Glycophorin A interferes in the agglutination of human erythrocytes by concanavalin A

Explanation of the requirement for enzymic predigestion

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Human erythrocytes become agglutinable with concanavalin A (Con A) after treatment with various proteinases or neuraminidase. The extent of agglutinability achieved with different enzymes is, however, different: Pronase, papain, trypsin, neuraminidase and chymotrypsin enhance the agglutinability in decreasing order, the last being barely effective. The actions of the enzymes on band 3, the Con A receptor, do not correlate with their abilities to increase the agglutinability: Pronase, papain and chymotrypsin cleave the protein, but not trypsin or neuraminidase. No significant differences are found in the number of Con A-binding sites or the affinities for the lectin between the normal and trypsin- or Pronase-treated cells. Thus the receptor does not seem to play a role in determining the Con A-agglutinability of erythrocytes. On the other hand, the cleavage of glycophorins, especially glycophorin A, and the release of sialic acid (in the peptide-bound form) are well-correlated with the enhancement in agglutination after the action of proteinases. The release of sialic acid by graded neuraminidase digestion and the increase in Con A-agglutinability show a correlation coefficient of 0.88. The major inhibitory role of glycophorin A in the process is indicated by the agglutination of En(a) heterozygous erythrocytes; the cells, known to bear about 50% glycophorin A molecules in their membrane, are agglutinated approximately half as well without proteolysis as are the trypsin-treated cells. Possible mechanisms by which glycophorin A could affect Con A-mediated agglutination are discussed.

INTRODUCTION

As a consequence of alterations occurring in their plasma membrane, tumour cells and cells transformed in vitro acquire a high agglutinability with plant lectins [1]. Extensive studies undertaken to elucidate the basis of the high lectin-agglutinability have identified several factors that influence the agglutinability of cells (reviewed in ref. [2]). The principal factors identified are: the number and characteristics of the lectin receptor, the cell-surface charge as determined by sialic acid residues, the mobility of the lectin–receptor complex in the membrane and the mobility-modulating agents such as cytoskeletal elements, and cellular deformability. Despite the recognition of these factors over a decade ago, we still lack details of the mechanism(s) by which they affect agglutination; in fact, the evidence implicating the role of some of the factors, e.g. the lectin receptor, is quite equivocal. The major reason for the lack of progress may be ascribed to paucity of information on the structure of the membrane of the cells used in the studies.

The best characterized membrane available at present is that of the human erythrocyte. Erythrocytes do not show shear-resistant agglutination with Con A, but acquire the agglutinability after treatment with a proteolytic enzyme [3], as do normal nucleated cells [4]. Thus normal and proteinase-treated erythrocytes offer an adequate model system to evaluate in detail the role of factors that influence agglutination. Additionally, significant information already exists on the Con A receptor (band 3 protein) [5], the negative charge-bearing glycophorins [6], the interaction between band 3 and glycophorin A [7,8] and the interaction between the receptor and the membrane skeleton [9]. The last is especially relevant, since it retards the mobility of the receptor in the membrane. Also, the erythrocyte is a highly deformable cell, and the factors affecting deformability are understood in reasonable detail [10].

In the present paper we analyse the roles of the receptor and the cell-surface charge in the agglutinability of erythrocytes with Con A. An increase in the number of lectin receptors [11,12], not universally found [13–16], is not likely to be of importance, since only a small fraction (~5%) of total receptors participates in agglutination [16,17]. However, the exposure of a qualitatively different receptor [18,19] on transformation [11,12] or enzymic treatment [20] may have different consequences. As far as the surface charge is concerned, no consistent increase or decrease on tumour cells is observed [21]. However, since increased sialylation of N-linked oligosaccharides of membrane proteins is consistently detected in transformed cells [22], the overall charge could be misleading. The importance of charge in lectin-mediated agglutination is indicated by the fact that neuraminidase treatment of normal and transformed cells increases their agglutinability [23–25].

