AFFINITY DENSITY PERTURBATION: A NEW FRACTIONATION PRINCIPLE AND ITS ILLUSTRATION IN A MEMBRANE SEPARATION

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1. Introduction

Many macromolecules and supramolecular structures carry specific receptors integral to, and characterizing their function. This is also true for membranes and, indeed, information proliferates that the plasma membranes of animal cells comprise a topologically extended array of diverse, but specific and genetically regulated, functional systems, each involving defined receptors; membranes thus playing biological roles far more intricate than their well studied participation in permeation and transport controls.

Many of the functions now recognized, i.e. cell–cell recognition, discrimination of self from not-self, binding of drugs, viruses and toxins, as well as the transfer of extracellular information into a code comprehensible to intracellular machinery, presumably involve specific biochemical cell surface specializations such as predicted very early by Ehrlich [1] and Weiss [2]. These are not necessarily distributed uniformly around the cell perimeter and perhaps nowhere in the "patchiness" of the cell surface illustrated more dramatically than in lymphoid cells, where, in the mouse, some specific antigenic determinants are distributed in widely separated clusters, while others have a more random distribution [3], and where dramatic changes in antigen distribution regularly accompany differentiation [4].

Recognizing the topological irregularities of plasma membranes, we had earlier developed fractionation methods in which the membranes are physically sheared into numerous minute, semi-permeable membrane sacs [5–10] which can thereafter be separated according to their physiochemical attributes. Some separations of certain plasma membrane antigens and enzymes have been achieved by this approach [11], but it has remained necessary to elaborate far more specific means by which given receptor-domain can be extracted from other membrane areas.

We have accordingly devised a fractionation system, where membrane fragments bearing a given receptor are rapidly separated ultracentrifugally according to the density increase caused by the combination of the receptor with its specific ligand, covalently coupled to a labelled particle of very high density which can also be seen by electron microscopy. We term this approach *"affinity-density-perturbation"* and herein present its essential principles. Using labelled ligands, particularly in combination with differently labelled receptors, provides the method with unusual quantitative discrimination. Moreover, electron microscopic study of the complexes between receptor-bearing membrane fragments and the density-perturbing particles creates the unique opportunity

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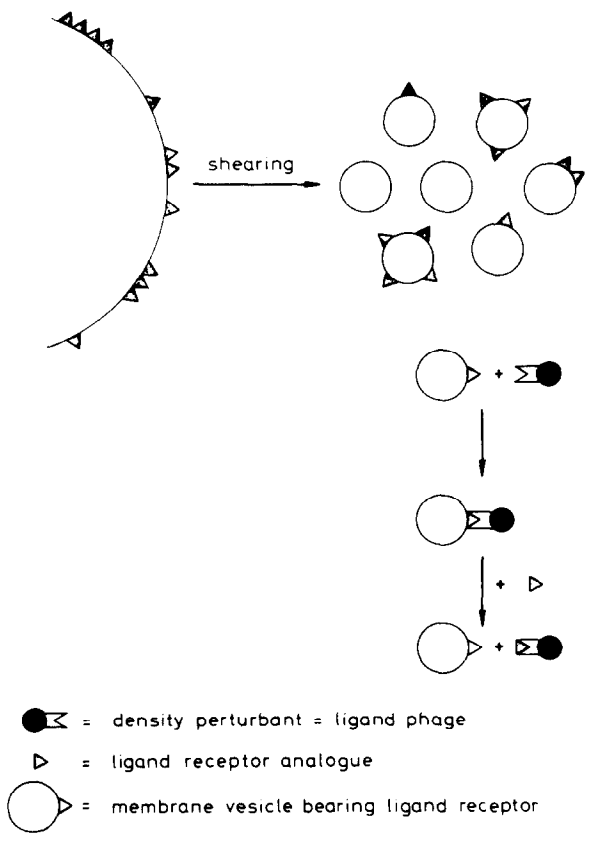

Fig.1. Schematic of the fractionation system: A plasma membrane bearing multiple receptors (△) is sheared into membrane fragments carrying different numbers of receptors in varying distributions. These are reacted with the ligand (Σ) coupled to the density perturbant, i.e. K29 phage (●), producing a membrane-receptor – ligand – phage complex of higher density than the membrane itself and of lower density than the density perturbant. Addition of a low molecular weight dissociating agent (▲) returns the membrane and density perturbant to their original densities.

to map receptor topology on isolated membranes and to compare this with the distribution of these sites on intact cells. Finally the method has a special advantage that many samples can be run simultaneously under optimally controlled environmental conditions. Although our application, illustrations and its major interest lie in the membrane field, this system has as wide a range of application as affinity chromatography and, in addition, offers several advantages.

