Carbohydrate induced modulation of cell membrane VII. Binding of exogenous lectin increases osmofragility of erythrocytes

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Abstract Due to their multivalent binding character, lectins when added exogenously will cross-link membrane surface receptors leading to lateral molecular reorganizations in the plane of the bilayer. This study reports for the first time that agglutination of rabbit erythrocytes by lentil lectin and concanavalin A increases their osmofragility. Increase in osmofragility was detected by measuring the hemolysis of erythrocytes in hypotonic as well as in isotonic solutions. It was also found that agglutination per se does not increase osmofragility but the binding of legume lectin is essential since human Rh^+ cells agglutinated by a monoclonal antibody do not exhibit hemolysis.

Key words: Lectin; Red cell membrane perturbation; Osmofragility; Hemolysis

1. Introduction

Plasma membranes control solute and solvent flow to establish defined environments. The barrier function of plasma membrane which prevents non-specific leakage of non-electrolytes and uncontrolled movement of ions between cells and their environments can be severed not only by exogenous agents forming transmembrane channels, e.g. toxins [1,2], ionophorous antibiotics [3–5] and endogenous peptides [6], but also by binding of exogenous proteins to membrane constituents which greatly perturb the mutual interaction of the membrane elements and induce leaks which may be irreconcilable with cell viability [7]. In the case of erythrocytes such perturbations of the barrier function are easily detectable by the hemolysis ensuing from the formation of leaks for small ions or even larger constituents [8].

Due to their multivalent binding character, lectins, when added exogenously, will cross-link membrane surface receptors leading to lateral molecular reorganizations in the plane of the bilayer [9]. Lectins have been used as membrane perturbing agents [10]. This report describes for the first time the formation and properties of leaks in rabbit erythrocytes upon agglutination by lentil lectin and concanavalin A. The changes in membrane permeability after binding of exogenous lectin were detected by measuring the osmofragility of agglutinated erythrocytes in hypotonic solution. Cells made leaky to ions swell and hemolyse when suspended in isotonic solution of salts capable to permeate the leaks [8]. The uptake of salt and water is driven by the now unbalanced colloid-osmotic pressure of intracellular impermeable solutes [8]. This colloid-osmotic lysis can be prevented by addition to the extracellular medium of non-electrolyte solutes capable to counterbalance the osmotic pressure of the intracellular macromolecules [8]. Such compounds will act protectively for an unlimited period only if they have virtually no access to the leak pathway. If they permeate slowly, they will protect for a limited period only. Normal red cells and red cells agglutinated with either a lectin or an antibody were thus suspended in isotonic solution and their hemolysis assessed. To confirm that this colloid osmotic lysis is due to the formation of membrane leaks, the ability of non-electrolyte solutes to prevent the hemolysis in isotonic solution was also assessed.

2. Materials and methods

2.1. Reagents

Trypsin, concanavalin A (Con A), ribose, galactose and lactose were purchased from Sigma Chemical Co., MO, USA. Monoclonal antibody against Rh antigen was from Span Diagnostics Private Ltd., India. All other chemicals used were of analytical grade. Blood was collected from healthy human donors in trisodium citrate as anticoagulant.

2.2. Methods

A blood sample from a healthy rabbit (adult, male, New Zealand white strain) was obtained by vein puncture in 3.8% trisodium citrate solution as an anticoagulant used in a ratio of 4:1. The erythrocytes were then centrifuged $3000\times g$ for 5 min, plasma and buffy coat were discarded and pelleted cells were washed tree times with 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). Finally, a 2% erythrocyte suspension was made (2 ml of packed erythrocytes in 100 ml PBS). One volume of erythrocyte suspension was incubated with two volumes of 200 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-trypsin in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl at 37°C for 1 h. The cells were washed four times in cold PBS and suspended as 2% erythrocyte suspension. The treatment with TPCK essentially inhibits the chymotrypsin which is usually present in trypsin. Chymotrypsin specifically cleaves band 3, a RBC membrane protein which acts as receptor for Con A [11]. The erythrocytes were treated with trypsin for the cleavage of glycophorin and release of sialic acid in peptide bond form to increase the agglutinatibility of cells with Con A [11]. The agglutinated cells were exposed to varying degrees of hypotonic solution to assess their osmofragility, as compared to unagglutinated cells [12].

Lentil lectin was prepared from *Lens culinaris* seeds as described by Tichy et al. [13] with some modifications [14].

