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Transformation of Neuraminidase-Treated Lymphocytes by Soybean Agglutinin

(mouse spleen/agglutination/membrane receptors/cell lines)

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ABSTRACT **Transformation of mouse-spleen lymphocytes in the presence of soybean agglutinin is markedly enhanced after their treatment with neuraminidase (EC 3.2.1.18). Incubation of the cells with the enzyme also facilitates their agglutination by the lectin. The soybean agglutinin-induced agglutination and transformation is inhibited specifically by *N*-acetyl-D-galactosamine. New specific binding sites for soybean agglutinin were shown to appear after neuraminidase treatment. It is postulated that the transformation of neuraminidase-treated mouse-spleen lymphocytes induced by soybean agglutinin is caused by the binding of the lectin to galactosyl residues exposed by the action of neuraminidase on the cell membrane.**

Soybean agglutinin (SBA) is known to agglutinate human and rabbit erythrocytes as well as malignant cells from different sources (1, 2). The agglutination is specifically inhibited by *N*-acetyl-D-galactosamine and also, but to a lesser degree, by D-galactose. Mouse lymphocytes are agglutinated by SBA only at relatively high concentrations (3). However, the lectin does not exhibit mitogenic activity. We showed (4) that extensive blastogenesis is induced in mouse-spleen lymphocytes by galactose oxidase (EC 1.1.3.9) only after incubation of the cells with neuraminidase (EC 3.2.1.18), suggesting that neuraminidase treatment exposes masked galactosyl residues within the cell membrane. These observations prompted us to investigate whether blastogenesis can be induced by SBA in lymphocytes after their incubation with neuraminidase.

MATERIALS AND METHODS

Soybean agglutinin, purified by affinity chromatography (5), was kindly supplied by N. Sharon, H. Lis, and R. Lotan of our department. ^{125}I-Labeled soybean agglutinin (37,000 cpm/μg) was prepared by the chloramine-T method of Greenwood *et al.* (6). Fluorescein-conjugated soybean agglutinin (A_{280}/A_{495} = 0.8) was a gift of Miles-Yeda Ltd., Rehovot, Israel. Saccharides were obtained from Pfanstiehl Laboratories Inc., U.S.A. *N*-Acetylneuraminic acid (crystalline, synthetic) was purchased from Sigma Chemical Co., U.S.A. Neuraminidase from *Vibrio comma* was supplied by Behringwerke AG, Germany, as a solution containing 500 units/ml (1 unit releases 1 μg *N*-acetylneuraminic acid from α-acid glycoprotein at 37° in 15 min at pH 5.5). [*methyl*-^{3}H]Thymidine (5 Ci/mmol) was obtained from the Nuclear Research Center, Negev, Israel.

Mouse-Spleen Cells were obtained from CBA/LAC female mice and cultured as described (7).

Cell Lines. Mouse secondary embryo, BHK, and L929 cells were grown in plastic petri dishes (Falcon Co.) containing Dulbecco's modified Eagle's medium with 10% fetal-calf serum. Rat myogenic cells and mouse neuroblastoma C-1300 cells were grown as described (8, 9). L1210 and P388 cells were maintained in the ascites form in DBA/2 mice.

[^{3}*H*]*Thymidine Incorporation into DNA.* Thymidine incorporation into the trichloroacetic acid (5%)-insoluble fraction of mouse-spleen cells was assayed at the time intervals given in the legends to figures and tables and as described (10).

Binding Assay. 10^7 Cells per ml in phosphate-buffered saline (pH 7.2) were incubated (when indicated) with neuraminidase (50 units/ml) for 30 min at 37° with shaking. The cells were then washed and suspended in phosphate-buffered saline (1 to 3 × 10^6/ml). Aliquots of the cell suspension (0.5 ml) were incubated with [^{125}I]SBA (20 μg/ml) for 30 min at 23° with shaking. The extent of [^{125}I]SBA binding to the cells was determined as described in the legend to Table 3. To calculate the amount of [^{125}I]SBA bound specifically, the amount of [^{125}I]SBA bound in the presence of D-galactose (10 mg/ml) was subtracted from the amount bound in its absence. The fraction of nonspecifically bound [^{125}I]SBA did not exceed 10% of the total.

