Erythropoiesis In Vitro: Enhancement by Neuraminidase

By Peter T. Rowley, Betsy M. Olsinson-Wilhelm, and Barbara A. Farley

Neuraminidase treatment of human fetal liver or adult marrow cells prior to culture results in an increased number of erythroid colonies and bursts. No increase occurs in the number of nonerythroid colonies. The number of bursts having more than eight subunits is increased preferentially. Individual burst subunits are also enlarged. Neuraminidase-treated cells yield erythroid bursts when cultured in concentrations of erythropoietin insufficient to produce bursts from untreated cells. It is proposed that (1) neuraminidase treatment of adult and fetal cell mixtures specifically stimulates differentiation of erythroid precursors, (2) the preferential stimulation of erythroid bursts having many subunits suggests a preferential susceptibility of more primitive BFU-Es, and (3) neuraminidase treatment enhances the response of erythroid precursors to erythropoietin.

We have previously reported that when a single cell suspension of human adult marrow or fetal liver is briefly treated with trypsin, the number of erythroid bursts and colonies arising in culture is increased. To determine the specificity of this phenomenon we have tested a variety of other enzymes on fetal liver. Although other enzymes tested, e.g., chymotrypsin, pronase, and phospholipase D, also had some stimulating action, the greatest effect was that produced by neuraminidase. In this report we show that neuraminidase enhances the effect of erythropoietin on erythroid precursors in both fetal liver and adult marrow, stimulates only erythroid development, and increases not only the number of bursts, but also the number of subunits per burst as well as the size of the individual burst subunit.

Materials and Methods

Human urinary erythropoietin (M-7-TaLSL, 16.2 IU/ml, and E 3-8 LSL, 52 IU/ml) was generously provided by Dr. Peter Dukes, Children's Hospital, Los Angeles, Calif., and by Dr. Anne Ball, Division of Blood Diseases and Resources, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Md. Neuraminidase (sialidase, N-acetyl-neuraminic glycohydrolase, E.C. No. 3.2.1.18, specific activity 39 U/mg protein with N-acetyl-neuraminic lactose substrate) from Cl. perfringens was obtained from Sigma Chemical Co. (N-9130) St. Louis, Mo. A neuraminidase inhibitor [compound 45, 1-(o-aminophenyl)-3,4-dihydroisoquinoline] was a generous gift of Dr. Theodore H. Haskell, Warner-Lambert Company, Ann Arbor, Mich.

The culture methods used have been previously described. Livers were obtained from midtrimester human fetuses aborted by dilatation and evacuation. Marrow was obtained from hematologically normal adults undergoing spinal surgery. The tissue was minced with scalpels in alpha medium not supplemented by serum. Disaggregation was promoted in the case of liver by mixing in a spinner bottle for 10 min and in the case of marrow by repeatedly pipetting with a Pasteur pipette. The resulting suspension of either tissue was passed through a mesh (200 perforations/sq cm) to remove residual cell clumps. To reduce the number of granulocytic colonies, the cell suspension was allowed to stand in a Falcon tissue culture flask at 10^6 nucleated cells/sq cm at 37°C in 5% CO_2 for 16 hr.

Cells were treated with neuraminidase in 0.145 M NaCl, 10 mM, HEPES, pH 7.4, at 0.5 U/ml, for 10 min or as stated in the text. The nucleated cell concentration during treatment was 1-4 x 10^7 cells/ml for fetal liver and 5 x 10^6 cells/ml for adult marrow. At the end of the incubation, 2 vol of cold fetal calf serum were added, and the cells were washed once in alpha medium/30% fetal calf serum. Viability, as assessed by trypan blue exclusion, was unaffected by the enzyme treatment. Untreated cells were incubated and washed identically except for the omission of neuraminidase.

Cells were plated at 10^5 nucleated cells/ml in alpha medium/0.8% methylcellulose/30% fetal calf serum/0.1 mM a-thioglycerol, containing (per ml) 1 µg Fe(NO_3)_3 - 9 H_2O, 100 U penicillin, 100 µg streptomycin, and erythropoietin (1 IU for fetal liver and 2 IU for adult marrow) except as otherwise indicated. Plates (35-mm diameter, Falcon) containing 1-ml cultures were incubated in 95% air/5% CO_2 at 100% relative humidity at 37°C.

A cell cluster was counted as an erythroid colony if it consisted of eight or more small, closely packed, pink or red cells and was not part of a burst. Colony counts were based on counts in 10 reticles selected at random along each of 6 diameters in each of 2 plates at 40 x magnification using a Nikon inverted microscope. Burst counts were done of the entire plate at 25 x magnification using a Nikon Zoom microscope Type 102. Whereas in cultures of adult marrow, colonies greatly outnumbered bursts, in cultures of fetal liver, their numbers were comparable.