Abbreviations used: Con A, Concanavalin A; MeMan, α-methyl d-mannopyranoside; TBS, Tris-buffered saline (0.01 M-Tris/HCl, pH 7.4, containing 0.15 M-NaCl); Tos-Lys-CH₂Cl, tosyl-lysylchloromethane ("TLCK"); Tos-Phe-CH₂Cl, tosylphenylalanilylchloromethane ("TPCK"); NeuAc, N-acetyleneuraminic acid.

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MATERIALS AND METHODS

Materials

Trypsin (twice crystallized), Tos-Phe-CH₂Cl-treated trypsin, papain (crystallized), Pronase ('protease'), chymotrypsin (crystalline), Tos-Lys-CH₂Cl-treated chymotrypsin, neuraminidase (type VI) from *Clostridium perfringens*, lactoperoxidase (from bovine milk), crystalline bovine serum albumin, MeMan, N-acetylneuraminic acid, Tos-Lys-CH₂Cl, Coomassie Brilliant Blue, p-rosaniline, Tris and SDS were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Con A was obtained either from Sigma (in which case it was type IV) or from Pharmacia Fine Chemicals, Uppsala, Sweden, which also supplied Sephadex G-75. Acrylamide was a product of Koch–Light Laboratories, Colnbrook, Bucks., U.K., and N,N'-methylenebisacrylamide and N,N,N'-tetramethylethylene diamine were products of Eastman Organic Chemicals, Rochester, NY, U.S.A. Carrier-free Na¹²⁵I was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were of reagent or better grade and were obtained from BDH, Bombay, India, or Sarabhai M. Chemicals, Baroda, India. Chymotrypsin (250 μg/ml) was treated with 100 μM-Tos-Lys-CH₂Cl for more than 30 min to inactivate trypsin [26].

Cells

Blood, irrespective of the group, was collected from healthy volunteers in acid/citrate/dextrose and used fresh. Cells were centrifuged at 1000 g for 5 min in the cold and washed four times in at least 10 vol. of TBS. After each centrifugation, a portion from the top of the cell pellet was discarded to ensure maximal removal of leucocytes.

Liquid-N₂-frozen En(a) heterozygous erythrocytes were obtained through the courtesy of Dr. H. M. Bhatia, Institute of Immunohaematology, Bombay, India. These, and similarly frozen normal cells, were thawed and washed as above.

Enzymic treatment of erythrocytes

Washed erythrocytes were suspended in 2 vol. of the enzyme solution and incubated at 37 °C. In order to establish the optimal conditions of their action on the Con A-agglutinability of cells, different concentrations of the enzyme were used, keeping the time of incubation constant at 90 min. The minimum concentration beyond which agglutination did not increase having been determined, cells were incubated with this enzyme concentration for different time periods. Thus the optimal conditions with respect to the concentration and time of incubation were established for trypsin, chymotrypsin, Pronase, papain and neuraminidase. The enzymes were dissolved in TBS, except neuraminidase, which was taken up in 0.1 M-Tris/maleate/0.11 M-NaCl, pH 5.6. After incubation, the cells were washed four times in large volumes of TBS in the cold.

Isolation of membranes

Buffer- and enzyme-incubated erythrocytes were lysed in cold 10 mM-Tris/HC1, pH 7.4. The membranes were sedimented at 22000 g for 15 min at 4 °C and washed in the same buffer three or four times to yield white ghosts.

Agglutination of cells

A 0.4% (v/v) suspension of erythrocytes was mixed with an equal volume of freshly prepared solution of Con A in TBS. Along with a range of lectin concentrations, a control was always included that contained Con A (250 μg/ml) and 50 mM-MeMan, with appropriate adjustments made in the buffer to maintain osmolarity. After incubation at 37 °C for 60 min, the tubes were tapped several times and the free cells and those in two-cell aggregates were counted under the microscope with a haemocytometer. The extent of agglutination was calculated as:

\[
100 - \left( \frac{\text{No. of unagglutinated cells}}{\text{No. of input cells}} \right)
\]

SDS/polyacrylamide-gel electrophoresis

The membranes were dissolved in a solution containing SDS and mercaptoethanol and electrophoresed in polyacrylamide gel in the presence of the detergent as described by Laemml [27]. Gradient (5–15%, w/w) or homogeneous polyacrylamide-gel slabs were used. Proteins were stained with Coomassie Blue, and glycoproteins were stained with the periodic acid–Schiff reagent.

