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MECHANISM OF THE MODULATION OF THE PHYTOHEMAGGLUTININ-P AGGLUTINATION OF THE HUMAN ERYTHROCYTE BY METABOLIC STATE

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Summary

The agglutination of human erythrocytes by phytohemagglutinin-P can be changed depending upon the metabolic state of the cell. The agglutination of intact cells depends upon the ATP content of the cell. Agglutination of resealed membranes from ATP depleted erythrocytes has been shown to be a function of the concentration of ATP in the resealing media. β,γ-Methylene-ATP competitively inhibits the effect of ATP within resealed membranes.

The route of entry of adenosine into the cell was also found to influence agglutinability; in nucleoside transport-inhibited cells, there was a distinct maximum of agglutination vs. adenosine concentration not observed in uninhibited cells.

The influence of cell shape on agglutination was also considered and agglutination was found to correlate with the percentage of cells with disc morphology. Alterations of the shape of erythrocytes produced by lysophosphatidylcholine treatment, however, were not accompanied by any change in agglutinability.

An attempt was also made to evaluate the relationship between agglutination and charge of the surface of the cell. Partition of resealed membranes in an aqueous two-polymer phase system which is primarily determined by surface charge decreased as a function of concentration of ATP in the lysis and resealing medium, while agglutination of these preparations on the other hand increased concomitantly.

Results suggest that transitions in morphology, surface charge, and phytohemagglutinin-P agglutination of human erythrocytes have at least one ATP-mediated event in common.

Introduction

Lectin mediated agglutination of cells is attributable to the interaction of the lectin with plasma membrane glycoproteins. The agglutination is sensitive to changes on the inside of the cell and can be modified by alterations of the metabolic state [1—6] or changes in the underlying membrane components [7].

Agglutination of human erythrocytes by concanavalin A [5] and phytohemagglutinin-P [4] is altered by changing the metabolic state, but the ways in which the two lectins interact with surface components of the cells appear to be distinct. The agglutination of red cells by both lectins is influenced by adenosine. In the case of phytohemagglutinin, this was most apparent with metabolically depleted erythrocytes, and seemed likely to be mediated via production of ATP [4]. On the other hand, concanavalin A agglutination was enhanced by adenosine even with fresh erythrocytes, and the enhanced agglutinability does not appear to involve ATP [5].

This report extends our observation on adenosine-modulated phytohemagglutinin-P agglutinability of human erythrocytes.

Materials and Methods

All chemicals were reagent grade unless otherwise stated. Adenosine (Sigma Grade), adenine, inosine, adenosine 5′-triphosphate, disodium salt (Sigma Grade) (ATP), β,γ-methylene adenosine 5′-triphosphate, sodium salt (β,γ-methylene-ATP), trypsin (crystalline, bovine pancreas, type III), soybean trypsin inhibitor (type I-S), Dextran (500 000 molecular weight), and dried Firefly Lanterns (50 mg vials) were obtained from Sigma Co. Phytohemagglutinin-P was from Difco Lab. Co. Lysophosphatidylcholine (egg) was from Supelco. Neuramidase (*Vibrio cholerae*, 500 units/ml) was obtained from Schwarz-Mann. Polyethylene glycol (6000—7500 molecular weight) was from Matheson, Coleman and Bell. *p*-Nitrobenzylthioguanosine was generously contributed by Dr. A.R.P. Paterson.

The procedures for preparation of washed erythrocytes, the metabolic depletion of the ATP of the cells, the trypsin treatment of erythrocytes, resealed membranes, agglutination assay, and sialic acid determination have been previously described [4].

The assay for ATP was essentially that described by Beutler [8], with modification to permit use of the Gilford spectrophotometer for the detection of emitted light.

