A CHEMICAL APPROACH FOR THE LOCALIZATION OF MEMBRANE SITES INVOLVED IN LYMPHOCYTE ACTIVATION

David Wynne, Meir Wilchek and Abraham Novogrodsky

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

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SUMMARY: The aldehyde groups formed on periodate oxidation of cell surface sialyl residues were used to insert a mitogenic site onto the lymphocyte membrane by attachment of biotin hydrazide or 2,4-dinitrophenyl hydrazine. The biotin- or 2,4-dinitrophenyl-conjugated cells were both agglutinated and stimulated when cultured with avidin or anti-2,4-dinitrophenyl antibody respectively. On the other hand, biotin or DNP-conjugated cells, modified via functional groups on the membrane proteins, were agglutinated but not stimulated when cultured with avidin or anti-DNP antibody respectively. Our results show that the specific interaction of a protein at the periodate oxidation site leads to blastogenesis.

INTRODUCTION

Lymphocytes are triggered to grow and divide upon interaction with a variety of agents including the phytomitogens and anti-immunoglobulins (1,2) and simple chemicals, such as sodium periodate (3,4). The explicit mechanism of the triggering process is largely unknown. However, it is generally considered that the triggering signal is confined to the cell membrane. It is important in understanding the mechanism of lymphocyte activation, to ascertain whether different mitogens trigger the cells by affecting different membrane sites, or whether the triggering signal is localized at a single, unique site.

We have used a chemical method for mapping the triggering sites on the lymphocyte membrane. Reagents were used to attach various ligands onto the cell via different functional groups on the membrane. It was expected that interaction of the anti-ligand with ligands conjugated to the lymphocyte triggering site(s) would stimulate the cells to undergo blastogenesis. Our results strongly suggest that the lymphocyte activation site is unique.

Abbreviations: Con A, concanavalin A; PBS, phosphate buffered saline, pH 7.2; Biotin-conjugated cells, cells sequentially treated with periodate, biotin hydrazide and borohydride; DNP, 2,4-dinitrophenyl; TNBS, 2,4,6-trinitrobenzene sulphonic acid; MNP, m-nitrophenyl; anti-DNP Ig, anti-DNP immunoglobulin.

MATERIALS AND METHODS

Concanavalin A (Con A) twice crystallized, was purchased from Miles-Yeda Ltd. [Methyl-^{3}H] thymidine (5 Ci/mmole) was obtained from the Nuclear Research Centre, Negev, Israel. Sodium metaperiodate was Merck proanalysis grade. Avidin (9.6 units/mg), d-biotin and 3,5-difluoro-2,4-dinitrobenzene were purchased from Sigma. 2,4,6-Trinitrobenzene sulphonic acid was purchased from Pierce.

Biotin N-hydroxysuccinimide ester was a gift from E. Bayer, or prepared as described previously (5). Biotin hydrazide was synthesized according to the method of Heitzmann and Richards (6). 2,4-Dinitro-N,N-di(2-chloroethyl)aniline (DNP-nitrogen mustard) was synthesized by the method of Ross (7). p-Arsanilic acid and m-nitroaniline were diazotized as described by Kagan and Vallee (8). N-DNP-ε-aminocaproic acid and N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide. Antisera, purified Ig and Ig fractions and Myeloma protein MOPC 315 were kindly provided by D. Givol.

Spleen Cells. Mice(CBA/LAC or Balb/c) aged 6-12 weeks, or rats (inbred Wistar), 200 g weight, were used in this study. The isolation and culture of the cells was carried out as described previously (4,9). Mouse spleen cells were cultured in Dulbecco modified Eagle's medium containing 5% fetal calf serum, heat inactivated at 56° for 30 min. Rat spleen cells were cultured in medium containing 10% horse serum.

[Methyl-^{3}H]-Thymidine Incorporation into DNA. Thymidine incorporation was assayed after 48 hr as described previously (4,9), unless otherwise specified Results are expressed as the mean value of triplicate cultures ± standard error.

