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DIRECT EVIDENCE FOR SIALIC ACID OXIDATION IN PERIODATE-INDUCED LYMPHOCYTE PROLIFERATION

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SUMMARY

Normal mouse spleen cells treated with periodate were stimulated to undergo blastogenesis. In contrast spleen cells from nude mice did not respond to periodate. Such a treatment of normal and nude spleen cells led to the oxidation of the cell surface sialic acid residues with formation of N-AN8. Prior treatment with neuraminidase of normal spleen cells greatly impaired their capacity to respond to periodate activation with a decrease in the amount of N-AN8 formed.

INTRODUCTION

Short-term periodate treatment of lymphocytes can induce blastogenesis similar to that caused by phytomitogens (1,2). It is commonly suggested that the mitogenic effect of periodate is due to the formation of aldehyde groups at the cell surface glycoproteins (1,3). Zatz et al (4) have speculated that the aldehyde groups are located on the carbon 7 of sialic acid residue after the removal of the two terminal carbons C9 and C8.

Moreover, Novogrodsky and Katchalski (1) have shown that the aldehydes formed can be reduced with tritiated borohydride and the radioactivity can be released by mild acid treatment. Sialic acid derivatives released from soluble glycoproteins can be identified by paper chromatography (5). In the present report, direct evidence is provided that the mitogenic effect of periodate on mouse spleen cells is concomittent with the formation of aldehyde groups on the lymphocyte membrane sialic acid residues.

MATERIALS AND METHODS

Animals - Inbred mice of DBA/2 and Balb/c strains 8 to 10 weeks old were obtained from Charles River, France. Athymic nu/nu (nude) mice, from outbred Swiss origin, 6 to 8 weeks of age were obtained from BD-Mérieux, France.

Chemicals - Sodium metaperiodate ($NaIO_4$) was from Merck, Germany. [^{3}H]Potassium borohydride (20 Ci/mmol) and [^{3}H]thymidine (1 Ci/mmol) were obtained from CEA, France. N-acetyl neuraminic acid (N-AN9) was a gift of Professor Montreuil.

Enzymes - Vibrio cholerae (VC) neuraminidase (500 units/ml) was obtained from Behringwerke,Germany. It was free of protease activity.

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Preparation and purification of N-AN9 derivatives = N-AN8 and N-AN7

The methyl ketosides of sialic analogues were synthetised as described by Yu and Ledeen (6), except that the molar ratio $NaIO_4$/N-AN9 was 1.5 and that the reduction step was achieved with tritiated borohydride (5). N-AN9 derivatives were released by acid treatment 0.1N H_2SO_4 at 80°C for 60 min. Tritiated N-AN8 and N-AN7 were separated by column chromatography on Dowex 1 x 8 (formiate, 200-400 mesh) resin. Elution was achieved with a linear formic acid gradient (0 to 1 N). Fractions corresponding to N-AN8 and N-AN7 were separated, concentrated and submitted to descending paper chromatography as described later, in order to control the ion exchange chromatography.

Periodate-borohydride modification of mouse spleen cells

Mouse spleen cells were teased apart in cold Hank's solution. Erythrocytes were lysed with an 0.83% aqueous solution of NH_4Cl. Cells were washed three times in cold phosphate-buffered saline (PBS) pH 7.2. They were then suspended in PBS at a concentration of 2×10^7 nucleated cells/ml and mixed with an equal volume of periodate in PBS for 10 min. at 0°C to give a final concentration of periodate ranging from 0.1×10^{-3} to 5×10^{-3}M. Control samples and samples to be incubated later with other mitogens were similarly treated for 10 min. at 0°C with PBS alone. Following this, if cells were not to be immediately treated with [^{3}H] borohydride, they were washed once in PBS and twice in culture medium. In studies where reduction followed oxidation cells were washed three times in PBS and resuspended at a concentration of 6×10^7 cells/ml in a freshly made [^{3}H] borohydride solution prepared as follows : 25 mCi K[^{3}H]$_4$ (20 Ci/mmol) were dissolved in 1 ml 0.1 N NaOH and then diluted to 20 ml in PBS. Cells were incubated for 30 min. at 23°C and then extensively washed in PBS.