This study was performed after approval by the Committee on Investigation of Human Subjects of the University of Rochester and in accord with an assurance filed with and approved by the Department of Health, Education, and Welfare.

Results

Studies on Fetal Liver

Effect on Erythroid Colony and Burst Number

Table 1 presents the results of the six experiments on the effect of neuraminidase treatment of human midtrimester fetal liver cells on erythroid colony and burst number on culture. Erythroid burst number in the treated cultures averaged 262% ± 48% (mean ± SE) of that in the untreated control cultures (p < 0.005). Erythroid colony number in the treated cultures averaged 178% ± 47% of that in the

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Supported in part by NIH Grant 5R01 AM-16847.

Submitted June 11, 1980; accepted October 29, 1980.

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0006-4971/81/5703-0014$01.00/0
Table 1. Effect of Neuraminidase Treatment on Erythroid Burst and Colony Formation in Cultures of Human Erythroid Tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Exp. No.</th>
<th>Cell Concentration (Nucleated Cells/ml Plated)</th>
<th>Enzyme Concentration (U/ml)</th>
<th>Maximum Number/10^6 Nucleated Cells Plated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Colonies</td>
</tr>
<tr>
<td>Liver</td>
<td>590</td>
<td>1 x 10^9</td>
<td>0.5</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>597</td>
<td>1 x 10^9</td>
<td>0.5</td>
<td>397</td>
</tr>
<tr>
<td></td>
<td>601</td>
<td>1 x 10^9</td>
<td>0.5</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>602</td>
<td>1 x 10^9</td>
<td>0.5</td>
<td>429</td>
</tr>
<tr>
<td></td>
<td>602</td>
<td>5 x 10^4</td>
<td>0.5</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td>648</td>
<td>5 x 10^4</td>
<td>0.5</td>
<td>1,481</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
<td></td>
<td>178% ± 47%</td>
</tr>
<tr>
<td>Marrow</td>
<td>577</td>
<td>1 x 10^9</td>
<td>2.0</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>582</td>
<td>1 x 10^9</td>
<td>0.2</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>606</td>
<td>1 x 10^9</td>
<td>0.5</td>
<td>148</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
<td></td>
<td>196% ± 55%</td>
</tr>
</tbody>
</table>

For details, see Materials and Methods.

untreated control cultures, but this increase was less consistent ($p < 0.2$).

Figure 1 compares the time course of cultures of neuraminidase treated and untreated cells at different concentrations of erythropoietin. Neuraminidase treatment increased erythroid colony and burst number. Large bursts (bursts with >8 subunits) showed a greater stimulation than did total bursts.

The interactions of the effects of neuraminidase and erythropoietin are better appreciated from Fig. 2, which shows colony and burst numbers as a function of erythropoietin concentration. Especially noteworthy is...
the fact that neuraminidase treatment resulted in appreciable erythropoiesis at erythropoietin concentrations that would have been otherwise ineffective.

When the concentration of neuraminidase for the 10-min, 25°C incubation was varied from 0.2 to 2.0 U/ml, treatment at all concentrations was found to increase burst number, but the maximum stimulation was obtained with 0.5 U/ml.

Neuraminidase increased burst number at all plating concentrations from 1.25 to 10 × 10^4 nucleated cells/ml. To a first approximation, relative stimulation was independent of cell concentration plated over the range tested.

**Effect on Burst Subunit Number and Size**

To examine whether neuraminidase treatment alters the number of subunits per burst, bursts were classified by number of subunits. In five experiments, the mean number of subunits per burst for the neuraminidase treated cultures (7.0 ± 1.3 SE) was significantly larger than the mean number for the untreated cultures (4.2 ± 0.9 SE) (p < 0.01). In other words, neuraminidase treatment shifted the burst subunit number distribution towards a larger number of subunits. Figure 3 presents the results of one representative experiment.

Neuraminidase treatment also increased the size of the individual burst subunit. Figure 4 shows bursts from untreated and treated cultures on the same day at the same magnification. At all erythropoietin concentrations tested, the average subunit size was greater in neuraminidase-treated cultures than in control cultures (p < 0.005), as shown in Fig. 5.

**Nonerythroid Colonies**

Nonerythroid colonies were not increased by neuraminidase treatment. This was true regardless of the erythropoietin concentration employed.
Effect of a Neuraminidase Inhibitor

In order to establish that the effects observed were due to enzymatic action and not to an impurity in the neuraminidase preparation, an inhibitor of neuraminidase was employed. In the experiment shown in Table 2, neuraminidase treatment of fetal liver cells resulted in a large increase in numbers of both colonies and bursts. Treatment with the inhibitor alone did not have any effect. When the inhibitor was added to the enzyme before the cells were exposed to the enzyme, no stimulation was observed. In fact, inhibition was observed; this may be due to an aspect of their interaction other than inhibition of enzymatic activity. These results support the claim that the effect of the enzyme preparation is due to its enzymatic action.