Our approach is schematized in fig.1. Membrane fragments bearing a specific receptor are combined with a density perturbant and the complex centrifuged to its isopycnic density, which is higher than that of the membranes but lower than that of the perturbant. For convenience of localization and quantification, the membranes and density perturbant are labelled with different radioisotopes. The formation of membrane – ligand complexes is blocked or reversed when desired by addition of reagents of higher affinity for the ligand than the receptor, or an excess of receptor analogues with similar affinity.

In these pilot studies we have used pure concanavalin A, (Con A), radioactively labelled with ^{125}I as ligand, converting it into a density perturbant by gluteraldehyde coupling [12] to purified coliphage K29 [13]. The use of Con A derives from a mutual interest in two aspects of this substance; namely, its mitogenicity for lymphocytes and its enhanced agglutination of neoplastically converted cells. Other ligands, e.g., antibodies, hybrid antibodies, peptide hormones, etc. are equally qualified. Similarly, other coupling methods can be used (e.g. [14]), as can other density perturbants, although the small size and stability of K29 offers an unusual advantage. K29 is an icosahedron about 450 Å in diameter and rather stable. Its density is 1.495. As dissociation agent we have employed trehalose which has a modest affinity for Con A, $K_A = 5.38 \times 10^{-3}$ l/mole [15]. Our membrane fragments were pig lymphocyte plasma membrane vesicles, prepared by surface shearing of the intact cells [16]. About 10^7 Con A receptors are expected per cell [17], i.e., one receptor per 100 $Å^2$ surface area, approximately 100× the number of vesicles produced by shearing. This large number of receptors has been advantageous in developing our system but future studies must take into account a presumably large variation in the affinity constants of diverse receptor sites as well as possible steric interference between receptors. Indeed, our own preliminary electron microscopic data on rat erythrocytes [18], the work of Edelman and Milette [17] and the studies of Nicolson and associates [19] indicate an irregular distribution of Con A receptors, depending on physiologic state.

2. Experimental

All reagents were of the highest available purity. Concanavalin A (Con A), 3 × recrystallized, was

obtained lyophilized from Miles-Seravac Ltd. (England), trehalose from Schuchardt GMBH (Germany) and glutaraldehyde and chloramine T from Fischer Scientific Co. (USA).

K29 coliphage was cultivated and purified according to [13] but using an initial multiplicity of 4–5, concentrating by dialysis in vacuo and resuspending on a slowly rotating disc. The phage preparations contained about 1.5×10^{14} PFU/ml.

Radioiodination was essentially as in [20]. For Con A, 40 mg of the protein in 4.0 ml 1 M NaCl, 0.01 M phosphate, pH 7.0, clarified by centrifugation (55,000 rpm, 45 min, Spinco Rotor SW56) was reacted at 0°, 0.4 mg $Na_2S_2O_5$ in 0.5 ml 0.01 M phosphate was added, the mixture dialysed for 24 hr against 5 changes of 2 l of 1 M NaCl and centrifuged (55,000 rpm, 45 min, Rotor SW56). The supernatant, containing about 3.4 mg protein and 2.8×10^7 cpm per ml, was stored at −20° until use.

Con A was coupled to K29 following the principles in [20]. 0.1 ml K29 (1.5×10^{13} PFU), plus 0.1 ml ^{125}I-Con A (2.8×10^6 cpm), plus 0.025 ml glutaraldehyde (0.05 mg), all in 0.15 M NaCl, pH 7.25 were reacted with gentle agitation for 2 hr at 4°. The labelled phages were then purified by repeated banding in continuous CsCl gradients of density 1.10–1.70 (30,000 rpm, 60 min, Rotor SW56), dialysed exhaustively against 0.15 M NaCl, large aggregates removed by low-speed spinning, concentrated by pelleting (55,000 rpm, 45 min, Rotor SW56) and gently resuspended in 0.15 M NaCl.

To test the activity of the coupled phages, 0.03 ml fresh, washed rat erythrocytes were incubated with ^{125}I-Con A–K29 ± 3×10^{-10} moles Con A and 1.5×10^{-5} moles α,α-trehalose, in 0.15 M NaCl for 1 hr at 20°. The cells, washed by repeated pelleting, bound 21% of the phage counts without trehalose and 2.2% after addition of the inhibitor. Membrane bound Con A–phage is easily recognized by electron microscopy.

Plasma membranes, isolated from pig lymph node cells [16], were tagged analogously with ^{131}I. 3.0 ml membrane (1.2 mg protein/ml) was reacted with 0.02 ml Na ^{131}I (0.2 mCi), 0.5 ml Chloramine T (0.1 mg) added dropwise, the mixture stirred 7 min at 0°, 0.5 ml (0.1 mg) $Na_2S_2O_5$ added and the membranes freed of the reagents by dialysis against 0.01 M phosphate, pH 7.0 and/or repeated washing in this buffer at 55,000 rpm for 30 min.