Culture conditions

Cells to be cultured were suspended in Eagle's minimal essential medium containing 10% Foetal bovine serum (GIBCO), 100 units penicillin streptomycin/ml, 2×10^{-3}M glutamine, 10^{-3}M sodium pyruvate, and adjusted to a cell density of 2.5×10^6/ml. One ml cultures were prepared in triplicate in Falcon 3033 tissue culture tubes and incubated in an atmosphere of 10% CO_2, 7% O_2, 83% N_2 for 48 hr. In cultures stimulated with phytohemagglutinin (PHA), PHA-M (DIFCO) was used at a protein concentration of 30 µg/ml, when stimulated with lipopolysaccharides (LPS). E. coli 055 : B5 LPS (DIFCO) was used at 10 µg/ml. The cells were pulsed with 1 µCi of [^{3}H] thymidine during the last six hours. Its incorporation into DNA was determined on the trichloracetic acid (5%) insoluble fraction. Radioactivity was determined with an Intertechnique Multimat liquid scintillation spectrometer. Results are expressed as counts per minute (cpm).

Identification of tritiated sialic acid derivatives

Spleen cells labelled by periodate [^{3}H] borohydride were treated with VC neuraminidase (20 U/10^8 cells) in PBS for 30 min. at 37°C, or with 0.1N H_2SO_4 at 80°C for 60 min. Following this, the samples were centrifuged at 3000 rpm for 10 min, the pellets were removed and pH of acid supernatants was adjusted to 6. All samples were adjusted to 2.4 ml with distilled water and counted for radioactivity. N-AN9 standard, tritiated N-AN8 and N-AN7 prepared as described above, the labelled products from the lymphocyte samples and polyols (erythritol, propane - 1.2 diol, glycerol) were chromatographied on Whatman n°3 paper with N-butyl alcohol : pyridine : 1N HCl, 5 : 3: 2 (v/v) The N-AN9 standard was visualized by the thiobarbituric acid method (7) and polyols by the periodate benzidine reagent (8). The paper strips containing the radioactive samples were cut into 1cm pieces, placed into scintillation vials and the radioactivity determined. Control samples were treated identically except for the addition of periodate.

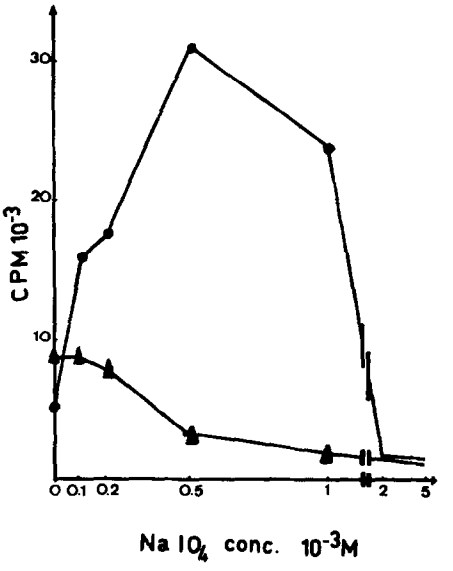

Fig.1 - Dose-response curve of [^{3}H] - thymidine incorporation in DBA/2 and nude mouse spleen cells after periodate treatment for 10 min. at 0°C. Cells were incubated for 48 hr and pulsed with 1 µCi of [^{3}H] - thymidine between 42 and 48 hr. (Mean of [^{3}H] - thymidine uptake of triplicate cultures, ●–●–● DBA/2, ▲–▲–▲ nude).

Prior treatment of cells with neuraminidase

In some experiments, spleen cells were incubated with VC neuraminidase before treatment. For the quantification of the sialic acid liberated by such a treatment, the method of Warren modified by Codington (9) was used.

RESULTS

Periodate induced lymphocyte proliferation. Splenic cells from DBA/2 and Balb/c mice exposed to periodate for 10 min. at 0°C, were stimulated to undergo blastogenesis and incorporate [^{3}H] -thymidine. Maximum proliferation was observed following treatment with 5 x 10^{-4}M $NaIO_4$. In contrast, spleen cells from nude mice did not respond to periodate oxidation over a wide concentration range from 10^{-4} to 5 x 10^{-3}M (Fig.1). Control samples from normal mice stimulated with PHA and LPS showed a strong proliferative response. Cells from nude mice showed no stimulation with PHA but a strong proliferation with LPS. Incubating periodate treated DBA/2 and Balb/c cells with both mitogens showed a definite increase in [^{3}H] - thymidine uptake. Since background values of periodates treated nude cells were lower it must be noted that such cells were equally stimulated by LPS (Table 1).

