Biochimica et Biophysica Acta, 406 (1975) 553–563

BBA 77103

EFFECT OF METABOLIC STATE ON PHYTOHEMAGGLUTININ-P AGGLUTINATION OF NORMAL HUMAN ERYTHROCYTES

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(Received May 20th, 1975)

SUMMARY

A reproducible quantitative assay for the lectin-mediated agglutination of human erythrocytes, depending on different rates of settling of agglutinated and non-agglutinated erythrocytes, was developed. This assay was used to study the aggregation of human erythrocytes by phytohemagglutinin-P. The aggregation of human erythrocytes by phytohemagglutinin-P was found to depend upon the metabolic state of the cells. Metabolically depleted erythrocytes agglutinated much less readily than did similar cells supplied with adenosine. This was not due to swelling and rigidity of the cells, since erythrocytes in hypotonic solution did not exhibit significantly altered phytohemagglutinin-P agglutination.

Metabolically depleted erythrocytes, or erythrocytes from blood stored 8 weeks, lysed and resealed in the presence of ATP, were agglutinated by phytohemagglutinin-P to a much greater extent than control samples without ATP. The presence of Mg^{2+}, either alone or with ATP, had little effect on the agglutinability of the resealed membranes. Low concentrations of Ca^{2+} (0.2 mM) had little effect on agglutinability, although high Ca^{2+} (5 mM) inhibited agglutinability of the resealed membranes somewhat.

Both metabolically depleted erythrocytes and depleted erythrocytes, previously treated with adenosine, when treated with trypsin released similar amounts of sialic acid. The agglutinability of the trypsinized adenosine-supplemented cells increased more readily than did that of trypsinized metabolically depleted cells.

The agglutination of erythrocytes was not affected by cytochalasin B (40 μg/ml). Vinblastine (0.2 mM) caused depleted erythrocytes to agglutinate similarly to adenosine-supplemented erythrocytes, but had no effect on the agglutination of adenosine-supplemented erythrocytes.

It is concluded that ATP in the human erythrocyte probably participates in the modulation of phytohemagglutinin-P agglutinability. This is not a consequence of the more rigid membrane known to accompany ATP depletion in the erythrocyte, or of the effect of ATP levels on Ca^{2+} or Mg^{2+} content. It appears likely that ATP modulates human erythrocyte phytohemagglutinin-P agglutinability through interaction, direct or indirect, with a membrane-associated component, which might also be sensitive to vinblastine.

INTRODUCTION

The ability of cells to modulate their surface architecture during biological transitions or in response to external stimuli is now well recognized, although the mechanisms involved are not understood. Lectins have been extensively used to probe the state of the glycoproteins on the cell surface. Pronounced differences in lectin-mediated agglutinability have been found when a cell is transformed [1], when treated mildly with protease [1, 2], with fixatives [3], or with cyclic AMP analogs [4]. The general finding that the number of lectin binding sites do not usually vary greatly as the result of these alterations of cells suggests that the agglutination is more complex than a simple cross-linking of cells by multivalent lectins.

The erythrocyte was chosen for our initial studies of the mechanisms by which the plasma membrane surface is modulated in lectin agglutinability. More is known concerning the membrane components, their structure, and position within the red cell membrane than for any other cell [5, 6].

The lectin-mediated agglutinability of some cultured normal and transformed cells was found to depend on the metabolic state of the cells and probably on the ATP content [7]. Red cell metabolism, since it is limited primarily to glycolysis, is less complicated than that of most other cell types. Thus, the interrelationship between metabolism of the cell and the architecture of the surface is also more readily studied in these cells. It was the object of this investigation to study the agglutinability by phytohemagglutinin-P of the human erythrocyte in different metabolic states.

MATERIALS AND METHODS

All chemicals employed were reagent grade unless otherwise stated. Adenosine (Sigma Grade), adenosine-5′-triphosphate, disodium salt (Sigma Grade), trypsin (crystalline, bovine pancreas, type III), and soybean trypsin inhibitor (Type I-S) were obtained from Sigma Co. Phytohemagglutinin-P was from Difco Lab Co., cytochalasin B was from Aldrich Co. and vinblastine sulfate was obtained from Lilly Co. Sialic acid was assayed using the thiobarbituric acid method after acid hydrolysis [8].

