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Membrane Site Modified on Induction of the Transformation of Lymphocytes by Periodate

(mouse spleen/concanavalin A/neuraminidase/papain)

ABRAHAM NOVOGRODSKY AND EPHRAIM KATCHALSKI

Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel

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ABSTRACT **Lymphocytes isolated from different sources undergo blastogenesis after mild treatment with periodate. Incubation of mouse spleen lymphocytes with neuraminidase or papain markedly reduces their response to periodate. Transformation induced by concanavalin A of the enzymically treated cells is not impaired. Blastogenesis induced by periodate is decreased when periodate-treated lymphocytes are reacted with borohydride, hydroxylamine, or semicarbazide. Treatment with these compounds does not affect transformation of cells by concanavalin A. It is suggested that different membrane sites are involved in lymphocyte transformation induced by periodate and by concanavalin A. The periodate target site seems to include a glycoprotein complex containing sialic acid that yields, on oxidation, an aldehyde moiety that is essential for transformation.**

Upon interaction with agents that affect cell membranes, lymphocytes undergo metabolic and morphological changes, referred to as transformation or blastogenesis (1). The binding and the blastogenic activity of the lectins, phytohemagglutinin and concanavalin A (Con A), can be reversed by specific saccharides, suggesting that a saccharide-containing site in the cell membrane is involved in lymphocyte transformation (2–4). We have previously (5) found that treatment of rat lymph-node lymphocytes with periodate induced blastogenesis, and suggested that the primary action of periodate in induction of lymphocyte transformation is the chemical modification of the cell membrane. Recently it has been shown that mouse lymph-node and spleen lymphocytes are stimulated by periodate, whereas mouse thymus cells are rendered responsive to periodate treatment upon addition of cell-free supernatant of mouse spleen-cell and macrophage cultures (6). Human lymphocytes are also stimulated by periodate, whereas chronic lymphatic leukemia lymphocytes are unresponsive to this treatment (7).

In an extension of the above studies we attempted to detect and characterize the membrane site modified on induction of transformation of lymphocytes by periodate. Our results suggest that the $NaIO_4$-target site includes a glycoprotein complex containing sialic acid that yields, on oxidation, an aldehyde moiety that is essential for transformation. Based on the properties of enzymically and chemically modified cells, we conclude that different membrane sites are involved in lymphocyte transformation induced by periodate and by Con A.

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

MATERIALS AND METHODS

Con A, twice crystallized, was obtained from Miles–Yeda Ltd. Con A labeled with ^{63}Ni ([^{63}Ni]Con A), specific activity of 4000 cpm/μg, was prepared by the procedure of Inbar and Sachs (8). [*Methyl*-^{3}H]Thymidine (5 Ci/mmol) was obtained from Nuclear Research Center, Negev, Israel. Sodium periodate ($NaIO_4$), analytical reagent, was obtained from BDH Chemicals Ltd., England. *N*-Acetylneuraminic acid (crystalline, synthetic) was obtained from Sigma Chemical Co. 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). Papain, purified by affinity chromatography (9) (22 units/mg), was obtained from Miles–Yeda Ltd.

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) (21). The large pieces of connective tissue were allowed to settle, and the supernatant suspension was harvested. Nucleated cells were counted after dilution in acetic acid (2%).

Mouse Spleen-Cell Cultures. Mouse spleen cells were suspended at a final concentration 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% CO_2 for 46 hr.

Treatment of Cells with $NaIO_4$. Spleen cells were suspended (13×10^6/ml) in PBS containing $NaIO_4$, at concentrations specified in the tables and figures, and incubated at 23° for 10 min.