A two-phase polymer system [9] was employed to assay changes in sealed membranes. Stock solution were prepared by mixing equal volumes of 11% Dextran and 8.8% polyethylene glycol in isotonic phosphate buffered saline, pH 7.4, and drawing off the upper and lower phases. For partitioning, equal volumes (usually 1 ml) of the two phases at 20°C were pipetted into small test tubes. Resealed membranes, in 0.25 ml isotonic phosphate-buffered saline were added, and the samples mixed by vortex for several seconds. The samples were allowed to stand until phase separation was complete, and 0.4 ml aliquots of the upper phase were withdrawn and added to 0.8 ml Drabkin's solution con-

taining 0.2% sodium dodecyl sulfate. Absorbance of these solutions at 540 nm was then determined.

For morphologic studies, 5 μl of a suspension of unfixed erythrocytes (40% v/v in phosphate buffered saline) was added to 0.2 or 0.4 ml of the plasma reserved from the prior metabolic depletion of the blood. 10 μl of this sample was placed on a slide, covered with a coverslip, and examined by light microscopy. Groups of cells suitable for counting were found at random with the focus adjusted so that the shapes of the cells could not be determined. Focus was then adjusted to give sharp images, and cells were scored as discs, spheres, or echinocytes. Any cell with spiky projections distinguishable was counted as an echinocyte.

Results

Effect of variation of ATP on agglutination

Erythrocytes metabolically depleted of ATP at 37°C were incubated with different concentrations of either adenosine or adenine plus inosine. The phytohemagglutinin-P agglutination and ATP content of these erythrocytes are given in Fig. 1. Agglutination increases directly with ATP concentration to about 110 μM and then becomes constant.

Metabolically depleted erythrocytes were lysed and resealed in media varying only in the concentration of ATP. The phytohemagglutinin-P agglutination of these resealed membranes as a function of ATP concentration during the course

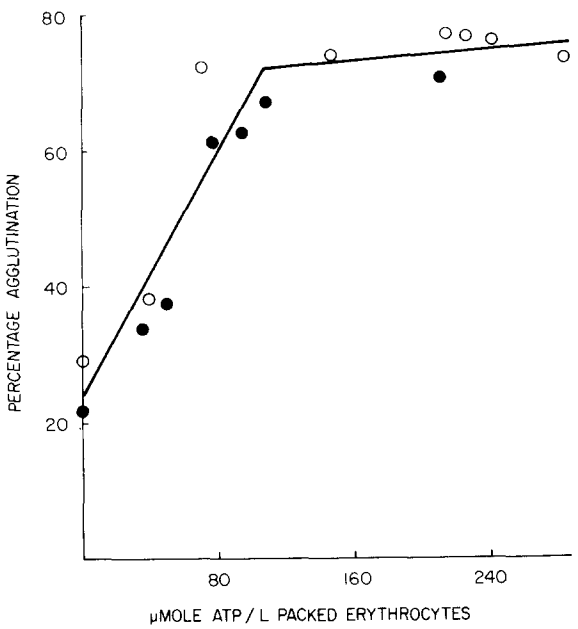

Fig. 1. Metabolically depleted, washed erythrocytes were incubated 110 min, 37°C, with different concentrations of adenosine (0—1 mg/ml) (●) or adenosine (0.5 mg) plus inosine (0—2.5 mg/ml) (○). Agglutination and ATP content were determined as described in the text. Packed erythrocytes contained approximately $8.45 \cdot 10^{12}$ cells/l.

of incubation with lectin is plotted in Fig. 2. At low concentrations of ATP (0 to 0.1 mM), there is no detectable agglutination. Agglutination rises sharply with increasing ATP to a maximum at about 0.7 mM ATP, and no further increase is obtained with higher concentrations of ATP. Although agglutination increases with time of incubation with the lectin, the shape of the curve of percentage agglutination vs. ATP concentration does not vary greatly with incubation time.

The effect of an ATP analog, β,γ-methylene-ATP, which cannot donate its γ-phosphoryl group, is shown in Fig. 3. Agglutination is not supported by β,γ-methylene-ATP at 0.3 or 0.9 mM in the lysis medium. Further, this analog of ATP inhibits the ATP supported agglutination competitively.

