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Neutral polymers elicit, and antibodies to spectrin, band 4.1 protein and cytoplasmic domain of band 3 protein inhibit the concanavalin A-mediated agglutination of human erythrocytes

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Abstract

Concanavalin A (Con A) is known to agglutinate human erythrocytes if the cells are pre-treated with a proteinase or neuraminidase. We report that untreated cells can also be made to agglutinate with the lectin if the lectin-bound cells are treated with anti-Con A antibodies, or if a neutral polymer such as serum albumin, polyvinylpyrrolidone or Ficoll is added. Thus, Con A falls in the category of 'incomplete' lectins. The polymer induces Con A-agglutinability without altering the receptor number, or deformability of the cells. If the polymer is sequestered within erythrocyte ghosts, Con A is unable to agglutinate them; but the presence of the polymer only on the outer surface (as in intact cells) or on both the surfaces permits agglutinability. Thus, the site of the polymer effect resides on the outer surface of the membrane. The polymer, however, is unable to induce agglutinability in erythrocyte vesicles, whose membrane lacks skeletal proteins. The result suggests a positive role for the membrane skeleton in the process of agglutination brought about by the polymer, as is true also for the agglutination of proteinase-treated cells. In order to obtain detailed information on the proteins participating in agglutination, monospecific antibodies to spectrins, band 4.1 protein, ankyrin and the cytoplasmic domain of band 3 protein were internalized in erythrocytes. It is found that anti-spectrin and anti-band 3 cytoplasmic domain, but not their Fab's, inhibit the Con A-mediated agglutinability partially, and anti-4.1 antibodies, as well as the Fab's, inhibit the agglutinability substantially. Anti-ankyrin, however, was without any effect. The results confirm a positive role for the membrane skeleton in the Con A-mediated agglutination of normal erythrocytes in the presence of a neutral polymer, or in proteinase treated cells. We also provide evidence for requirement of Mg-ATP in the agglutination process.

Keywords: Incomplete lectin; Lectin; Antibody; Incomplete antibody; Membrane skeleton; Erythrocyte membrane; Concanavalin A; (Human erythrocyte)

1. Introduction

Antibodies and plant lectins are called 'complete' or 'incomplete' depending on whether they can directly agglutinate washed human erythrocytes or not [1,2]. The incomplete antibodies/lectins bind to erythrocytes, but fail to agglutinate them unless pre-treated with a proteinase or further treated with anti-lectin antibodies or a neutral polymer. Concanavalin A, perhaps the best studied and the most widely used lectin, binds to erythrocytes but requires prior proteolysis of erythrocytes to agglutinate them. We show here that the untreated cells can also be agglutinated by Con A on the addition of anti-Con A antibodies, or in the presence of BSA, Ficoll or PVP. This places Con A in the category of incomplete lectins.

In our earlier work we found that the pre-treatment of red cells with a proteinase causes the removal of surface charge in the form of glycophorin-associated sialic acid [3]. The agglutinability was found to rise in proportion to the removal sialic acid in peptide-bound form. Neuraminidase-treatment also had a similar effect on the increase in agglutinability. Also, contrary to earlier belief, the membrane skeleton was found to be necessary for agglutination to occur [4]. We now provide confirmation of the role of the membrane skeleton in the process of agglutination by showing that sequestration of antibodies to skeletal proteins inhibits the ability of Con A to agglutinate the cells.

Abbreviations: BSA, bovine serum albumin; Con A, concanavalin A; methyl-α-man, methyl-α-D-mannopyranoside; PVP, polyvinylpyrrolidone; RCA, *Ricinus communis* agglutinin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; WGA, wheat germ agglutinin.

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How does the neutral polymer bring about agglutinability in the cells in the presence of intact glycophorin? And, does the polymer-induced agglutinability also depend on the membrane skeleton? Our results show that a factor on the outer cell surface plays an important role, and the membrane skeletal proteins also participate in the polymer-aided agglutination. The results, additionally, show that physiological concentrations of Mg-ATP are necessary for complete agglutination to occur.

2. Materials and methods

2.1. Materials

The fine chemicals were purchased from Sigma, St. Louis, MO, USA, except the following: Con A, Sepharose 4B, protein A-Sepharose, DEAE-Sephadex, Sephadex G-75 and Ficoll, which were products of Pharmacia-LKB, Uppsala, Sweden. Nitrocellulose membrane was purchased from Radiochemical Centre, Amersham, Bucks., UK. Freund's adjuvant was obtained from Difco Laboratories, Detroit, MI, USA. The laboratory reagents were obtained from local sources. Normal rabbit serum was obtained from an unimmunized animal from our Institute animal house.

Several grades of (human and bovine) serum albumins were tried. Only those that did not form visible precipitate with Con A, and lead to the formation of rouleaux with an erythrocyte suspension of 10% hematocrit were used. These were either fatty acid-free, or were made so by the procedure of Chen [5].

2.2. Methods

The collection of blood, washing of erythrocytes, determination of lectin-mediated agglutination of red cells [3] and vesicles [4], determination of deformability [6], and iodination of Con A, and its binding to red cells [4] have been described earlier as indicated. Ghosts were prepared according to Bennett [7], or Hanahan and Ekholm [8] and resealed. The large membrane skeleton-free vesicles were obtained as described by Leonard and Ohki [9], and the small vesicles according to Lutz et al. [10]. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [11]. Protein was estimated according to Peterson [12] using BSA as the standard.

2.3. Pronase / trypsin treatment of erythrocytes

Five times washed erythrocytes were incubated with 2 vol. of 50 μg/ml Pronase or 200 μg/ml TPCK-trypsin in 0.01 M Tris-HCl/0.15 M NaCl (pH 7.4) (buffer 1) at 37° C for 1 h. The cells were washed four times in TBS in the cold. The washing was adequate to remove the proteinases, as the ghosts prepared from these cells had intact skeletal proteins; and also, the addition of TLCK to the cells after trypsin treatment and before washing, did not alter any membrane proteins, or affect the subsequent results of agglutinability.

2.4. Preparation of monospecific antibodies

The collection of human blood, washing of red cells and the preparation of ghosts were done essentially as described by Bennett [7]. After washing the ghosts in buffer 1 to remove band 6 protein [13], spectrin was extracted by incubation in low ionic strength medium and purified by gel filtration chromatography [7]. Ankyrin was extracted from inside-out vesicles by incubation in 1 M KCl. The supernatant was dialyzed and passed through DEAE-Sepharose according to Tyler et al. [14]. The vesicular pellet was then incubated in 10 vol. of 3 mM *p*-chloromercuriphenylsulfonic acid in 5 mM phosphate buffer, pH 8.5, for 30 min at 37° C to remove band 4.2 [15]. The cytoplasmic domain of band 3 was cleaved by α-chymotrypsin at 0° C and purified on DEAE-Sepharose [7]. Protein 4.1 was purified from Triton shells of erythrocytes by the method of Ohanian and Gratzer [16]. The purity of the isolated proteins was checked by SDS-PAGE [11]. The purified proteins were coupled to CNBr-activated Sepharose [17].