Determination of Sialic Acid Released by Neuraminidase. Mouse spleen cells were suspended in NH_4Cl (0.8%) and incubated for 7 min at 23°. Under these conditions, erythrocytes were selectively lysed. After centrifugation, erythrocyte ghosts that overlayered the pellet were removed by aspiration. The cells were then washed with PBS and suspended at a final concentration of 70×10^6 cells per ml in PBS or sodium acetate (50 mM, pH 5.5) containing NaCl (9 mg/ml) and $CaCl_2$ (1 mg/ml). Neuraminidase was added, and incubation proceeded for 60 min at 37°. After centrifugation, the sialic

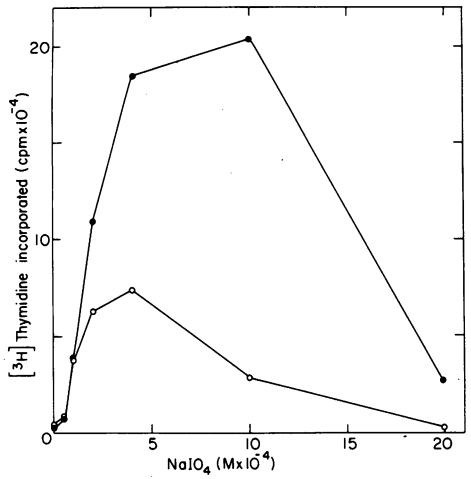

FIG. 1. Response of mouse spleen cells to treatment with $NaIO_4$ at different concentrations after incubation with neuraminidase. Mouse spleen cells were suspended at a final concentration of 70×10^6/ml in PBS containing neuraminidase (50 units/ml) and incubated for 60 min at 37°. The cells were then washed and treated with $NaIO_4$ at concentrations specified on the *abscissa*. Cells were suspended in culture medium (5×10^6/ml), and [^{3}H]thymidine incorporation was determined after incubation for 46 hr. Neuraminidase-treated cells (O——O); untreated cells (●——●).

acid in the supernatant was determined by the method of Warren (10) as modified by Codington *et al.* (11).

Binding of [^{68}Ni]Con A to Mouse Spleen Cells. Mouse spleen cells, treated with NH_4Cl (0.8%) as described above, were washed, suspended (12×10^6 ml) in PBS, and incubated for 30 min at 23° with [^{68}Ni]Con A at different concentrations. The cells were then centrifuged, washed twice with 2-ml portions of PBS, and suspended in 1 ml of trichloroacetic acid (5%). After incubation for 15 min at 23°, the precipitate was centrifuged, and the supernatant (containing ^{68}Ni released from [^{68}Ni]Con A upon acidification) was mixed with 11 ml of Bray's scintillation solution (12) and counted. For calculation of the amount of [^{68}Ni]Con A bound specifically, the amount of [^{68}Ni]Con A bound in the presence of methyl α-D-mannopyranoside (16 mg/ml) was subtracted from the amount bound in the absence of the saccharide.

Reduction of $NaIO_4$-Treated Mouse Spleen Cells with Tritiated Borohydride. Mouse spleen cells that had been treated with NH_4Cl (0.8%) for removal of contaminating erythrocytes were washed, suspended in PBS, and treated with $NaIO_4$ (0.4 mM). The cells were washed, suspended (60×10^6/ml) in PBS containing [^{3}H]KBH_4 (4.4 nmol/ml, 2×10^5 cpm/nmol), and incubated for 30 min at 23°. The cells were then centrifuged, washed, and suspended (20×10^6/ml) in PBS. Cells in 1 ml were collected on glass filters and washed twice with 5-ml portions of 1% NaCl and twice with 5-ml portions of 5% trichloroacetic acid. The filters were dried and counted.

[^{3}H]Thymidine Incorporation into DNA. 2.5 μCi of [^{3}H]-thymidine was added to 1 ml of cell cultures that had been incubated for 46 hr. After incubation of the cell cultures for an additional 2 hr with agitation, incorporation of [^{3}H]thymidine into DNA was determined (13). In each experiment, cultures were incubated in triplicate, and the results were expressed as the mean value of three determinations of [^{3}H]thymidine incorporation. The range of deviation from the mean value was usually less than 10%.

RESULTS

Response of mouse spleen cells to $NaIO_4$ and Con A after incubation with neuraminidase

The stimulation of [^{3}H]thymidine incorporation in mouse spleen cells after treatment with $NaIO_4$ at different concentrations is given in Fig. 1. The data also show that the response of spleen cells to $NaIO_4$ is markedly reduced after treatment with neuraminidase.

