erythropoietin *in vitro*, but prevents exogenous and endogenous erythropoietin from stimulating erythropoiesis *in vivo*. The magnitude of the depression of the erythropoietic response of plethoric mice depends upon the time of enzyme injection after the initiation of a wave of erythropoietic activity. If neuraminidase is given immediately after the injection of exogenous erythropoietin, the erythropoietic response is either abolished or markedly reduced. If the enzyme is injected into plethoric mice immediately before a hypoxic exposure, the erythropoietic response is again either abolished or markedly reduced. These results suggest that the enzyme has acted on the hormone itself or, in the case of the hypoxic stimulation of endogenous erythropoietin production, on those reactions involved in the synthesis of erythropoietin.

The depression in the erythropoietic response observed when 40 units of neuraminidase were injected 24 hours after the initiation of an erythropoietic wave was particularly evident when 0.8 unit of erythropoietin had been given. A complex interplay between the half-lives of erythropoietin and neuraminidase probably contributes to this result, and the possibility of a direct action of neuraminidase on developing nucleated erythroid cells cannot be excluded. The plasma half-life of erythropoietin has been estimated by a number of investigators[13] to be in the range of 2-5 hours. Our data on the disappearance of ^{125}I-labeled neuraminidase, and the data of Gasic *et al.*[14] suggest that the plasma half-life of the enzyme is also 3-5 hours. Thus, all of the larger doses of injected erythropoietin may not have disappeared from the plasma by 24 hours, and that remaining may

be acted upon by neuraminidase, preventing an erythropoietic stimulus. Some evidence suggests that the biological life of erythropoietin is much longer than the plasma half-life.[15] It is also possible that erythropoietin molecules are on the surface of receptor cells, and neuraminidase can still destroy the biological activity of the hormone by sialic acid removal before the cell is actually triggered. It seems unlikely that the reduction in the ^{59}Fe incorporation observed in these experiments was the result of some interference in iron transport to hemoglobin-synthesizing cells, since neuraminidase-treated transferrin[16] binds iron as untreated transferrin.[17]

The destruction of circulating plasma erythropoietin in rats by intravenously administered neuraminidase more definitively indicates that, in spite of the enormous number of receptors available to the enzyme, the major effect on erythropoiesis is the actual destruction of the hormone itself.

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Table I

Effect of neuraminidase on the erythropoietic response of plethoric LAF_1 mice to erythropoietin

Group		Number of mice	72-hr. ^{59}Fe incorporation
1	0.4 units erythropoietin	22**	5.6 ± 0.51*
2	0.4 units erythropoietin + 20 units neuraminidase	13**	1.2 ± 0.19
3	0.4 units erythropoietin + 20 units neuraminidase 24 hr.later	7	5.6 ± 0.30
4	0.4 units erythropoietin + 40 units neuraminidase	7	0.88 ± 0.17
5	0.4 units erythropoietin + 40 units neuraminidase 24 hr. later	7	3.8 ± 0.20
6	0.8 units erythropoietin	6	9.3 ± 0.67
7	0.8 units erythropoietin + 40 units neuraminidase	7	1.2 ± 0.17
8	0.8 units erythropoietin + 40 units neuraminidase 24 hr. later	7	5.6 ± 0.68
9	Saline	8	0.56 ± 0.04

* Standard error of the mean.

** Average of at least 2 experiments (Neuraminidase was injected intravenously 2 min. after the intravenous injection of erythropoietin).

Table II

Effect of neuraminidase on the erythropoietic response of plethoric LAF_1 mice to hypoxia

Group		Number of mice	72-hr. ^{59}Fe incorporation
1	6 hr. hypoxia***	19**	6.7 ± 0.52*
2	20 units neuraminidase + 6 hr. hypoxia	12**	2.8 ± 0.43
3	6 hr. hypoxia then 20 units neuraminidase	6	2.4 ± 0.50
4	6 hr. hypoxia + 20 units neuraminidase 18 hr. later	7	6.0 ± 0.64
5	40 units neuraminidase + 6 hr. hypoxia	7	0.43 ± 0.06
6	6 hr. hypoxia + 40 units neuraminidase 18 hr. later	7	3.5 ± 0.50
7	Saline	8	0.56 ± 0.04

* Standard error of the mean.