Agglutination Assay. Cells were treated with neuraminidase where indicated and incubated with SBA at the concentrations given in Table 4, as described above. Agglutination was recorded after 30 min. The agglutination was inhibited in all cases when galactose (10 mg/ml) was included in the incubation mixture.

RESULTS

Transformation and agglutination of neuraminidase-treated mouse-spleen cells by soybean agglutinin

The amount of [^{3}H]thymidine incorporated into mouse-spleen cells treated with SBA at different concentrations, before and after incubation with neuraminidase, is given in Fig. 1. The cells showed a markedly enhanced response to SBA after treatment with neuraminidase under the specified conditions. Maximal stimulatory effect was observed at an SBA concentration of about 100 μg/ml. The effect decreased considerably at higher SBA concentrations; for example, at a concentration of 200 μg SBA per ml, thymidine incorporation decreased to about one-third of the maximal value, while no stimulatory effect was noted at a concentration of 400 μg/ml.

Morphological analysis of mouse-spleen cells treated with neuraminidase and incubated with SBA (100 μg/ml), under

Abbreviation: SBA, soybean agglutinin.

TABLE 1. *Inhibition of neuraminidase action on mouse-spleen cells by N-acetylneuraminic acid*

Treatment		[^{3}H]Thymidine incorporated (cpm) into cells after additional treatment with		Agglutination of cells treated with SBA
Neuraminidase	*N*-Acetylneuraminic acid	None	SBA	
−	−	1,620	5,090	−
+	−	2,180	15,810	+
−	+	2,180	4,930	−
+	+	3,430	6,190	−

8×10^7 Mouse-spleen cells per ml in phosphate-buffered saline were treated with neuraminidase (50 units/ml) in the presence or absence of *N*-acetylneuraminic acid (6 mg/ml) and incubated for 30 min at 37° with shaking. The cells were then washed with phosphate-buffered saline and suspended in culture medium (5×10^6/ml). SBA was added to a final concentration of 20 μg/ml. After incubation for 72 hr, [^{3}H]thymidine incorporation during 2 hr was measured. Agglutination was recorded after 5 hr of incubation.

the experimental conditions outlined in Fig. 1, revealed that about 30% of the cells had undergone blast transformation, whereas only few blast cells could be detected when the mouse-spleen cells were not previously treated with neuraminidase and incubated with SBA as above.

Mouse-spleen cells pretreated with neuraminidase (Fig. 1) and suspended in phosphate-buffered saline, pH 7.2 (22) (5×10^6 cells per ml), were quantitatively agglutinated after incubation with SBA (20 μg/ml) at room temperature (24°) for 60 min. No agglutination occurred under these conditions when incubation of the cells with neuraminidase was omitted.

Incubation of mouse-spleen cells with neuraminidase in the presence of *N*-acetylneuraminic acid, a competitive inhibitor of the enzyme (11), did not increase their response to SBA (Table 1). Cells thus treated also could not be agglutinated by the lectin (Table 1). It is thus reasonable to assume that the modification of the mouse-spleen lymphocytes by incubation with the neuraminidase preparation is due to neuraminidase activity itself and not to possible contaminating enzymes.

TABLE 2. *Effect of various saccharides on SBA-induced agglutination and stimulation of neuraminidase-treated mouse-spleen cells*

Additions		[^{3}H]Thymidine incorporated (cpm)	Agglutination
SBA	Saccharide		
−	−	1,740	−
+	+	21,200	+
+	D-Galactose	4,340	−
+	*N*-Acetyl-D-galactosamine	4,010	−
+	D-Glucose	22,100	+
+	*N*-Acetyl-D-glucosamine	23,400	+
+	α-Methyl-D-glucose	16,100	+
+	α-Methyl-D-mannose	21,200	+
+	L-Fucose	24,900	+

8×10^7 Mouse-spleen cells per ml in phosphate-buffered saline were treated with neuraminidase (50 units/ml) for 30 min at 37° with shaking. The cells were then washed with phosphate-buffered saline and suspended in culture medium (5×10^6/ml). SBA was added to a final concentration of 20 μg/ml and the specified saccharide to a final concentration of 5 mg/ml. After incubation for 72 hr, [^{3}H]thymidine incorporation during 2 hr was measured. Agglutination was recorded after 5 hr of incubation.

