Biochimica et Biophysica Acta, 685 (1982) 347–354
Elsevier Biomedical Press

BBA 71065

CONTROL OF HIGH AFFINITY LECTIN BINDING TO AN INTEGRAL MEMBRANE GLYCOPROTEIN IN LIPID BILAYERS

NIKA V. KETIS and CHRIS W.M. GRANT *

Department of Biochemistry, University of Western Ontario, London, Ontario, N6A 5C1 (Canada)

(Received August 3rd, 1981)

Key words: Glycophorin; Lectin; (Wheat germ agglutinin)

High affinity binding of wheat germ agglutinin to glycophorin is demonstrated to be potently affected by non-specific interaction of the receptor with other protein and oligosaccharide structures present at the membrane surface. It is suggested that this may represent a significant general mechanism of receptor control.

Introduction

The function of membrane glycoproteins as cell surface recognition sites has attracted considerable interest in recent years [1,2]. Specific binding to the cell periphery is often a key early event in external modulation of cell metabolism: for instance in the case of macromolecular effectors such as antibodies, lectins, and polypeptide hormones or toxins. Obviously, a primary factor in determining cellular response to such an effector is the presence of appropriate receptors on the membrane; and high affinity receptors are felt to be particularly important (see, for example, Refs. 3 and 4). As a result, there have been numerous studies directed toward quantitation of high affinity binding sites, their distribution over the cell surface, and changes in their numbers and distribution subsequent to specific binding events. Typically these studies employ radiolabel assays and fluorescence microscopy. Although this is clearly an instructive approach, there are certain aspects which may be rewardingly attacked in model membranes. We report here an analogous study of lectin binding to the integral membrane glycoprotein, glycophorin, in large liposomes. The system provides a single population of receptors which in the native membrane function as the MN blood group determinant and the receptor for wheat germ agglutinin (Ref. 5 and references therein).

There exists in the literature the concept that ligand binding characteristics of receptors may depend to some extent on their situation in the membrane. Thus for instance a considerable fraction of the glycolipid population in cell membranes can be inaccessible; and their accessibility may be modified indirectly (for review, see Ref. 6). A very interesting analogous situation of 'cryptic' oligosaccharide chains has recently been found in IgG and IgM; in which case antigen binding is claimed to expose terminal sugar residues [7,8]. As far as membrane glycoproteins are concerned, relatively little data exist with regard to crypticity other than crypticity brought about by direct chemical modification. However virus binding to erythrocytes has been shown to modify sialic acid exposure [9]; and insulin receptors in adipocytes show increased affinity for hormone in the presence of anti-receptor antibody [10]. The latter observation makes the point that receptors can be more subtly affected than simply being buried (cryptic) or unmasked: their binding affinity may be subject to local control. We have observed a related phenomenon in our model membranes,

* To whom correspondence should be addressed.

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and have tentatively attributed it to nonspecific headgroup interactions.

Materials and Methods

Materials

Glycophorin was isolated from outdated bank blood by the method of Marchesi and Andrews [11] and extensively ethanol washed. The concanavalin A receptor glycoprotein of human erythrocytes was isolated by a modification of the method of Findlay [12] as described earlier by one of us [13]. Lipids were obtained from Sigma and were pure as judged by thin-layer chromatography on silica gel plates. 2-Chloroethanol and unlabelled wheat germ agglutinin were from Sigma. ^{3}H-Labelled wheat germ agglutinin was from New England Nuclear and was typically 62% trichloroacetic acid precipitable with a specific activity of $5 \cdot 10^8$ dpm/mg of protein. ^{125}I-Labelled wheat germ agglutinin was made according to the general method of Markwell [14] and was typically 71% trichloroacetic acid precipitable with a specific activity of $4 \cdot 10^5$ cpm/μg of protein. ^{125}I was from Amersham. Bovine serum albumin was either ultra pure globulin free, or the 96–99% fraction V from Sigma (both of which gave the same results). Dextran T500 was from Pharmacia.