** Average of at least 2 experiments.

*** Simulated altitude of 22,000 ft. (321 torr.)

Table III

Erythropoietic activity of plethoric LAF_1 mice injected with serum taken from rats given neuraminidase and exposed to hypoxia or injected with erythropoietin

Treatment	72-hr. ^{59}Fe incorporation
Hypoxic exposure**	8.18 ± 1.34*
Hypoxic exposure + neuraminidase	0.59 ± 0.05
Erythropoietin injected	6.17 ± 0.57
Erythropoietin injected + neuraminidase	0.38 ± 0.03
Normal serum	0.49 ± 0.04

* Standard error of the mean. 6-8 mice/group.

** Male rats exposed to a simulated altitude of 22,000 ft (321 torr.) for 5 hours.

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These experiments were undertaken to determine whether neuraminidase *in vivo* prevents the biological activity of endogenous and exogenous erythropoietin.

Materials and Methods

Female LAF_1/JAX mice weighing about 25 gm. were made plethoric by exposure to increasing amounts of carbon monoxide for 3 weeks as described by Fogh.[8] The mice were used 7 days after removal from the CO chamber when erythropoiesis was maximally suppressed. Erythropoiesis was stimulated either by intravenous injection of sheep plasma erythropoietin or by a 6-hour exposure to a simulated altitude of 22,000 ft. (pressure of 321 torr). The erythropoietin was a Step 1 preparation containing about 0.4 I.R.P. units per mg. dry weight obtained from Connaught Medical Research Laboratories, Toronto, Canada. 0.5 μCi of ^{59}Fe as the citrate was injected intravenously either 56 hours after erythropoietin injection or 56 hours after the beginning of the hypoxic exposure. The mice were bled 72 hours after the ^{59}Fe injection, and the radioactivity in 0.5 ml. blood was measured. The results are expressed as the per cent of the injected ^{59}Fe in the calculated blood

volume which was assumed to be 7 per cent of the body weight. All hematocrits were above 60 per cent at the time of sacrifice. Each experimental group contained at least 6 mice.

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In one experiment 1 ml. aliquots containing 4 I.R.P. units erythropoietin were incubated with various concentrations of neuraminidase in vitro for 1 hour at 37°C. After incubation the solutions were placed in a boiling water bath for 5 minutes, cooled, diluted to 5 ml. with Gey's solution, and 0.5 ml. was given subcutaneously to plethoric mice to assay for erythropoietic activity.

A group of Sprague-Dawley rats weighing about 400 gm. was injected intravenously with 160 units of neuraminidase per 100 gm. body weight (250 units per ml. saline). Control rats were injected with an equivalent volume of saline. The rats were immediately placed in an altitude chamber at 22,000 ft. for 5 hours and then sacrificed. Two groups of rats were injected intravenously with 8 units of sheep plasma erythropoietin, and 10 minutes later one group was injected intravenously with neuraminidase, and the control group was injected with saline. One hour after the erythropoietin injection they were sacrificed.

All rats were bled from the dorsal aorta under ether anesthesia. The

blood was chilled, centrifuged immediately, and the plasma removed. Ten ml. of the plasma were placed in 30 ml. of distilled water and adjusted to pH 5.0 with 1 N HCl. The solutions were placed in a boiling water bath for 5 minutes, cooled, centrifuged for 10 minutes at 5°C. The supernatant was collected, lyophylized, and reconstituted to 10 ml. The final solution contained 5 per cent human serum albumin. The erythropoietic activity of the supernatant was assayed in plethoric mice after the subcutaneous injection of 1 ml.

Results

The biological activity of 1 I.R.P. unit of sheep erythropoietin was completely destroyed after incubation in vitro with 25, 12.5 or 6.25 units of neuraminidase, whereas neuraminidase concentrations of 0.125 units or less had no detectable effect. Neuraminidase concentrations of 2.5 and 1.25 units destroyed 88 per cent and 36 per cent of the biological activity, respectively.