TABLE 3. *Increase in binding of ^{125}I-labeled soybean agglutinin to N-acetyl-D-galactosamine-like sites in mouse-spleen cells treated with neuraminidase*

Saccharide added	^{125}I-Labeled soybean agglutinin (cpm) bound per 10^6 cells	
	Cell treatment	
	None	Neuraminidase
—	2,300	7,820
N-Acetyl-D-glucosamine	1,860	7,810
N-Acetyl-D-galactosamine	243	305

A suspension of mouse-spleen cells in NH_4Cl (0.8%) was incubated for 7 min at 23°. Under these conditions erythrocytes are selectively lysed. After centrifugation, erythrocyte ghosts overlying the pellet were removed by aspiration. Cells were then washed, suspended in phosphate-buffered saline (8×10^7/ml), treated with neuraminidase (50 units/ml) for 30 min at 37° with shaking, washed again, and suspended in phosphate-buffered saline (10^7/ml). Aliquots (0.5 ml) of the lymphocyte suspension were then incubated with ^{125}I-labeled SBA (20 μg/ml) and the saccharide specified (20 mg/ml) for 30 min at 23° with shaking. After centrifugation, the cells were washed twice with 2-ml portions of phosphate-buffered saline, filtered on a glass filter, washed twice with 4-ml portions of phosphate-buffered saline, dried, and counted in a Packard scintillation counter

Preliminary experiments have shown that mouse-thymus cells similarly treated with neuraminidase undergo transformation on incubation with SBA in the presence of a macrophage culture supernatant (12). Neuraminidase-treated human blood lymphocytes were found also to undergo blastogenesis on incubation with SBA.

[^{3}H]Thymidine incorporation into neuraminidase-treated mouse-spleen cells, after incubation with SBA for different time intervals

The amount of [^{3}H]thymidine incorporated into mouse-spleen cells treated with neuraminidase and incubated with SBA for time intervals of 1–4 days is shown in Fig. 2. Cells were incubated with [^{3}H]thymidine for 2 hr after the time intervals specified on the abscissa. Increase in the time of incubation with the lectin caused a corresponding increase in the amount of thymidine incorporation. [^{3}H]Thymidine incorporation into cells similarly treated but without prior incubation with neuraminidase remained low after 3 days of incubation with SBA, and increased only slightly after the fourth day of incubation.

Effect of saccharides on the agglutination and stimulation by SBA of neuraminidase-treated mouse-spleen cells

The SBA-induced agglutination and stimulation of neuraminidase-treated mouse-spleen cells are inhibited markedly by D-galactose and *N*-acetyl-D-galactosamine, and to some extent by α-methyl-D-glucoside (Table 2). The other saccharides tested had no effect (Table 2). Under the given experimental