Receptor-bearing liposomes

Lipid bilayer structures used in this work were multilamellar liposomes and, unless otherwise stated, their composition was 37:14:1 mole ratio dimyristoyl phosphatidylcholine/cholesterol/glycophorin. Their preparation has been described by us previously [15]. The basic approach was to employ 1:1 (volume ratio) 2-chloroethanol/water to dissolve lipid plus protein, and then to remove the solvent to leave a film on the inside of a round bottom flask. Subsequent hydration of this film produced liposomes bearing receptor glycoproteins. For the work described here we selected that subpopulation of liposomes which was large enough to sediment when centrifuged at $850 \times g$ for 5 min. Glycophorin and lipids were readily stored in forms which could be measured into 2-chlorethanol/water. The concanavalin A receptor was freshly prepared and precipitated from detergent solution by dialysis against 5 mM Hepes buffer after adding a 0.5 mole ratio of dimyristoylphosphatidylcholine [13]. The precipitate was then added to the 2-chloroethanol/water solution of other membrane components.

Binding assay

For binding studies a given batch of large liposomes was separated into identical portions to be followed under different conditions of temperature or surface coating. If liposomes were to be coated with a non-specific layer of macromolecules [16–19] they were subsequently handled in buffers containing 3 mg/ml serum albumin or Dextran T500. Labelled lectin was added in (Ca^{2+}, Mg^{2+})-containing phosphate-buffered saline and allowed to incubate for 15 min prior to any washing procedure (in our hands wheat germ agglutinin binding to liposomes is essentially complete within this time). Individual samples had a final volume of 300 μl and contained between 10^6 and 10^7 liposomes.

Following the incubation outlined above, liposomes were washed in 15 ml Corning centrifuge tubes containing 10 ml of phosphate-buffered saline (with or without serum albumin or Dextran depending upon the nature of the surface coat desired). In order to do this the incubation mixture was carefully layered on top of the wash buffer and the sample centrifuged for 5 min at $850 \times g$. Supernatants were aspirated off and the pellet was resuspended and washed with a further 10 ml of buffer. Washed pellets were added to scintillation vials for counting. Bound lectin was determined by subtraction of counts in an identical sample to which inhibitory sugar had been added prior to washing. Nonspecific binding never exceeded 10% of specific over the range of concentrations tested.

For fluorescence microscopy, liposomes (10^7/ml) were exposed for 15 min to fluorescein-labelled wheat germ agglutinin (Miles-Yeda) at 1 mg/ml. Samples were viewed with a Zeiss fluorescence microscope.

Intact cells

Intact erythrocytes tended to stick to the walls of the centrifuge tubes used in the above assay. Hence a modification of the differential centrifugation technique of Bittiger and Schnebli [20] was employed as follows.

Human red blood cells, freshly drawn in citrate saline, were washed four times with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} (no serum albumin). For incubation with lectin each sample contained 10^7 cells and had a final volume of 300 μl. Samples were incubated at 22°C for 35 min prior to being layered carefully onto 1.0 ml of buffer (with or without serum albumin) in microfuge tubes (Brinkman Instruments Inc., 1.5 ml size). These tubes had been previously centrifuged at $12500 \times g$ for 2 min to eliminate air bubbles. The cells were washed twice in this manner by centrifuging at $12500 \times g$ for 5 min; and following the second centrifugation, supernatants were aspirated and the microfuge tube bottoms containing the cell pellets were cut off with a hot razor blade and placed in scintillation vials for counting. Under these conditions, if the cells were left out, no appreciable counts (such as ^{125}I-labelled wheat germ agglutinin aggregates) could be sedimented either in the presence or absence of serum albumin.

Results and Discussion

Basic binding studies

A variety of techniques for assembly of glycophorin into lipid bilayers have been described in the literature [21–23], however, it was important for this particular study that the model cells be large and stable. Hence an approach was chosen that involved hydration of mixed lipid/protein films to produce liposomes [22]. The liposomes employed in this work were all multilamellar structures, 3–50 μm in diameter. However, some were made of lipid alone, others were designed with receptor glycoproteins assembled into their walls; and both types could be used 'as is', or first coated with a surface layer of non-specific protein or polysaccharide.