Under the experimental conditions outlined in the legend to Fig. 1, 1.7 μg of sialic acid is released per 70×10^6 cells. Under optimal conditions for neuraminidase activity on glycoproteins [50 mM sodium acetate (pH 5.5), containing NaCl (9 mg/ml) and $CaCl_2$ (1 mg/ml)], 1.9 μg of sialic acid per 70×10^6 cells was liberated upon incubation of the cells with the enzyme (100 units/ml) for 60 min at 37°.

The data presented in Fig. 2 show that the response of spleen cells to $NaIO_4$ after treatment with neuraminidase is markedly reduced, whereas the response of neuraminidase-treated cells to Con A stimulation remains practically unimpaired. A slight enhancement of the response to Con A stimulation was observed in cells treated with neuraminidase at a concentration of 40 units/ml.

The purified neuraminidase used was stated by the manufacturer to be free of lecithinase C, and neither protease nor aldolase activity could be demonstrated. However, it seemed desirable to determine whether the effect of neuraminidase on

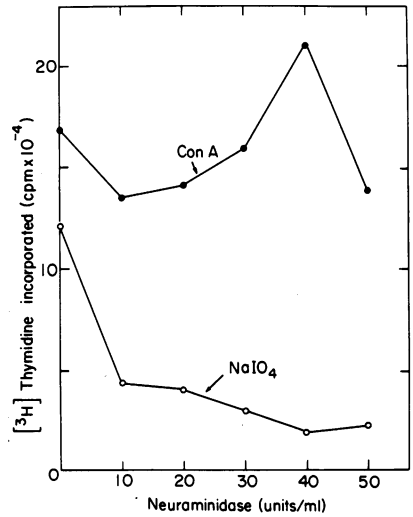

FIG. 2. Response of mouse spleen cells to treatment with periodate and Con A after incubation with neuraminidase at different concentrations. Mouse spleen cells at a final concentration of 70×10^6/ml in PBS were treated with neuraminidase at different concentrations for 60 min at 37°. The cells were then washed and treated with $NaIO_4$ (0.4 mM). They were suspended in culture medium (5×10^6/ml) and Con A was added, as indicated, to a final concentration of 2 μg/ml. [^{3}H]Thymidine incorporation was determined after incubation for 46 hr. The extent of [^{3}H]thymidine incorporation into cells that were not treated with $NaIO_4$ and Con A, but were incubated with neuraminidase at concentrations of 0, 10, 20, 30, 40, and 50 units/ml was 586, 450, 460, 551, 967, and 682, respectively.

mouse spleen cells is specific or is due to the activity of contaminating enzymes. Incubation of mouse spleen cells with neuraminidase in the presence of *N*-acetylneuraminic acid, a competitive inhibitor of the enzyme (14, 15), inhibited the enzyme's reduction of the cell response to $NaIO_4$ stimulation (Table 1). Incubation with neuraminidase of cells that had been treated with $NaIO_4$ does not affect their transformation.

By the use of a trypan-blue exclusion test and by following the release of ^{51}Cr during incubation of ^{51}Cr-labeled mouse spleen cells (16), we could show that the cells are not damaged by the combined treatment with neuraminidase and $NaIO_4$. Moreover, mouse spleen cells incubated with neuraminidase followed by $NaIO_4$ treatment can be stimulated almost maximally by Con A (Table 2, Exp. 1).

Induction of lymphocyte transformation by periodate involves incubation of the cells with the inducing agents for a short period (10 min). In contrast, transformation of cells by Con A was achieved in our experiments by prolonged incubation of the cells with the lectin (48 hr). We, therefore, wanted to determine whether the number of binding sites for Con A is altered soon after incubation of the cells with neuraminidase, which impairs the response of the cells to periodate. Mouse spleen cells, incubated with [^{63}Ni]Con A (see *Methods*) at concentrations of 30, 60, and 120 μg/ml, bound specifically 2.0×10^6, 2.3×10^6, and 2.7×10^6 molecules of [^{63}Ni]Con A per cell, respectively. Under similar conditions, neuraminidase-treated cells (50 units of enzyme/ml; see Table 1) bound 2.4×10^6, 2.6×10^6, and 3.1×10^6 molecules of [^{63}Ni]Con A per cell. These findings show that neuraminidase treatment does not remove Con A-specific binding sites from the cells.

