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COOPERATIVITY OF LECTIN BINDING TO LYMPHOCYTES, AND ITS RELEVANCE TO MITOGENIC STIMULATION

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Summary

The relationship between the binding patterns of soybean agglutinin, peanut agglutinin (both in their native (unaggregated) form and in their polymerized form), and of *Phaseolus vulgaris* leucoagglutinin, to neuraminidase-treated lymphocytes from different sources, and the mitogenic activity of these lectins, was studied. In all cases investigated, binding of a lectin to lymphocytes which resulted in stimulation was a positive cooperative process. Our findings support the assumption that clustering of receptors and conformational changes in membrane structure are prerequisites for mitogenic stimulation.

Introduction

The mitogenic stimulation of lymphocytes by lectins requires the binding of the lectin to the cells. This binding, however, is not, by itself, sufficient to cause cell activation, since there are many lectins that bind to lymphocytes but do not stimulate them. It is generally believed that for mitogenic stimulation to occur, lectins must induce specific changes in membrane structure, such as crosslinking and clustering. Consequently a series of metabolic events is initiated which culminates in an increase of DNA synthesis and cell division. Recently it has been concluded that lectins can indeed induce conformational changes in lymphocyte membranes, since it has been found that binding of concanavalin A to rat thymocytes [1,2] or to plasma membranes isolated from rabbit thymocytes [3] exhibits positive cooperativity.

In this communication we describe studies on the relationship between the mitogenic activity and the binding patterns of various lectins to neuraminidase-treated lymphocytes from mouse and rat. The lectins studied include soybean agglutinin and peanut agglutinin, both in their native (unaggregated) form and in their polymerized form, and of *Phaseolus vulgaris* leucoagglutinin. In all cases investigated, stimulation occurred only when the binding of the lectin to

cells exhibited positive cooperativity. Evidence for the requirement of clustering for mitogenic activity is also presented.

Materials and Methods

Chemicals. D-Galactose and *N*-acetyl-D-galactosamine were obtained from Pfanstiehl, thyroglobulin from Sigma, chloramine-T from Fluka, Switzerland, sodium metabisulphite from BDH, U.K. di-*n*-butylphtalate, and di-*n*-octylphtalate from Eastman Organic Chemicals, hydrocortisone acetate from Frederiksberg Chemical Lab., Denmark, glutaraldehyde (70%) from Ladd Research Industries, Inc., U.S.A., Sephadex G-25 and G-150 (superfine) from Pharmacia, [^{125}I]Na carrier-free from The Radiochemical Center, Amersham, [*Me*-^{3}H]thymidine (5 Ci/mmol) from Nuclear Research Center, Negev, Israel. Neuraminidase (*Vibrio comma*) was obtained from Boehringwerke AG, Germany, as a solution containing 500 units/ml (1 unit releases 1 μg *N*-acetylneuraminic acid from α_1-acid glycoprotein at 37°C in 15 min at pH 5.5). Fetal calf serum was from Biolab, Israel.

Lectins. Leucoagglutinin was obtained from Pharmacia; soybean agglutinin and peanut agglutinin were prepared by affinity chromatography [4,5]. Fractionation of self aggregated soybean agglutinin to polymeric soybean agglutinin ((soybean agglutinin)$_n$) and unaggregated (monomeric) soybean agglutinin was achieved by gel filtration on Sephadex G-150 (superfine) [6]. Polymerization of peanut agglutinin with glutaraldehyde and separation of the polymeric material ((peanut agglutinin)$_n$) from the unmodified lectin were carried out essentially as for soybean agglutinin [7,8] and will be described in detail elsewhere (Prujansky, A., Ravid, A., Lis, H. and Sharon, N. (1977), unpublished).