Preparation of erythrocyte and resealed membrane samples

Human (Type A, Rh positive) blood was collected in either acid citrate dextrose or citrate phosphate dextrose solution, and used within a week of collection. The cells were metabolically depleted by incubation of whole blood at 37 °C for 25–30 h. Penicillin, 100 units/ml and streptomycin, 0.1 mg/ml, were added to blood to minimize possibility of bacterial growth. Adenosine when used was added to obtain a concentration of 4 mg per ml for the last 2 h of 37 °C incubation unless otherwise indicated. Erythrocytes were washed just prior to assay 3 times with 0.9 % saline and once with Solvent I (1 volume isotonic phosphate buffer pH 7.4 and 9 volumes of 0.9 % NaCl), with removal of buffy coat.

Resealed membranes were prepared from cells washed 3 times with 0.9 % saline at 2–5 °C and once with Solvent II. Solvent II was 0.9 % saline containing 10 mM Tris, adjusted to pH 7.4 at room temperature. Four ml of packed washed cells were added to 12 ml 10 mM Tris, pH 7.4 (pH at room temperature) containing

indicated materials at 0 °C. After 10 min, 1.6 ml 1.42 M KCl/0.28 M NaCl was added, and the preparation was incubated at 37 °C for 30 min. The resealed membranes were then washed twice with Solvent II and once with Solvent I by centrifugation at $4500 \times g$ for 30 min at 2–5 °C. The packed, resealed membranes which still contain hemoglobin were made up quantitatively to 10 ml with Solvent I.

Trypsin treatment

Both depleted and adenosine-treated erythrocytes from recently outdated blood were washed 3 times with 0.9 % saline and once with Solvent I. Samples were treated with trypsin in Solvent I at concentrations indicated in Fig. 3, and incubated 30 min at 37 °C. At the end of this time, a five fold excess by weight of trypsin inhibitor was added. The suspension was centrifuged ($1500 \times g$, 10 min, 2–5 °C), supernatants were collected and stored at −20 °C until they could be assayed. The pellets were washed twice with Solvent I.

Quantitative assay for phytohemagglutinin-P agglutination of erythrocytes

Washed erythrocytes were diluted to 50 % hematocrit in Solvent I. 0.05 ml of the cell suspension was added to 1 ml of varying concentrations (50–80 μg/ml) of phytohemagglutinin-P in Solvent I at 0 °C to give approximately $2.5 \cdot 10^8$ cells/ml. Samples were mixed, and incubated at the desired temperature with gentle shaking.

At various times, samples, usually in duplicate or triplicate, were cooled to 0 °C, resuspended by agitating uniformly in a Vortex mixer, for 10–15 s, to give a homogeneous suspension. The tubes were allowed to stand upright at 0 °C for 7–10 min. The supernatant only was gently mixed to give a homogeneous suspension, without disturbing the settled pellet and 0.4 ml aliquots were then withdrawn from the supernatant with an automatic micropipet. Alternatively, sample tubes, 1 cm × 7.5 cm, were marked at uniform height (7 mm from the bottom) to facilitate taking samples from the same depth of supernatant and unstirred supernatants were used. The results were not significantly different using either procedure.

The supernatant samples were added to 2 or 3 ml Drabkins' solution containing 0.2 % sodium dodecyl sulfate, and were read at 540 nm.

The percentage of cells agglutinated was calculated by dividing absorbance at 540 nm of the agglutinated sample supernatants by the corresponding reading from a similar sample with phytohemagglutinin-P omitted and subtracting this percentage from 100 %. In practice, it was found that the values obtained in the absence of lectin did not vary greatly over the incubation time used.

All samples were matched to contain a similar number of erythrocytes. This was verified by determination of hemoglobin concentration of the washed erythrocyte suspension.