The antiserum was obtained by injecting a rabbit subcutaneously at several sites with 2 mg protein-equivalent of ghosts emulsified in Freund's complete adjuvant. After 10 days a booster of 1 mg ghost protein in the incomplete adjuvant was given, and blood was collected after 10 more days. The rabbit was intermittently boosted, and bled similarly. In order to increase the titer of anti-ankyrin and anti-4.1 protein, the same rabbit was later injected with the proteins of 1 M KCl-extract of inside-out vesicles [14]. The antisera were stored at −20° C after addition of 0.02% azide. Anti-4.1 protein was also obtained by injecting another rabbit with 100 μg of the purified protein in complete adjuvant, followed 2 weeks later with 80 μg protein in the incomplete adjuvant.

The monospecific antibodies were prepared by passing the antiserum against whole ghosts serially through 10 ml columns of spectrin-Sepharose (2 mg protein/ml), ankyrin-Sepharose (0.4 mg/ml), protein 4.1-Sepharose (0.1 mg protein/ml), and Sepharose coupled to the cytoplasmic domain of band 3 (0.6 mg/ml). The columns were separated and washed extensively with 0.01 M Tris-HCl/0.5 M NaCl (pH 7.4). The monospecific antibodies were eluted with 4 M $MgCl_2$ and dialyzed against distilled water, the pH of which was adjusted to 7.4 with ammonium bicarbonate. After measuring the protein concentration (A_{280nm} = 13.5 [18]), aliquots were lyophilized and stored at −20° C.

Antibodies to protein 4.1 were also obtained by absorbing the anti-4.1 antibodies to 80–100 μg of 4.1 protein bound to nitrocellulose strips. The latter were prepared by

electrophoretic transfer of purified 4.1 protein from 8% SDS-polyacrylamide gels run according to Laemmli [11], and carefully cutting out the 4.1a and b region after staining with Ponceau S. Purified spectrin was also electrophoresed and blotted on nitrocellulose. The nitrocellulose was cut to remove all polypeptides smaller than spectrin. This was used to pick up anti-spectrin antibodies. The antibody-absorbed strips were washed extensively with the high salt buffer, and the antibodies were extracted and processed similar to the column-purified antibodies.

The Fab fragments were prepared by papainization of the IgG fraction of the antiserum obtained by ammonium sulfate precipitation at 40% saturation [19]. The monospecific Fab fragments were isolated in the same manner as the intact antibodies. The residual intact antibodies were removed by absorption on protein A-Sepharose.

2.5. Specificity of the antibodies / Fab fragments

The erythrocyte membrane proteins were separated in several lanes on 5–15% polyacrylamide gel according to Laemmli [11], and were blotted on to nitrocellulose [20]. The lanes were cut out, and one of them was stained with Ponceau S to visualize the separated proteins. After overnight blocking in 3% BSA in buffer 1, a nitrocellulose strip bearing the erythrocyte proteins was incubated in 0.5 μg/ml monospecific antibody in buffer 1 containing 0.3% BSA, 0.05% (v/v) NP-40 and 0.02% azide for 2 h. After five washes in buffer 1 it was incubated for 2 h with (1:1000 dil) goat antiserum prepared against rabbit IgG. The strip was washed, and further incubated with peroxidase-(rabbit) antiperoxidase conjugate (1:3000 dilution) for 1 h. After three washes, it was finally incubated with 0.5 mg/ml 4-chloronaphthol and 0.015% H_2O_2 for development of color.

2.6. Entrapment of antibodies in erythrocytes and agglutination with Con A

1 vol. of normal or Pronase/trypsin-treated erythrocytes was added to 4 vol. of a chilled solution of the monospecific antibody or its Fab fragment in 2 mM Mg-ATP/5 mM Tris-HCl (pH 7.4). The cells were dispersed by tapping the tubes gently and were kept on ice for 10 min for the completion of lysis. The control cells were lysed in the same solution devoid of any antibody. The suspension was made isoosmotic by the addition of an appropriate volume of 3 M KCl. The cells were incubated for 1 h at 37° C to reseal and to allow the antibodies to bind. They were then washed thrice in buffer 1 in the cold. The Con A-mediated agglutination of control and antibody-entrapped cells was carried out as described earlier [3].

The more agglutinable Pronase-treated cells were used to study the effects of all, except the anti-spectrin, antibodies. Preliminary experiments indicated slight stimulation of agglutination at low concentrations of the anti-spectrin antibody. For this reason, the slightly less agglutinable trypsinized erythrocytes were employed.

In order to rule out the possibility that the differences in agglutination were due to any contaminating antibodies for extracellular determinants, intact trypsinized erythrocytes were treated with each of the monospecific antibodies in buffer 1 for 60 min. After washing, the Con A-agglutinability of the cells was determined.

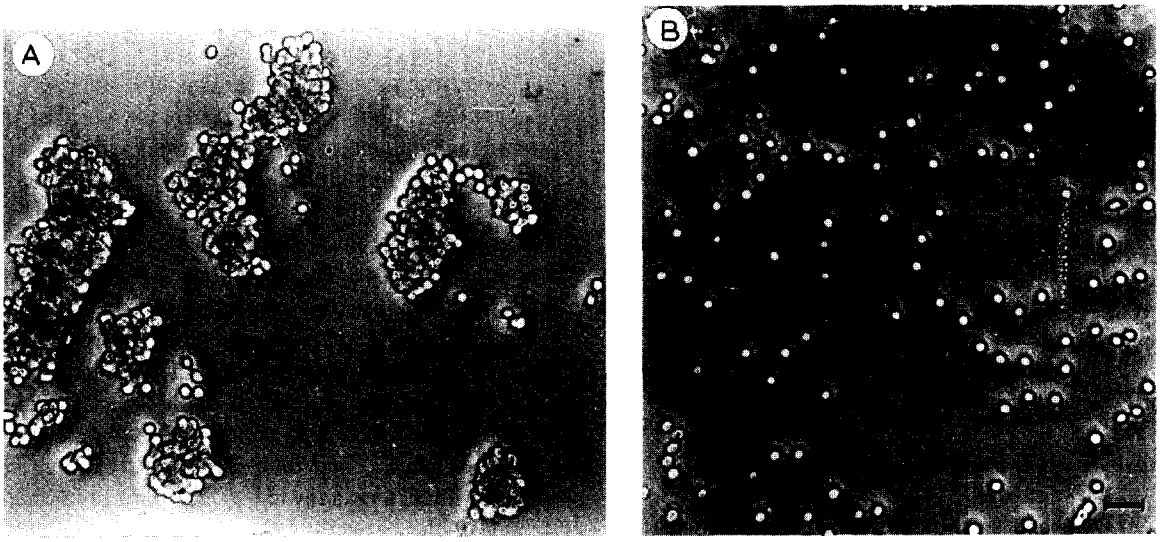

Fig. 1. Con A-mediated agglutination of human erythrocytes in the presence of anti-Con A antibodies. Washed human erythrocytes were suspended at 10% hematocrit in buffer 1, and Con A was added at the final concentration of 250 μg/ml. After incubation at 37° C for 1 h, the cells were centrifuged at 400 × g for 5 min. The supernatant was discarded, and the cells were washed thrice. The cells were resuspended at 10% hematocrit, and the anti-Con A antibody was added in 1:1, 1:4, 1:16 and 1:64 dilutions of the solution obtained from the supplier. To the second set, normal rabbit serum was added in similar dilutions. The agglutination was examined after 1 h at 37° C. (A) 1:4 dilution of the anti-Con A antiserum; (B) undiluted non-immune serum. The bar in B corresponds to 10 μm.