Labelling of lectins. Lectins were iodinated according to the chloramine-T method [9] as previously described [10], using 10 mg lectin dissolved in 0.4 ml of 1 M inhibitor solution (D-galactose for peanut agglutinin and soybean agglutinin, thyroglobulin for leucoagglutinin) and 2 mCi ^{125}I; iodination was for 1 min. Both the [^{125}I]peanut agglutinin and [^{125}I]soybean agglutinin obtained were repurified by affinity chromatography, and the purified products had the same specific hemagglutinating activity as the native lectins [4,5]. The specific radioactivity of the labelled lectins was 20—130 · 10^3 cpm/μg.

Animals. Mice, BALB/c male (6—12-weeks old); rats, Wistar inbred strain (80—100 g).

Preparation of lymphocyte suspensions. Isolation of cells from experimental animals was carried out as described [11]. Hydrocortisone-resistant rat thymocytes were obtained by the same procedure 48 h after intraperitoneal injection of 15 mg hydrocortisone acetate to each animal. The separation of mouse splenocytes into B and T cells was carried out according to Reisner et al. [12], a method based on different agglutinability of these two populations with soybean agglutinin, and separation of the agglutinated cells from the unagglutinated cells by gravity sedimentation on 50% fetal calf serum.

Treatment of cells with neuraminidase. Cells (10^8/ml) in phosphate-buffered saline, pH 7.4, were treated with neuraminidase (50 units/ml) for 30 min at 37°C with shaking, and washed twice with phosphate-buffered saline.

Binding of iodinated lectins to mouse and rat lymphocytes. Cells ($5 \cdot 10^7$/ml) were suspended in 0.2 ml phosphate-buffered saline containing 0.1 mg bovine serum albumin (to reduce nonspecific binding) and were incubated with different concentrations of iodinated lectins. The incubation was carried out in Beckman microtubes containing 100 μl of an oil mixture (di-*n*-butylphtalate/di-*n*-octylphtalate, 1 : 1 v/v). Since the tubes are very narrow, the reaction solution could be layered above the oil phase with an air bubble separating them, and no contact between the reaction solution and the oil phase was formed during incubation. After incubation of 60 min, at room temperature, the tubes were centrifuged in a Beckman Microfuge for 30 s. The tubes were cut above the sedimented cell pellet and both the pellet and the supernatant were counted using a Packard Gamma Counter. The amount of iodinated lectin bound specifically was calculated by subtracting the amount of iodinated lectin in the presence of its inhibitor (0.3 M D-galactose for peanut agglutinin and soybean agglutinin, and 5 mg/ml thyroglobulin for leucoagglutinin), from that bound in its absence. The nonspecific binding did not exceed 10% of the total binding, over the whole range of lectin concentrations used. All experiments were carried out in duplicate, and the results given are the average values; the individual results did not differ by more than 10%.

Stimulation. Lymphocytes were cultured at 37°C in an atmosphere of 5% CO_2/95% air in microtest plates with 96 flat bottomed wells, each well containing $5 \cdot 10^5$ cells in 200 μl of Dulbecco's modified Eagle's medium containing 5% heat-inactivated fetal calf serum and supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and different concentrations of lectin. The cells were incubated for 48 h. [^{3}H]Thymidine (1 μCi) was added to the cell cultures 6 h before harvesting. At the end of the culture period the cells were collected with a MASH III cell harvester (Microbiological Associates), and washed on filters with water. The filters were dried and counted in a toluene scintillation liquid using a Packard Tri-Carb Scintillation Spectrometer.

Results

The results of binding of soybean agglutinin and peanut agglutinin, both in their monomeric and polymeric form, and of leucoagglutinin, to neuraminidase-treated mouse T splenocytes are shown in Fig. 1. Two types of binding curves can be distinguished: hyperbolic curves, which characterize the binding of peanut agglutinin and soybean agglutinin (Figs. 1A, 1B), and sigmoidal ones which characterize the binding of $(\text{peanut agglutinin})_n$, $(\text{soybean agglutinin})_n$ and leucoagglutinin (Figs. 1C, 1D, 1E). This distinction is made clearer by plotting the data according to Scatchard [13] (Fig. 2). Peanut agglutinin and soybean agglutinin gave linear Scatchard plots indicating a single association constant for all lectin-cell receptor interactions, while $(\text{soybean agglutinin})_n$, $(\text{peanut agglutinin})_n$ and leucoagglutinin binding give biphasic Scatchard plots typical of positive cooperativity. These two groups of lectins differ also in their mitogenic activity towards these cells: non-mitogenic (peanut agglutinin, soybean agglutinin) and mitogenic ($(\text{peanut agglutinin})_n$, $(\text{soybean agglutinin})_n$, leucoagglutinin) (Figs. 3, 4).