The supernatants obtained after settling of cells were always checked for hemolysis. If visible hemolysis occurred, 0.4 ml of the cell-free supernatant were added to 2 or 3 ml Drabkins' solution and read at 540 nm. The contribution due to hemolysis was subtracted from both readings used to obtain the percentage of cells agglutinated. Using lower concentrations of phytohemagglutinin-P and relatively gentle agitation with the Vortex mixer of the agglutinated samples, aids in minimizing hemolysis.

Quantitative assay for phytohemagglutinin-P agglutination of resealed membranes

The assay for phytohemagglutinin-P agglutination of erythrocytes was modified for use with resealed membrane preparations. 0.1 ml of resealed membranes were used, and supernatants were sampled after standing at 0 °C for 40, 44, 48 and 52 min, to ensure that settling of agglutinated membranes was complete. Control samples without phytohemagglutinin-P were always run simultaneously. The assay for the hemoglobin concentration of the supernatant was modified slightly. The 0.4 ml sample of supernatant was added to 0.8 ml Drabkins' solution containing 0.2 % sodium dodecyl sulfate in these experiments.

Filterability of cells

The barrel of a 3 ml disposable syringe was mounted in a Pop-Top holder (General Electric) containing a Nuclepore filter (General Electric) with a 5 μm pore and a 13 mm diameter.

The flow rate of suspensions of cells (at identical concentrations for compared samples) through this apparatus was determined. No negative pressure was applied for flow determination, but the filter was washed between samples with Solvent I under moderate suction. Flow rate of Solvent I was determined between samples to check for clogging or other changes in the filter. Samples were added as the last drops of Solvent I were filtering to ensure continuity of flow.

RESULTS

Characteristics of the quantitative agglutination assay

Fig. 1 shows the increase in agglutination of erythrocytes obtained by incubat-

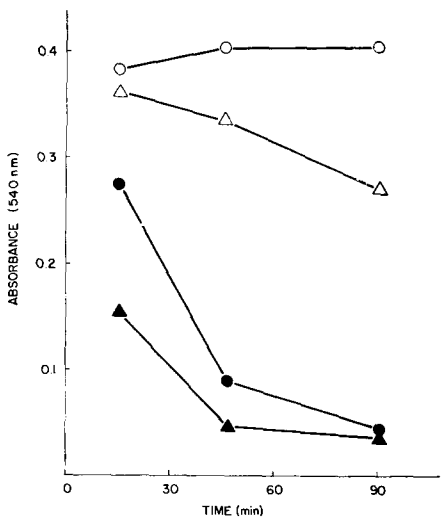

Fig. 1. The effect of lectin concentration and time on agglutination of human erythrocytes by phytohemagglutinin-P. The extent of agglutination is a function of the number of cells remaining suspended in supernatant. The number of cells suspended is measured by assaying for hemoglobin content of supernatant as described in Materials and Methods. The points are the averages of values obtained after settling of the samples for 5 and for 15 min; no significant difference was observed for values obtained using these two settling times. The concentration of lectin added to red cell suspension was: (○) no lectin; (△) 31 μg/ml; (●) 125 μg/ml; (▲) 500 μg/ml.

ing for various times with several concentrations of phytohemagglutinin-P. The agglutination depends on both phytohemagglutinin-P concentration and time of incubation with phytohemagglutinin-P. There is little settling of the erythrocytes unless they are treated with the lectin.

Effect of metabolic state and hypotonic media on agglutination of intact erythrocytes

The metabolic state of erythrocytes was varied generally by the method of Weed et al. [9]. Cells were depleted of metabolites by incubation for 25–30 h at 37 °C. This treatment [9] results in loss of almost all ATP in these cells. If the cells are then incubated with adenosine for 2 h, ATP is regenerated [9]. In the present studies, the incubations were usually in plasma, rather than serum [9], which was found to have little effect on lectin agglutinability. Phytohemagglutinin-P agglutination of depleted erythrocytes at 37 °C varies as a function of adenosine concentration with which the cells were treated prior to agglutination over a wide range, as shown in Fig. 2. Incubation of the cells with lectin at room temperature (19 °C) causes slower agglutination, but the difference in depleted cells previously incubated with and without adenosine is maintained. At 0 °C, agglutination is so slight that its quantitative estimation is not feasible.