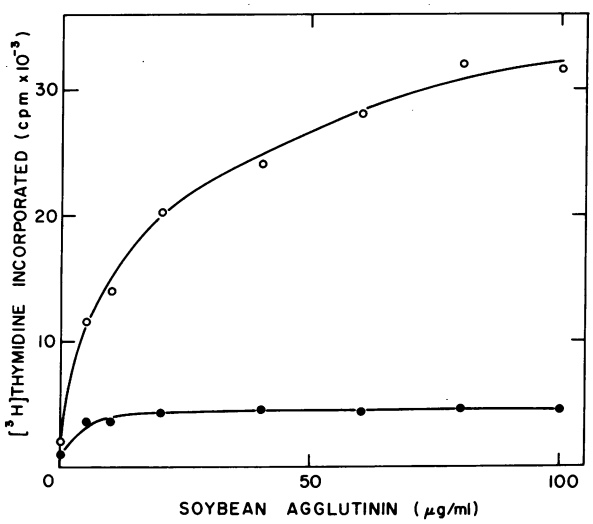

FIG. 1. Stimulation by soybean agglutinin of mouse-spleen cells treated with neuraminidase. 8 × 10^7 Mouse-spleen cells per ml in phosphate-buffered saline were treated with neuraminidase (50 units/ml) for 30 min at 37° with shaking. The cells were then washed with phosphate-buffered saline and suspended in culture medium (5 × 10^6/ml). SBA was added to the final concentration indicated and the cells were incubated for 72 hr. [³H]Thymidine incorporation during 2 hr was then determined. Neuraminidase-treated cells (O——O); untreated cells (●——●).

conditions at concentrations of 40 μg/ml and 200 μg/ml, D-galactose inhibited [³H]thymidine incorporation by 10% and 51%, respectively, and *N*-acetyl-D-galactosamine by 38% and 70%, respectively.

Increase in binding of ^{125}I-labeled soybean agglutinin to *N*-acetyl-D-galactosamine-like sites in mouse-spleen cells treated with neuraminidase

Neuraminidase-treated mouse-spleen lymphocytes bind about 3.5 times as much ^{125}I-labeled SBA as the untreated cells. The binding of the labeled lectin to the cells is inhibited almost completely by *N*-acetyl-D-galactosamine (Table 3). Using fluorescence microscopy we found that neuraminidase-treated mouse-spleen lymphocytes bind more fluorescein-conjugated SBA as compared to untreated cells. The binding was inhibited by *N*-acetyl-D-galactosamine.

Effect of neuraminidase treatment of different cell lines on their binding to and agglutination by SBA

The effect of neuraminidase treatment of different cell lines on the specific binding of ^{125}I-labeled SBA is illustrated in Table 4. In most cases neuraminidase treatment increased the specific binding of the cells to SBA. However, in some cell lines (e.g., mouse secondary embryo cells) the change in the specific site for SBA after neuraminidase treatment was rather small. No correlation was found between the total number of SBA-specific sites of a particular cell line and agglutinability of the cells by the lectin. In a few cell lines (mouse secondary embryo cells and L929), neuraminidase renders the cells agglutinable by SBA without affecting markedly the extent of binding of the lectin by the cells.

DISCUSSION

The transformation of mouse-spleen lymphocytes in the presence of SBA is seen to be markedly enhanced after treatment with neuraminidase, and incubation of the cells with the enzyme also facilitates their agglutination by the lectin. The SBA-induced agglutination and transformation is inhibited specifically by *N*-acetyl-D-galactosamine. New specific binding sites for SBA appeared after neuraminidase treatment.

Sialic acid in glycoproteins is always found to occupy a non-reducing terminal position and to be glycosidically linked either to D-galactose or to *N*-acetyl-D-galactosamine (13). It is thus plausible to assume that the transformation of mouse-spleen lymphocytes induced by SBA is caused by the binding of the lectin to galactosyl residues exposed by the action of neuraminidase on the cell membrane. We have shown recently (4) that extensive blastogenesis is induced in mouse-spleen cells treated with galactose oxidase after incubation with neuraminidase. On the basis of this observation, we have suggested 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. Previously, we found that lymphocyte transformation can be induced by periodate (7, 14). Evidence was presented to show that the membrane sites modified by the periodate include a glycoprotein(s) containing sialic acid, which yields on oxidation an aldehyde moiety that is essential for transformation. Since sialic acid in glycoproteins is known to be glycosidically linked to galactose or *N*-acetylgalactosamine, one may postulate that the chemical oxidizing agent, periodate, the enzymic oxidizing agent, galactose oxidase, and the chemically inert lectin, SBA, trigger lymphocytes to undergo transformation by affecting the same membrane site. In this connection it is pertinent to