Reaction of Spleen Cells with Different Reagents. Spleen cells (20 x 10^6/ml), in phosphate buffered saline pH 7.4 (PBS) were treated with the reagent (usually 10^{-4} M) for 30 min at room temperature. In some cases DMSO (1%) was added to aid solubility of the reagent, but did not effect cell viability. After treatment the cells were centrifuged, washed with PBS and resuspended in culture medium.

Treatment of spleen cells with 2,4,6-trinitrobenzene sulphonic acid (TNBS) was carried out as above, over a concentration range between 10^{-3} M and 10^{-7} M. In some cases TNBS treatment was also carried out in borate buffered saline, pH 9 .

Treatment of Cells with Diazotized Compounds. Cells (20 x 10^6/ml) were suspended in ice cold borate buffered saline and the freshly prepared diazotized compound added to a final concentration of 10^{-4} M. After 30 min reaction in ice the cells were centrifuged, washed once with PBS and resuspended in culture medium.

Treatment of Spleen Cells with Periodate. Cells (70 x 10^6 ml), suspended in PBS, were treated with periodate (1 mM) for 30 min in ice, centrifuged and washed with PBS. After recounting, the cells were resuspended in culture medium or in PBS for further reaction.

Reaction of Periodate Treated Cells with other Reagents. Periodate treated cells (20 x 10^6/ml) suspended in PBS, were treated with biotin hydrazide (1 mM), DNP-hydrazine (10^{-4} M) or KBH_4 (2 mM) for 30 min at room temperature. The cells were then centrifuged, washed with PBS and resuspended in culture medium. Cells treated sequentially with periodate, biotin hydrazide and borohydride were designated biotin-conjugated cells.

Preparation of ^{125}I-Labelled Avidin. ^{125}I-Labelled avidin was prepared by the chloramine-T method (10). Radioactivity of the avidin solution was determined in a

Packard Gamma counter. Protein concentration was determined from the absorbance at 282 nm (11). Biotin binding was determined by the 4-hydroxyazo-benzene-2'-carboxylic acid displacement method of Green (12). The labelled avidin obtained had a specific activity of 18500 cpm/μg of active avidin.

Binding of ^{125}I-Labelled Avidin to Biotin-Conjugated Spleen Cells. Cells (5×10^6/ml), suspended in PBS, were shaken with ^{125}I-labelled avidin (30 μg/ml) for 30 min at room temperature. An aliquot (200 μl) of the cell suspension was then layered onto 100 μl of an oil consisting of a 1:1 (v/v) mixture of dibutylphthalate and dioctylphthalate in plastic Beckman microtubes and centrifuged for 15 sec in the Beckman microfuge. The tubes were then cut above the cell pellet and the pellet counted in a Packard Gamma counter. The amount of ^{125}I-labelled avidin specifically bound was calculated by subtracting the amount bound in the presence of biotin (10 μg/ml) from that bound in its absence.

RESULTS

In order to study the location of the lymphocyte activation site, reagents listed in Table 1, Group B, were attached to different functional groups of lymphocyte membrane proteins. Under the coupling conditions used, less than 10% of the cells were killed as estimated by the trypan blue exclusion test (13), and by their ability to respond to Con A.

The cells modified with these ligands (Table 1) interacted with the anti-ligand, since they were agglutinated in the presence of the anti-ligand. Furthermore, DNP-conjugated cells were lysed in the presence of anti-DNP antibody and complement. Similarly to anti-DNP Ig, the myeloma protein MOPC 315 also agglutinated DNP-conjugated cells. However, in spite of the demonstrable interaction of the anti-ligand with the modified cells, such cells were not stimulated by the anti-ligand (Table 1, Group B).

Avidin Induced Stimulation of Biotin-Conjugated Cells. Previous studies have shown that periodate oxidation of cell surface sialyl residues (3,4) introduces an aldehyde group onto the membrane, leading to blastogenesis. The blastogenesis can be inhibited by aldehyde reagents. These aldehyde groups can also be utilized to introduce a ligand onto the cell surface. Thus periodate treated cells were reacted with biotin hydrazide or DNP-hydrazine to introduce the biotin or DNP moieties, respectively.

