Induction of Lymphocyte Transformation by Sequential Treatment with Neuraminidase and Galactose Oxidase

(mouse spleen/concanavalin A/aldehyde/membrane sites/papain)

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ABSTRACT Treatment of mouse-spleen cells with galactose oxidase (EC 1.1.3.9) after incubation with neuraminidase (EC 3.2.1.18) induced extensive blastogenesis. Treatment of the cells with galactose oxidase before incubation with neuraminidase had very little stimulatory effect. Either of these enzymes alone had practically no effect on the cells. The lymphocyte transformation induced by galactose oxidase in neuraminidase-treated cells was decreased upon reacting the cells with borohydride or hydroxylamine, whereas treatment with these compounds did not affect transformation of cells by concanavalin A. It is suggested that galactosyl residues exposed by the action of neuraminidase on the cell membrane are oxidized by galactose oxidase, and the aldehyde moiety thus formed is involved in the induction of blastogenesis.

Recently, we reported that treatment of lymphocytes with periodate induced extensive blastogenesis (1). The membrane site modified by the periodate seems to include a glycoprotein(s) containing sialic acid that yields, on oxidation, an aldehyde moiety that is essential for transformation (2, 3). Sialic acid in glycoproteins always occupies a nonreducing terminal position and is glycosidically linked either to D-galactose or to N-acetyl-D-galactosamine (4). Galactose exposed by the action of neuraminidase (EC 3.2.1.18) on plasma glycoproteins could be oxidized at the carbon 6 position by galactose oxidase (EC 1.1.3.9) to yield the 6-aldehyde analogue (5, 6).

In the present study, we have found that extensive blastogenesis was induced in mouse-spleen cells treated by galactose oxidase after incubation with neuraminidase. Our results suggest that galactosyl residues exposed by the action of neuraminidase on the cells are oxidized by galactose oxidase and that the aldehyde moiety formed is essential for the induction of blastogenesis.

MATERIALS AND METHODS

Concanavalin A (Con A), twice crystallized, was obtained from Miles-Yeda Ltd. [*Methyl-³H*]Thymidine (5 Ci/mmol) was obtained from the Nuclear Research Center, Negev, Israel. NaIO₄, analytical reagent, was obtained from BDH Chemicals Ltd., England. Neuraminidase from *Vibrio comma* was obtained from Behringwerke AG, Germany, as a solution containing 500 units/ml (1 unit releases 1 μg of N-acetylneuraminic acid from α-acid glycoprotein at 37° in 15 min at pH 5.5). Galactose oxidase, 25 units/mg, was obtained from Worthington Biochemical Corp., U.S.A. (a unit is the quantity of enzyme that yields an absorbance of 1.0 at 420 nm, by the

peroxidase-chromogen assay). Catalase from beef liver, twice crystallized, 32,000 units/mg, was obtained from Sigma Chemicals Co., U.S.A. (1 unit decomposes 1 μmol of H₂O₂ per min at 25°). Bis(2-hydroxyethyl)dithiocarbamate was synthesized according to Fritz and Sutton (7).

Isolation of Mouse-Spleen Cells. CBA/LAC female mice, aged 6-12 weeks, were used. The mice were killed by ether. Spleens were removed and minced in phosphate-buffered saline (PBS; pH 7.2) (8). The large pieces of connective tissue were allowed to settle, and the supernatant suspension was harvested. Nucleated cells were counted after dilution in 2% acetic acid.

Mouse-Spleen Cell Cultures. Mouse-spleen cells were suspended at a final density of 5×10^6 /ml in Dulbecco's modified Eagle's medium, containing human serum (5%, heat-inactivated at 56° for 30 min), and supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Cultures of 1 ml were prepared in triplicate in polystyrene tubes (17 × 100 mm), loosely capped, and incubated at 37° in an atmosphere of 95% air-5% CO₂ for 46 hr.

[³H]Thymidine Incorporation into DNA. [³H]Thymidine (2.5 μCi) was added to cell cultures (1 ml) that had been incubated for 46 hr. After additional incubation of the cell cultures for 2 hr with shaking, incorporation of [³H]thymidine into DNA was determined (9). In each experiment, cultures were incubated in triplicate; results are expressed as the mean value of three determinations. The range of deviation from the mean value was usually less than 10%.

Inactivation of Galactose Oxidase with Bis(2-hydroxyethyl)-dithiocarbamate. Galactose oxidase (2 mg/ml) in PBS, Ca⁺⁺- and Mg⁺⁺-free, was treated with bis(2-hydroxyethyl)dithiocarbamate (2 mM) for 5 min at 23°. Under these conditions the enzyme lost essentially all its activity, as measured by the peroxidase-chromogen assay (10).