TABLE 1

BLASTOGENIC RESPONSE OF UNTREATED AND PERIODATE TREATED SPLEEN CELLS FROM NUDE AND NORMAL MOUSE SPLEEN CELLS TO MITOGENIC AGENTS (PHA and LPS)

Mitogen	[^{3}H] - thymidine incorporation		
	Nude	DBA/2	Balb/C
	Control (PBS, 10 min)		
None	8,755	5,030	2,283
PHA	6,150	40,507	39,841
LPS	57,335	65,819	-
	$NaIO_4$ treated ($5x10^{-4}$M, 10 min)		
None	3,070	32,094	23,754
PHA	3,340	88,942	110,032
LPS	23,685	110,537	-

Spleen cells were incubated with PBS alone or with periodate ($5x10^{-4}$M) for 10 min at 0°C, washed and cultured for 48 hr as described in methods. Cultures were pulsed with 1 μCi [^{3}H] - thymidine between 42 and 48 hr. Results are expressed as CPM (mean of three experiments).

Identification of products liberated by mild acid hydrolysis of periodate-[^{3}H]- borohydride modified spleen cells

The effect of incubation of DBA/2 spleen cells for 10 min. at 0°C with periodate, at different concentrations, is shown in Fig. 2. Radioactivity was located in the migration area of N-AN9 derivatives. With 10^{-4}M and 2×10^{-4}M concentrations, only N-AN8 was identified (Fig 2a). With higher concentrations (from 5×10^{-4}M to 5×10^{-3}M), two radioactive peaks were detected as N-AN8 and N-AN7. Few amounts of N-AN7 were released. In contrast CPM values located in the N-AN8 peak increased with increasing $NaIO_4$ concentrations. Furthermore, no radioactivity was detected in the areas corresponding to typical degradation products of other carbohydrate components such as glycerol, erythritol and propane 1-2 diol (5). The same set of experiments was carried out with Balb/c and nude spleen cells. Similar results were observed with Balb/c spleen cells. N-AN8 was always the major N-AN9 analogue released from chemically modified cells. However, when comparing CPM values obtained with 5×10^{-4}M periodate oxidation of DBA/2 and nude

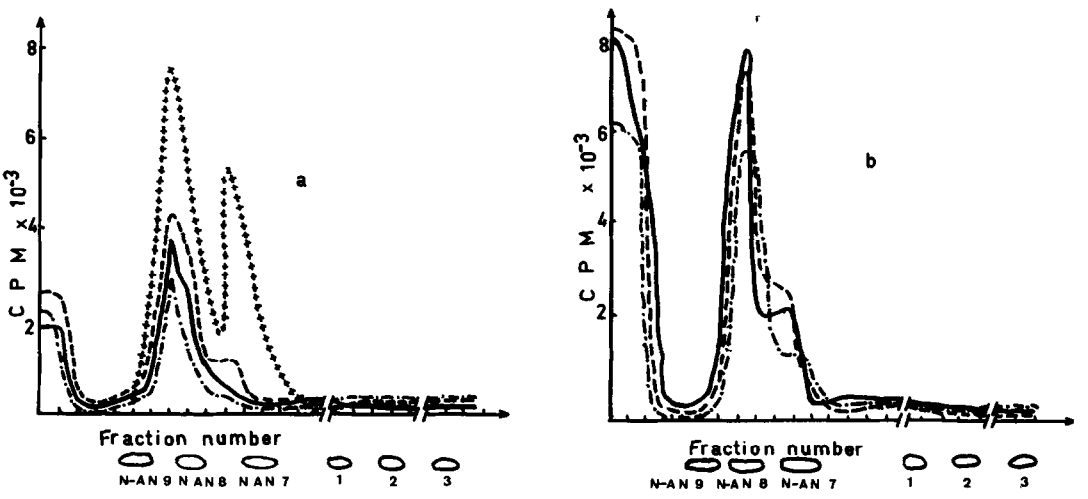

Fig.2 - Paper chromatography of acidic hydrolysis products of DBA/2 spleen cells treated as outlined in methods. Each point is a mean of two experiments. a xxx : N-AN8 and N-AN7,— · — · 10^{-4}M, ——— 2 x 10^{-4}M, --- 5 x 10^{-4}M . b— — 10^{-3}M,——— 2 x 10^{-3}M ---- 5 x 10^{-3}. No CPM values were detected on control chromatogram (acid hydrolysis of cells treated by [^{3}H] borohydride only).

spleen cells, nude maximum CPM values were much lower and showed no further increase when using higher periodate concentrations (Fig. 3).