2.3. Determination of osmofragility of lectin agglutinated erythrocytes and protection of their lysis by addition of non-electrolytes

For the determination of osmofragility of erythrocytes [15] from the cell suspension prepared as above, aliquots of 200 μl were made in different tubes. To these aliquoted cell suspensions were added 100 μl of either lectin or PBS and the mixture was incubated for a period of 1 h at room temperature (28°C) for the agglutination to take place. After this, 1.5 ml of respective hypotonic solution (i.e. 0.35–0.75% NaCl) or isotonic solution (0.85% NaCl) was added. The cells were then incubated for a period of 1–4 h at room temperature with intermittent gentle mixing. At the end of the incubation period, the tubes were centrifuged at $3000\times g$ for 5 min and the supernatant was collected. Extent of hemolysis was measured by taking absorbance at

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540 nm. Results are reported as percent lysis, where the lysis of identical volume of erythrocytes with identical amount of distilled water is taken as 100%.

For protection of osmotic lysis, the lectin agglutinated erythrocytes were incubated with 1.5 ml of isotonic solution containing KCl (75 mM), NaCl (35 mM), Na_2HPO_4/NaH_2PO_4 (12.5 mM), pH 7.4. In addition, 30 mM of either ribose, galactose, mannose or lactose were added. The final osmolarity was 280 mosmol/l [8]. The cells were incubated at room temperature for 6 h with gentle mixing. Hemolysis was subsequently quantified as described above. To establish whether erythrocyte osmofragility is linked to the process of agglutination per se or to the agent inducing agglutination, trypsinized human blood group O Rh^+ cells agglutinated with either Con A or with anti-Rh monoclonal antibody were suspended in the isotonic solution described above. After incubation at room temperature for 6 h the hemolysis was measured as described above.

3. Results and discussion

The osmotic fragility of rabbit erythrocytes in the presence and absence of the lectin was studied by measuring their hemolysis in hypotonic solutions. The results are presented in Figs. 1–5. Fig. 1a presents the hemolysis of Con A agglutinated erythrocytes after 1 h and 4 h incubation in hypotonic solution. It is evident from the figure that the Con A agglutinated cells exhibit more hemolysis than unagglutinated cells in hypotonic solution. This enhanced hemolysis is more pronounced at 0.75% NaCl and 0.65% NaCl concentration. The Con A agglutinated erythrocytes in 0.75% NaCl concentration exhibit 29.6% and 47.3% lysis against 4.6% and 5.6% ($P < 0.001$) lysis of unagglutinated cells in 1 and 4 h, respectively. Similarly, at 0.65% NaCl concentration agglutinated erythrocytes exhibit 62.3% and 72.33% lysis against 34% and 40% lysis ($P < 0.001$) of unagglutinated cells in 1 and 4 h, respectively. At NaCl concentrations lower than 0.55%, the hypotonic lysis in agglutinated and unagglutinated cells was almost equal. Surprisingly, at 0.85% NaCl, i.e. isotonic solution, where the unagglutinated erythrocytes show no hemolysis, the agglutinated erythrocytes exhibited 36.5% hemolysis after the lapse of 4 h ($P < 0.001$). Hemolysis of Con A agglutinated rabbit erythrocytes on prolonged incubation even in isotonic solution indicates that binding of Con A to rabbit erythrocytes is somehow perturbing the erythrocyte membrane thereby increasing its osmotic fragility. It has been known earlier that binding of Con A to pronase treated human erythrocytes results in concomitant partial hemolysis [16]. To ensure if this effect is specific to Con A or could be achieved by other plant lectins, we studied the hemolysis of erythrocytes agglutinated with lentil lectin, another plant lectin with similar sugar specificity. The results are presented in Fig. 1b. On agglutination with lentil lectin a similar increase in hemolysis of erythrocytes in hypotonic solution is also observed. The effect of lectin concentration on the increased hemolysis in hypotonic solution was also studied. For this, the erythrocytes were agglutinated with increasing concentrations of Con A and then incubated in 0.65% NaCl concentration for 4 h. The results are presented in Fig. 2. It is found that with increase in Con A concentration, the % hemolysis also increases. At 1.3 μg Con A, only 42% hemolysis is observed but at 333.3 μg Con A about 89.5% hemolysis is ob-