The simple presence of glycolipid or glycoprotein receptors in model membranes is often demonstrated by showing that the structures involved may be agglutinated with some specific agent directed against them. Alternatively their ability to inhibit agglutination of erythrocytes may be measured. Both of these criteria were fulfilled by the liposomes used in this work: if (and only if) they bore glycophorin they were readily agglutinated by wheat germ agglutinin, and inhibited wheat germ agglutinin-mediated hemagglutination. Similar experiments have been performed with other bilayer systems containing glycophorin in the past by one of us and by other workers [21–24]. However, direct agglutination experiments do not provide information concerning the number of receptor sites available for binding. Hemagglutination inhibition experiments provide an estimate of the number of sites actually occupied at the agglutination end-point; but, as described below, both low and high affinity sites are lumped together in such measurements. When workers quantitate lectin binding sites on cell membranes, they refer to numbers obtained in an assay which involves incubation with lectin followed by extensive washing in lectin-free buffers to remove 'unbound' material. It should be noted that this approach only counts those binding sites of such high affinity that the dissociation rate is slow on a time-scale of minutes, and may ignore large numbers of significant but lower affinity sites. In the work described here lectin binding sites were quantitated in exactly this manner, counting the amount of ^{3}H- or ^{125}I-labelled wheat germ agglutinin bound (see Materials and Methods). Briefly, duplicate aliquots were incubated with labelled lectin; then inhibitory sugar was added to one sample as control and both were washed extensively by differential centrifugation to remove 'unbound' lectin. Counts in control pellets were used to subtract non-specific binding (which was never more than 10% of specific).

Not surprisingly, liposomes of lipid alone (no glycophorin) failed to bind wheat germ agglutinin regardless of the presence or absence of a surface layer of serum albumin or Dextran. They did not agglutinate with wheat germ agglutinin, did not inhibit wheat germ agglutinin-mediated hemagglutination, and did not show bound counts in the assay described above. Liposomes possessing glycophorin as a surface receptor, but no surface layer of albumin or dextran, were highly agglutinable by wheat germ agglutinin and competed very potently for wheat germ agglutinin in hemagglutination inhibition assays. Furthermore, such liposomes retained measurable amounts of fluorescent lectin or radiolabelled lectin when collected by simple centrifugation subsequent to in-

cubation with wheat germ agglutinin, in agreement with the results in Refs. 22 and 23. However, when washed extensively by repeated differential centrifugation in lectin-free buffers (as is typically done in studies of intact cells), very little lectin remained bound (Fig. 1, B). This seemed odd to us at first since glycophorin in the intact cell retained wheat germ agglutinin during lengthy washing procedures (see section on 'Relevance to intact cells' and Fig. 6). Apparently the switch of receptor situation had somehow selectively impaired high affinity binding. Of course, a critical difference is that cells possess a considerable surface layer of material including peripheral proteins, glycolipid and glycoprotein headgroups, and adsorbed species (up to 50% of the latter may not be removed by washing procedures, (Brooks, D., personal communication)). Indeed, when we coated glycophorin-bearing liposomes with albumin or dextran, the situation reverted to that which one finds in cells: a large population of very low affinity sites, and a smaller population of sites not displacable by extensive washing (Figs. 1, A and 2, A). This phenomenon has been mentioned by us in an article dealing with (+)-cooperativity in lectin binding [15], but its explanation must be considered speculative. Certainly serum albumin and dextran are well known to adhere extensively to membranes and lipid bilayers [16–19], and no resultant disruption of membrane integrity has been reported. In our hands the surface coat does not induce significant changes in liposome state of aggregation/disaggregation, or in their size, or appearance at the level of light or electron microscopy. Model building of the structures present in our bilayer systems strongly suggests that one phenomenon to be considered is simple non-specific collisional interaction of the glycophorin headgroup with adsorbed macromolecules. The glycophorin headgroup is a conformationally highly mobile structure [25–27] whose behaviour/conformation might be influenced by such interactions. The potential relevance of these considerations to binding studies is that high affinity binding typically depends upon stable, optimal ligand orientation.