Neuraminidase hydrolysis of normal and periodate treated spleen cells

Balb/c spleen cells were incubated at a final concentration of 10^{8}/ml with VC neuraminidase (20 U/10^{8} cells) for 30 min. at 37°C. The release of sialic acid from normal cells was achieved within 30 min. Under our conditions, 3.6 µg per normal 10^{8} cells were liberated (Codington method).

In contrast using periodate treated spleen cells, we could not measure any sialic acid in the supernatants obtained from neuraminidase hydrolysis. This could be due either to a lack of the sensibility of the method or to the absence of sialic acids. So the radioactivity was evaluated : negligible CPM values were released by neuraminidase treatment (Table 2). The data suggest that periodate oxidized sialic acid, forming N-AN8 and N-AN7 which are not significantly released by VC neuraminidase as proved by Suttajit and Winzler (10).

Effect of prior treatment with neuraminidase on the mitogenic effect of periodate

The data presented in table 3 show that pretreatment of cells by VC neuraminidase markedly reduced the proliferative action of periodate.

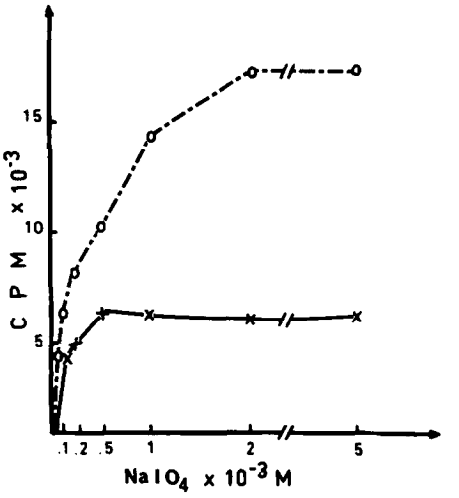

Fig. 3 - Comparison of CPM values obtained from DBA/2 and nude spleen cells treated as outlined in methods. CPM values represent radioactivity in the migration area of N-AN8 and N-AN7. Each point is a mean of two experiments (O—·—·—O DBA/2, —x—x— nude)

Table 2

COMPARISON OF CPM VALUES DETECTED IN THE MIGRATION AREA OF N-AN 8 AND N-AN 7 OF AN ENZYMATIC AND THEN ACIDIC HYDROLYSIS OF $5x10^{-4}$M $NAIO_4$ [^{3}H] BOROHYDRIDE Balb/c SPLEEN CELLS

	Time	Neuraminidase treatment	Subsequent acidic hydrolysis	Direct acidic hydrolysis
CPM	15 min	280	5,420	6,600
	30 min	320	5,865	

Spleen cells were incubated with periodate and [^{3}H] borohydride, and then sequentially treated with neuraminidase and H_2SO_4 0.1 N at 80°C for 60 min., or directly hydrolysed by H_2SO_4. Each CPM value is a mean of two experiments.

Viability determined by the dye-exclusion test with Trypan blue showed no cytotoxic effect of the combined treatment with VC neuraminidase and periodate. Moreover mouse spleen cells incubated with VC neuraminidase and then treated with periodate responded well to PHA stimulation.

Table 3

RESPONSE OF NORMAL MOUSE SPLEEN CELLS TO PERIODATE AND PHA AFTER TREATMENT BY NEURAMINIDASE

Treatment	[^{3}H] - thymidine uptake of cells treated with			
	None	PHA	$NaIO_4$	$NaIO_4$ + PHA
PBS	2,375	30,208	21,664	91,170
Neuraminidase	3,291	30,972	7,278	30,495

Mouse spleen cells were suspended (10^8/ml) in PBS alone or in PBS containing neuraminidase (20 U/ml) and incubated for 30 min., at 37°C. Following this, the cells were washed, aliquots were incubated with periodate and then cultured as described in methods. Results are expressed as CPM (mean of three experiments).

Effect of prior treatment with neuraminidase on the chemical modifications induced by periodate and borohydride treatment

The results obtained from periodate - [^{3}H] borohydride treated Balb/c spleen cells with or without preincubation with VC neuraminidase are illustrated in table 4. Treatment with VC neuraminidase before periodate was followed by a pronounced decrease of CPM values. When comparing migration areas of N-AN9 derivatives, neuraminidase treated cells showed 38% of the CPM values of the untreated cells, when comparing acid supernatants (that is to say total radioactivity) the percentage was the same (table 4). Furthermore, no radioactivity was detected in another area of paper chromatogram. These data suggest that neuraminidase susceptible and unsusceptible sialic acids are oxidized by periodate. An enzymatic removal of a portion of the sialic acid residues is accompanied by a decrease of formation of periodate induced aldehyde groups and of labeling by [^{3}H] borohydride.