Response of spleen cells to $NaIO_4$ and Con A after incubation with papain

In Table 2, Exp. 2 we show that the response of spleen cells to $NaIO_4$ was markedly reduced after incubation with papain. The response of the papain-treated cells to Con A stimulation was hardly affected. Moreover, spleen cells can be stimulated almost maximally by Con A after incubation with papain, followed by $NaIO_4$ treatment. Preliminary experiments have indicated that papain, in contrast to neuraminidase, when applied to cells first treated with $NaIO_4$, markedly reduced their stimulation. Treatment of spleen cells with papain (0.5 mg/ml, see Table 2, Exp. 2) resulted in a reduction of about 20% in their specific binding sites for [^{63}Ni]Con A.

In some experiments, treatment of mouse spleen cells with papain alone (0.5 mg/ml, under the conditions outlined above) resulted in a notable stimulation of the cells, corresponding to about 10% of that of Con A-treated cells. This finding is in accord with the report on blastogenesis of human lymphocytes induced by papain treatment (17).

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

Treatment	*N*-Acetylneuraminic acid (mg/ml)	[^{3}H]Thymidine incorporated (cpm) into cells treated with: None	Con A	$NaIO_4$
PBS	0	2474	221000	177200
PBS	1.5	1717	204200	170100
PBS	6.0	2414	160000	196000
Neuraminidase	0	1639	204800	26780
Neuraminidase	1.5	1781	157000	85290
Neuraminidase	6.0	2900	221300	158800

Mouse spleen cells at a final concentration of 70×10^6 cells per ml in PBS were treated with neuraminidase (50 units/ml) in the presence of *N*-acetylneuraminic acid at the concentrations specified above. The cells were then centrifuged, washed, and treated with $NaIO_4$ (1 mM) as indicated. After suspension in culture medium (5×10^6/ml), Con A was added, as indicated, to a final concentration of 2 μg/ml, and the cells were cultured for 46 hr.

TABLE 2. *Response of mouse spleen cells to periodate and Con A after treatment with neuraminidase or papain*

Exp.	Treatment	[^{3}H]Thymidine incorporated (cpm) into cells treated with: None	Con A	$NaIO_4$	$NaIO_4$ + Con A
1	PBS	1086	136900	130400	143900
	Neuraminidase	1156	159200	27380	131100
2	PBS	2761	173700	118100	121200
	Papain	3251	145600	28490	127400

Exp. 1. Experimental conditions are outlined in Table 1.

Exp. 2. Mouse spleen cells were suspended (100×10^6/ml) in PBS containing fetal-calf serum (10%, heat inactivated for 30 min at 56°) and papain (0.5 mg/ml), and incubated for 60 min at 37°. The cells were then washed and treated with $NaIO_4$ and Con A as indicated in Table 1.

Decrease of $NaIO_4$-induced transformation by reaction of $NaIO_4$-treated cells with borohydride, hydroxylamine, or semicarbazide

In Table 3 we show that $NaIO_4$-induced transformation is markedly reduced when $NaIO_4$-treated cells are reacted with borohydride, hydroxylamine, and, to a lesser degree, with semicarbazide. Cells treated as above can still be stimulated by Con A. $NaIO_4$-treated cells that have been reduced with borohydride are not stimulated, however, by repetition of the treatment with $NaIO_4$. Treatment of mouse spleen cells with borohydride and semicarbazide before $NaIO_4$ treatment did not affect the response of the cells to the oxidizing agent. Under similar conditions, hydroxylamine, when applied to untreated cells, did reduce the response of the cells to $NaIO_4$ stimulation to some extent. The response of the cells to Con A stimulation was, however, not reduced.