Effect of a specific nucleoside transport inhibitor

p-Nitrobenzylthioguanosine, an inhibitor of facilitated adenosine transport in the erythrocyte which allows increased utilization of adenosine for ATP production by means of protection of adenosine from adenosine deaminase proximal to the transport site [10], produces a marked increase in phytohemagglutinin-P agglutination of metabolically depleted erythrocytes in the presence of about 0.5 to 2 mM adenosine as is seen in Fig. 4. The influence of the inhibitor at low or high concentration of adenosine is considerably less.

Correlation of agglutination and surface charge of cells

Depleted erythrocytes were lysed and resealed in media containing various concentrations of ATP. Their phytohemagglutinin-P agglutination and their aqueous two-polymer phase partitioning were determined. The results are shown in Fig. 5 as a function of ATP concentration. Agglutination increases, while partitioning into the upper phase decreases. Both parameters reach a plateau at the same ATP concentration.

Metabolically depleted erythrocytes were lysed and resealed in media con-

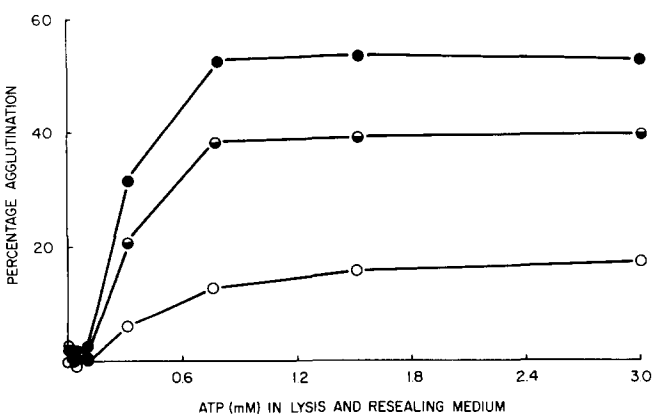

Fig. 2. Agglutination of resealed erythrocyte membranes containing different concentrations of ATP. Metabolically depleted, washed erythrocytes were lysed and resealed in media containing the indicated concentrations of ATP. Agglutination was determined following 75 min (○), 110 min (◒), and 155 min (●) incubation with phytohemagglutinin-P.

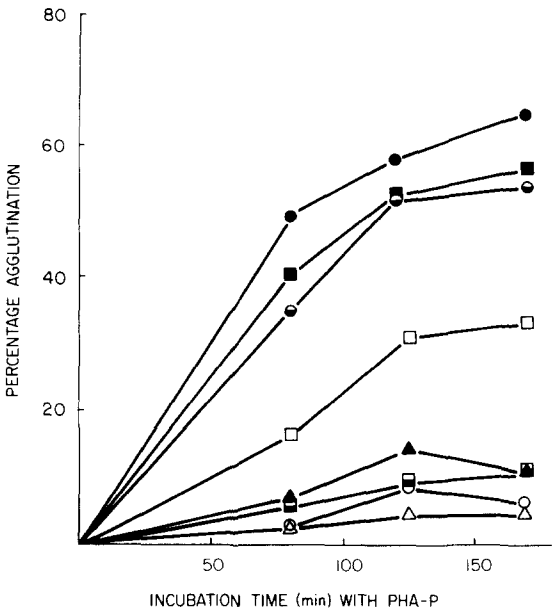

Fig. 3. Agglutination of resealed erythrocyte membranes containing different concentrations of ATP and/or β,γ-methylene-ATP. Metabolically depleted, washed erythrocytes were lysed and resealed in media containing the following additions: 0.3 mM ATP (◒); 0.9 mM ATP (●); 0.3 mM β,γ-ATP (◠); 0.9 mM β,γ-ATP (▲); 0.3 mM ATP + 0.3 mM β,γ-ATP (□); 0.3 mM ATP + 0.9 mM β,γ-ATP (◒); 0.9 mM ATP + 0.9 mM β,γ-ATP (■) and no additions (○), with subsequent determination of agglutination.