RESULTS

Response of mouse-spleen cells to treatment with neuraminidase and galactose oxidase

Treatment of mouse-spleen cells with galactose oxidase after incubation with neuraminidase markedly stimulated [³H]thymidine incorporation into the cells (Table 1). On the other hand, treatment of the cells with neuraminidase after incubation with galactose oxidase had very little stimulatory effect. Incubation with either one of these enzymes alone had practically no effect on the cells. Mouse-spleen cells, treated

Abbreviations: Con A, concanavalin A; PBS, phosphate-buffered saline (pH 7.2).

with neuraminidase and galactose oxidase and incubated in media containing sera from various sources, differed in the extent of their response to the enzymic treatment (Table 2). A small, but significant stimulation of [^3H]thymidine incorporation was found in the enzymically-treated cells when incubated in serum-free medium. The data given in the table include for comparison the response of NaIO_4 -treated cells when incubated in the presence of the various sera specified.

Neuraminidase- and galactose oxidase-treated mouse-spleen cells are agglutinated upon incubation in media containing sera from various sources (Table 2); small agglutinates of the treated cells form after a few hours. The agglutinates increase in size as the incubation proceeds, and at 48 hr large clumps containing blast cells are visible. During the third day of culture, the cell clumps are reduced in size, and many single large blast cells can be seen. Morphological analysis of mouse-spleen cells treated with neuraminidase and galactose oxidase under the experimental conditions specified in Table 1 and incubated for 72 hr, revealed that more than 50% of the cells had undergone blast transformation. Many mitoses were also observed. Preliminary experiments have shown that human blood lymphocytes also undergo blastogenesis upon treatment with neuraminidase and galactose oxidase under the experimental conditions specified in Table 1.

Evidence for the specificity of the action of galactose oxidase on mouse-spleen cells

To clarify whether the action of the galactose oxidase preparation used on mouse-spleen cells is specific or whether the effect might be due to the activity of contaminating enzymes, we investigated the effect of excess D-galactose and of bis(2-hydroxyethyl)dithiocarbamate, an inhibitor of galactose oxidase, on the induction of blastogenesis by galactose oxidase in the neuraminidase-incubated mouse-spleen cells.

(a) *Inhibition by D-Galactose of the Action of Galactose Oxidase on Neuraminidase-Treated Mouse-Spleen Cells.* D-Galactose markedly inhibited the stimulation of the cells induced by galactose oxidase (Table 3). The fact that the rate of [^3H]thymidine incorporation into cells treated with galactose oxidase and D-galactose was lower than the incorporation into untreated cells, suggested that hydrogen peroxide formed during the enzymic reaction caused cell

TABLE 1. Response of mouse-spleen cells to treatment with neuraminidase and galactose oxidase

Treatment		[^3H]Thymidine incorporated (cpm)
First	Second	
—	—	889
Galactose oxidase	—	1,425
Neuraminidase	—	1,059
Neuraminidase	Galactose oxidase	93,995
Galactose oxidase	Neuraminidase	4,380

Mouse-spleen cells ($20 \times 10^6/\text{ml}$) were incubated either with neuraminidase (50 units/ml) for 30 min at 37° or with galactose oxidase (0.25 unit/ml) for 30 min at 23° . After centrifugation the cells were washed, suspended ($20 \times 10^6/\text{ml}$) in PBS, and treated as indicated either with neuraminidase or galactose oxidase. After washing with PBS, the cells were suspended in culture medium ($5 \times 10^6/\text{ml}$). [^3H]Thymidine incorporation was determined after incubation for 46 hr (9).

TABLE 2. Effect of different sera on [^3H]thymidine incorporation into mouse-spleen cells treated with sodium periodate or neuraminidase and galactose oxidase

Serum	[^3H]Thymidine incorporated (cpm) into cells treated with	
	NaIO_4	Neuraminidase and galactose oxidase
None	26,330	15,430
Human	182,490	99,380
Fetal calf	99,530	109,200
Bovine	57,640	27,540
Human cord blood	113,310	81,120

Treatment of cells with periodate. Mouse-spleen cells suspended in PBS ($16 \times 10^6/\text{ml}$) were incubated with NaIO_4 (1 mM) for 30 min at 0° .