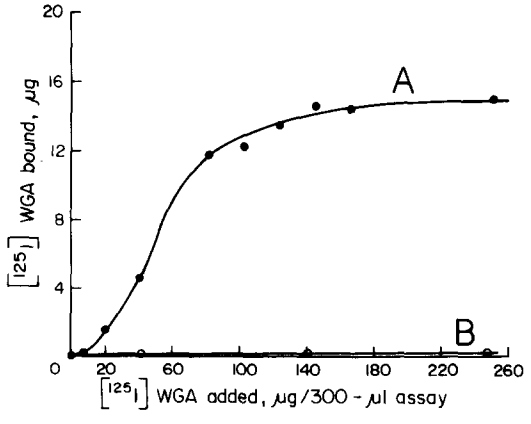

Fig. 1. High affinity binding of ^{125}I-labelled wheat germ agglutinin ([^{125}I]WGA) to liposomes bearing the integral membrane receptor glycoprotein, glycophorin. Curve A is for liposomes which also possessed a surface layer of serum albumin, while those used in deriving curve B had no such surface coat. Glycophorin was from N-type blood. Each assay tube contained $1.36 \cdot 10^6$ liposomes. Curve A liposomes had on average $9.3 \cdot 10^6$ high affinity sites * (see Footnote on p. 351).

Fluorescence studies

Experiments with fluorescein-labelled lectin independently confirmed the radiolabel results of the previous section. Liposomes without glycophorin did not bind fluorescent wheat germ agglutinin. Liposomes with glycophorin, but without a non-specific layer of serum albumin or Dextran, lost their fluorescence quite readily upon repeated washing. Fluorescent lectin could be displaced from high affinity binding sites (coated liposomes bearing receptor glycoprotein) by inhibitory sugar. Fig. 3 illustrates the stained appearance of liposomes bearing glycophorin as a receptor in addition to a surface layer of serum albumin. They were exposed to fluorescent wheat germ agglutinin at 23°C and then extensively washed in lectin-free buffers (containing serum albumin). Figs. 3A, B dimyristoylphosphatidylcholine/cholesterol liposomes which are fairly fluid at room temperature [28,29]. The appearance is reminiscent of 'receptor patching' in intact cells and presumably reflects headgroup cross-linking by the multivalent lectin. Receptor redistribution can be prevented by replacing dimyristoylphosphatidylcholine with the more rigid dipalmitoylphosphatidylcholine. The distribution of high affinity bound lectin then becomes diffuse (Figs. 3C, D). Clearly, high affin-

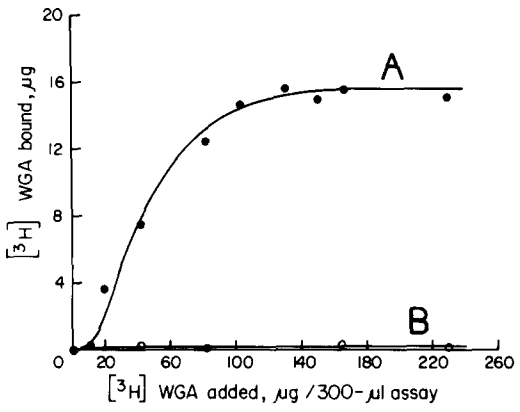

Fig. 2. High affinity binding of ^{3}H-labelled wheat germ agglutinin ([^{3}H]WGA) to glycophorin-bearing liposomes: in the presence of a surface coat of Dextran T500 (Curve A), and in the absence of a surface coat (Curve B). Glycophorin was from N-type blood. Each assay tube contained $1.4 \cdot 10^7$ liposomes. Curve A liposomes had on average $2.4 \cdot 10^6$ high affinity sites (see Footnote on p. 351).

ity binding does not depend upon massive lateral redistribution of receptors.