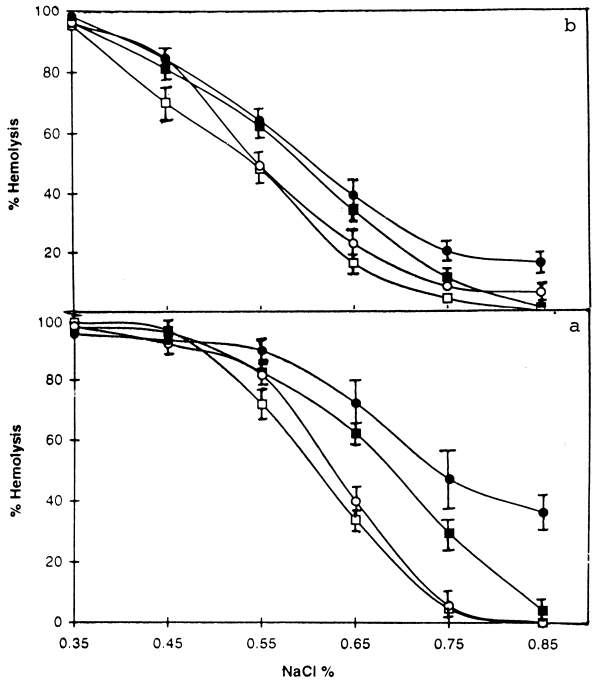

Fig. 1. Hemolysis of trypsinized erythrocytes at varying NaCl concentrations. Trypsinized rabbit erythrocytes (control) after 1 h incubation (– □ –), after 4 h incubation (– ○ –); trypsinized rabbit erythrocytes agglutinated with (a) Con A, (b) lentil lectin after 1 h incubation (– ■ –), after 4 h incubation (– ● –) in different NaCl solutions.

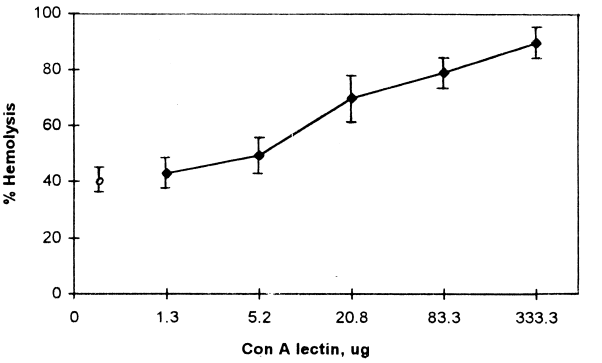

Fig. 2. Effect of change in concentration of Con A on hemolysis of trypsinized rabbit erythrocytes in 0.65% NaCl. Control (○), unagglutinated cells.

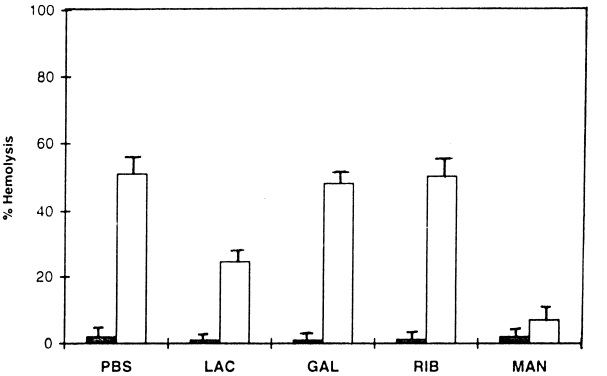

Fig. 3. Protection of colloid-osmotic lysis of Con A agglutinated trypsinized rabbit erythrocytes in PBS by addition of non-electrolytes. PBS, phosphate buffered saline; Lac, lactose 30 mM; Gal, galactose 30 mM; Rib, ribose 30 mM; Man, mannose 30 mM. Filled bars represent control, i.e. unagglutinated cells.

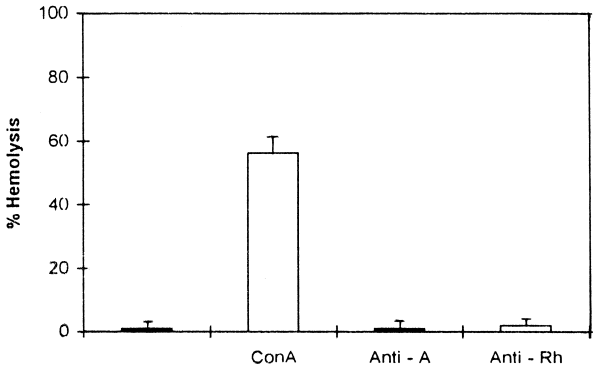

Fig. 4. Hemolysis of trypsinized human O Rh^+ red blood cells in isotonic phosphate buffered saline after agglutination with Con A anti-Rh antibody. Filled bars represent control, i.e. unagglutinated cells and cells with anti-A antibody. Trypsinization enhances agglutination both with Con A and anti-Rh antibody.