Adenosine in a final concentration of 4 mg/ml in the solution of phytohemagglutinin-P did not enhance the agglutination of freshly drawn and washed erythrocytes, and in fact slightly depressed the degree of agglutination.

Both depletion of ATP [9] and hypotonic medium [10] cause slower filtration of erythrocytes through filters with pores in the range of the cell diameter. Agglutination of the cells by phytohemagglutinin-P in phosphate buffered saline (pH 7.4) at

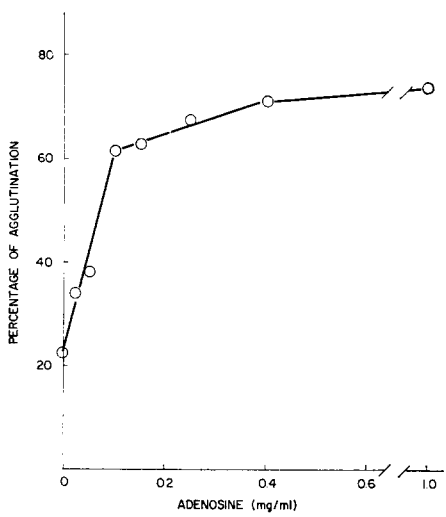

Fig. 2. Agglutination by phytohemagglutinin-P as a function of adenosine concentration previously incubated with metabolically depleted erythrocytes. Depleted erythrocytes were washed, suspended at 30 % hematocrit in Solvent I, added to an equal volume of adenosine in Solvent I to give the indicated final concentration, incubated for 110 min at 37 °C, and were then washed and resuspended in Solvent I. They were then assayed for agglutination with 75 μg phytohemagglutinin-P/ml, 37 °C, 105 min as described in Materials and Methods. Values are corrected for hemolysis.

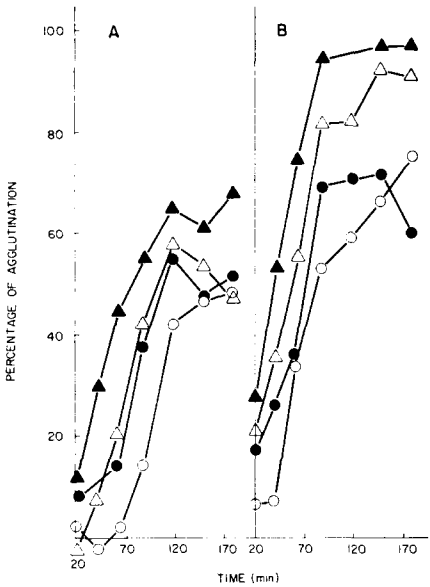

Fig. 3. Agglutination by phytohemagglutinin-P of metabolically depleted erythrocytes, with and without adenosine treatment, followed by trypsin treatment, as described in Materials and Methods. Agglutination was with 50 μg phytohemagglutinin-P/ml, 37 °C. (A) depleted cells; (B) depleted cells plus adenosine. The final concentrations of trypsin were: (○) 0 μg/ml; (●) 0.1 μg/ml; (△) 0.5 μg/ml; (▲) 2.5 μg/ml.

210 milliosmolarity, which is sufficient [10] to cause a sharp decrease in filterability of erythrocytes, was not significantly different from that in the usual isotonic solvent I.

Effect of trypsin treatment on agglutination of erythrocytes in different metabolic states

Protease treatment of cells has a marked effect on lectin agglutinability [1, 2] and was investigated. Depleted cells were divided into two aliquots, and one aliquot was treated with adenosine. After washing, both samples were treated with trypsin and agglutinability with the lectin determined. Results are shown in Fig. 3. Both groups of erythrocytes respond to trypsinization with increased agglutinability, and this was a function of the extent of digestion. The adenosine-treated cells show a greater agglutination at a particular trypsin concentration than do the depleted cells. The differences between cells incubated with and without adenosine are greater with increase in protease treatment of the cells. Cells treated with 2.5 μg trypsin/ml released about 30 % of the total sialic acid of the cell in 30 min and this release was not influenced by presence or absence of adenosine.