Labelled membranes were reacted with 0.15 aliquots of ^{125}I-Con A–K29, containing 3×10^{-10} moles Con A, both in 0.15 M NaCl, for 2 hr at 20°, ± 10^{-4} mole α,α-trehalose in a total vol of 0.5 ml, 0.4 ml of the mixture layered on a continuous 4.0 ml CsCl gradient (density = 1.10–1.60) and immediately centrifuged to isopycnic equilibrium (40,000 rpm, 45 min, Rotor SW56). Unreacted membranes and phage conjugates were similarly treated. Thereafter the gradients were "cut" into 0.4 ml samples and, after density determination, were freed of CsCl by dialysis against 0.15 M NaCl and counted in a Packard Autogamma scintillation spectrometer to separately determine the ^{125}I and ^{131}I distributions. Non-particulate material remained above the gradient.

3. Results

The basic results of the system are illustrated in fig. 2. As noted before [5, 11] salt density gradients can be used effectively for certain membrane fractionations. Aggregation does not occur if the concentration of multivalent ions is kept low, and our previous studies showed no loss of protein or lipid at these and higher Γ/2.

Untreated membranes equilibrate rather symmetrically about a density of 1.18, somewhat higher than that found with ascites tumor membrane by Wallach and Kamat [5]. As anticipated, pure, Con A–K29 phage conjugate (not shown) pellets, its density, when iodinated, approaching 1.6.

Membrane (~1 mg protein), reacted with Con A–K29, containing 3×10^{-10} moles Con A (2×10^{13} ligands), distributes in a broad, irregular layer, with maximal density about 1.30–1.34, overlapping only minimally (~10%) with the normal membrane distribution and exhibiting a distinct shoulder at density = 1.4. Although 15% of the reacted membrane reaches densities greater than 1.45, no membrane material is trapped in the pellet. At the same time a peak for the Con A–K29 conjugate appears at the same density as the new membrane layer and 76% of the conjugate moves to lower densities. Since there is no membrane label associated with the remaining high density conjugate, we presume that the latter represents unreacted or unreactive material. However, since independent tests showed more than 85% of the Con A–K29 reactive with dextran, it is reasonable to assume that there is an excess of reac-

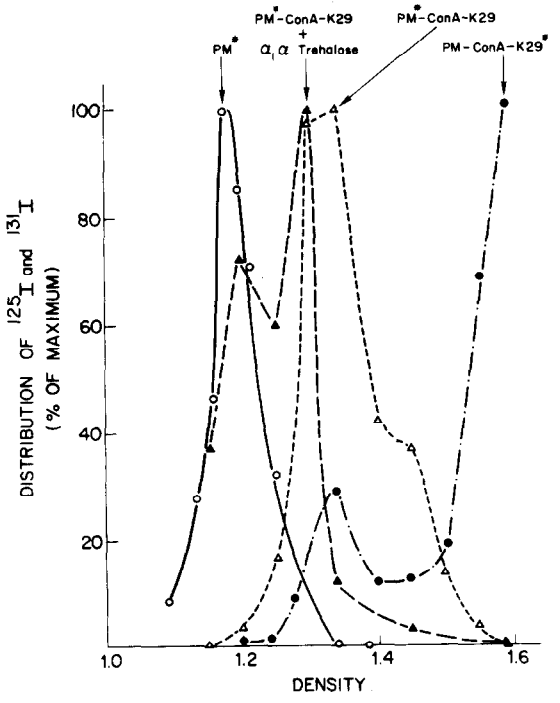

Fig.2. Density distribution in a CsCl gradient of a) untreated lymphocyte membranes (PM)* (○—○—○); b) the label of the membrane* Con A – K29 complex (△--△--△); c) Con A – K29*, after reaction with membrane (●-●-●) and d) membrane* Con A – K29, after reaction with α,α-trehalose (▲— —▲— —▲).
* Indicates the label, ^{125}I for the density perturbant and ^{131}I for the membrane.

tive Con A – K29 in the system. 99% of the Con A – K29 label and membrane lables were recovered.

The irregularity and width of the membrane distribution, as well as the shoulders in both membrane and Con A – K29 distributions point to a heterogeneity in the number and/or affinities of the Con A binding sites. According to the Law of Mass Action, the separate sedimentation rates and densities of the Con A – K29 and its membrane complexes would foster some dissociation of the latter. This would skew the distribution of the complexes to lower densities, but the rapidity of the centrifugal separation minimizes the effect and still further improvement can be anticipated with higher velocity rotors.