Peanut agglutinin has been reported to stimulate neuraminidase-treated rat

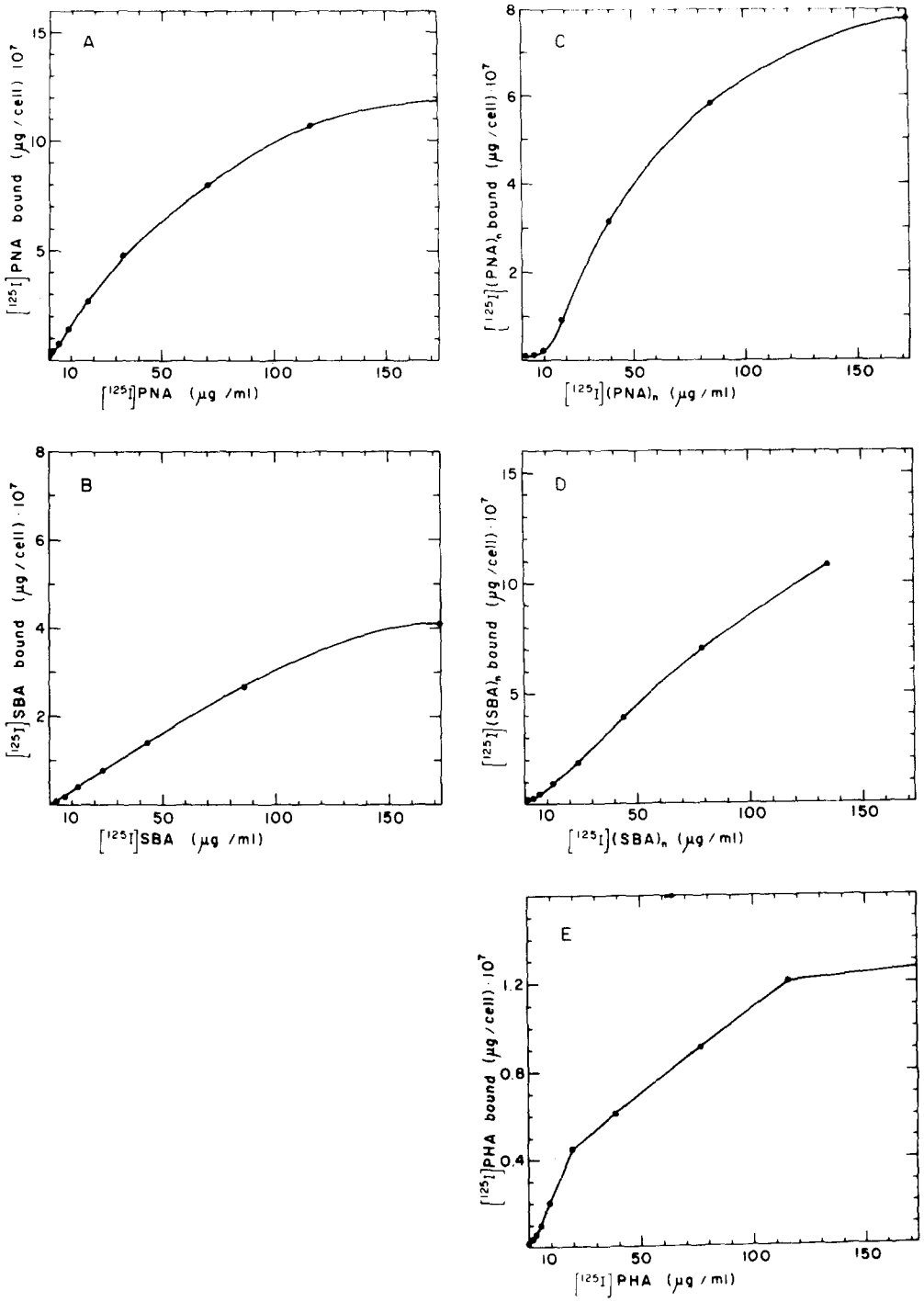

Fig. 1. Binding of iodinated lectins to neuraminidase-treated mouse T spleen cells. Experimental details as described in text. A, [^{125}I]peanut agglutinin (PNA); B, [^{125}I]soybean agglutinin (SBA); C, [^{125}I] $(PNA)_n$; D, [^{125}I] $(SBA)_n$; E, [^{125}I]leucoagglutinin (PHA). Each point is the mean of at least three separate binding experiments which differed by no more than 10%.