Reduction of $NaIO_4$-treated mouse spleen cells with tritiated borohydride

Recently (18), a general method was described for radioactive labeling of glycoproteins containing sialic acid, consisting of periodate oxidation followed by tritiated borohydride reduction. We attempted to use this technique for labeling $NaIO_4$-treated mouse spleen cells. Reduction of $NaIO_4$-treated cells with [^{3}H]KBH_4 (see *Methods*) resulted in incorporation of 2409 cpm into the Cl_3CCOOH-insoluble fraction, whereas the untreated cells incorporated only 431 cpm. Incubation of cells with neuraminidase before periodate treatment resulted in a decrease of about 40% in the incorporation of tritium into the acid-insoluble fraction. About 50% of the tritium incorporated into the acid (5%)-insoluble fraction of $NaIO_4$-

TABLE 3. *Decrease of periodate-induced transformation by reaction of periodate-treated cells with borohydride, hydroxylamine, or semicarbazide*

Treatment		[^{3}H]Thymidine incorporated (cpm) into cells treated with			
I	II	None	Con A	$NaIO_4$	$NaIO_4$ + Con A
PBS	PBS	1303	151900	183600	162800
PBS	KBH_4	1965	150500	215500	178000
PBS	NH_2OH	1766	266400	101200	194000
PBS	Semicarbazide	2218	225900	249900	224000
$NaIO_4$	PBS	172800	192400	130100*	—
$NaIO_4$	KBH_4	13480	135500	15130*	—
$NaIO_4$	NH_2OH	10820	255800	—	—
$NaIO_4$	Semicarbazide	119000	197400	—	—

Mouse spleen cells were treated with $NaIO_4$ (1 mM) as indicated. The cells were then washed and suspended (20×10^6/ml) in PBS containing potassium borohydride (1 mM), hydroxylamine (1 mM), or semicarbazide (5 mM), and incubated for 30 min at 23°. The cells were then washed and treated with $NaIO_4$ (1 mM) as indicated. Cells were suspended in culture medium, treated with Con A, and cultured under conditions oultined in Table 1.

* Cells were treated with $NaIO_4$ at a final concentration of 0.4 mM.

treated cells could be extracted with ethyl alcohol (95%); about 70% of this tritium was rendered acid (5%)-soluble on heating in 0.1 N H_2SO_4 for 60 min at 80°. The above data indicate that sialic acid residues are being oxidized upon treatment of spleen cells with $NaIO_4$ and that a fraction of the $NaIO_4$-target sites of the cell is located in a lipid-complex fraction. Recently, Blumenfeld *et al.* (19) have introduced tritium label into erythrocyte-membrane sialoglycoprotein on reduction of periodate-treated erythrocytes with [^{3}H]-KBH_4.

DISCUSSION

Our results suggest that the membrane site, modified on induction of lymphocyte transformation by periodate, includes a glycoprotein-complex containing sialic acid. The evidence for this assumption is based on the finding that the response of mouse lymphocytes to periodate is markedly reduced after previous treatment of the cells with neuraminidase or papain. Neuraminidase treatment did not reduce the response of $NaIO_4$-treated cells when applied *after* treatment with periodate. This is probably due to failure of the enzyme to hydrolyze the oxidized sialic acid derivative in the cell membrane.

We have previously shown (20) that treatment of lymphocytes with $NaIO_4$, at concentrations inducing maximal transformation, does not reduce the specific binding of [^{63}Ni]Con A to cells. It was thus suggested that Con A-binding sites and $NaIO_4$-target sites, essential for induction of blastogenesis, are different. The latter conclusion is substantiated by the following observations: (*a*) Blastogenesis of spleen lymphocytes induced by periodate is markedly reduced after treatment with neuraminidase or papain, whereas Con A-induced blastogenesis of the enzymically treated cells remains practically unimpaired. (*b*) Periodate-induced blastogenesis is practically eliminated when periodate-treated cells are reacted with borohydride or hydroxylamine. Such treatments do not affect blastogenesis of the cells induced by Con A. It is plausible to assume that $NaIO_4$-target sites and Con A-receptor sites, essential for induction of blastogenesis, are located on the same cells. This conclusion is supported by the observation that the effects of $NaIO_4$ and Con A on stimulation of [^{3}H]thymidine incorporation in mouse spleen cells are not additive. It is possible that the aldehyde moiety formed upon oxidation of sialic acid residues in the cell membrane is essential for induction of lymphocyte transformation by $NaIO_4$. Reagents that are known to react with aldehyde moieties have, therefore, reduced the transformation induced by periodate.

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