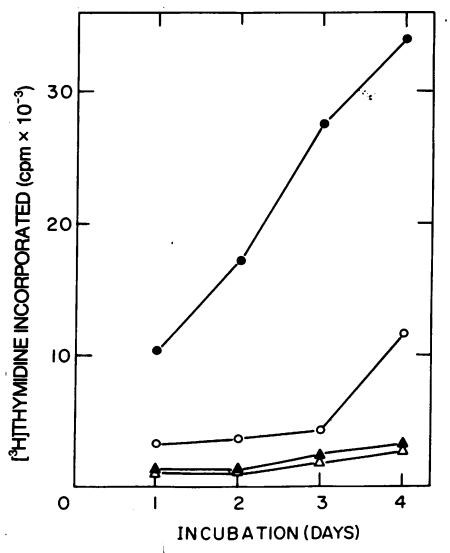

FIG. 2. [³H]Thymidine incorporation into mouse-spleen cells treated with SBA for different time intervals. 8 × 10^7 Mouse-spleen cells per ml in phosphate-buffered saline were treated with neuraminidase (50 units/ml) for 30 min at 37° with shaking. The cells were then washed with phosphate-buffered saline and suspended in culture medium (5 × 10^6/ml). SBA was added to a final concentration of 20 μg/ml. [³H]Thymidine incorporation within 2 hr was determined after the incubation times specified on the abscissa. Neuraminidase-treated cells incubated with SBA (●——●); untreated cells incubated with SBA (O——O); neuraminidase-treated cells incubated without SBA (▲——▲); untreated cells incubated without SBA (△——△).

TABLE 4. *Effect of neuraminidase treatment of different cell lines on their binding to and agglutination by SBA*

Cell line	Neuraminidase	Binding assay ^{125}I-labeled SBA (cpm) bound specifically per 10^6 cells	Agglutination assay: SBA (μg/ml)	Agglutination assay: Agglutination
C_3H mouse secondary embryo cells	−	10,500	20	−
	+	13,000	20	+
SWR mouse secondary embryo cells	−	18,800	20	−
	+	13,400	20	+
BHK	−	130	400	−
	+	3,530	200	−
L929	−	17,300	20	−
	−	—	80	+
	+	29,800	5	+
Rat myogenic cells	−	83,900	20	−
	+	41,000	20	−
Mouse neuroblastoma C-1300	−	30,900	20	−
	+	66,200	20	−
L1210	−	1,210	20	−
	+	4,980	20	+
P388	−	2,930	20	−
	+	14,200	20	+

Cells in culture, 3 days after subculturing, were washed with phosphate-buffered saline, Ca^{++} and Mg^{++} free, and removed from the petri dishes by washing with the same buffer containing 0.02% disodium EDTA. L1210 and P388 cells which were maintained in the ascites form in DBA/2 mice were treated with NH_4Cl (0.8%), under the conditions specified in the legend to Table 3, in order to obtain a cell suspension free of erythrocytes. For details of the binding and agglutination assays, see *Methods*.

note that the terminal sequence sialic acid → galactose → *N*-acetylglucosamine was suggested for the receptor of the red kidney phytohemagglutinin on human erythrocytes (15) as well as for the receptor sites of various phytomitogens on human lymphocytes (16).

In some of the cell lines tested (see Table 4), it was found that treatment with neuraminidase facilitates the agglutination of the cells by SBA without the occurrence of any marked change in the number of the binding sites for the lectin. Recently, it was suggested that cell agglutination by various lectins depends on the relative distribution of the saccharide receptors on the cell surface, rather than on their total number (17, 18). It is, therefore, plausible to assume that the incubation of different cells with neuraminidase facilitates the patching of the SBA-binding sites induced by the lectin. Moreover, the observed increase in the agglutinability of different cell lines by various lectins after treatment with proteolytic enzymes (2, 19, 20) might be due in part also to the removal of some of the membrane sialoglycoproteins. Patching of specific membrane sites might be involved as well in the triggering of lymphocytes to undergo transformation (21). It is, therefore, possible that neuraminidase renders lymphocytes responsive to SBA by two mechanisms: (*a*) exposure of new sites for the lectin, and (*b*) facilitation of the patching of the saccharide-containing sites after their binding to the lectin.

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