Radioiodination of Con A and binding to cells

Con A was iodinated with ¹²⁵I essentially as described by Phillips et al. [28], Con A adsorbed on Sephadex G-75 beads being used. The specific radioactivity of the Con A obtained was 48000 c.p.m./μg of protein. With 10% (w/v) trichloroacetic acid, 98.6% of the radioactivity was precipitated, and 90–98% of the Con A binding to erythrocytes was inhibited in the presence of 50 mM-MeMan. For binding studies, 1 × 10⁷ cells in 0.1 ml of TBS were placed in silicone-treated tubes and incubated with ¹²⁵I-Con A in the concentration range 0–1000 μg/ml. After 60 min (by which time the binding was complete; results not shown) at 37 °C, the suspension was transferred to 0.3 ml of 5% bovine serum albumin dissolved in TBS in polypropylene tubes. After centrifugation at 13000 g for 2 min, the tips of the tubes containing the cells were cut off and counted for radioactivity in a γ-radiation counter.

Analytical methods

Protein-bound sialic acid was hydrolysed by heating in 50 mM-H₂SO₄ at 80 °C for 1 h and was determined as described by Warren [29]. Protein determination was by the method of Lowry et al. [30], with bovine serum albumin as the standard. SDS was included in the samples to the extent of 1% (final concn.).

RESULTS

Enhancement of Con A-agglutinability by enzymes

The optimal conditions of enzymic treatment, with respect to the ability to enhance Con A-agglutinability of erythrocytes, were found to be as follows: Pronase, 10 μg/ml; papain, 50 μg/ml; trypsin, 100 μg/ml; chymotrypsin, 250 μg/ml; and neuraminidase 0.01 unit (as defined by the supplier)/ml. An incubation period of 60 min was used for all enzymes except neuraminidase, for which the incubation was 45 min.

The effect of various enzymes on Con A-agglutinability of erythrocytes at various concentrations of the lectin is
shown in Fig. 1. Pronase and papain were the most effective enzymes, followed by trypsin and neuraminidase (which showed roughly equal effects in different experiments), whereas chymotrypsin was the least effective. Normal cells incubated for 60 min in TBS failed to show any agglutination. Treatment of cells with Tos-Phe-CH₂Cl-treated trypsin had an effect identical with that shown by trypsin. Similarly, inclusion of albumin at 5 mg/ml during the incubation of cells with neuraminidase did not alter the agglutinability produced by neuraminidase alone. The effect of chymotrypsin, however, was substantially diminished when the enzyme was pretreated with Tos-Lys-CH₂Cl, a trypsin inhibitor. Thus the enzymes differ considerably, even when used under the optimal conditions of their action, in their abilities to bring about Con A-agglutinability in erythrocytes.

The action of enzymes on band 3

Pronase and Tos-Lys-CH₂Cl-treated chymotrypsin substantially degraded band 3; papain degraded it partially, whereas Tos-Phe-CH₂Cl-treated trypsin and neuraminidase (in the presence of albumin) had no effect (Fig. 2a). When trypsin (not treated with Tos-Phe-CH₂Cl) or neuraminidase (without albumin) were used, a small, but detectable, effect on band 3 degradation was observed (results not shown). The effects of the enzymes on band 3 are consistent with reports in the literature (e.g. [31–33]).

Thus there seems to be no correlation between band 3 degradation/intactness and Con A-agglutinability.