2.7. Statistical analysis

The agglutination differences between normal and antibody/Fab-entrapped erythrocytes were statistically evaluated by paired t-test. The significant differences ($P < 0.05$) are denoted by asterisks in the figures.

3. Results

3.1. Agglutination of Con A-bound erythrocytes by anti-Con A antibodies

Anti-Con A antibodies added in dilutions of 1:1, 1:4, 1:16, 1:64 and 1:256 of the antiserum to Con A (250 μg/ml)-bound erythrocytes, produced strong agglutination of the cells (Fig. 1A). Large clumps were seen at all dilutions with maximum clumping evident at 1:4 dilution. The clumps were resistant to vigorous vortexing, but inclusion of 0.05 M methyl-α-man abolished the agglutination (obviously by dissociating the cell-bound Con A). The addition of normal rabbit serum in place of the anti-Con A antiserum did not produce agglutination (Fig. 1B).

3.2. Con A-mediated agglutination of normal erythrocytes in the presence of albumin, Ficoll or PVP

The addition of serum albumin, Ficoll or PVP to the red cells treated with 250 μg/ml Con A produced shear-resistant clumps. In all cases, the presence of 0.05 M methyl-α-man did not permit agglutination. With bovine (or human) serum albumin, maximum agglutination was obtained at 100 mg/ml (Fig. 2A). With Ficoll, agglutination was maximum at 50 mg/ml (the highest concentration used) (Fig. 2B); while it nearly peaked at 50 mg/ml PVP (Fig. 2C).

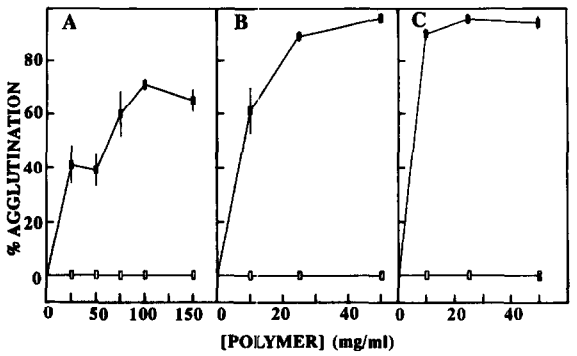

Fig. 2. Agglutination of untreated human erythrocytes by Con A in the presence of neutral polymers. Washed human erythrocytes were incubated with 250 μg/ml Con A (■) for 1 h at 37° C. Serum albumin (A), PVP (B) or Ficoll (C) was added at various concentrations and further incubated for 1 h. The tubes were tapped firmly 15 times to break loose clumps, and the agglutination was quantitated microscopically. Each point indicates the mean ± S.E. of five experiments. In each series a control was included that contained 250 μg/ml Con A and 0.05 M methyl-α-man (□), and the indicated concentrations of the polymer.

In the above experiments the cells were first treated with Con A, and the polymer was added subsequently. Even when the sequence of addition of Con A and the polymer was reversed, or if the two were added simultaneously, the agglutination was not affected (not shown).

3.3. Binding of Con A in presence of the polymer

The binding of ^{125}I-labeled Con A (250 μg/ml, which saturates the binding sites) to erythrocytes was studied in the presence of albumin (100 mg/ml), Ficoll (50 mg/ml) and PVP (25 mg/ml). PVP was found to have no significant effect of Con A binding to red cells: the number of binding sites (means ± S.D. × 10^5) was 1.90 ± 0.29 per cell in buffer 1 and 2.02 ± 0.18 in the presence of PVP ($P > 0.2$). On the other hand, albumin was found to enhance the binding by about 50%, and Ficoll decrease it by almost 75%. However, we consider the albumin and Ficoll effects to be suspect for the following reasons: albumin, although 'essentially globulin-free', does contain 1–3% globulins, most of which should be glycoproteins. These could bind to ^{125}I-Con A, and the aggregates, although not visible to the naked eye, could sediment through dibutylphthalate along with the cells. This could explain the higher number of sites. Sucrose is known to bind to Con A [21], and Ficoll which is polysucrose, may possess some affinity for the lectin. It could thus compete for Con A, reducing its binding to cells.

3.4. Effect of polymers on deformability of erythrocytes

Ficoll and PVP, at the concentrations used, produced rouleaux when added to an erythrocyte suspension of 10% hematocrit used in the deformability studies. Hence the deformability study was carried out only with albumin. It was found that 100 mg/ml albumin brought about a slight, but statistically significant, decrease in the deformability of red cells. The deformability index of untreated cells was 0.698 ± 0.06 (means ± S.D.; $n = 3$). In the presence of 100 mg/ml albumin the index (after correcting for the filtration time of BSA solution), was 0.606 ± 0.044 ($n = 3$; $P < 0.05$).

To further assess the role of deformability in polymer-mediated Con A-agglutinability, the cells treated with 5 mM diamide were used. Diamide is known to drastically reduce the deformability of erythrocytes [22]. The nearly non-deformable cells, however, agglutinated with Con A in the presence of albumin, PVP or Ficoll as well as the normal erythrocytes (not shown). Thus, the polymers do not appear to bring about agglutinability by affecting the deformability of the cells.

3.5. The site of polymer effect

Is the effect of the polymer on agglutinability restricted to the outer cell surface, or can it also occur from the

cytoplasmic side of the membrane? Or, can the agglutination occur if the polymer is present on both the surfaces? In order to test this, ghosts (which agglutinate well with Con A in the presence of any one of the three polymers), were suspended in a solution of 25 mg/ml PVP in buffer 1 in the cold. The ghosts were then sealed by incubation at 37° C for 1 h. Con A was added directly to these ghosts, which were exposed to the polymer on both the sides of the membrane. Alternatively, after resealing, the ghosts were washed with buffer 1 to remove the extracellular PVP and Con A was added. In the latter case, PVP was present only on the internal membrane surface. After incubation, the ghosts were examined by dark field microscopy for agglutination.