Studies on Adult Marrow

Table 1 also presents the results of experiments on the effect of neuraminidase treatment of human adult marrow cells on erythroid colonies and bursts observed upon culture. Erythroid burst number in the treated cultures average 210% ± 16% (mean ± SE) of that in the untreated control cultures (p < 0.05). Erythroid colony number in the treated cultures averaged 196% ± 55% of that in the untreated control cultures but, as in the case of fetal liver, was less consistent (p < 0.2). The relative increase in burst number was generally greater for larger bursts (>8 subunits) (not shown).

Figure 6 presents the time course of the neuraminidase effect on cultures of human adult marrow at five different concentrations of erythropoietin. Neuraminidase...
Table 2. Neuraminidase Inhibitor Blocks Erythroidic Effect of Neuraminidase

<table>
<thead>
<tr>
<th>Colonies/Plate (Day 5)</th>
<th>Bursts/Plate (Day 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>60</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>569</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>58</td>
</tr>
<tr>
<td>Inhibitor + neuraminidase</td>
<td>16</td>
</tr>
</tbody>
</table>

Nonadherent fetal liver cells, $5 \times 10^8$ nucleated cells/ml, were incubated 10 min at 25°C in HEPES buffer containing neuraminidase (0.5 U/ml), inhibitor [1-(o-aminophenyl)-3,4-dihydroisoquinoline, 1 mg/ml], both, or neither. In the case of both, the enzyme (5 U in 0.1 ml) and inhibitor (10 μl containing 100 μg in 95% ethanol) were preincubated 15 min at 25°C.

Neuraminidase increased the number of erythroid colonies and bursts in nearly all cases.

No increase occurred in nonerythroid colonies (not shown).

**DISCUSSION**

The results presented demonstrate that brief neuraminidase treatment of a single cell suspension of human fetal liver or adult marrow cells results in an increased number of erythroid colonies and bursts.

The results of neuraminidase treatment reported here are compared with our previously reported results of trypsin treatment in Table 3. With both enzymes, stimulation is erythroid specific. In both cases, burst number is increased, burst number is increased more than colony number, and burst subunit size is increased. These increases are more marked with neuraminidase than with trypsin. Neuraminidase, but not trypsin, results in a differential increase in number of large bursts, a prolongation of burst redness, and a lowering of the minimal concentration of erythropoietin needed to produce colonies or bursts.

![Time course of appearance of erythroid colonies and bursts in cultures of neuraminidase treated and untreated human adult marrow cells at different concentrations of erythropoietin.](image)

Fig. 6. Time course of appearance of erythroid colonies and bursts in cultures of neuraminidase treated and untreated human adult marrow cells at different concentrations of erythropoietin. Human adult marrow cells, following overnight adherence, were suspended in buffer with or without neuraminidase, 0.5 U/ml, for 10 min at 25°C, washed, and plated at $10^4$ nucleated cells/ml. Mean ± SE.
These effects of neuraminidase treatment prompt a number of questions.

1. *Is the effect observed a specific one or merely the consequence of a general stimulation of mitosis?*

In our previous report on the erythroid stimulating effect of trypsin, it was necessary to consider that the enzyme is known to stimulate mitosis in density-inhibited cells. Neuraminidase has been shown to cause proliferation of density-inhibited chick embryo fibroblasts. We show that neuraminidase causes an increase, not only in number of colonies and bursts, but also in burst subunit size. To what extent can the erythropoietic effect of neuraminidase be explained by its action as a nonspecific mitogen?

Although increased mitosis must be involved in the effects described here, there are three reasons why the neuraminidase effect cannot be discounted as simply a nonspecific mitogenic one. First, the effect is erythroid specific. The number of nonerythroid colonies is unaltered. Second, the distribution of bursts by subunit number is shifted towards a larger number of subunits. Perhaps the precursors of bursts having many subunits are differentially susceptible to the action of neuraminidase or perhaps precursors of bursts of smaller numbers of subunits are in some way altered to develop more subunits. Third, the relative increase in burst number is greatest at low erythropoietin concentrations. This finding indicates some interaction between erythropoietin action on the one hand and response to neuraminidase treatment on the other, allowing neuraminidase-treated precursors to plate more efficiently than untreated precursors. Thus, in these senses, the neuraminidase effect observed is a specific one not accounted for by a general stimulation of mitosis.