Con A binding to normal and proteinase-treated cells

The number of Con A-binding sites/cell and the affinity constants for the interaction between the lectin
Table 1. Con A-binding characteristics of normal, trypsin- and Pronase-treated erythrocytes

Normal, trypsin- and Pronase-treated erythrocytes were incubated with 0–1000 µg of ¹²⁵I-labelled Con A/µl. The cell-bound radioactivity was sedimented through an albumin cushion and counted. The binding was found to be saturated at and above 500 µg of Con A/µl. The data were plotted as described by Scatchard [34], where a linear relationship between the ‘bound’ and the ‘bound/free’ radioactivities was observed. Regression lines were drawn by using the method of least squares, and the number of binding sites and the affinity constants were calculated.

The values represent mean ± S.D. for three experiments. Significance of the difference between normal and trypsin/Pronase-treated cells: *P > 0.5, **P > 0.2

<table>
<thead>
<tr>
<th>Cells</th>
<th>10⁻⁴ x Number of sites</th>
<th>10⁻⁴ x Affinity constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.37 ± 0.22</td>
<td>1.20 ± 0.50</td>
</tr>
<tr>
<td>Trypsin-treated</td>
<td>1.15 ± 0.32*</td>
<td>0.94 ± 0.40*</td>
</tr>
<tr>
<td>Pronase-treated</td>
<td>1.07 ± 0.16**</td>
<td>0.84 ± 0.35*</td>
</tr>
</tbody>
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and the receptor(s) on the normal, trypsin-treated and Pronase-treated cells are presented in Table 1. There are only small, statistically insignificant, differences in the number of receptor sites between the agglutinable and unagglutinable cells. Likewise, the binding affinities of the normal and the two proteinase-treated cells for Con A are not significantly different. The values of the binding sites/cell and of the affinity constants for normal cells obtained in these studies compare well with those reported in the literature for Con A binding to normal cells (see, e.g., refs. [16,35]).

**Effect on glycophorins**

When the effect of various enzymes on the glycophorin profile was analysed (Fig. 2b), papain was found to degrade all glycophorins; Pronase was nearly as effective, except that, in some experiments, glycophorin C was left apparently intact. Tos-Phe-CH₂Cl-treated trypsin degraded glycophorin A (dimer and monomer) and C, whereas neuraminidase treatment led to a diffused appearance of all the glycophorins on the acrylamide gel, indicating the removal of sialic acid from them. Of the enzymes used, Tos-Lys-CH₂Cl-treated chymotrypsin had the least effect: glycophorin A (dimer) remained partially undegraded, with the rest being degraded to two fragments possessing somewhat higher mobility on SDS/polyacrylamide-gel electrophoresis, whereas the other species of glycophorin appeared slightly, or not at all, affected. The same results were obtained with commercial Tos-Lys-CH₂Cl-treated chymotrypsin and the chymotrypsin treated with Tos-Lys-CH₂Cl in the laboratory [26].

Thus Pronase, papain and trypsin, each of which has a large effect on Con A-agglutinability, degrade glycophorins substantially. Neuraminidase, which also has a considerable effect on the agglutinability, appears to affect all the species, as indicated by the diffused nature of the bands on SDS/polyacrylamide-gel electrophoresis. Tos-Lys-CH₂Cl-treated chymotrypsin, the least effective of the enzymes in bringing about agglutinability, has the minimum effect on glycophorins.

**Fig. 3. Relationship between the removal of sialic acid and Con A-agglutinability of cells treated with various enzymes**