It was found that the ghosts exposed to PVP on both the surfaces were agglutinated with Con A (Fig. 3B,C), but those with PVP only on the inside were not (Fig. 3A). Thus, the polymer effect appears to be confined to the external surface of erythrocytes. The simultaneous presence of the polymer on the inner surface, however, does not interfere with its action on the outer surface of the cell. This result also shows that the polymer-induced Con A-agglutinability of erythrocytes is not a consequence of osmotic pressure differences across the membrane.

In order to check if the polymer is indeed internalized, white ghosts were exposed to 10, 20 and 40 mg/ml BSA, and sealed. After washing, the ghosts were subjected to SDS-PAGE. A dose-dependent increase in the albumin content of the ghosts was found. At 40 mg/ml albumin, about 10% of the total ghost protein was BSA (not shown).

3.6. The role of the membrane skeleton

The microscopically visible 'large' vesicles lack the cytoskeletal proteins, bands 1, 2 (spectrins), 2.1 (ankyrin), 4.1a and b, 4.9 and 5 (actin) (Fig. 4A). These vesicles were not agglutinated with Con A (Fig. 4B), even when albumin, Ficoll or PVP was added at concentrations that produce heavy clumping in whole cells (Fig. 2). The vesicles, however, bound Con A at the same density as whole cells: the number of Con A binding sites (means $\pm$ S.D. $\times 10^5$) per cell-equivalent of phospholipids were 1.21 $\pm$ 0.34 for whole cells and 1.51 $\pm$ 0.45 for vesicles ($P > 0.4$). The vesicles yielded large clumps with RCA and WGA (Fig. 4C).

The small vesicles obtained by in vitro aging of erythrocytes [10] produced identical results (not shown).

3.7. Specificities of the purified antibodies / Fab fragments

The purified spectrin used for spectrin-Sepharose preparation did not show any contamination by SDS-PAGE (Fig. 5A, lane b). The anti-spectrin antibodies isolated using the affinity column showed strong staining of the two spectrin bands (Fig. 5B, lanes b and c) but also light staining of a few low molecular weight peptides in ghost proteins (Fig. 5B, lane b). The anti-spectrin antibodies purified using nitrocellulose strips impregnated with pure spectrin (obtained by electrophoresis and blotting of purified spectrin) also gave a similar picture (not shown). Since the nitrocellulose strip did not contain any proteins smaller than spectrins, the lower molecular weight peptides on the Western blot (Fig. 5B, lane b) most probably represent small amounts of spectrin degradation products occurring naturally in the erythrocyte membrane.

The purified ankyrin appeared to be virtually pure by SDS-PAGE (Fig. 5A, lane c), but showed a small amount of band 2.2 protein by immunostaining (Fig. 5B, lane e). The antibodies to ankyrin stained all the three erythrocyte isoforms, i.e., bands 2.1, 2.2 and 2.3 (Fig. 5B, lane d), which are immuno-crossreactive [23].

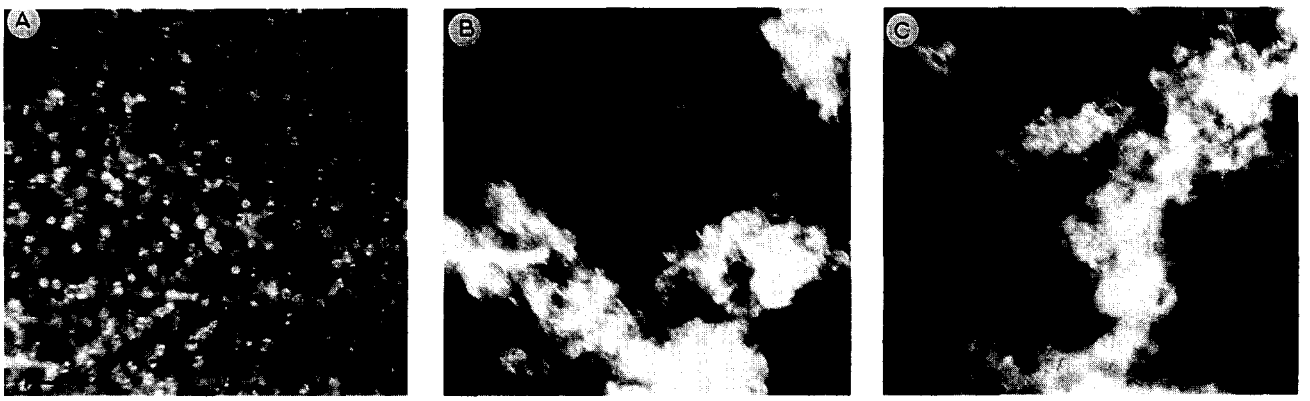

Fig. 3. The sidedness of PVP action on the Con A-mediated agglutination of erythrocytes. White ghosts were incubated with 25 mg/ml PVP or buffer 1 alone and sealed by incubation at 37° C for 1 h. In the former, the ghost membrane is exposed to PVP on both its sides. An aliquot of this was washed thrice to obtained ghosts, which have PVP only on the inside. To a portion of the ghosts sealed without PVP, 25 mg/ml PVP was added. Con A was added at 250 μg/ml to all preparations. After incubation for 1 h, agglutination was assessed by dark field microscopy. (A) PVP only inside (or ghosts without PVP on either side); (B) PVP only on outside; and (C) PVP on both sides. The bar in A is equivalent to 10 μm.