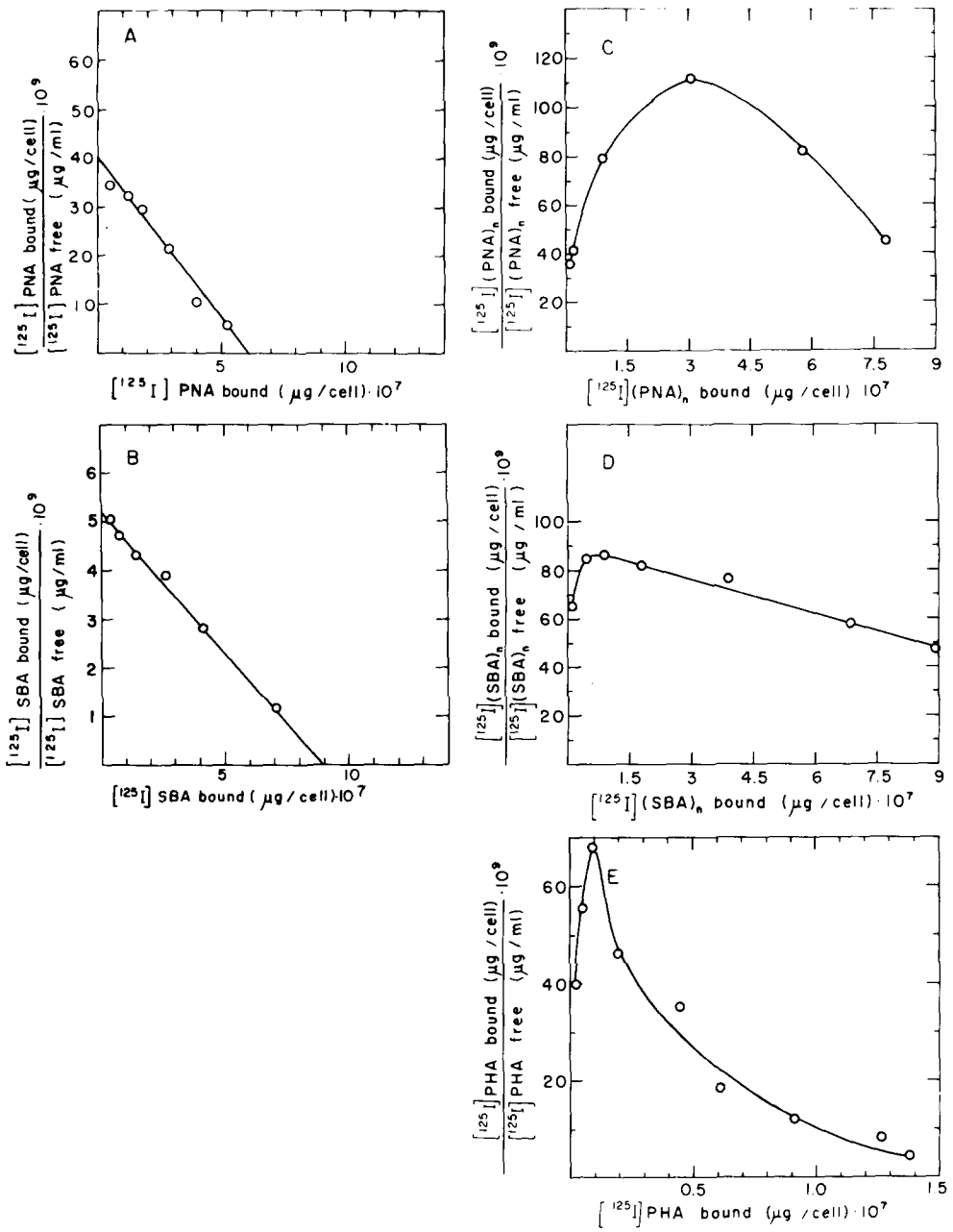

Fig. 2. Scatchard plots of specific binding of lectins to neuraminidase-treated mouse T spleen cells. Experimental details as described in text. A, [^{125}I]peanut agglutinin (PNA); B, [^{125}I]soybean agglutinin (SBA); C, [^{125}I] $(PNA)_n$; D, [^{125}I] $(SBA)_n$; E, [^{125}I]leucoagglutinin (PHA).

spleen and thymus cells [11]. Since both spleen and thymus cell preparations are heterogeneous, the binding of peanut agglutinin and its mitogenic effect were examined on a more homogeneous subpopulation of lymphocytes, the hydrocortisone-resistant thymocytes. The results given in Figs. 5 and 6 show