2. *Is the effect described produced by a specific enzymatic action of neuraminidase?*

To determine whether a contaminant in the enzyme preparation was responsible for the effect, an inhibitor of the enzymatic action of neuraminidase was employed. The inhibitor completely blocked the erythropoietic effect of neuraminidase treatment (Table 2). This finding suggests that the effect is not only due to the neuraminidase molecule, but the result of neuraminidase acting enzymatically.

3. *Is the primary site of action of neuraminidase a serum component or a cell?*

Neuraminidase hydrolyzes the glycosidic bond joining the keto group of N-acetylneuraminic acid (sialic acid) to a carbohydrate, glycoprotein, or glycolipid. Erythropoietin itself contains sialic acid. Removal of sialic acid from erythropoietin may even enhance activity in vitro. Could erythropoietin be the primary site of the neuraminidase effect described here?

The treatment procedure used here makes a serum constituent a less likely target for neuraminidase action than a cell constituent. The reason is that, prior to treatment, the serum is removed by washing the cells, and treatment with neuraminidase is done in serum-free medium. If the target for neuraminidase action is a serum constituent, it must be one which is bound to the cell.

When mouse bone marrow is treated with neuraminidase and then injected intravenously into irradiated mice, spleen colonies are reduced by one-half. This reduction could represent clearance from the circulation, as is seen after neuraminidase treatment of plasma glycoproteins or of mature erythrocytes.

4. *If the site of action is cellular, on which cell type is neuraminidase acting?*

The effects of neuraminidase treatment on cells have been most extensively studied with mature erythrocytes. Neuraminidase treatment shortens in vivo survival as noted above. It reduces negative charge and electrophoretic mobility. It eliminates reactivity to M and N blood group antisera. It prevents agglutination by Sendai and Newcastle viruses. It results in agglutination by concanavalin A. The reaction of neuraminidase with mature red cells is inhibited by the addition of concanavalin A or phytohemagglutinin.

If neuraminidase has its effect by an action directly on erythroid precursors, one mechanism might be to facilitate the interaction of the precursors with erythropoietin. Removal of sialic acid, a strong anion, from the precursors might enhance interaction with erythropoietin, which itself contains multiple sialic acid residues.

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### Table 3. Comparison of Effects of Neuraminidase and Trypsin

<table>
<thead>
<tr>
<th>Similarities</th>
<th>Neuraminidase</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythroid specific stimulation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Burst number increased</td>
<td>++ (Figs. 1, 6)</td>
<td>+</td>
</tr>
<tr>
<td>Bursts increased more than colonies</td>
<td>+ + (Figs. 1, 6)</td>
<td>+</td>
</tr>
<tr>
<td>Burst subunit size increased</td>
<td>+ + (Fig. 5)</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Differences</th>
<th>Neuraminidase</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large bursts (→8 subunits) preferentially stimulated</td>
<td>Yes (Fig. 3)</td>
<td>No</td>
</tr>
<tr>
<td>Bursts red longer</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Minimal erythropoietin concentration lowered</td>
<td>Yes (Fig. 2)</td>
<td>No</td>
</tr>
</tbody>
</table>
With regard to surface constituents of erythroid cells containing sialic acid, detailed information is available only for mature erythrocytes. Of all identified erythrocyte membrane proteins, only two are believed to protrude through the lipid bilayer, band 3, and glycophorin. Glycophorin contains nearly all the sialic acid. Information on nucleated erythroid cells is much more limited. In marrow there is evidence that only erythroid cells contain surface glycophorin.

Since our experiments were done with cell mixtures, the erythroid effect observed may have been mediated by neuraminidase action on some type of nonerythroid cell. A role for T lymphocytes in erythropoiesis in vitro has been demonstrated. Neuraminidase treatment of sensitized lymphocytes renders them more susceptible to lectin stimulation and to lysis by certain antibodies. Following serial exposure to neuraminidase and galactose oxidase, T lymphocytes from primed mice proliferate and differentiate.

Monocytes have been implicated in erythroid differentiation as one source of burst-promoting activity. Neuraminidase treatment of monocytes decreases the charge, increases deformability, and increases ability to phagocytize particles.

In conclusion, the effect of neuraminidase treatment on human hematopoietic precursor cells appears to be specific, in that stimulation occurs only of erythroid colonies and bursts, bursts with a large number of subunits are preferentially increased, and the relative colony and burst increase is greatest at low erythropoietin concentrations. The effect appears to be the result of neuraminidase acting enzymatically. Identification of the target cell type and specific surface constituent acted upon is the subject of continuing investigation.

REFERENCES


Erythropoiesis in vitro: enhancement by neuraminidase

PT Rowley, BM Ohlsson-Wilhelm and BA Farley