The data in Table I show that by selectively modifying the variables of the dose of the hormone or enzyme and the time interval between injections, the biological activity of erythropoietin can be unaffected, reduced, or abolished. An injection of 0.4 unit erythropoietin immediately followed by an injection of 20 units neuraminidase drastically reduced the erythropoietic response (Groups 1 and 2); while the same amount of neuraminidase injected 24 hours later had little effect (Group 3). Similar results were observed when 40 units of neuraminidase were given (Groups 4 and 5). When 40 units of neuraminidase were given 24 hours after erythropoietin (Groups 1 and 5),

a slight decrease, of doubtful significance ($P > 0.1$), in ^{59}Fe uptake occurred. In contrast, the injection of 40 units of neuraminidase 24 hours after injection of 0.8 unit of erythropoietin significantly reduced ($P < 0.005$) the 72-hour ^{59}Fe uptake (Group 6 compared to Group 8). When 40 units of neuraminidase were injected immediately after this increased erythropoietin dose, the erythropoietic response was almost completely abolished (Groups 6 and 7).

The data in Table II illustrate that the erythropoietic response of plethoric mice to a 6-hour exposure to a simulated altitude of 22,000 ft. can be intentionally altered by varying the time of injection and dose of neuraminidase. There was a significant depression in erythropoietic response ($P < 0.02$) when 20 units of neuraminidase were injected immediately before or immediately after the hypoxic exposure (Groups 2 and 3). There was no significant decrease in erythropoietic response when the same amount was injected 18 hours after the end of the hypoxic exposure (Group 4). Injection of 40 units of neuraminidase immediately before or immediately after the hypoxic exposure resulted in an erythropoietic response indistinguishable from that seen in the saline-injected controls (Groups 5 and 6). Injection of 40 units of neuraminidase 18 hours after the end of hypoxic exposure significantly ($P < 0.01$) decreased the erythropoietic response (Groups 6 and 1).

The data presented in Table III indicate that circulating erythropoietin, produced in rats either by a hypoxic exposure or by the injection of exogenous erythropoietin, is inactivated <u>in vivo</u> by the injection of neuraminidase. Other experiments indicate that the acidification and heat treatment of the rat plasma completely inactivates neuraminidase, but such treatment has little

effect on plasma erythropoietin.[9]

Discussion

It is evident from these experiments that neuraminidase not only destroys the biological activity of erythropoietin *in vitro*, but prevents exogenous and endogenous erythropoietin from stimulating erythropoiesis *in vivo*. The magnitude of the depression of the erythropoietic response of plethoric mice depends upon the time of enzyme injection after the initiation of a wave of erythropoietic activity. If neuraminidase is given immediately after the injection of exogenous erythropoietin, the erythropoietic response is either abolished or markedly reduced. If the enzyme is injected into plethoric mice immediately before a hypoxic exposure, the erythropoietic response is again either abolished or markedly reduced. These results suggest that the enzyme has acted on the hormone itself or, in the case of the hypoxic stimulation of endogenous erythropoietin production, on those reactions involved in the synthesis of erythropoietin.

The depression in the erythropoietic response observed when 40 units of neuraminidase were injected 24 hours after the initiation of an erythropoietic wave was particularly evident when 0.8 unit of erythropoietin had been given. A complex interplay between the half-lives of erythropoietin and neuraminidase probably contributes to this result, and the possibility of a direct action of neuraminidase on developing nucleated erythroid cells cannot be excluded. The data of Gasic *et al.*[10] indicate that the plasma half-life of the enzyme is 3-5 hours. The plasma half-life of erythropoietin has been estimated by a number of investigators[11] to be in the range of 2-5 hours. Some evidence suggests that the biological life of erythropoietin is much longer than the

plasma half-life.[12] Thus, all of the larger dose of injected erythropoietin may not have disappeared from the plasma by 24 hours, and that remaining may be acted upon by neuraminidase, preventing further erythropoietic stimulation. It is also possible that erythropoietin molecules are on the surface of receptor cells, and neuraminidase can still destroy the biological activity of the hormone by sialic acid removal before the cell is actually triggered. It seems unlikely that the reduction in the ^{59}Fe incorporation observed in these experiments was the result of some interference in iron transport to hemoglobin-synthesizing cells, since neuraminidase-treated transferrin binds iron just as does untreated transferrin.[13]