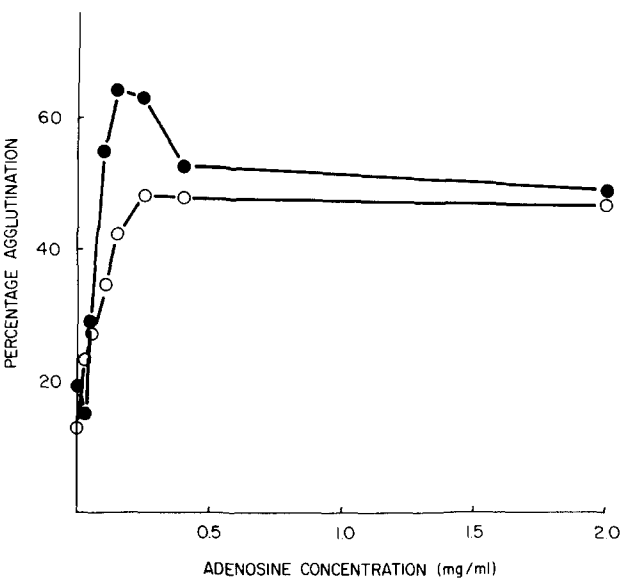

Fig. 4. Effect of a specific nucleoside transport inhibitor, *p*-nitrobenzylthioguanosine on the agglutination of metabolically depleted erythrocytes incubated with adenosine. Metabolically depleted, washed erythrocytes were incubated with the indicated concentrations of adenosine in the presence (●) or absence (○) of 15 μM *p*-nitrobenzylthioguanosine for 130 min, at 37°C. Aliquots of these samples were added to 5 volumes of buffer or phytohemagglutination-P solution, and agglutination was determined after 120 min incubation with the lectin.

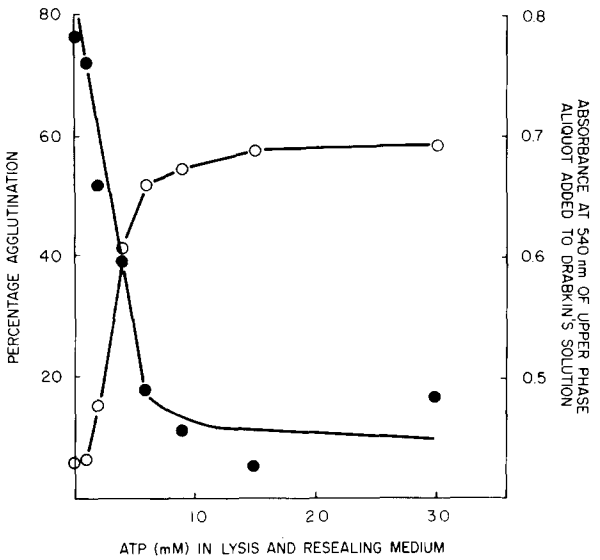

Fig. 5. Comparison of agglutination and aqueous two-polymer phase partitioning of resealed membranes containing different concentrations of ATP. Metabolically depleted, washed erythrocytes were lysed and resealed in media containing the indicated concentrations of ATP. Agglutination (○) and aqueous two-polymer phase partitioning (●) of the resealed membranes were then determined.

taining various concentrations of magnesium and ATP. The variation of magnesium concentration was found to produce only modest changes in both the agglutination and the partitioning, but there was an approximately linear relation between the partitioning and agglutination.

Resealed membrane preparations were treated with neuraminidase. The aqueous two-polymer phase partitioning of these samples showed that as the sialic acid was removed, there was a moderate effect on partitioning; however, the phytohemagglutinin-P agglutination of all neuraminidase treated samples in this experiment was so extensive that they would all have to be considered 100% agglutinated.

