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STERIC FACTORS IN LYMPHOCYTE STIMULATION BY ORGANOMERCURIALS

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SUMMARY

Resting human lymphocytes were stimulated to initiate DNA synthesis by divalent mercury ions or by the divalent organomercurial, 1,4-bismercury-3, 4-dihydroxybutane. Monovalent methylmercury was ineffective, as was mercury-substituted dextran which is a polyvalent compound where the mercury atoms are farther apart than in the divalent butane derivative. These findings suggest that for organomercurials to stimulate lymphocyte DNA synthesis, they must crosslink protein sulfhydryl groups and bring these groups into close proximity.

INTRODUCTION

The elements of the cell surface have considerable lateral mobility (1-4) and their aggregation seems to be a critical step in the response of cells to external signals (5-7). Aggregation of surface receptors occurs in such diverse processes as lymphocyte stimulation (8-11), mast cell degranulation (12,13) and insulin binding (14). The steric aspects of the binding and aggregation phenomena are not clearly defined. This is in part due to the fact that the reagents used to investigate these phenomena are largely confined to antibodies, peptide hormones, and plant lectins. The information that can be obtained with these probes is restricted by both their preparative difficulties and the complexity of their structures. We have previously shown that the introduction of organic spacer groups between two metal ions could be used to design well defined chemical models for the kind of selectivity required by biochemical processes (15). Since inorganic mercury ions can stimulate resting lymphocytes to initiate DNA synthesis (16,17) and since the synthesis of

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organomercurials is straightforward, we undertook the development of a series of organomercurials which could stimulate lymphocyte mitogenesis.

MATERIALS AND METHODS

Preparation of Mercurials. Compound 1 (Fig. 1) was prepared by adding a suspension of methylmercury chloride (500 mg) in 30 ml of ethanol to a solution of cysteine (242 mg) in ethanol-water (10 ml). A homogeneous solution formed, which upon standing started to separate crystals of compound 1. Compound 1 decomposes slowly above 170°C.

Compound 2 was prepared by saturation of a solution of mercuric acetate (10 g) in water (70 ml) by butadiene at room temperature for 30 min., after which time the test for Hg^{2+} was negative. Crystals separated from this solution after it was maintained at 4°C overnight. For the diacetate corresponding to compound 2 ($C_8H_{14}O_6Hg_2$), the calculated mercury content is 66.06%; the obtained crystals had 65.89% mercury. The diacetate form was converted into the cysteine form as follows. Part of the crystals (427 mg) were dissolved in 0.3 M NaOH (20 ml) and treated with a solution of cysteine (90 mg) in water. When the resulting homogeneous solution was neutralized with acetic acid (1 M), the less soluble compound 2 separated. Extraction of the precipitate with hot water (30 ml) and subsequent cooling yielded 180 mg of compound 2. This compound decomposes slowly above 155°C. Purity of compound 2 was checked by thin layer chromatography on silica gel G using a mixture of isopropanol (2 vol), triethylamine (1 vol), and water (1 vol) as an eluent. Mercury derivatives were detected by spraying the plate with a solution of sodium borohydride in 0.1 M NaOH; the associated reduction led to the formation of black spots of elementary mercury. In this system the cysteine complex of mercury had rf 0.4 and compound 2 had rf 0.0.

Compounds 3a and 3b were prepared from dextran of average molecular weight 170,000 and purified as previously described (18).

Lymphocyte Response to Mercurials. Normal human peripheral blood lymphocytes were prepared by isopycnic centrifugation on Ficoll-Hypaque gradients and cultured at 2 x 10^6 cells/2 ml in MEM alpha, 10% autologous serum and 25 mM HEPES pH 7.2 at 37°C in the presence of the compounds indicated in the table. DNA synthesis was measured on the third day of culture by a four hour pulse with 1 μCi [^{3}H] d thymidine/2 ml culture. Incorporation of radioactivity into trichloroacetic acid precipitates was determined as previously described (17).

RESULTS

The chemical structures of the organomercurials used in this study are shown in Fig. 1. Human lymphocytes in tissue culture were exposed for 3 days to either inorganic mercury, an organomericurial, phytohemmaglutinin, or different combinations of the lectin plus a mercurial. DNA synthesis was measured on the 3rd day of culture using a 4 hour pulse with

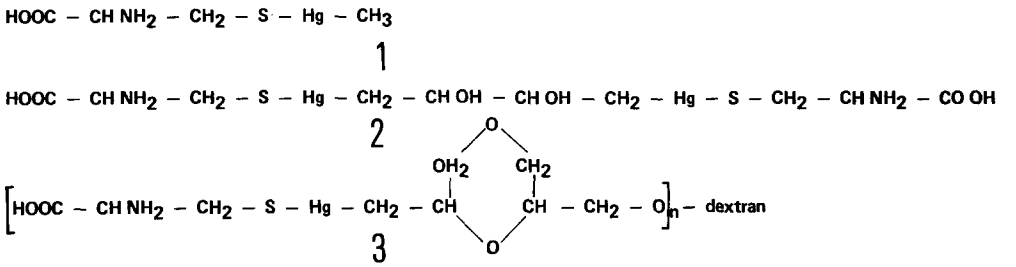

Figure 1.

[^{3}H] thymidine. After 3 days in culture, the lymphocytes from all donors showed an increase in DNA synthesis in response to PHA stimulation. At a concentration of 10 μM, mercuric chloride stimulated a 50-fold increase in DNA synthesis (Table I). Higher concentrations inhibited the spontaneous level of DNA synthesis in these cells.