Treatment of cells with neuraminidase and galactose oxidase. Mouse-spleen cells in PBS ($80 \times 10^6/\text{ml}$) were incubated with neuraminidase (50 units/ml) for 30 min at 37° . The cells were then washed, suspended in PBS ($20 \times 10^6/\text{ml}$), and treated with galactose oxidase (0.5 unit/ml) for 30 min at 23° .

After treatment of the cells with periodate or with galactose oxidase, they were washed and suspended ($5 \times 10^6/\text{ml}$) in media containing different sera at a final density of 5%. All sera were heat-inactivated at 56° for 30 min. The cells were cultured under the conditions outlined in the legend to Table 1. The incorporation of [^3H]thymidine into untreated cells ranged from 1050 to 2100 cpm.

damage. This was corroborated by microscopic observation as well as by the fact that the cells, treated as above, respond poorly to Con A. The damaging effect of hydrogen peroxide could be eliminated by including catalase in the incubation

TABLE 3. Inhibition by D-galactose of the action of galactose oxidase on neuraminidase-treated mouse-spleen cells

Treatment	[^3H]Thymidine incorporated (cpm) into cells after additional treatment with	
	None	Con A
—	1,314	100,030
D-Galactose	2,565	97,890
Catalase	1,098	—
Galactose oxidase	93,740	104,690
Galactose oxidase + catalase	119,420	—
Galactose oxidase + D-glucose	100,500	120,690
Galactose oxidase + D-galactose	223	13,150
Galactose oxidase + D-galactose + catalase	1,655	93,930

Mouse-spleen cells suspended in PBS ($80 \times 10^6/\text{ml}$) were incubated with neuraminidase (50 units/ml) for 30 min at 37° . The cells were then washed, resuspended in PBS ($20 \times 10^6/\text{ml}$) to which were added, as indicated, the following: D-galactose (20 mg/ml), D-glucose (20 mg/ml), catalase (320 units/ml), galactose oxidase (0.5 unit/ml). After incubation for 30 min at 23° , the cells were washed with PBS and suspended in culture medium ($5 \times 10^6/\text{ml}$). Con A was added where indicated to a final concentration of $2 \mu\text{g}/\text{ml}$. [^3H]Thymidine incorporation was determined after incubation for 46 hr.

TABLE 4. *Decrease in neuraminidase- and galactose oxidase-induced transformation of mouse-spleen cells on reaction of the enzymically treated cells with borohydride or hydroxylamine*

Treatment of cells after incubation with neuraminidase and galactose oxidase	[³ H]Thymidine incorporated (cpm) into cells after additional treatment with	
	None	Con A
—	124,220	188,340
KBH ₄	58,780	157,550
NH ₂ OH	28,390	175,750

Mouse-spleen cells suspended in PBS (80×10^6 /ml) were incubated with neuraminidase (50 units/ml), catalase (320 units/ml), and galactose oxidase (0.5 unit/ml) for 30 min at 37°. The cells were then washed twice, resuspended in PBS, Ca⁺⁺- and Mg⁺⁺-free, containing hydroxylamine (1 mM) or potassium borohydride (1 mM), and incubated for 30 min at 23°. The cells were then washed, suspended in culture medium, treated as indicated with Con A, and cultured under the conditions outlined in the legend to Table 3.

mixture. Cells treated with neuraminidase and subsequently incubated with galactose oxidase in the presence of D-galactose and catalase did not undergo transformation, but were fully responsive to Con A (Table 3, last line). Galactose oxidase, D-galactose, and catalase, when added together to neuraminidase-incubated mouse lymphocytes after treatment with galactose oxidase alone, did not eliminate the stimulation of the cells induced by the latter.

(b) *Inhibition by Bis(2-hydroxyethyl)dithiocarbamate of the Galactose Oxidase-Induced Stimulation of Neuraminidase-Treated Mouse-Spleen Cells.* Galactose oxidase is a copper enzyme that can be inhibited by treatment with the chelating agent, diethyldithiocarbamate (11). As shown in Fig. 1, treatment of galactose oxidase with 2 mM bis(2-hydroxyethyl)dithiocarbamate markedly inhibited its stimulatory activity on neuraminidase-incubated mouse-spleen cells. However, the cells retained their responsiveness to Con A. Galactose oxidase treated as above lost essentially all its activity, as measured by the peroxidase-chromogen assay (10).

Decrease of galactose oxidase-induced transformation by reaction of the enzymically-treated cells with borohydride or hydroxylamine

When neuraminidase-incubated and galactose oxidase-treated cells are reacted with borohydride or hydroxylamine, under the experimental conditions specified in Table 4, transformation is reduced. Cells so treated can still be stimulated by Con A (Table 4). Reaction of mouse-spleen cells with borohydride and hydroxylamine before the enzymic treatment do not affect the response of the cells to subsequent treatment with neuraminidase and galactose oxidase.