Agglutinability of erythrocyte membranes prepared by lysing and resealing in the presence of ATP and/or divalent cations

The agglutination by phytohemagglutinin-P of washed, resealed red cell membranes was studied in order to determine the effect of various materials on the agglutination.

Incorporation of Ca^{2+} at concentrations of 0, 0.2, 1 and 5 mM, into mem-

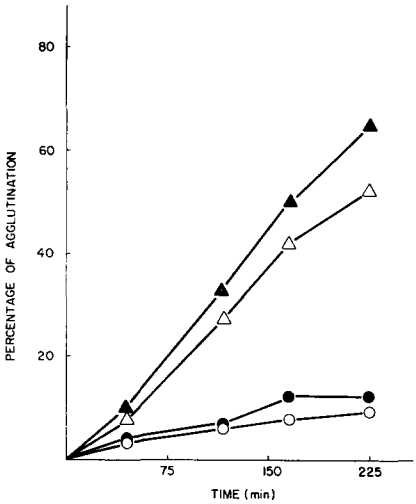

Fig. 4. Agglutination by phytohemagglutinin-P at 37 °C of resealed membranes prepared from metabolically depleted erythrocytes, in the presence and/or absence of ATP and/or Mg^{2+} in the lysis and resealing medium, as described in Materials and Methods. Additions to lysis media were: (●) no additions to lysis medium; (○) 2 mM Mg^{2+} in lysis medium; (△) 2 mM disodium ATP in lysis medium; (▲) 2 mM disodium ATP+2 mM Mg^{2+} in lysis medium.

branes from adenosine-treated erythrocytes was found to have little influence on the agglutinability of the resealed membranes with the exception of a moderately decreased agglutinability of the membranes resealed in 5 mM Ca^{2+}. A similar experiment on the same sample depleted without adenosine added yielded somewhat more varied responses. The lowest agglutination was still obtained with the membranes resealed in 5 mM Ca^{2+}. The membranes sealed in 1 mM Ca^{2+}, however, appeared to agglutinate somewhat more extensively than the membranes resealed without Ca^{2+} or in 0.2 mM Ca^{2+} and much more than membranes resealed in 5 mM Ca^{2+}. Agglutination of membranes resealed in 0 or 0.2 mM Ca^{2+} was, however, quite similar.

The effect on agglutination of Mg^{2+} and ATP is presented in Fig. 4. Under the conditions employed, the membranes from depleted cells agglutinated only slightly if resealed in buffer or in a buffer containing 2 mM Mg^{2+}. Incorporation of 2 mM ATP in the resealing medium had a pronounced effect on agglutinability. Similar membranes resealed in medium containing both 2 mM ATP and 2 mM Mg^{2+} showed a similar increase in agglutinability, possibly a little greater than that produced by ATP alone.

Membranes from depleted erythrocytes resealed in either 1 or 5 mM ATP agglutinated similarly to each other. The agglutination of resealed membranes from erythrocytes stored cold for 8 weeks in acid citrate dextrose plasma was also greatly increased by the presence of ATP in the lysing and resealing medium.

Effects of cytochalasin B and vinblastine on agglutination and filtration of metabolically depleted and adenosine-treated erythrocytes

Fig. 5A shows that cytochalasin B, at 40 μg/ml, has no effect on the agglutination of either metabolically depleted or adenosine-treated erythrocytes. On the