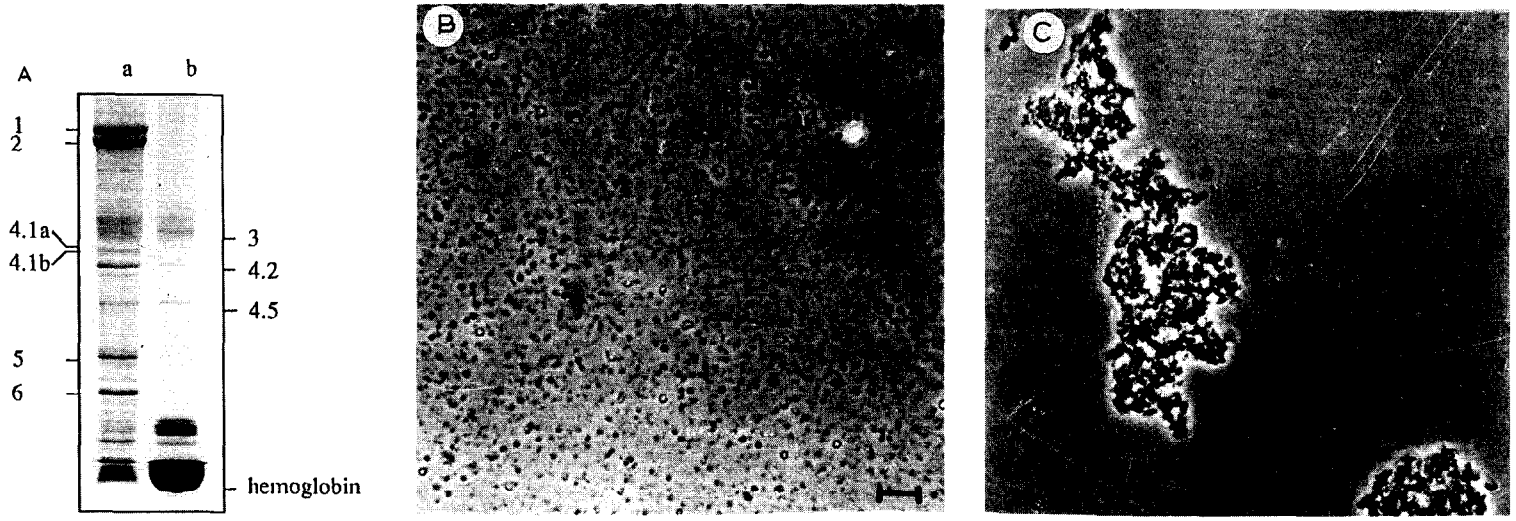

Fig. 4. The agglutination of membrane skeleton-free vesicles. Large vesicles were prepared from washed erythrocytes according to [9]. The suspension containing the vesicles, intact cells and ghosts was centrifuged at 400 × *g* for 10 min. The supernatant was recentrifuged until microscopic examination revealed absence of intact cells. The vesicles were then sedimented at 15000 × *g* for 10 min. The loose, pale upper layer of ghosts was separated by gentle agitation, and discarded. The vesicles were washed twice, repeating the above procedure. The purity of the vesicles was assessed by the absence of the skeletal proteins, spectrins (bands 1 and 2), band 4.1a and b, and actin (band 5) on a 5 to 12% acrylamide gel in the presence of SDS and mercaptoethanol. The gel was stained with Coomassie brilliant blue (A): a, ghosts (50 μg protein); b, whole vesicles (300 μg protein). For agglutination, the vesicles were suspended in 10 vol. of buffer 1 and incubated with 250 μg/ml Con A. After 1 h, albumin (100 mg/ml), Ficoll (50 mg/ml) or PVP (25 mg/ml) was added. Following an additional hour, the vesicles were examined by phase contrast microscopy. The control vesicles, and those treated with Con A and albumin/Ficoll/PVP appeared free. Only the vesicles treated with PVP are shown (B). An aliquot of the vesicle suspension was treated with 10 μg/ml WGA or RCA for 1 h. The clumps obtained with RCA are shown (C). The bar in B corresponds to 10 μm.

The purified 4.1 protein migrated in SDS-PAGE predominantly as a doublet of 80 and 78 kDa; but minor amounts of other bands were also found to be present (Fig. 5A, lane d). These were polypeptides of about 85, 73, 53 and 42 kDa. The monospecific antibodies obtained using the affinity column (Fig. 5B, lane f) or the nitrocellulose-bound pure 4.1 protein also stained the same bands. These bands correspond to the isoforms/degradation products of the protein reported in the literature (e.g., [24]).

The purified cytoplasmic domain of band 3 protein migrated as a doublet on SDS-PAGE in the 43 kDa region (Fig. 5A, lane e). The purified antibodies stained the intact band 3 protein, besides the bands of M_r 60 000 and 42 000 in ghosts (Fig. 5B, lane h). Band 3-related polypeptides of M_r about 64 000, 60 000, 42 000 and 18 000–26 000 have been identified in erythrocyte ghosts prepared in the presence of proteinase inhibitors [25].

The presence of antibodies to other inappropriate proteins in each of the purified antibody preparation is ruled out from Fig. 5A (lanes b, d, f and h). Also, none of the antibodies reacted with inappropriate purified proteins when checked by immunoblotting (data not shown). When intact proteinase-treated erythrocytes were treated with each of the four monospecific antibodies and washed, none of the antibodies caused any change in the Con A-agglutinability of the cells. Thus, the observed effects of the antibodies were not due to any contaminating activity towards extracellular determinants.

In order to rule out the possibility that residual proteinase activity rather than the antibodies was responsible for the observed effect on agglutinability, the trypsinized erythrocytes, prior to antibody internalization, were treated with 20 μg/ml TLCK to inactivate the enzyme. When Con A-agglutinability was determined after washing, no difference was observed. Also, no changes were seen in the apparent intensity of any of the membrane skeletal (or other) proteins of the trypsin/Pronase-treated red cells on SDS-PAGE following antibody internalization and resealing. Thus, the proteinase used for digestion of intact cells apparently does not persist after washing, nor does the process of lysis and resealing affect the membrane proteins.

The purified antibodies were internalized in normal or Pronase/trypsin-treated erythrocytes. The Con A-mediated agglutination of the cells was examined (after addition of 25 mg/ml PVP to normal cells). The results with both sets of the cells were nearly identical. Only the results with proteinase-treated cells are given below.

3.8. Effect of anti-spectrin antibodies and the Fab fragments

Anti-spectrin antibodies were internalized in trypsinized erythrocytes at 0, 0.2, 0.4, 0.8, 2.0, 4.0 and 6.0 mg/ml (Fig. 6). While 0.2 mg/ml produced a small stimulation, and 0.4 mg/ml had no apparent effect (not shown), the remaining four antibody concentrations produced inhibition. The maximum effect, a little over 30%, was observed at 2.0 mg/ml. The extent of inhibition with the four