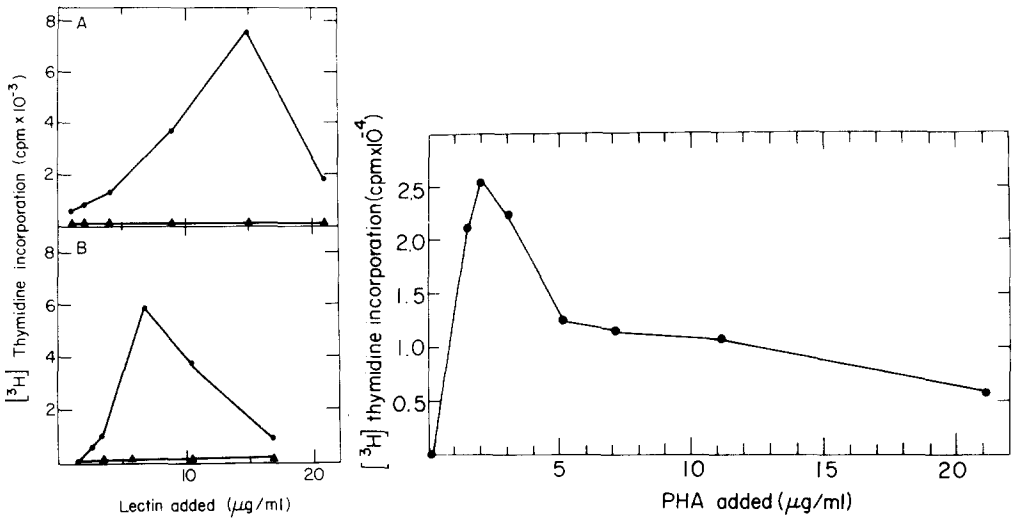

Fig. 3. Stimulation of [^{3}H]thymidine incorporation into neuraminidase-treated mouse T spleen cells by monomeric and polymeric lectins. Experimental details as described in text. A, soybean agglutinin (▲——▲); (soybean agglutinin)$_n$ (●——●); B, peanut agglutinin (▲——▲); (peanut agglutinin)$_n$ (●——●).

Fig. 4. Stimulation of [^{3}H]thymidine incorporation into neuraminidase-treated mouse T spleen cells by leucoagglutinin (PHA). Experimental details as described in text.