Relationship between shape and agglutination

The morphology of depleted erythrocytes after incubation with varying concentrations of adenosine and the corresponding agglutination of these cells are plotted in Fig. 6. The percentage of cells exhibiting the disc shape shows a correlation with the percentage agglutination, with virtually parallel dependencies on adenosine concentration through the lower range (0 to 0.4 mg/ml). Spheres and echinocytes decrease more or less similarly to each other as a function of adenosine concentration. The effect of trypsin treatment on the shape of depleted erythrocytes was also investigated and found to be quite small in relation to corresponding increases in agglutination.

Agglutination of depleted and adenosine-restored cells showed no response to lysophosphatidylcholine from 0 to 3 μg/ml, a range over which disc morphology of cells fixed by glutaraldehyde in the presence of lysophosphatidylcholine decreased substantially for the adenosine-restored cells.

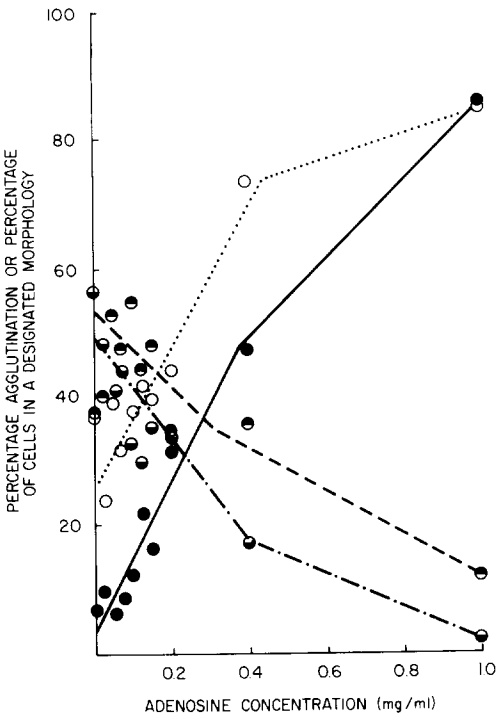

Fig. 6. The relationship of shape of erythrocyte and agglutination by phytohemagglutinin. Metabolically depleted, washed erythrocytes were incubated with the indicated concentrations of adenosine, 2 h, 37°C, and stored at 2—5°C overnight. Agglutination (○.....○) and morphologies as echinocytes (○- - - - -○), spherocytes (◒— · — ·◒) or discs (●———●) of aliquots of each sample were then determined.

Discussion

Our earlier work clearly demonstrated that lectin mediated agglutination of human erythrocytes can be altered by changes in the metabolism or metabolites of the cell [4,5]. Similar demonstration of a dependency of lectin agglutinability on the metabolic state of the cell has been made for eucaryotic cells [1,2,3,6]. Neither concanavalin A or wheat germ agglutinin mediated agglutination of human erythrocytes, however, was reported responsive to metabolic activity [11]. The effect of adenosine in enhancing phytohemagglutinin-P agglutination of metabolically depleted erythrocytes has been established by this study to arise from its role as a substrate for ATP production rather than as a ligand itself. This conclusion is supported by the observation that the agglutination of erythrocytes depends on concentration of ATP generated either from adenosine or from adenine plus inosine. The curve of agglutination vs. ATP concentration produced metabolically in intact cells also is similar in general shape to the curve of agglutination of resealed membranes from metabolically depleted erythrocytes which were resealed with ATP.

It has recently been shown that erythrocytes treated with the competitive nucleoside transport inhibitor *p*-nitrobenzylthioguanosine produce higher levels of ATP from adenosine [10] indicating an influence of route of entry on subse-

quent adenosine metabolism. The increased agglutination of erythrocytes in the presence of this inhibitor is compatible with adenosine simply enhancing ATP generation. However, the distinct optimum in adenosine concentration observed in the nucleoside transport inhibited erythrocytes for phytohemagglutinin agglutination is an unusual feature not observed with adenosine alone or with ATP incorporated into resealed membranes. These results indicate that the route of entry of adenosine into the ATP-depleted erythrocytes has an important influence on its effect on the phytohemagglutinin-P agglutination.