Response of mouse-spleen cells to neuraminidase and galactose oxidase after treatment with papain

Previously it had been shown that incubation of mouse-spleen cells with papain reduces markedly their response to periodate stimulation (2). We tested [³H]thymidine incorporation into cells treated with papain after incubation with neuraminidase and galactose oxidase under the experimental conditions given in Table 4. [³H]Thymidine incorporation decreased by about

80% when compared to the corresponding value for cells that were not treated with the proteolytic enzyme, but were incubated with neuraminidase and galactose oxidase. Spleen cells incubated sequentially with papain [under the conditions described (2)], neuraminidase, and galactose oxidase as above could be fully stimulated by Con A.

DISCUSSION

Treatment of lymphocytes with neuraminidase and galactose oxidase induces extensive blastogenesis, the sequence in which the enzymes are applied to the cells affecting markedly the extent of transformation. Thus, treatment of mouse-spleen cells with galactose oxidase after incubation with neuraminidase stimulates the cells, whereas treatment in the reverse order has little effect (Table 1). This would suggest that treatment of the cells with neuraminidase exposes sites that are susceptible to the action of galactose oxidase, and that oxidation of these sites by the latter enzyme is essential for triggering the cells. Since galactose oxidase is known to oxidize specifically the C-6 primary hydroxyl group of D-galactose or N-acetyl-D-galactosamine (10), one might assume that one of these saccharides becomes accessible to galactose oxidase after the removal of sialic acid by neuraminidase. Sialic acid in glycoproteins always occupies a nonreducing terminal position and is glycosidically linked either to D-galactose or to N-acetyl-D-galactosamine (4). In this connection it is pertinent to note that the terminal sequence sialic acid → galactose → N-acetylglucosamine was suggested for the receptor site of the red kidney bean phytohemagglutinin on human erythrocytes (12), as well as for the receptor sites of various phytomitogens on human lymphocytes (13).

The stimulation of the neuraminidase-treated lymphocytes by galactose oxidase seems to be specific since D-galactose, in

FIG. 1. Inhibition by bis(2-hydroxyethyl)dithiocarbamate of galactose oxidase-induced stimulation of [³H]thymidine incorporation into neuraminidase-treated cells. Mouse-spleen cells suspended in PBS (80×10^6 /ml) were incubated with neuraminidase (50 units/ml) for 30 min at 37°. The cells were then washed, resuspended in PBS (20×10^6 /ml), and treated with galactose oxidase at different concentrations. After incubation for 30 min at 23° the cells were washed, suspended in culture medium, and cultured under conditions outlined in the legend to Table 1. Galactose oxidase previously treated with bis(2-hydroxyethyl)dithiocarbamate (○—○) (see *Methods*); untreated enzyme (●—●).

the presence of catalase or bis(2-hydroxyethyl)dithiocarbamate, eliminates the enzyme-induced blastogenesis. Catalase did not reduce the stimulation of the cells by galactose oxidase. The possibility that hydrogen peroxide, which is formed upon incubation of the cell with the oxidase, is involved in the triggering process can thus be ruled out.

Blastogenesis induced by the treatment of lymphocytes with neuraminidase and galactose oxidase is reduced when the enzymically treated cells are reacted with borohydride or hydroxylamine (Table 4). It is possible that the aldehyde moieties, formed upon oxidation of the galactosyl residues exposed by neuraminidase treatment, are involved in the induction of lymphocyte transformation. The possible role of an aldehyde moiety in the induction of lymphocyte transformation was also postulated in studies on blastogenesis induced by periodate (2). The function of the aldehyde moiety in the induction of blastogenesis is not known. It may be that the aldehyde groups react with free amino groups in the cell membrane to yield a Schiff base, and a "cross-linked" structure is formed. Cross-linking of membrane sites might play a role in lymphocyte transformation induced by lectins and by anti-immunoglobulin antibody (14).

Lymphocytes treated with neuraminidase and galactose oxidase are agglutinated during incubation in media containing various sera. The agglutination of the cells under these conditions might result from interaction of an agglutinin present in those sera with a membrane site exposed during the enzymic treatment. Alternatively, the agglutination of the cells might be directly related to the alterations in the cell

membrane induced by the enzymic treatment. These alterations might also be involved in the triggering of the cell to undergo blastogenesis.

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