Temperature studies

The role of structural changes secondary to lectin binding in determining the phenomena described in this paper was further investigated as follows. While the lipid mixture, dimyristoylphosphatidylcholine/cholesterol, is semifluid at 22°C, it is rigid at 4°C [28,29]. Several experiments were designed to take advantage of this: given batches of liposomes were divided in half and tested at two different temperatures with all other conditions held constant. As Fig. 4 illustrates, the basic shape of the binding curves remained much the same. The only consistent difference at 4°C was that the number of bound lectin molecules was 30–50% smaller than at 22°C ($8.6 \cdot 10^5$ sites per liposome at 4°C vs. $2.3 \cdot 10^6$ sites per liposome at 22°C in the experiment shown *). Such an observation is consistent with the logic that high affinity binding is optimal if some receptor rearrangement is permitted; but clearly membrane fluidity is not an absolute requirement for this rearrangement. Hence, although gross lateral redistribution of receptors may increase the number of high affinity binding sites in our system, it is not necessary for high affinity binding.

Effect of the presence of a second family of receptor glycoproteins

The 2-chloroethanol/water mixture used for preparing lipid/protein dry films in this work dissolves a wide variety of membrane components. Hence one may theoretically increase the complexity of the bilayer structures by adding more than one species. This is potentially desirable since in recent years investigators have attached considerable potential importance to non-covalent interactions amongst membrane components. We have experimented with wheat germ agglutinin binding to membranes possessing the concanavalin A receptor glycoprotein from human erythrocytes in addition to glycophorin. The concanavalin A receptor is a subpopulation of 'band 3', an integral membrane glycoprotein thought to associate with glycophorin in the intact cell [30,31]. This second major erythrocyte membrane glycoprotein may be selectively isolated [12,13] and separately incorporated into lipid bilayer structures as described previously [13,24]. The resultant preparation is readily adapted to the present experiments by simply adding it to the 2-chloroethanol/water solution of lipid and glycophorin (see Materials and Methods) prior to solvent removal and rehydration. Liposomes containing glycoprotein receptors for wheat germ agglutinin and concanavalin A compete successfully for both lectins in hemagglutination inhibition assays.

Incorporation of a second receptor population into the glycophorin liposomes would be expected to increase the density of the liposome 'glycocalyx', and possibly to alter the conformation/behaviour of glycophorin via some specific association. Unfortunately, given the shortcomings of the liposome system (most notably poor control of liposome size and physical characteristics) any observed change in binding would have to be very large to be confidently considered significant. High affinity lectin binding curves for these more complex liposomes are shown in Fig. 5.

* The average numbers of high affinity lectin binding sites per liposome were calculated from Scatchard plots of binding curve data. K_a for high affinity lectin binding to glycophorin in liposomes and intact cells was in the range of 10^5 to $10^6\ M^{-1}$.