(a) Erythrocytes were treated with either buffered saline or enzymes as described in the legend to Fig. 1. After incubation, the cells were centrifuged and the supernatants were separated. The cells were washed and their agglutinability was determined at 100 µg of Con A/µl. The proteinase digestes were made 50 mM with respect to H₂SO₄ and heated at 80 °C for 1 h. Sialic acid was determined in the hydrolysates and neuraminidase digest. The symbols are the same as those used in Fig. 1. (b) Washed erythrocytes were incubated in 2 vol. of neuraminidase (0.01 unit/ml) at 37 °C. Samples were drawn at different times and cooled. The cells were sedimented and the supernatant was separated. Con A-agglutinability of the cells (△) (at 100 µg/ml) was determined after they had been washed. Sialic acid was determined in the supernatant. Membranes from an equivalent number of cells were heated at 80 °C for 1 h in 50 mM-H₂SO₄ to liberate all the sialic acid. The sialic acid released by the neuraminidase action is expressed as percentage of the total sialic acid (△).
The actions of various proteinases on glycophorins observed here are consistent with those reported by other workers (see, e.g., ref. [31]). The chymotrypsin effect is a matter of some controversy: the early studies show no effect, whereas Dzandu et al. [36] have recently shown slight degradation by di-isopropyl fluorophosphate-treated chymotrypsin. In our study, also, a slight effect is observed with Tos-Lys-CH₂Cl-treated chymotrypsin.

**Removal of sialic acid and agglutinability**

Since the enzymes that have much greater effect on Con A-agglutinability also have greater degradative action on the sialic acid-rich glycophorins, and since neuraminidase also brings about agglutinability, it appeared possible that the differential abilities of the proteinases to affect agglutinability might be related to the amount of sialic acid (NeuAc) released by them from the erythrocyte surface. To test this, the Con A-agglutinability of the cells digested with each of the various enzymes was compared with the amount of sialic acid released in the digests. The proteinases would cleave NeuAc-containing glycopeptides, whereas neuraminidase would liberate sialic acid in the free form. The proteinase digests were hydrolysed and the released NeuAC was estimated. A proportionality between the removal of NeuAc and the increase in agglutinability was observed (Fig. 3a), the correlation coefficient for which was 0.88.

In order to assess further the relationship between the release of sialic acid and agglutinability, erythrocytes were digested with neuraminidase for different periods of time to remove graded amounts of sialic acid. The agglutinability pattern of the digested cells was nearly superimposable on the pattern of sialic acid released from them (Fig. 3b). When the release of NeuAc and the rise in agglutinability were compared directly, a correlation coefficient (again) of 0.88 was obtained, suggesting a close dependence of agglutinability on the removal of sialic acid from the membrane.

**Con A-agglutination of En(a) heterozygous cells**

The En(a−) erythrocytes lack glycophorin A in their membrane. The cells from heterozygous subjects are known to contain ~ 50% of the normal amounts of glycophorin A [37,38]. The En(a) heterozygous cells were found to exhibit about 45% agglutinability without any enzymic treatment (Fig. 4b). On treatment with trypsin, the cells acquired as much agglutinability as the trypsin-treated normal cells (Fig. 4a).

**DISCUSSION**

Pronase, papain and trypsin, the enzymes active in bringing about Con A-agglutinability in erythrocytes, differ in their effects on band 3. Pronase and papain each cleave the protein, whereas trypsin has no apparent effect. On the other hand, Tos-Lys-CH₂Cl-treated chymotrypsin, which cleaves band 3, hardly enhances

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![Fig. 4. Con A-agglutinability of En(a) heterozygous erythrocytes](image)

Liquid-N₂-frozen normal and En(a) heterozygous cells were thawed, washed, and a portion of each one of them was treated with trypsin (100 µg/ml). The cells were washed after 1 h at 37 °C. The agglutination of untreated and trypsin-treated normal and En(a) heterozygous cells was determined at different concentrations of Con A.
agglutinability. Thus the agglutinability of cells is not related to intactness or degradation of the receptor after proteolysis. The effect of neuraminidase on agglutinability also emphasizes the same point, since band 3 contains very little, if any, sialic acid [39]. Pronase and papain cleave band 3 in its exofacial domain with the fragments still remaining united and the oligosaccharide chain unaffected (see, e.g. [33]). Thus, despite proteolysis, there is no loss of the Con A receptor. The existence of almost the same number of Con A-binding sites on the normal, trypsin- and Pronase-treated cells, and nearly the same affinities with which the lectin binds the two categories of cells, suggest that no new receptors are exposed after proteolysis. Of the two potential receptors in the membrane besides band 3, the N-linked oligosaccharide of glycophorin A [40] is unlikely to be available, owing to degradation of glycophorin in the agglutinable cells (Fig. 2b; also see below), and glucosyl- and lactosyl-ceramide [41] exposure, besides adding to the number of available receptor molecules, might be expected to exhibit a binding affinity different from that of band 3. In a study in which erythrocyte ghosts were extensively digested with Pronase [42], the resultant vesicles devoid of any intramembranous particles were found to be unagglutinable by Con A. Thus the glycolipid receptors appear incapable of bringing about agglutination with the lectin. It can therefore be concluded that agglutinability can arise in the cells even in the absence of any alterations in the receptor molecule available on the normal cell.