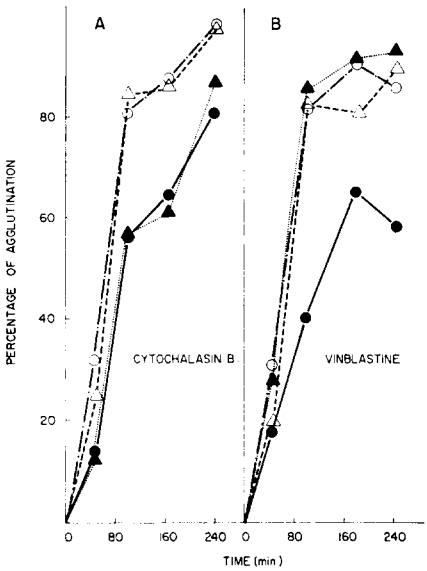

Fig. 5. Effect of cytochalasin B and vinblastine on agglutination of metabolically depleted and adenosine-treated depleted erythrocytes. Agglutination was by 70 μg phytohemagglutinin-P/ml, 37 °C. Assays were prepared as described under Materials and Methods with correction for hemolysis. Erythrocytes were pre-incubated with the drug for a few minutes prior to their addition to the phytohemagglutinin-P solution, which also contained the drug. Control samples without phytohemagglutinin-P were run to provide values for zero agglutination for each point. Samples were as follows: metabolically depleted erythrocytes, without (●) and with (▲) the drug; adenosine-treated erythrocytes without (○) and with (△) the drug. (A) Effect of cytochalasin B, 40 μg/ml. All samples contained equal concentration of dimethylsulfoxide, used as solvent for cytochalasin B. (B) Effect of vinblastine sulfate, 0.2 mM.

other hand, Fig. 5B shows that vinblastine, at 0.2 mM, has a pronounced effect on the agglutination of metabolically depleted erythrocytes, causing them to agglutinate like adenosine-treated erythrocytes. Agglutination of adenosine-treated erythrocytes, however, showed no response to vinblastine.

Vinblastine also exerted different effects on filtration of depleted and aden-

TABLE I

EFFECT OF VINBLASTINE ON THE DEFORMABILITY OF METABOLICALLY DEPLETED AND ADENOSINE-TREATED ERYTHROCYTES

Vinblastine, where present, was at 0.2 mM in the filtration medium. Erythrocytes in plasma were diluted 100-fold in the medium 10 min prior to filtration at room temperature. Filtration time is for passage of 2.1 ml as described in Materials and Methods.

Metabolic state of erythrocytes	Vinblastine	Filtration time (s)
Depleted	—	220
Depleted	+	159
Depleted plus adenosine	—	160
Depleted plus adenosine	+	313

osine-treated erythrocytes (Table I). This reagent caused a decrease in the filtration time of approximately 30 % with metabolically depleted erythrocytes. There is approximately a two fold increase in filtration time of adenosine-treated erythrocytes. This is comparable to the change in filtration time obtained with 0.2 mM vinblastine in fresh erythrocytes [11]. The addition of vinblastine to depleted erythrocytes caused them to filter almost identically to adenosine-treated erythrocytes.

DISCUSSION

The quantitative assay employed in these studies of lecithin agglutination of of human erythrocytes affords a more quantitative estimation of agglutination than the commonly employed visually scored assay. Although several other reports employing spectrophotometric or other techniques [12–16] for the quantitative study of aggregation have appeared, none of these seem easier to perform than the method reported.

It also readily allows comparison of the agglutination as a function of time of incubation with lectin and allows flexibility in selection of several other parameters such as concentration of erythrocytes and lectin. In selecting exact protocols for this assay, it is necessary that the resuspension protocol should be reproducible, vigorous enough to disperse any obvious large clumps in the depleted cell plus lectin samples, but not so vigorous as to obliterate differences between depleted and adenosine supplemented samples or to cause severe hemolysis. Settling time selected should be long enough to allow the agglutinated cells to settle fairly completely, but not so long as to allow extensive settling of unagglutinated erythrocytes. Any reagent that alters the settling time of erythrocytes would affect the results of this assay. This is readily compensated for by the inclusion of all reagents employed singly or in combination in controls, but visual examination should be made to establish that aggregation of cells is in fact taking place.

Using this assay, it was possible to show that the agglutination of human erythrocytes by phytohemagglutinin-P is markedly influenced by the metabolic state of the cells. Erythrocytes metabolically depleted by incubation at 37 °C for 25–30 h, show a decreased agglutinability compared to the same cells to which adenosine had been added prior to washing and agglutination.