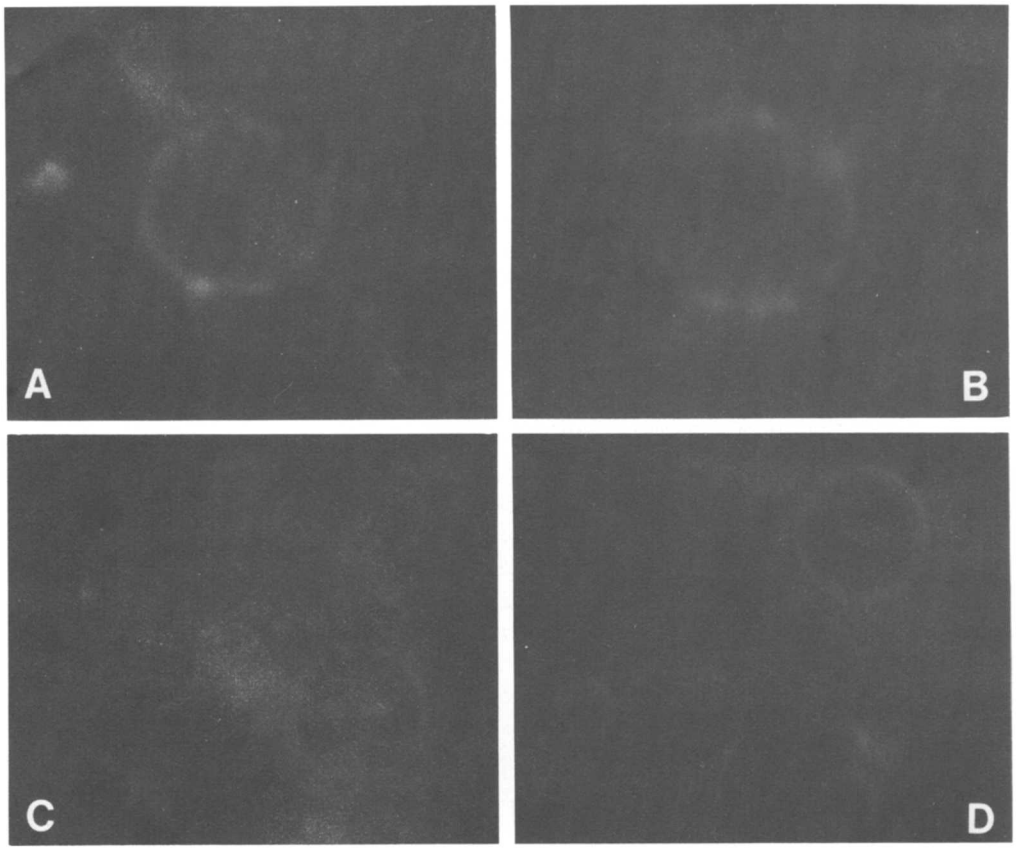

Fig. 3. Fluorescein-wheat germ agglutinin binding to liposomes bearing glycophorin as a receptor plus a surface layer of serum albumin. Without the latter surface coat, lectin bound to glycophorin was removed by subsequent washing. In A, and B the liposomes were fluid (4:1.5 mole ratio dimyristoylphosphatidylcholine/cholesterol); in C and D, they were rigid (4:1.5 mole ratio dipalmitoylphosphatidylcholine/cholesterol). Temperature, 23°C. Binding was reversed by *N*-acetylglucosamine.

The basic nature of wheat germ agglutinin binding to glycophorin was unchanged by the presence of concanavalin A receptor when the liposomes were coated with a surface layer of adsorbed serum albumin (Fig. 5, A). However, in three out of four separate complete experiments with uncoated liposomes, there were also significant numbers of high affinity wheat germ agglutinin binding sites (sites which didn't exist with glycophorin on its own as demonstrated earlier), see Fig. 5, B. Presumably this small population of sites not requiring a layer of serum albumin or dextran for high affinity binding is attributable to the presence of the second glycoprotein. That the concanavalin A receptor by itself does not bind wheat germ agglutinin was readily confirmed by control experiments with liposomes containing it but not glycophorin. These results are consistent with the mechanisms described already whereby nonspecific headgroup interactions could influence ligand binding affinity of an existing receptor headgroup; although it is also possible that they reflect some specific interaction between the two families of glycoproteins. We have previously claimed an interaction between glycophorin and the concanavalin A receptor in model membranes [24].

Relevance to intact cells

Although the erythrocyte has lost its functional responsiveness to specific external contacts, glycophorin is considered a classic model for integral membrane glycoproteins. It is our claim that many receptors at the eucaryotic cell surface may be importantly influenced in their affinity for ligands

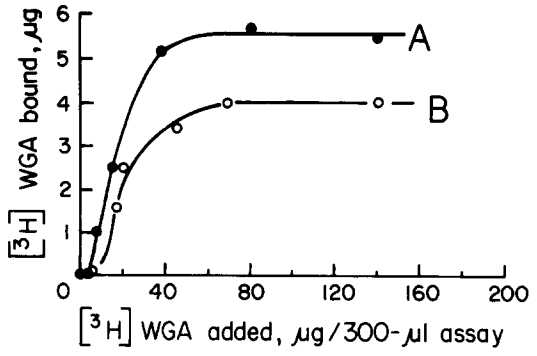

Fig. 4. Temperature effects on binding of ^{3}H-labelled wheat germ agglutinin ([^{3}H]WGA) to glycophorin-bearing liposomes coated with serum albumin (uncoated liposomes did not bind wheat germ agglutinin at either temperature). Indentical liposomes were handled at 22°C (Curve A) or 4°C (Curve B). Glycophorin was from N-type blood. Each assay tube contained $1.5 \cdot 10^{6}$ liposomes. There were $2.3 \cdot 10^{6}$ high affinity binding sites per liposome at 22°C and $8.6 \cdot 10^{5}$ at 4°C (see Footnote on p. 351).