That enzymatic degradation of glycophorins parallels the rise in Con A-mediated agglutination (Fig. 2b) and that a quantitative relationship (r = 0.88) exists between the removal of sialic acid and the increase in agglutinability (Figs. 3a and 3b) suggests a negative role of glycophorin-associated sialic acid in the determination of Con A-agglutinability of erythrocytes. The roughly 50% agglutination of En(a-) heterozygous cells (Fig. 4), known to possess approx. 50% of the normal number of glycophorin A molecules in the membrane [37,38], underlines the importance of this glycophorin in Con A-induced agglutination. Previously, Bird & Wingham [43] and Tanner & Anstee [44] also observed that En(a-) cells possess a higher agglutinability with several lectins, including Con A [44]. The glycosylation of band 3 has been reported to be increased in En(a-) cells [37,38]. This could slightly affect the affinity of the receptor for Con A [45]. However, in view of the enhanced agglutinability of the cells with lectins of diverse specificities, it is unlikely that this could be the cause of higher agglutinability of En(a-) cells with Con A.

From the results presented here it is clear that the increase in Con A-agglutinability is related to a decrease in the charge borne by glycophorin A molecules. The results of Schnebli and co-workers [3,16], indicating an inverse correlation between the cell-surface charge (measured by electrophoretic mobility) and Con A-agglutinability of enzyme-treated cells, and the enhancing effect of basic proteins on agglutinability, are consistent with this conclusion. How can glycophorin A interfere in the process of agglutination? There is evidence for the association of glycophorin A with band 3, the Con A receptor, in the membrane [46,47]. For shear-resistant agglutination, it is generally found that the agglutinating cells interact with each other via pre-aggregated lectin–receptor complexes, forming multiple bridges between them [2]. Clearly, an association with a negatively charged protein would interfere in the aggregation of receptor molecules, as the associated negative charges would tend to repel each other. The removal of the charge-bearing molecule would remove the repulsive barrier. It is known that the intramembranous particles containing band 3 and glycophorin A [48] aggregate easily in En(a-) cells [49] and in the membranes pre-treated with neuraminidase [50]. An additional important effect that the charge-bearing molecules can have on agglutination is in preventing a close approach [within 25 nm (25 Å)] between the cells participating in agglutination. This is "initially impossible because of the potential energy repulsive barriers" [51]. In order to overcome these, "sensing of surface regions having lower than average charge density would be needed" [21]. Proteolysis or neuraminidase treatment can help to provide such regions. This manner of influence of charge on agglutination is likely to be important for all lectins whether or not glycophorin A is associated with the respective receptors.

Although glycophorin-like molecules do exist in the membranes of some nucleated cells [52], they are probably not universally distributed. It is likely that other molecules rich in sialic acid [53,54] could play a role similar to glycophorin in the process of agglutination. The treatment of cells with proteinase or neuraminidase can aid in receptor aggregation and/or cell–cell interaction. The proteinase-treated normal cells, like transformed cells, show the ability to aggregate their lectin–receptor complexes [2]. It is interesting to note that some normal cell types which show high agglutination with lectins exhibit surface proteolysis [55].

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Glycophorin and the agglutination of erythrocytes by concanavalin A


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