Neuraminidase is widely distributed in microorganisms, and its presence has been demonstrated in a number of mammalian tissues such as kidney, liver, intestinal mucosa, and brain.[14-17] Warren and Spearing[18] first demonstrated the occurrence of the enzyme in mammals using commercial preparations of bovine and human plasma glycoproteins (Cohn Fraction VI), suggesting to us that some neuraminidase normally occurs in plasma. They could not, however, detect the enzyme in unprocessed plasma or serum with their assay methods. Baba et al.[19] demonstrated a marked elevation of neuraminidase activity in the livers of mice bearing tumors, and suggest that neuraminidase may be an adaptive enzyme whose production is induced when blood glycoprotein levels are elevated.

Ward et al.[20] have recently shown that the serum erythropoietin levels of patients with iron deficiency or primary hematopoietic anemias vary directly with the degree of anemia, whereas in patients with chronic infection or malignancy no correlation exists between serum erythropoietin levels and the severity of anemia. We are unaware of any measurements of plasma neuraminidase

levels in patients with chronic infection or malignancy. The observations in experimental animals[19] suggest that increases in plasma neuraminidase levels might be expected in these conditions and measurements should be made. The present experiments clearly indicate that injected bacterial neuraminidase destroys the biological activity of circulating erythropoietin. Increases in mammalian plasma neuraminidase in disease would presumably also inactivate the circulating hormone.

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Group		Number of Mice	72-hr. ^{59}Fe incorporation
1	0.4 units erythropoietin	22**	5.6 ± 0.51*
2	0.4 units erythropoietin + 20 units neuraminidase	13**	1.2 ± 0.19
3	0.4 units erythropoietin + 20 units neuraminidase 24 hr. later	7	5.6 ± 0.30
4	0.4 units erythropoietin + 40 units neuraminidase	7	0.88± 0.17
5	0.4 units erythropoietin + 40 units neuraminidase 24 hr. later	7	3.8 ± 0.20
6	0.8 units erythropoietin	6	9.3 ± 0.67
7	0.8 units erythropoietin + 40 units neuraminidase	7	1.2 ± 0.17
8	0.8 units erythropoietin + 40 units neuraminidase 24 hr. later	7	5.6 ± 0.68
9	Saline	8	0.56± 0.04

* Standard error of the mean.

**Average of at least 2 experiments.

Group 1 vs 2, $P < 0.001$; Group 1 vs 4, $P < 0.001$; Group 1 vs 5, $0.05 < P < 0.1$; Group 6 vs 7, $P < 0.001$; and Group 6 vs 8, $P < 0.005$.

Table II

Effect of neuraminidase on the erythropoietic response of plethoric LAF_1 mice to hypoxia

Group		Number of mice	72-hr. ^{59}Fe incorporation
1	6 hr. hypoxia***	19**	6.7 ± 0.52*
2	20 units neuraminidase + 6 hr. hypoxia	12**	2.8 ± 0.43
3	6 hr. hypoxia then 20 units neuraminidase	6	2.4 ± 0.50
4	6 hr. hypoxia + 20 units neuraminidase 18 hr. later	7	6.0 ± 0.64
5	40 units neuraminidase + 6 hr. hypoxia	7	0.43± 0.06
6	6 hr. hypoxia + 40 units neuraminidase 18 hr. later	7	3.5 ± 0.50
7	Saline	8	0.56± 0.04

* Standard error of the mean.

** Average of at least 2 experiments.

***Simulated altitude of 22,000 ft. (pressure of 321 torr)

Group 1 vs 2 or 3, $P < 0.02$; Group 1 vs 5, $P < 0.001$; and Group 1 vs 6, $P < 0.01$.

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