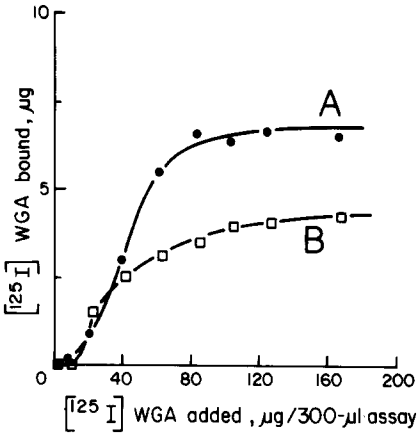

Fig. 6. Binding of ^{125}I-labelled wheat germ agglutinin ([^{125}I]WGA) to intact human erythrocytes in the presence (A) and absence (B) of a surface coat of serum albumin. Blood was M-type. Each assay tube contained $3.3 \cdot 10^{7}$ cells. The number of wheat germ agglutinin binding sites per erythrocyte was $4.0 \cdot 10^{5}$ in the presence and $2.2 \cdot 10^{5}$ in the absence of the serum albumin coat (see Footnote on p. 351).

by interactions with other surface structures. The layer of nonspecific protein (serum albumin) or polysaccharide (dextran) applied to our liposomes has its counterpart in all eucaryotic cells (the glycocalyx, a region populated by oligosaccharide

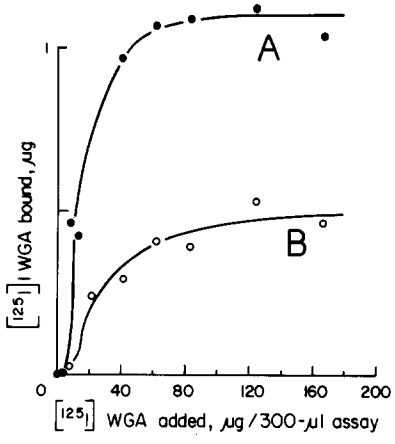

Fig. 5. Binding of ^{125}I-labelled wheat germ agglutinin ([^{125}I]WGA) to liposomes bearing a second family of receptor glycoproteins ('band 3') in addition to glycophorin (1 : 1 mol ratio). The total lipid/protein ratio was kept the same as in the previous figures (2 : 1, by weight). Curves A and B are for liposomes with and without a surface layer of serum albumin, respectively. Glycophorin was from N-type blood. Each assay tube contained $1.3 \cdot 10^{6}$ liposomes. Curve A liposomes had on average $6.0 \cdot 10^{5}$ high affinity sites (see Footnote on p. 351). Curve B liposomes had 50% fewer sites.

headgroups, peripheral proteins, and material adsorbed from the surrounding interstitial fluid or growth medium). Hence the forces acting upon the receptors in this system are not peculiar to an artificial membrane.

Fig. 6 shows, for purposes of comparison with our model system, wheat germ agglutinin binding curves for intact erythrocytes. Cells were collected fresh in citrate/saline to avoid contamination with macromolecules such as heparin, and then used within hours. They were washed extensively with phosphate-buffered saline and split into identical batches, of which one was coated with serum albumin. Even in intact cells the presence of a layer of exogenous macromolecules influences lectin binding. In this case the number of high affinity sites is $4.0 \cdot 10^{5}$ in the presence of albumin and $2.2 \cdot 10^{5}$ in its absence. However, it is noteworthy that, while high affinity wheat germ agglutinin binding to glycophorin on its own (i.e. in our model membranes) is critically dependent upon the presence of an added surface coat of macromolecules, binding to intact membranes is not. This may be explained by the fact that, as mentioned earlier, intact cells still retain most of their glycocalyx, including some adsorbed species, after washing.

Acknowledgements

This work was supported by grants from the National Cancer Institute and the Medical Research Council of Canada.

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