The sharp increase in phytohemagglutinin-P agglutinability of resealed membranes from metabolically depleted erythrocytes, or from erythrocytes stored cold in acid citrate dextrose plasma for 8 weeks, which is elicited by the presence of ATP in the lysis and resealing medium, as well as the response of depleted cells to adenosine, suggests that ATP is involved in the control of phytohemagglutinin-P agglutinability of human erythrocytes.

Agglutination of cells by lectins requires the specific binding of lectins to carbohydrate moieties of cell surface glycoproteins. Most studies of cells in different states which exhibit quite different lectin agglutinabilities, however, show either little difference in binding of lectin, or else differences which seem too small to account for the differences in agglutinabilities. It would appear then that differences in lectin mediated agglutination cannot be attributed to differences in the amounts of lectin bound. Other factors currently thought to influence lectin agglutinability have been recently discussed by Nicolson [17] and Raff et al. [18]. These factors include possible

enhancement of agglutination through lectin-induced clustering of lectin-receptor complexes [17, 18], thought more likely to be influential when low numbers of binding sites are occupied [17], and apparently not a predominant factor in several cases [18], surface morphology [17, 18], cell deformability [17], cell charge repulsion forces [17], and the mode of satisfaction of the binding sites of the polyvalent lectin molecule, which may bind receptors on either single or multiple glycoproteins [18].

One of the most likely of these factors, decrease in erythrocyte deformability, was known to be produced by depletion of ATP [9]. However, little difference in lectin agglutinability was produced by treatments observed by others to decrease deformability of the erythrocyte membrane, including vinblastine treatment [11] or incorporation of Ca^{2+} at 0.2 mM in resealed membranes [9]. Further, lysis and resealing of membranes from depleted erythrocytes in the presence of Mg^{2+}, which was shown to greatly increase deformability of these membranes [9], has no influence on agglutinability. These findings are a convincing indication that ATP does not mediate its effect on agglutinability through an effect on membrane deformability or on the control of transport or binding of Ca^{2+} or Mg^{2+}. Hypotonic media which produce a sharp decrease in filterability of erythrocytes [10] also do not greatly influence the agglutination. Because lectin-induced clustering of lectin receptors does not appear to occur in the intact human erythrocyte [19], this factor can be disregarded in explaining the effect of metabolic state on agglutinability of human erythrocytes.

Vinblastine and adenosine restoration have quite similar effects on agglutinability of depleted erythrocytes, while vinblastine does not affect agglutination of adenosine-treated erythrocytes. Because the agglutination curve for vinblastine-treated depleted erythrocytes is so similar to that for adenosine-treated erythrocytes, it is conceivable that the interaction of ATP which modulates the agglutination might involve a vinblastine-sensitive component. Proteins sensitive to cytochalasin B do not appear to participate in modulation of human erythrocyte phytohemagglutinin-P agglutination.

The mechanism by which trypsinization elicits increased lectin agglutinability is not yet understood, but is of considerable interest in light of the magnitude of the effect often produced and of the similarity of responses to lectins of transformed cells and of trypsinized normal counterpart cells. Clearly, in the studies reported here, the metabolic modulation of phytohemagglutinin-P agglutinability is maintained and even enhanced in the trypsinized human erythrocyte.

ATP also appears to play a role in lectin agglutinability of cultured normal and transformed hamster cells [7]. Interestingly, high ATP is associated with low concanavalin A agglutinability in these cultured cells, in contrast to association of high ATP with high phytohemagglutinin-P agglutinability of human erythrocytes reported here. That such diverse types of cells as human erythrocytes and cultured and transformed hamster cells show an influence of metabolic state on lectin agglutinability suggests that cellular metabolism may in general influence lectin agglutinability.

The human erythrocyte, with its comparatively simple metabolism and relatively well understood membrane structure, is an attractive cellular system in which to pursue a more detailed understanding of the metabolic modulation of lectin agglutinability and surface expression.

ACKNOWLEDGEMENTS

This investigation was supported in part by grants from NIH, CA 13534 and CA 08480.

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