Upon addition of excess α,α-trehalose (3×10^4 moles of sugar/mole of conjugated Con A), the membrane Con A – K29 complex dissociates notably, the whole membrane distribution shifting to lower density and splitting into 2 bands, one coincident with the density of unreacted membranes. This phenomenon, particularly the emergence of the low density component, most likely reflects the distribution of binding sites, particularly those of low affinity. As stated earlier, α,α-trehalose can effect more than 90% dissociation of Con A binding; that this is not seen in fig. 2 is attributed to the fact that the sugar remains at the top of the gradient, while the particulate components quickly sediment. This process, favoring persistence of the complexes, is what allows us to point out the presence of low affinity binding sites. It appears nonetheless that all membranes have lost some ligand, and some membrane fragments have lost all of the ligand.

4. Discussion

Using ^{125}I-labelled Con A – K29 conjugates as density perturbants, we have demonstrated selective, but specifically reversible density changes when the conjugate reacts with lymphocyte membrane fragments; reaction of the conjugate increases the density of the membrane vesicles and lowers that of the phage – Con A conjugate. Since each lymphocyte bears ~10^7 Con A receptors on its membranes and the membrane is unlikely to yield more than 10^5 fragments under our conditions, each fragment probably bears more than one receptor, and others may carry more. This is substantiated by our finding (shown in fig. 2) that reaction with Con A – K29 conjugate increases the density of essentially all membrane fragments. An extension of the present study should allow a subfractionation into particles bearing 0, 1, 2 or more receptors, but this is not our aim. It is also possible that there are intracellular Con A receptors and/or that some of the receptor-bearing membranes invert, becoming ligand-inaccessible. This possibility, first emphasized by Wallach [11] should be tested in all cell systems; however, in lymphocytes Con A binding sites appear concentrated at the cell surface. Finally, one must consider a reorientation of the receptors during the fractionation; our system provides an unusual opportunity to test this micromorphologically.

We wish to introduce a vitally needed innovation to biology, one allowing i) elucidation of membrane

topology at the biochemical, as well as micromorphologic levels and ii) isolation of receptor domains which can "trigger" a cell upon reaction with a specific ligand. Obvious examples of the latter are a) the membrane loci of the action of membranes with peptide hormones or neurotransmitters, b) antigen receptors on immunocytes and c) antigen clusters. For these examples, we would require the density perturbant couples to a) hormones or transmitters; b) specific antigen, and c) specific immunoglobulins.

We emphasize that our interest here lies not merely in the isolation of the receptor, but in the *membrane machinery* which translates membrane–ligand binding into biological action.

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References

[1] P. Ehrlich, in: Gesammelte Arbeiten, ed. F. Himmelwelt (Springer, London, New York, Paris, 1957) p. 178.
[2] P. Weiss, Proc. Natl. Acad. Sci. U.S. 46 (1960) 993.
[3] T. Aoki, V. Hammerling, E. de Harven, E. Boyse and L.J. Old, J. Exp. Med. 130 (1969) 979.
[4] V. Hammerling, in: The Dynamic Structure of Cell Membranes, eds. D.F.H. Wallach and H. Fischer (Springer, New York, Heidelberg, Berlin, 1971) p. 51.
[5] D.F.H. Wallach and V.B. Kamat, Proc. Natl. Acad. Sci. U.S. 52 (1964) 721.
[6] V.B. Kamat and D.F.H. Wallach, Science 148 (1965) 1343.
[7] D.F.H. Wallach and V. Vlahovic, Nature 216 (1967) 182.
[8] T.L. Steck and D.F.H. Wallach, in: Methods in Cancer Research Vol. V, ed. H. Busch (Academic Press, New York, 1970) p.92.
[9] T.L. Steck, R.S. Weinstein, J.H. Straus and D.F.H. Wallach, Science 168 (1970) 225.
[10] T.L. Steck, J.H. Straus and D.F.H. Wallach, Biochim. Biophys. Acta 203 (1970) 385.
[11] D.F.H. Wallach, in: Specificity of Cell Surfaces, eds. B. Davis and L. Warren (Prentice Hall, Englewood Cliffs, N.J., 1967) p. 129.
[12] F. Wold, in: Methods in Enzymology, eds. S.P. Kolowick and N.O. Kaplan (Academic Press, New York, 1967) p. 638.
[13] S. Stirm, W. Bessler, F. Fehemel and E. Freund Molbert, J. Virology 8 (1971) 343.
[14] A.F. Schick and S.J. Singer, J. Biol. Chem. 236 (1961) 2477.
[15] L.L. So and F.F. Goldstein, Biochim. Biophys. Acta 165 (1968) 398.
[16] W. Imm, K. Resch, E. Ferber and D.F.H. Wallach, submitted to Biochim. Biophys. Acta.
[17] G. Edelman and C.F. Milette, Proc. Natl. Acad. Sci. U.S. 68 (1971) 2436.
[18] V. Speth, E. Ferber and D.F.H. Wallach, to be published.
[19] G.L. Nicolson, personal communication, to be published.
[20] P.F. Mconahey, Int. Arch. Allergy 29 (1966) 185.