Rat spleen cells were sequentially treated with periodate, biotin hydrazide and borohydride (biotin-conjugated cells) as outlined in Materials and Methods. Avidin markedly stimulated such cells, the stimulation being specific for the attached biotin moiety since a mixture of biotin and avidin did not stimulate the modified cells (Table 2). Avidin had no effect on control cells.

TABLE 1. Specific interaction of proteins with lymphocytes modified by conjugation of chemical groups onto the cell surface

Reagents	Functional group on cell	Chemical group introduced	Specific binding protein	Stimulation
Group A				
Biotin hydrazide	Aldehyde ($NaIO_4$ generated)	Biotin	Avidin	+
DNP-hydrazine		DNP	Anti-DNP Ig	
Group B				
Trinitrobenzene sulphonic acid	ϵ-NH_2	TNP	Anti-DNP Ig[b]	-
N-DNP-ϵ-aminocaproyl N-hydroxysuccinimide ester		DNP		
α-N-Bromoacetyl-ϵ-N-DNP-lysine[a]				
1,3-difluoro-2,4-dinitrobenzene				
2,4-dinitro-N,N-di(2-chloroethyl)aniline (N-DNP-nitrogen mustard)				
Diazotized m-nitroaniline	Tyrosine	MNP		
Diazotized p-arsanilic acid		Arsanilic acid	Anti-arsanilic acid Ig	
Biotin N-hydroxysuccinimide ester	ϵ-NH_2	Biotin	Avidin	

[a] Also reacts with -SH groups; [b] TNP and MNP also interact with anti-DNP Ig.

TABLE 2. Avidin induced DNA synthesis into Biotin conjugated spleen cells

Source of spleen cells	Cells treated sequentially with IO_4^-	BioH[a]	KBH_4	[^{3}H] Thymidine incorporation (cpm ± s.e.) into cells cultured with -	Avidin[b]	< p[c]	Avidin-biotin mix[d]	Con A (2 μg/ml)
Rat	-	-	-	3410 ± 410	5040 ± 180		-	40380 ± 4470
	-	-	+	2850 ± 250	3540 ± 390		-	32010 ± 310
	+	-	-	182010 ± 16060	174830 ± 21420		-	191990 ± 8580
	+	-	+	10780 ± 990	9960 ± 550		-	68820 ± 2560
	+	+	-	19240 ± 810	26170 ± 2610			60220 ± 4350
	+	+	+	21550 ± 1160	92020 ± 1880	0.01	19330 ± 2940	67560 ± 5740
Mouse	-	-	-	2960 ± 480	1750 ± 250			83200 ± 17850
	-	-	+	2880 ± 610	2500 ± 390			74050 ± 10630
	-	+	+	2860 ± 260	2450 ± 330			56920 ± 8580
	+	-	-	43110 ± 1330	25260 ± 2550			92960 ± 4470
	+	+	-	19070 ± 2460	20440 ± 2260			47840 ± 2020
	+	+	+	2440 ± 290	7210 ± 1280	0.05	1250 ± 130	60560 ± 6420

[a] Biotin hydrazide; [b] 20 μg/ml rat cells; 45 μg/ml mouse cells ; [c] p, level of significance of observed differences from controls; [d] avidin premixed with biotin (10 μg/ml).

Similarly to rat spleen cells, biotin-conjugated mouse spleen cells were also stimulated by avidin (Table 2). In this case, however, the avidin response was lower than that in the rat but was statistically significant and has been confirmed in at least 15 experiments. In an attempt to improve the response of biotin-conjugated mouse spleen cells to avidin, the modification with biotin hydrazide was carried out at lower pH. However, to offset the increased reagent toxicity at that pH, the reaction had to be carried out at lower concentration. Thus biotin-conjugated mouse spleen cells prepared at pH 6.5 with 0.4 mM biotin hydrazide gave a similar avidin response as normally prepared biotin-conjugated cells.