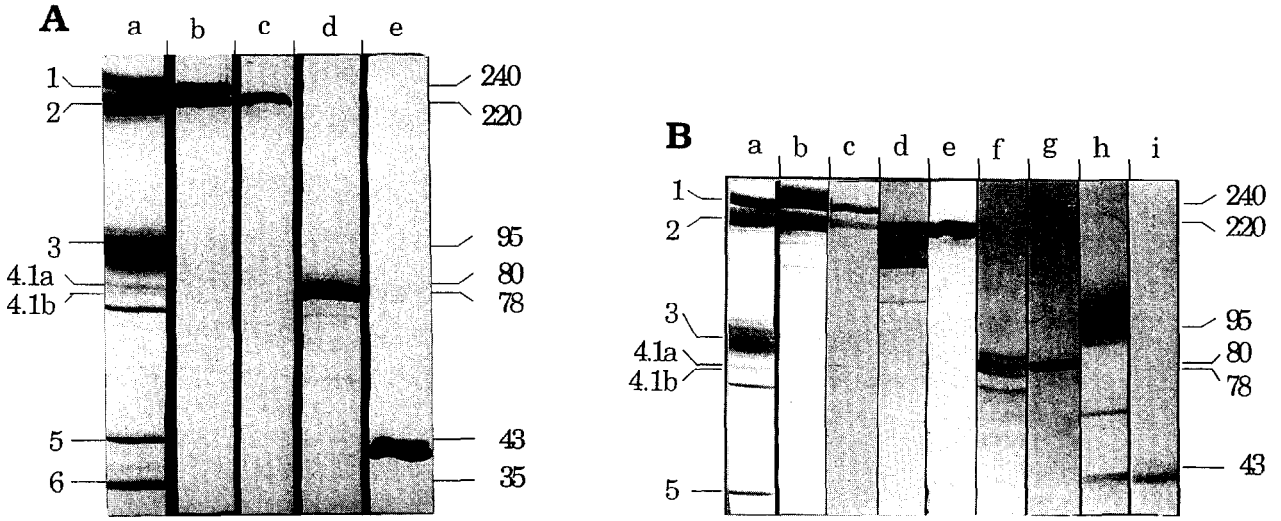

Fig. 5. (A) Purity of the isolated proteins and specificity of the purified antibodies. Electrophoresis of the isolated proteins was carried out on 8% polyacrylamide gels in the presence of SDS and mercaptocthanol. Lane a, ghost proteins (40 μg protein, Coomassie blue staining); lanes b–e, approx. 10 μg protein per lane, silver staining. b, spectrin; c, ankyrin; d, band 4.1 protein; e, cytoplasmic domain of band 3 protein. (A composite picture of electrophoretic patterns obtained at different times.) (B) Specificities of the antibodies using erythrocyte ghosts and purified proteins (a composite picture). Lane a, Coomassie blue stained pattern of ghost proteins (same as Fig. 1a, lane a), lanes b–i, immunoblots. 30 μg ghost protein (lanes b, d, f and h), 4 μg spectrin (lane c), 5 μg ankyrin (lane e), 5 μg 4.1 protein (lane g) and 10 μg cytoplasmic domain of band 3 protein (lane i) were electrophoresed and transblotted. The blots were blocked and incubated with the corresponding monospecific antibodies (0.2 μg/ml) and visualized as described under Materials and methods. Lanes b and c developed with anti-spectrin antibodies; d and e with anti-ankyrin; f and g, with anti-4.1 protein; and h and i with anti-cytoplasmic domain of band 3. The polypeptides are numbered on the left according to [10], and molecular weights are indicated on the right hand side.

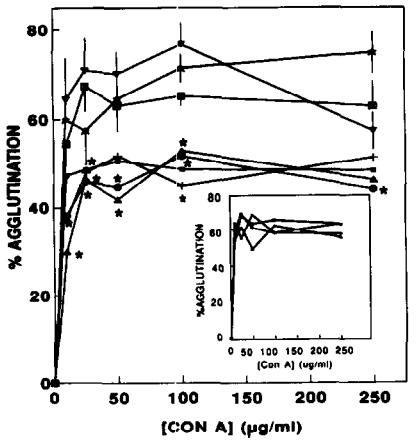

Fig. 6. Effect of internalized anti-spectrin antibodies and their Fab fragments on the Con A-mediated agglutination of erythrocytes. Trypsinized erythrocytes were lysed in 4 volumes of chilled 0.005 M Tris-HCl (pH 7.4), containing the antibody or its Fab fragment and 2 mM Mg-ATP. After 10 min on ice an aliquot of 3 M KCl was added to make the solution isoosmotic. Resealing of the cells was carried out by incubation at 37° C for 1 h. Subsequently, the cells were washed thrice with buffer 1 in the cold. Agglutination was carried out with various concentrations of Con A. Antibody concentrations used were 0 (⊠); 0.2 (▼); 0.8 (■); 2.0 (▲); 4.0 (●) and 6.0 (+) mg/ml. 2.2 mg anti-spectrin were pre-incubated with 2.0 mg purified spectrin and internalized (★). The results plotted are means ± S.E. of 4–7 experiments. In some cases the S.E. bars are not shown to avoid confusion. The inset shows the results obtained with the Fab fragment of the anti-spectrin antibodies. The same symbols are used as in the case of intact antibodies. Results are average of four experiments.

antibody concentrations varied within 10% of each other; however, the standard errors of their mean effects varied considerably more. Incubation of 2 mg/ml anti-spectrin with 2 mg/ml purified spectrin before internalization completely neutralized the agglutination-inhibiting ability of the antibody. The Fab fragments of anti-spectrin antibodies failed to produce any effect on agglutination up to a concentration of 4.0 mg/ml (Fig. 6, inset).

Identical results were obtained with the antibodies obtained using the affinity column or the nitrocellulose strip bearing purified spectrin.

3.9. Effect of anti-4.1 protein antibodies / Fab fragments

The anti-4.1 protein antibodies inhibited Con A-mediated agglutinability of Pronase-treated red cells in the range 0.4 to 4.0 mg/ml in a concentration-dependent manner (Fig. 7A). At the highest concentration used, the inhibition was nearly 75%. If the antibody was neutralized by pre-treatment with purified 4.1 protein, the inhibitory activity was totally abolished. The Fab fragments of the monospecific antibodies appeared to show even a greater effect, with the agglutination decreasing to about 10% at 2.0 mg/ml (Fig. 7B). On a molar basis, however, the intact antibodies and Fab fragments had similar effects.

The antibodies to 4.1 protein isolated using the affinity column or the 4.1 protein-impregnated nitrocellulose gave the same inhibition of agglutination.

3.10. Effect of anti-ankyrin antibodies / Fab fragments

Due to their low yield, the anti-ankyrin antibodies could be used only over the concentration range of 0.4 to 1.6 mg/ml (Fig. 8). No significant stimulation or inhibition of agglutinability was observed at any of the concentrations. Since no progressive concentration-dependent change was observed, it is doubtful if increasing the concentration beyond 1.6 mg/ml would have produced a significant effect. Thus, it is concluded that anti-ankyrin antibodies have no effect on Con A-mediated agglutinability of erythrocytes. The Fab fragments of the antibody were also ineffective up to the concentration of 1.6 mg/ml (Fig. 8, inset).

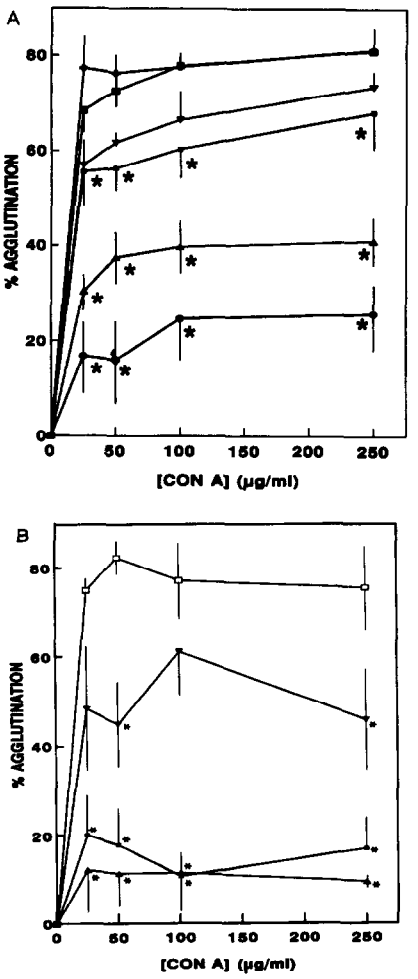

Fig. 7. Con A-agglutinability of erythrocytes following internalization of anti-4.1 protein antibodies/Fab fragments. (A) Anti-4.1 antibodies (a) at 0 (⊠); 0.4 (▼); 0.8 (■); 2.0 (▲); and 4.0 (●) mg/ml. 4 mg/ml antibody was pre-incubated with 4 mg/ml purified 4.1-protein, and the complex was internalized (+). (B) The Fab fragments at 0 (□); 0.4 (▼); 0.8 (■) and 2.0 (▲) mg/ml were internalized in Pronase-treated erythrocytes, and the Con A agglutinability of the cells was determined after resealing and washing. Values are means ± S.E. of 3–7 experiments.