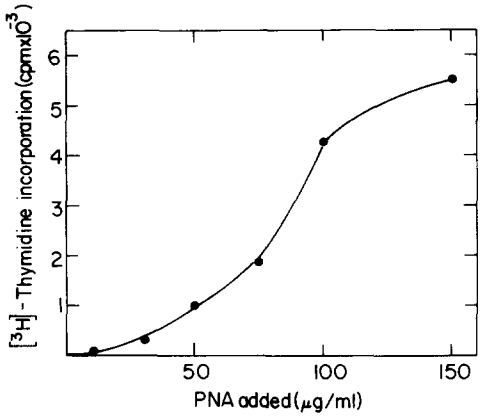

Fig. 5. Stimulation of [^{3}H]thymidine incorporation by peanut agglutinin (PNA) into neuraminidase-treated hydrocortisone-resistant rat thymocytes. Experimental details as described in text.

TABLE I

MITOGENIC ACTIVITY AND MODE OF BINDING TO LYMPHOCYTES OF DIFFERENT LECTINS

Lectin	Lymphocyte *	Mitogenic activity	Cooperativity in binding
Peanut agglutinin	Mouse T splenocytes	—	—
Peanut agglutinin	Rat hydrocortisone-resistant thymocytes	+	+
Soybean agglutinin	Mouse T splenocytes	—	—
(Peanut agglutinin)$_n$	Mouse T splenocytes	+	+
(Soybean agglutinin)$_n$	Mouse T splenocytes	+	+
Leucoagglutinin	Mouse T splenocytes	+	+

* All cells were pretreated with neuraminidase.

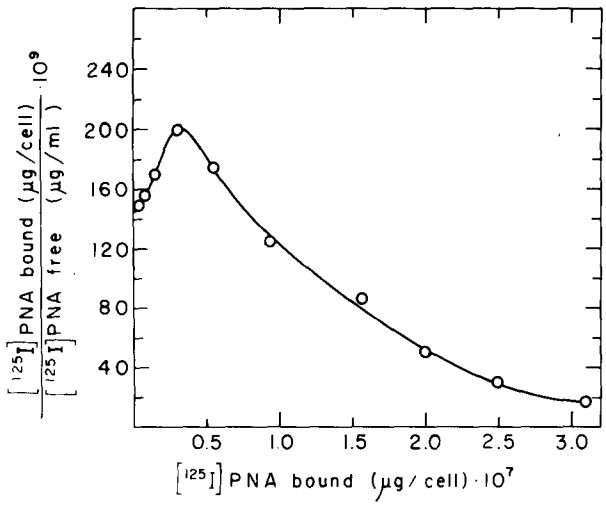

Fig. 6. Scatchard plot of binding of [^{125}I]peanut agglutinin (PNA) to neuraminidase-treated hydrocortisone-resistant rat thymocytes. Experimental details as described in text.

that peanut agglutinin stimulates this subpopulation and its binding to these cells exhibits positive cooperativity. The results obtained are summarized in Table I.

Discussion

Our data clearly demonstrate that the binding of a lectin to a lymphocyte surface that leads to mitogenic stimulation is a positively cooperative process, while the binding of lectin which is not followed by stimulation is non-cooperative (Table I).

In all cases examined, no cooperativity was observed in binding of lectins to saccharides in solution [14,15]. Assuming that this is also true for the interaction between lectins and polymeric ligands (e.g. glycoproteins), we may safely conclude that the cooperativity observed by us is a reflection of changes occuring in the membrane.

We have further shown that cooperative binding is not solely a function of the properties of the lectin but it is dependent on the properties of the cell to which it binds. Binding of the different lectins investigated to the same type of cell (mouse T lymphocytes) exhibits different patterns depending on whether or not the cell undergoes stimulation; this is also true for binding of the same lectin (peanut agglutinin) to cells from different sources (lymphocytes of mouse and rat). The binding experiments cannot be done under the same conditions as the stimulation experiments: a larger number of cells ($5 \cdot 10^7$/ml) is needed in order to get significant binding at low lectin concentrations than is used in the stimulation experiments ($2.5 \cdot 10^6$/ml). However, comparison of the binding and stimulation curves shows that the concentration range where the positive cooperative effect occurs, falls below or within the mitogenic concentration range of the corresponding lectin.