It is important to determine whether the avidin stimulation of biotin-conjugated cells is due to specific interaction of avidin with the biotin attached to the cell surface, or perhaps due to avidin-induced re-exposure of aldehyde groups during culture. It has been shown that cysteine can inhibit periodate-induced stimulation of spleen cells (14), possibly by the formation of a thiazolidine derivative with aldehyde groups (15). Fig. 1 shows that there is little or no effect of cysteine on the avidin stimulation of biotin-conjugated cells, or on the Con A stimulation of untreated cells. However, the

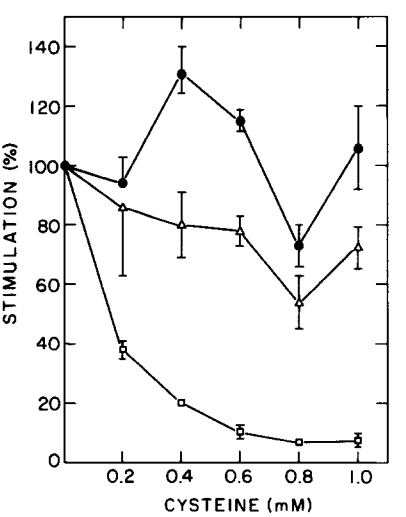

Fig. 1. Effect of cysteine on the induction of DNA synthesis by different agents. Mouse spleen cells were treated with various reagents, as outlined in Materials and Methods and cultured, as indicated, in the presence of different concentrations of cysteine. (o - o), untreated cells cultured with Con A (2 μg/ml). (Δ - Δ), biotin-conjugated cells, cultured with avidin (45 μg/ml). (□ - □), periodate treated cells. 100% Stimulation was 102500 ± 6500 cpm, 12250 ± 1750 cpm and 32250 ± 2750 cpm, respectively.

inhibitory effect of cysteine on periodate-induced stimulation is quite marked, even at low concentrations.

Fig. 2 shows the effect of avidin at different concentrations on the stimulation of biotin-conjugated rat spleen cells. Avidin stimulated such cells maximally at concentrations of 3-5 μg/ml. Concentrations of avidin up to 45 μg/ml did not lower this maximum (data not shown). A similar pattern of avidin stimulation of biotin-conjugated mouse spleen cells was also observed.

Specific binding of avidin to mouse spleen cells was found only with cells treated sequentially with periodate and biotin hydrazide and was found to be 1 - 1.5 μg ^{125}I-avidin/10^6 cells. Cells treated sequentially with periodate, biotin hydrazide and borohydride specifically bound avidin to the same extent.

DISCUSSION

In this study we have attempted to map the lymphocyte activation site by chemical techniques. Ligands were attached to the cell surface membrane at different sites and the mitogenicity of the appropriate anti-ligand studied. The DNP group was attached to amino groups, sulfhydryl groups or tyrosyl residues on cell surface proteins. Interaction of DNP-conjugated cells with the antibody was demonstrated by agglutination of the modified cells with anti-DNP antibody and by lysis of the modified cells in the presence of antibody and complement. Despite observed agglutination of such cells with antibody, they were not stimulated. No difference was observed by the introduction of the DNP group directly onto the membrane with TNBS or via a spacer using N-DNP-ε-aminocaproyl N-hydroxysuccinimide ester. Furthermore, although TNBS modifications were carried out over a wide range of pH and reagent concentrations, the modified cells were not stimulated by anti-DNP antibody. Likewise, modification of surface proteins with other ligands (arsanilic acid, biotin) caused agglutination by the appropriate anti-ligand, but failed to stimulate the cells.

Conversely, modifications of cell surface carbohydrates resulted in marked stimulation upon interaction with the appropriate anti-ligand. Cell surface carbohydrate residues have previously been shown to be important in the interaction of many mitogens with the cell surface (16, 17) and may well place a key role in the activation of lymphocytes. Unfortunately, they are not readily available to simple chemical reactions, that will not affect cell viability. Nevertheless, we have utilized the aldehyde group, formed after mild periodate oxidation of cell surface sialyl residues, to attach various ligands onto the cell membrane.