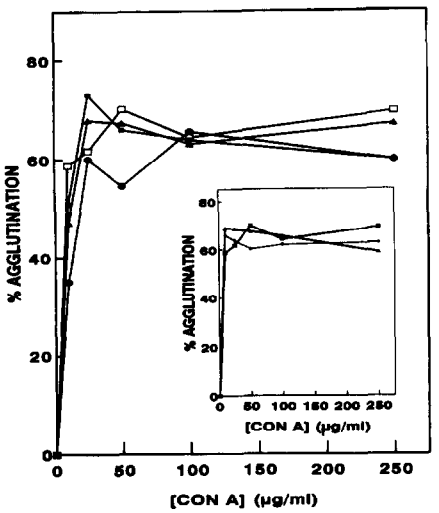

Fig. 8. Effect of internalized anti-ankyrin antibodies on the Con A-mediated agglutination of erythrocytes. Anti-ankyrin antibodies were internalized in Pronase-treated erythrocytes at 0 (□); 0.4 (●); 0.8 (■); and 1.6 (▲) mg/ml. The Con A-agglutinability of the cells was determined after resealing and washing. The inset shows the results obtained with the Fab fragment of the anti-ankyrin antibodies. (The same symbols as those used for the intact antibodies.) Values are means of three or four experiments, the error bars are not shown.

3.11. Effect of antibodies / Fab's to the cytoplasmic domain of band 3 protein

Internalization of antibodies to the cytoplasmic domain of band 3 protein at 0, 0.8, 2.0 and 4.0 mg/ml in Pronase-treated cells produced a progressive concentration-dependent inhibition of Con A-mediated agglutination (Fig. 9). At 4.0 mg/ml of the antibody, 50% inhibition of agglutination was observed (at 50 μg/ml Con A). Incubation of 4 mg/ml of the antibody with equal amount of the purified band 3 cytoplasmic domain completely abrogated the effect of the antibody. The Fab fragments of the antibodies had no effect even at 4.0 mg/ml (Fig. 9, inset).

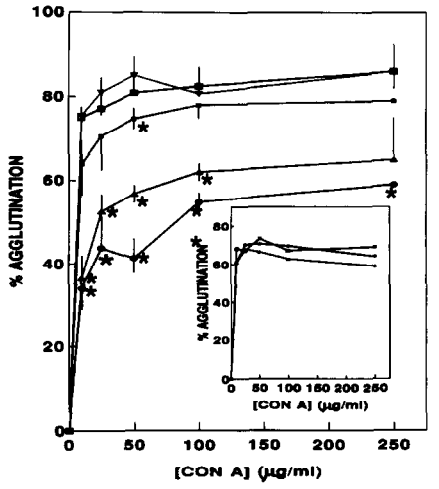

Fig. 9. Con A-agglutinability of erythrocytes following internalization of antibodies/Fab to the cytoplasmic domain of band 3 protein. Antibodies to the cytoplasmic domain of band 3 protein were internalized in Pronase-treated erythrocytes at 0 (⊠); 0.8 (■); 2.0 (▲) and 4.0 (●) mg/ml. 4mg/ml antibody and 2.0 mg/ml purified band 3 cytoplasmic domain were incubated before internalization (▼). The Con A-agglutinability of the cells was determined after resealing and washing. Values are means ± S.E. of 4–7 experiments. Inset shows results obtained with the Fab fragments of these antibodies. (Symbols are the same as in the case of intact antibodies. Values are means ± S.E. of four experiments.)

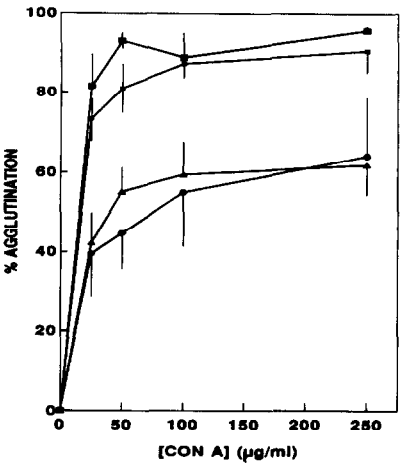

Fig. 10. Effect of intracellular Mg-ATP on the Con A-agglutinability of erythrocytes. Pronase-treated cells were lysed in hypotonic medium lacking (▲) or containing (■) 2 mM Mg-ATP or 2 mM Mg-ATP-analogue (●). After resealing their agglutinability by Con A was determined. The agglutinability of fresh unlysed Pronase-treated erythrocytes is also shown (⊠). Values are means ± S.E. of five experiments. The difference between the agglutinabilities of ghosts without ATP and with ATP internalized was statistically significant at all Con A concentrations. The difference between the intact cells and the ATP internalized ghosts was not significant. The difference between the agglutination of ATP-internalized and the ATP-analogue-internalized cells was also statistically significant.

3.12. Role of Mg-ATP

When Pronase-treated erythrocytes were lysed in 4 vol. of buffer and the agglutination of the cells was assessed after resealing, a substantial decrease (varying between 40–50% at different concentrations of Con A) was observed (Fig. 10). When the lysis was carried out in the presence of 2 mM Mg-ATP (the approximate intracellular concentration in red cells), the decrease in agglutination did not occur. If instead of Mg-ATP, Mg^{2+} and β,γ-methylene adenosine triphosphate (a non-hydrolyzable analogue of ATP) were included at the same concentration, the decrease persisted. The decrease was again observed if Mg^{2+} or ATP alone was present. Thus, for normal Con A-mediated agglutination of erythrocytes, the presence of Mg-ATP is required.

4. Discussion

It has been known for a long time [26] that Con A is unable to agglutinate normal human erythrocytes, but pretreatment of the cells with proteinase/neuraminidase makes them agglutinable with the lectin. The results reported here

that anti-Con A antibodies and neutral polymers elicit agglutinability in normal cells, place Con A in the category of 'incomplete' lectins [1].