On the basis of the above observations we postulate that the positive coop-

erativity in the binding of mitogenic lectins reflects alterations in cell membrane structure which are an essential event in the stimulation process.

In binding phenomena, positive cooperativity implies that the binding constant of the ligand-receptor association increases as the extent of occupancy of receptor sites increases. In the interaction of lectins with cell surfaces, positive cooperativity can be explained either by an increase in the affinity of the receptors to the lectin or by an increase in the number of available binding sites caused by unmasking of cryptic receptors. Both types of change may be the result of either conformational changes in membrane components or of their redistribution in the membrane, facilitated by the fluid character of the latter [16].

The requirement of multivalency for stimulation of mouse lymphocytes, as previously demonstrated with soybean agglutinin [17], and recently with peanut agglutinin (Prujansky, A., Ravid, A., Lis, H. and Sharon, N. (1977), unpublished), strongly suggests that the generation of the triggering signal requires the crosslinking and clustering of lectin · receptor complexes. The clustering process also affects the mode of lectin binding in a way which increases the binding constant and results in the observed cooperativity.

The crucial step in the formation of clusters is "nucleation", where the first crosslinked receptors are obtained. This step appears to require a minimal number of lectin molecules bound to cell receptors and some degree of receptor mobility in the membrane.

Cluster formation is most likely the critical step leading to alterations in membrane structure which may serve as the triggering signal for the mitogenic process, for example by increasing Ca^{2+} influx through pore formation [18]. Clustering also enables the lectins to bind with increased association constant to the cell surface, as a result of higher localized concentrations of available receptors and, thus, multivalent attachment of the lectin to the cell surface.

Since native peanut agglutinin stimulates neuraminidase-treated rat lymphocytes, we postulate that in this case the clustering process is accomplished more easily than in mouse lymphocytes. This may reflect a difference in the nature of the receptors of these two types of cell, as was also observed in other studies (Ravid, A. (1977), unpublished and ref. 19): the stimulation of biotin-conjugated rat lymphocytes and dinitrophenyl-conjugated rat lymphocytes by the crosslinking agents avidin and anti-dinitrophenyl antibodies, respectively, was 10 times higher than that of mouse lymphocytes after the same treatment.

It is possible that the rat lymphocyte membrane is more fluid than that of mouse lymphocyte, leading to high mobility of its lectin receptors. Another possibility is that the lectin receptors on rat cells carry more available saccharide chains, and thus effective crosslinking, cluster formation and cooperativity can be obtained by divalent lectins.

Triggering of cell stimulation requires formation of clusters of a suitable size which can be achieved with low concentration of multivalent lectins ((soybean agglutinin)$_n$, (peanut agglutinin)$_n$, leucoagglutinin), whereas higher concentration of the divalent lectin are needed, as can be seen from comparison of stimulation of the mouse and rat cells (Figs. 3—5). It is possible that low concentrations of divalent lectins form clusters which are too small to initiate stimulation but can affect lectin binding. Therefore, the cooperative effect appears in rat

cells at lower concentrations than the mitogenic concentrations, while in mouse cells the two phenomena appear together. Our conclusions are in line with previous results of Schmidt-Ullrich et al. [3] who have found that the binding of mitogenic concanavalin A to purified plasma membrane of rabbit thymocytes induces a dimerization of its receptors (55000 dalton glycoprotein) through a cooperative process that leads to an increased affinity for the lectin. This cooperativity, which occurs at low concentrations of concanavalin A, is not observed in glutaraldehyde-treated membrane in which the binding proteins have been artificially brought into their high affinity state by crosslinking.