The inhibition of agglutination of membranes resealed in the presence of relatively low concentrations of ATP produced by concomitant incorporation of β,γ-methylene-ATP, an analog which cannot donate its γ-phosphoryl group, and the relief of this inhibition obtained by increasing ATP concentration strongly indicate that phosphorylation may be involved in the modulation of phytohemagglutinin-P agglutination by ATP.

Although phytohemagglutinin-P agglutination of depleted erythrocytes incubated with several concentrations of adenosine shows a direct correlation with disc morphology, the insensitivity of agglutination to lysophosphatidylcholine in concentrations which substantially decrease disc morphology clearly demonstrate that the disc morphology is not essential to agglutination by phytohemagglutinin-P. Also, the lack of response of phytohemagglutinin-P agglutination to this echinogenic agent argues against the generality of the suggested [12] correlation of increased agglutinability of erythrocytes with the echinocytic morphology, and additionally suggests that any alterations in the membrane fluidity induced by lysophosphatidylcholine do not influence agglutinability in this instance. It was further noted that extensive trypsin treatment does produce some increase in disc shape, as was previously reported [13], but that trypsin treatment which results in only a small increase of disc-shaped cells causes a very large increase in agglutination, again showing a dissociation between morphology and agglutination in this system. Although the disc morphology itself does not seem to be directly related to phytohemagglutinin-P agglutination, it appears probable that there are common surface components involved in shape and agglutination modulated by ATP-mediated events.

The change in cell surface charge was monitored by aqueous two-polymer phase partitioning [9] and was found to vary with metabolic state concomitantly with metabolic state-governed increases in agglutination. A number of physicochemical factors including charge, steric restrictions and spacing of binding sites, and mobility of receptors probably influence lectin agglutinability and in turn may be influenced by the metabolic state of the cell (for review, see ref. 7). The correlation we observed between charge and agglutination suggests that a major physicochemical basis for the increase in phytohemagglutinin-P agglutination which is elicited by ATP may be the decrease in cell surface charge. That the correlation is not merely fortuitous is supported by the similar relation between partitioning and agglutination observed with variations in Mg^{2+} content, although the range covered in this experiment was limited. It should be noted that while charge is not necessarily the sole determinant of partitioning, it is generally the predominant factor [9]. Additionally, we have actually demonstrated in our system a linear relation between partitioning and amount of sialic acid removed by neuraminidase.

When very little sialic acid is removed and there is little change in partitioning behavior of the cell, a marked change in agglutination can be observed. Thus, accessibility of receptor sites may be the most important factor governing agglutination. These results preclude using partial sialic acid removal to probe the relation between surface charge and agglutination, and point out that effects of neuraminidase on cell surface phenomena cannot uncritically be interpreted as arising from reduction of overall surface charge. They further suggest that changes in a relatively minor fraction of a cell surface component can produce substantial changes in cell surface properties. Both the changes in sialylation of glycopeptides [14] and the changes in metabolic patterns are characteristic of malignant cells [15], and may well play an important and interacting role in producing the high lectin agglutinability and other altered cell surface properties associated with malignancy [16].

It is concluded from this study that the modulation of phytohemagglutinin-P agglutination of metabolically depleted human erythrocytes by adenosine is mediated through ATP, with probable involvement of the transfer of the γ-phosphorus, and that this modulation is sensitive to the route of entry of adenosine into the cell. ATP mediates an increase in disc morphology and decrease in surface charge, both of which occur concomitant with an increase in phytohemagglutinin-P agglutination. Thus, it would appear that the three parameters of phytohemagglutinin-P agglutination, morphology, and surface charge appear likely to involve common elements in their modulation by cellular ATP in the human erythrocyte.

Acknowledgements

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