DISCUSSION

Treatment of normal mouse spleen cells with various concentrations of periodate causes an enhancement of lymphocyte proliferation, while the spleen cells from the congenitally athymic nude mice do not respond to periodate stimulation. These data lend further support for the convincing evidence that periodate is a specific T cell mitogen (11, 12).

The mitogenic effect of periodate is related with the formation of aldehyde groups on cell surface glycoproteins. But the exact mechanism of

Table 4

EFFECT OF NEURAMINIDASE ON THE LABELLING OF Balb/c SPLEEN CELLS SEQUENTIALLY TREATED BY PERIODATE AND [^{3}H] BOROHYDRIDE

	N-AN 8 and N-AN 7 expressed as CPM		Acid supernatant	
Treatment	none	$NaIO_4$	none	$NaIO_4$
PBS	0	2,668	20,090	131,940
neuraminidase	0	1,028	20,100	49,389

Balb/c spleen cells are incubated (10^8/ml) in PBS alone or in PBS containing neuraminidase (20U/ml) for 30 min. at 37°C and then treated by 5×10^{-4}M periodate .[^{3}H] borohydride as described in methods. Each value is a mean of two experiments.

this oxydative activation is not yet known. Hughes (13) postulated that it could be likened to the cross-linking effect of multivalent ligands : the reactive aldehyde groups could form either Schiff bases with free amino groups or acetal bonds with other hydroxyl groups on adjacent glycoproteins. It is plausible to assume that, at low concentrations of oxidant, the most susceptible residues are the acyclic C7-C9 positions of sialic acids. We directly prove that sialic acid residues are involved in the mitogenic effect. Indeed when DBA/2 spleen cells are treated with periodate, we show that aldehyde groups are formed principally on the carbon 8 and at a lower extent on the carbon 7 of sialic acid residues. This phenomenom is accompanied by the proliferation of spleen cells and an increase of [^{3}H] thymidine incorporation. It should be noted that, in these data, the assumption has been made that periodate oxidizes cell surface components only. In fact, it has been shown recently that oxidation at 0°C with low concentrations of periodate during a short reacting time is rationale for specific labeling of cell surface sialic acids by periodate .[^{3}H] borohydride (14). It can be seen that, at 5×10^{-4}M periodate concentration, the mitogenic effect is maximal whereas complete oxidation of sialic acid residues is not achieved. We assume that a sufficient number of oxidized groups is generated to induce an optimal proliferation and with higher periodate concentrations, the increase of aldehyde groups leads to abortive interactions with a concomittent decrease of [^{3}H] thymidine incorporation. Normal and nude mouse lymphocytes seem to be similarly affected at the cell surface by periodate oxidation since sialic acid derivatives are released from normal and nude mice, but

the difference in their responses could indicate a disparity in the biological means of blastogenic induction between T and B cells. The absence of response of B cells to periodate could be due either to a lack of a particular set of glycoproteins bearing the sialic acid residues involved in the lymphocyte activation or to a defavorable spatial configuration of the oxidized oligosaccharidic receptor. More interesting are the results obtained with the sequential treatment of cells with neuraminidase and periodate : the mitogenic effect is nearly abolished confirming the results previously reported by Novogrodsky and Katchalsky (1). Besides, we show that simultaneously, there is an obvious decrease of the aldehyde groups generated at the cell surface. But the question is to know whether sialic acids only are oxidized, or are other aldehyde groups formed by oxidation of internal sugar residues on the glycoproteins? In fact, oxidation and cleavage of such components are minimal as it is proved by the absence of polyols after periodate -[^{3}H] borohydride treatment. So, periodate oxidation does not generate aldehyde groups on internal sugar residues susceptible to trigger mitogenesis. This is comforted by the results indicated in Table 4. When comparing periodate -[^{3}H] borohydride cells with and without prior neuraminidase treatment, CPM values decreases are very similar in acid supernatants and in N-AN9 derivatives migration areas. So even when a part of sialic acids is removed by neuraminidase, periodate[^{3}H] borohydride treatment did not alter any other carbohydrate components.

The results in this study indicate that periodate induced mitogenesis is related with the generation of aldehyde groups on sialic acid derivatives essentially N-AN8 and at a lower extent N-AN7.

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