An apparent exception to the requirement of alterations in membrane structure for mitogenic stimulation, as is suggested here, is that of succinyl-concanavalin A binding to rat thymocytes. While this binding was non-cooperative, the cells were stimulated [1]. It should be noted, however, that in this case binding was measured at 4°C, a temperature at which cell stimulation does not occur.

Positive cooperativity in binding of lectins to cells is not confined to lymphocytes. Cuatrecasas [20] has observed the same phenomenon in the binding of concanavalin A and wheat germ agglutinin to fat cells and in the range of concentrations where insulin-like effects are observed.

The conclusion that multivalency of lectins is a prerequisite for mitogenic activity seems to be in contradiction to the recent findings that monovalent fragments of antibodies (which react with cell surface carbohydrates) and monovalent concanavalin A are mitogenic for mouse spleen lymphocytes [21—23]. However, according to Sela et al. [21] the receptor crosslinkage necessary for cell activation may, in these cases, be achieved by the adsorption of the monovalent mitogen on the surface of macrophages, which then present them in a multivalent form to the responding cells.

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References

1 Bornens, M., Karsenti, E. and Avrameas, S. (1976) Eur. J. Biochem. 65, 61—69
2 Karsenti, E., Bornens, M. and Avrameas, S. (1977) Eur. J. Biochem. 75, 251—256
3 Schmidt-Ullrich, R., Wallach, D.F.H. and Hendricks, J. (1976) Biochim. Biophys. Acta 443, 587—600
4 Gordon, J.A., Blumberg, S., Lis, H. and Sharon, N. (1972) FEBS Lett. 24, 193—196
5 Lotan, R., Skutelsky, E., Danon, D. and Sharon, N. (1975) J. Biol. Chem. 250, 8518—8523
6 Lotan, R., Lis, H. and Sharon, N. (1975) Biochem. Biophys. Res. Commun. 62, 144—150
7 Lotan, R., Lis, H., Rosenwasser, A., Novogrodsky, A. and Sharon, N. (1973) Biochem. Biophys. Res. Commun. 55, 1347—1355
8 Lotan, R., Lis, H., Rosenwasser, A., Novogrodsky, A. and Sharon, N. (1974) Biochem. Biophys. Res. Commun. 56, 1100
9 Greenwood, F.C., Hunter, H.H. and Glover, J.S. (1965) Biochem. J. 89, 114—123
10 Reisner, Y., Lis, H. and Sharon, N. (1976) Exp. Cell Res. 97, 445—448
11 Novogrodsky, A., Lotan, R., Ravid, A. and Sharon, N. (1975) J. Immunol. 115, 1243—1248
12 Reisner, Y., Ravid, A. and Sharon, N. (1976) Biochem. Biophys. Res. Commun. 72, 1585—1591
13 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660—672

14 Van Landschoot, A., Loontiens, F.G., Clegg, R.M., Sharon, N. and de Bruyne, C.K. (1977) Eur. J. Biochem. 79, 275—283
15 Loontiens, F.G., Clegg, R.M. and Jovin, T.M. (1977) Biochemistry 16, 159—166
16 Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720—731
17 Schechter, B., Lis, H., Lotan, R., Novogrodsky, A. and Sharon, N. (1976) Eur. J. Immunol. 6, 145—149
18 Naino, V.C., Green, N.M. and Crumpton, M.J. (1974) Nature 251, 324—327
19 Wynne, D., Wilchek, M. and Novogrodsky, A. (1976) Biochem. Biophys. Res. Commun. 68, 730—739
20 Cuatrecasas, P. (1973) Biochemistry 12, 1312—1322
21 Sela, B.A., Wang, J.L. and Edelman, G.M. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1127—1131
22 Beppu, N., Terao, T. and Osawa, T. (1975) J. Biochem. 79, 1113—1117
23 Fraser, A.R., Heraperly, T.T., Wang, J.L. and Edelman, G.M. (1976) Proc. Natl. Acad. Sci. U.S. 73, 790—794