Compound 1, in which mercury is substituted by a methyl group showed no ability to stimulate lymphocytes; only the general toxic effects of mercury were seen as manifested by a decrease in thymidine incorporation to values lower than those demonstrated by untreated cells. The addition of compound 1 to PHA-stimulated lymphocytes also inhibited the mitogenic response of those cells (Table I).

Compound 2, an organomercurial derived from butane, contains two mercury atoms per molecule. It was an effective stimulator of DNA synthesis with maximum stimulation occurring in the same concentration range as was found for inorganic mercury ions (10 μM). When lymphocytes were stimulated by PHA in the presence of compound 2, the amount of DNA synthesis was always less than after stimulation by PHA alone. Thus only the toxicity of organomercurial 2 was manifested in this combination.

Compounds 3a and b are organomercurials derived from a polysaccharide, dextran, of average molecular weight 170,000, i.e. containing about 1,000 glucose units (14). Compound 3a contained approximately 800 mercury atoms per average polysaccharide molecule. Compound 3b contained about 30 mercury atoms per molecule. Both compounds were ineffective as stimulators.

TABLE I

Lymphocyte Stimulation Index†

Stimulators	$HgCl_2$	Compound 1*	Compound 1 + PHA‡	Compound 2	Compound 2 + PHA	Compound 3a	Compound 3a + PHA	Compound 3b	Compound 3b + PHA
Conc. Hg µgatoms/ml									
100	.09	.031	.028	.24	.17	.012	.37		
50	.25	.031	.038	12	35.5	.009	.66		
10	53	.036	.032	122.7	321	.025	2.4		
5	20.4	.034		89.4	439	.99	16.8		
1	3.49	.031	23.6	30.8	528	1.05	18	.82	20.17
0.5		.45	38.2			1.07	28.6	1.3	32.7
0.1		1.07						1.5	34.5
.05		1.03						1.38	31.6
.025								1.17	32.6
PHA Alone‡			33.0		592		21.0		33.7

† Lymphocyte Stimulation Index = (DPM[^{3}H]dThd Incorporated by 2 x 10^6 treated cells/DPM[^{3}H]dThd incorporated by 2 x 10^6 control cells) Values < 1 = Inhibition, 1 = No effect, > 1 = Stimulation.

‡ PHA used at 1.7 µg/ml

*Formulas of compounds in Figure 1

Only the toxic effects could be demonstrated when these mercury compounds were used alone or in combination with PHA (Table I). This is in agreement with a previous observation that mercury-containing polymers did not stimulate lymphocytes (M. Wilchek and L.A. Loeb, private communications).

DISCUSSION

Binding of mercury ions or organomercurials to sulfhydryl groups is very strong and can be made quite selective. The association constant of methylmercury with cysteine is about $10^{16}{}_{M}{}^{-1}$, while the association constants for methylmercury with other amino acids are lower by a factor of 10^{8} or more (19). Consequently, when organomercurials are used in the form of cysteine complexes, only the sulfhydryl-containing molecules are important in any subsequent equilibria (18-21, and J. Pitha, manuscript in preparation). Thus the organomercurials 1, 2, and 3 used in this study represent selective sulfhydryl reagents. There are free sulfhydryl groups on the surface of cells (22 and J. Pitha, manuscript in preparation) and these represent a primary binding site for reagents 1, 2, and 3; this was at least rigorously established for compound 3 (18).

Interestingly, the effects of organomercurials 1, 2, and 3 on lymphocytes are quite different. Monovalent compound 1 and multivalent compounds 3a and b showed toxic but no stimulatory effects. Compound 2 which is a divalent sulfhydryl reagent (26), is a potent stimulator.

The ineffectiveness of the monovalent sulfhydryl reagent, compound 1, and the effectiveness of the divalent reagent, compound 2, correlate well with other findings on lymphocyte stimulation. Lectins or antibodies used for lymphocyte stimulation are basically divalent reagents for glycoproteins. Using those compounds it was shown that cross-linkage or multipoint attachment of a compound to the cell surface is necessary for stimulation to occur (8-11). Thus there is an analogy between the protein-based and the mercury-derived systems, the former reacting with the sugar moieties

of surface glycoproteins and the latter reacting with the sulfhydryl groups on the protein portions.

The lack of stimulatory activity of the polyvalent reagent, compound 3, is quite surprising. This compound is effectively bound to cell surfaces (18); thus the absence of stimulatory activity can hardly be due to the lack of binding. Also, since compound 3 is polyfunctional, there is no lack of cross-linking ability. The failure of compound 3 to stimulate DNA synthesis may, however, be based on the geometry of the cross-links formed. Cross-linking by mercury ions leads to close contact, as two sulfhydryl-containing proteins are then separated by just a single mercury atom. After the cross-linking by organomercurial compound 2, the proteins are separated by two mercury atoms and four carbon atoms. After cross-linking with organomercurial 3 the minimal separation is by two mercury atoms, four oxygen atoms and ten carbon atoms. Moreover, the actual separation may be much larger as many more distant mercury atoms are available for binding. It is probable that cross-linking and immobilization of sulfhydryl-containing elements of the cell surface at some large arbitrary distance is not satisfactory to achieve lymphocyte stimulation. Apparently, the effective stimulators must not only cross-link two or more elements of the cell surface, but also allow them to achieve geometrical proximity.

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