served ($P < 0.001$). Presuming that the increased hemolysis of lectin agglutinated cells is due to the formation of membrane leaks to small ions, attempts were made to prevent this colloid osmotic lysis by addition of non-electrolytes to the extracellular medium. The results are presented in Fig. 3. On 6 h incubation in PBS, Con A agglutinated cells exhibited 51% hemolysis whereas unagglutinated cells exhibited only 2% lysis ($P < 0.001$). In the presence of 30 mM mannose, a sugar inhibiting the binding of Con A to the erythrocytes, the hemolysis decreased to 6.9% only. Addition of D-galactose (30 mM) or D-ribose (30 mM) had no effect with Con A agglutinated cells exhibiting 48% and 50% hemolysis, probably because D-galactose and D-ribose were able to penetrate the leak. However, addition of 30 mM lactose, a disaccharide, reduces the hemolysis of Con A agglutinated erythrocytes to 24.5% only, thus exhibiting about 50% protection from hemolysis. It is also established that erythrocyte osmofragility is not linked to the process of agglutination per se but is specifically linked to lectin treatment since agglutination of human erythrocytes with a monoclonal antibody does not induce hemolysis (Fig. 4). To study the effect of temperature on this lectin induced hemolysis of erythrocytes, Con A agglutinated cells were incubated in PBS at different temperatures and hemolysis measured after 6 h. The results are represented in Fig. 5. It is found that hemolysis of Con A agglutinated rabbit erythrocytes increases with the rise in incubation temperature. At 4°C only

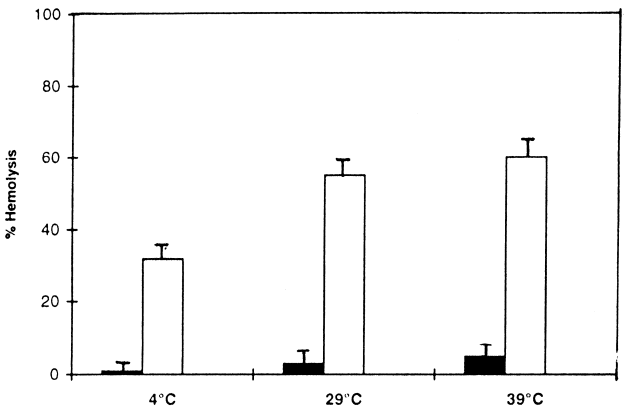

Fig. 5. Effect of temperature on colloid-osmotic lysis of Con A agglutinated trypsinized rabbit erythrocytes in PBS. Filled bars represent control, i.e. unagglutinated cells.

32% lysis is obtained which increases to 55% and 60% lysis at 29°C and 39°C, respectively.

The fact that antibody agglutinated erythrocytes do not show hemolysis but lectin agglutinated cells do, clearly indicates that erythrocyte osmofragility is linked to the agent inducing agglutination which in this case is a legume lectin. The hydrophobic cavity of Con A is known to be well conserved in all legume lectins [17]. The aggregation of membrane proteins by exogenously added lectin produces lipid areas free of membrane proteins [18]. The non-specific interactions of lipid domains with hydrophobic sites on legume lectins probably perturbs the membrane structure resulting in induction of leaks. Soybean agglutinin (SBA) mediated vesicle agglutination induces leakage of vesicle content followed by membrane fusion [19]. A massive disruption of the membrane permeability barrier is triggered when glycophorin incorporated in a bilayer consisting of unsaturated phosphatidyl ethanolamine (PE) is clustered upon addition of wheat germ agglutinin (WGA) which allows PEs to undergo the bilayer to non-bilayer that is hexagonal H_{II} phase transition [20].

Our earlier results [14] indicated that agglutination of cells with exogenous lectins increases their susceptibility to superoxide anion damage. Possibly, formation of leaks on membrane perturbations by binding of the exogenous lectin facilitates the entry of superoxide anion through the hydrophobic lipid bilayer of the cell membrane which is otherwise impermeable.

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