The proteinase treatment does not bring about the agglutinability by altering the number of Con A binding sites [3] or the deformability of the cells [6]. The polymer-induced agglutinability also does not arise by altering these parameters. The increase in Con A-agglutinability following proteinase/neuraminidase treatment is correlated ($r = 0.88$) with the removal of sialic acid, predominantly associated with glycophorin A [3]. The latter is non-covalently complexed with band 3 in the membrane [27,28]. Since aggregation of lectin-receptor complexes is required for shear-resistant agglutination [29], the highly negatively charged glycophorin can interfere with the aggregation of Con A-band 3 complexes, thus preventing agglutination. The removal of charge leads to aggregation of band 3 molecules following Con A binding [30,31]. How is the neutral polymer then able to bring about agglutinability in the presence of intact glycophorin? Pollack et al. [32] studying the polymer-induced agglutination of erythrocytes by anti-Rh antibody, showed that the polymer decreases the zeta potential on the membrane by increasing the dielectric constant of the medium. This would, in effect, amount to reduction in surface charge. Indeed, the site of the polymer effect is located on the external surface of the cell membrane (Fig. 3). (However, another report [33] failed to find any effect of neutral polymers on the zeta potential.)

The vesicles lacking skeletal proteins in their membrane fail to agglutinate with Con A whether isolated from proteinase-treated [4] or normal erythrocytes (Fig. 4). Thus, despite a 50-fold increase in the lateral mobility of the Con A receptor [34], the removal of skeletal proteins leads to loss of agglutinability with Con A. The inhibition of agglutination by anti-spectrin and anti-4.1 protein reported here, confirms the participation of the membrane skeleton in the process of agglutination. This requirement, again, is common for the proteinase- and polymer-mediated agglutinations.

Since only the intact anti-spectrin antibodies, and not the Fab's, inhibit agglutination, the inhibitory activity is due to cross-linking of spectrin molecules. Also, the extent of inhibition is no more than 30%, and beyond 2 mg/ml antibody concentration (Fig. 6), there is no further effect. This suggests the involvement of a very small number of epitopes on the spectrin molecules, and that the epitopes are not uniformly accessible to the antibodies. The inhibition by anti-4.1 antibodies is quite substantial, and concentration dependent (Fig. 7A). Since the Fab fragments of the antibodies are as effective (Fig. 7B), evidently a domain on the 4.1 protein participates in the agglutination process.

Anti-ankyrin antibodies/Fab's have no effect on agglutination (Fig. 8), but antibodies to band 3 cytoplasmic domain are inhibitory (Fig. 9). How could the cytoplasmic domain of the Con A receptor influence agglutination? In view of the participation of the membrane skeleton, and the lack of involvement of ankyrin, we envisage a Con A-induced interaction between the cytoplasmic tail of band 3 and the membrane skeleton. Such a linkage is supported by the fact that Con A binding prevents the extraction of the spectrin-actin complex from the membrane under hypotonic conditions [31]. The reported effects of Con A on erythrocytes, such as stabilization of the shape [35], decrease in deformability [6], and the resistance to fragmentation on heating at 49° C [31] can also be consequences of such an interaction. Since 49° C is the denaturation temperature of spectrin [36], an alteration in physicochemical properties of spectrin is suggested as a result of Con A binding to the cell. This possibly indicates an interaction between the cytoplasmic domain of band 3 with spectrin, although interaction with other components is also possible. For example, the cytoplasmic domain of band 3 possesses a binding site for 4.1 protein [24,37].

The concentrations of the various antibodies required for inhibition of agglutination is apparently quite large: 0.4 to 6.0 mg/ml, corresponding to about $2.5 \cdot 10^{-6}$ to $4 \cdot 10^{-5}$ M. During hypotonic lysis, proteins of high molecular weight (10^6) can get into the cell, but the pores in the membrane exist for only about 10 sec [38]. Whether equilibration between extra- and intra-cellular materials occurs during this time is not clear. (The indicated antibody concentrations would thus be only notional.) Further, since the antibodies used are polyclonal, the concentration of the antibody to each of the antigenic epitopes will be much smaller. Thus, although apparently large, the concentration of the epitope-specific antibody available within the cell may not be very large. The extent of antibody binding will be further determined by its affinity for the antigen.

Shear-resistant agglutination of cells requires a ligand-induced pre-aggregation of receptor molecules [29]. The aggregation of not only band 3 molecules, but also of skeletal proteins is observed in agglutinated cells, as detected by chemical cross-linking [30,31]. The membrane skeleton is associated with contractile proteins [39] that could help in the process of aggregation. (The slight stimulation of agglutination seen at 0.2 mg/ml anti-spectrin (Fig. 6) may be due to a mild aggregation of spectrin produced at the low concentration of the antibody.) Some reports indicate that lectin-induced patches of receptor molecules coaggregate with spectrin/ankyrin in nucleated cells [40–42]. Fodrin (non-erythrocyte spectrin) has been reported to stimulate actomyosin-associated Mg-ATPase [43]. It is conceivable that the physicochemically altered spectrin molecule in the Con A-bound cells (discussed above), could bring about the stimulation. The ensuing contraction of the skeletal structure could account for the aggregation of skeletal proteins, and together with them, of the band 3 molecules putatively attached to them. Also, 4.1 protein, apart from being associated with spectrin and actin, and possessing a binding site for band 3 molecules [24,37], is reported to be complexed with myosin, and to

regulate the actin-mediated activation of myosin-ATPase [44]. Possibly the binding of anti-4.1 antibody/Fab to the 4.1 protein molecule interferes with this activation, and the subsequent force generation. The substantial inhibitory effect of anti-4.1 antibody, and its Fab, is thus not surprising.

If contraction of the skeleton helps in aggregation of band 3-Con A complexes, and eventually in agglutination, the latter should be dependent on ATP. The preliminary evidence presented here on the requirement of physiological concentrations of Mg-ATP for full agglutination indicates such a dependence. Also, the phosphorylation of the tyrosine residue at position 418 in the amino acid sequence of the 4.1 molecule is reported to reduce the latter's ability to promote spectrin-actin assembly, and to bind to band 3 protein [45]. ATP thus may influence the agglutinability in more than one way.

The conditions bringing about agglutination of cells by incomplete lectins and antibodies are identical. It should therefore be surprising if the two groups of ligands did not produce agglutination by the same mechanism. The involvement of the membrane skeleton in the Con A-mediated, but not RCA- or WGA-mediated [4], agglutination suggests that the membrane skeleton may be required for the agglutination by the incomplete (anti-Rh) but not the complete antibody. While the present results rule out any role for deformability in the polymer-induced agglutination by Con A, an earlier report has implicated a major role for deformability in anti-Rh-mediated agglutination [46]. A reassessment of this role may be worth while. Interestingly, recent results have shown that the Rh antigen is a component of a complex of several polypeptides, including glycophorin B [47]. The inhibitory role of the latter, similar to that of glycophorin A in the Con A-mediated agglutination, can easily be envisaged.

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