Cells treated sequentially with periodate, biotin hydrazide and borohydride

(biotin-conjugated cells) were stimulated with avidin. Unlike lectin-induced lymphocyte stimulation, in which an optimal concentration of the given lectin stimulates maximally, avidin-induced stimulation reached a plateau and did not decline upon increasing concentrations of avidin up to 45 μg/ml. Furthermore, binding studies showed that only cells sequentially treated with periodate and biotin hydrazide (with or without borohydride), specifically bound ^{125}I-labelled avidin. The avidin stimulation of biotin-conjugated cells was not due to re-exposure of the periodate generated aldehyde groups since stimulation was still observed in the presence of concentrations of cysteine that markedly inhibited the periodate response (Fig. 1).

It is of interest to note that cells treated sequentially with periodate and biotin hydrazide respond poorly to avidin, whereas further treatment with borohydride strongly potentiated the avidin response. The explanation for this observation is not

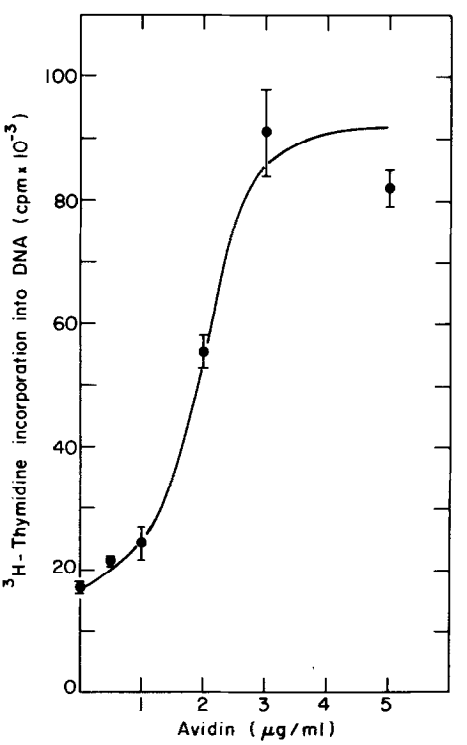

Fig. 2. Effect of avidin concentration on the induction of DNA synthesis in biotin-conjugated cells. Biotin-conjugated rat spleen cells were prepared as outlined in Materials and Methods, and cultured with various amounts of avidin. Thymidine incorporation into biotin-conjugated cells cultured with avidin (5 μg/ml) which was premixed with biotin (10 μg/ml) was 21600 ± 4000 cpm.

known, although two possibilities may be considered: borohydride reduces the hydrazone formed on reaction of biotin hydrazide and the aldehyde group, or reduces free aldehyde groups that did not react initially with biotin hydrazide. The avidin response of cells treated sequentially with periodate and biotin hydrazide was also potentiated by further treatment with cysteine (data not shown). In this case, cysteine acts only as an aldehyde reagent. It is therefore likely that borohydride also potentiates the avidin response of biotin-conjugated cells by reacting with the free aldehyde groups on the cell.

The experimental results obtained with rat spleen cells outlined above, have also been verified in the mouse. Preliminary results have also shown that rat spleen cells, sequentially treated with periodate, DNP-hydrazine and borohydride, could be stimulated by anti-DNP Ig. In addition, cells treated with neuraminidase, and galactose oxidase followed by biotin hydrazide, were also stimulated by avidin.

We have shown in this study that only ligands conjugated to cells via surface carbohydrate residues were transformed by the anti-ligand. To our knowledge this report is the first demonstration of the insertion of a mitogenic site onto the lymphocyte membrane.

On a more general basis, it can be seen that the lymphocyte will be triggered by chemical modification only if the chemical group introduced is attached in the proper position. Attachment of the ligand to other parts of the cell surface, leads to an abortive interaction of the ligand binding protein, which includes cell agglutination and perhaps perturbation of the cell membrane but does not result in stimulation. On the other hand, it has been proposed that the enzymes neuraminidase, galactose oxidase, the lectin soybean agglutinin and the chemical agent periodate, all act in the same general site, leading to blastogenesis (18). Furthermore, it has been suggested that phytomitogens may bind to the cell surface via a common receptor (19). The results in this study strongly substantiate this hypothesis and indicate that only a specific, cell surface interaction at a particular "triggering" site will lead to stimulation of the cell.

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