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EFFECT OF METABOLIC STATE ON AGGLUTINATION OF HUMAN ERYTHROCYTES BY CONCANAVALIN A

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SUMMARY

Intact freshly drawn or stored human erythrocytes, which show little agglutination by concanavalin A, become agglutinable by this lectin in the presence of adenosine. α-Methylglucose (10 mM) completely inhibits this agglutination. The concanavalin A agglutination shows no sensitivity to vinblastine or cytochalasin B.

Resealed membranes prepared with ATP in lysing and resealing medium give modest agglutinability, while the presence of adenosine in both the lysing and the resealing medium results in a substantial agglutinability of the resealed membranes.

Mild trypsin treatment of the erythrocytes causes an enhanced sensitivity to adenosine activation of the concanavalin A agglutination, while extensive trypsin treatment produced highly agglutinable erythrocytes that show no response to the presence of adenosine in the lectin solution. The extensively treated erythrocytes also show concanavalin A agglutination at temperatures below 37 °C, under conditions in which intact or moderately treated erythrocytes do not agglutinate, with or without adenosine present.

Results suggest that the adenosine activation of concanavalin A agglutination of intact human erythrocytes is mediated through a metabolic conversion of adenosine to a rapidly turned over metabolite which participates directly in the activation of agglutination. The agglutinability does not appear to depend on whole cell ATP levels, but may involve a particular pool of ATP.

The effect of variation of cellular metabolic state and the response of particular systems involved in lectin-mediated agglutinability to cellular metabolism seem to be worth consideration in explaining the frequently large differences in agglutinability of und in cells in different biological states, such as those encountered in normal and transformed cells.

INTRODUCTION

We have previously reported that the agglutination of human erythrocytes by the lectin phytohemagglutinin-P reflects the metabolic state of the cell [1]. In the human erythrocyte the receptors for phytohemagglutinin have been shown to be

largely distinct from those for the lectin concanavalin A. The major sialoglycoprotein is a phytohemagglutinin receptor, but does not bind concanavalin A tightly [2], while a fraction of protein 3 is tightly bound by concanavalin A [3].

It has been reported that concanavalin A does not agglutinate intact human erythrocytes appreciably, although prior proteolytic treatment of the cells causes them to be highly agglutinable by concanavalin A [4].

Employing a modification of the quantitative assay for agglutination described earlier [1], we have found conditions under which intact human erythrocytes are highly agglutinable by concanavalin A, and have compared several aspects of the modulation of cancanavalin A agglutination of human erythrocytes with their previously reported [1] phytohemagglutinin-P agglutination.

METHODS

Preparation of washed erythrocytes and resealed membranes

Plasma was removed ($1500 \times g$, 3 min, 2–5 °C) from human Type A, Rh positive blood in acid-citrate-dextrose anticoagulant or citrate-phosphate-dextrose anticoagulant. The erythrocytes were washed ($1500 \times g$, 10 min, 2–5 °C) three times with 0.9 % saline and once with Solvent I (1 vol. isotonic sodium phosphate, pH 7.4, and 9 vol. 0.9 % saline). Buffy coat was removed during washing.

Resealed membranes were prepared as previously described [1].

Quantitative assay for concanavalin A agglutination of erythrocytes

Method I. 0.1 ml aliquots of washed erythrocytes, diluted to 40 % hematocrit, were added to 1×7 cm test tubes containing 1 ml of Solvent I or concanavalin A (Sigma, Type IV) in Solvent I and containing 4 mg adenosine (Sigma, Sigma Grade) where indicated. The cell suspension was incubated at 37 °C with gentle shaking. At various times, samples were transferred to ice-water and resuspended by mixing in a Vortex mixer for 3 s. The samples were then allowed to settle at 0 °C for about 50 min. The supernatant was then gently stirred to produce a homogeneous suspension without disturbing the settled pellet. Aliquots of 0.2 ml of the supernatant were withdrawn with an Eppendorf pipet and added to 1 ml Drabkin's solution containing 0.2 % sodium dodecyl sulfate. Control samples without the lectin were run concurrently with each concanavalin A-containing sample. Absorbance of samples at 540 nm was determined on Zeiss spectrophotometer. Percentage of agglutination was $100\ \% - 100(A_{\text{sample with lectin}})/(A_{\text{sample without lectin}})$.

Where necessary, corrections were made for any hemolysis [1], but corrections were rarely necessary. Determinations were usually performed in triplicate and were always handled so as to obtain equal concentrations of erythrocytes in each assay solution.

Method II. Method II was similar to Method I, except that the step involving stirring of the supernatant was omitted. Uniform 1×7 cm test tubes were calibrated so that supernatants were withdrawn with the Eppendorf tip exactly 7 mm from the bottom of the tube. In our hands, Methods I and II gave similar results, with respect to both actual values and average deviations of replicates.

Concanavalin A agglutination of resealed membrane was carried out as described for cells, except that aliquots of 0.2 ml resealed membranes [1] were used as

samples and settling time was increased to 2 h. An aliquot of 0.4 ml of the supernatants was added to 0.8 ml Drabkin's solution containing 0.2 % sodium dodecyl sulfate.

Trypsin treatment of erythrocytes

Varying concentrations of trypsin (Sigma, Type III) were added to washed erythrocytes with a 50 % hematocrit in Solvent I and incubated at 37 °C for 40 min with gentle shaking. At the end of that time an excess (5-fold, by weight) of trypsin inhibitor (Sigma, Type I-S) was added. Samples were then washed by centrifugation at $1500 \times g$ for 10 min at 5 °C three times with Solvent I.

RESULTS

Effect of metabolite variation on agglutination

Incubation of washed human erythrocytes, with concanavalin A at concentrations ranging from 62.5 to 1000 μg/ml, at 37 °C, produced only weak agglutination, which was difficult to detect. On the other hand, erythrocytes incubated with concanavalin A in the presence of 4 mg adenosine/ml are agglutinated to a much greater extent. The concanavalin A was essential for the agglutination, as incubation of the erythrocytes with adenosine alone did not cause agglutination. α-Methylglucose at 10 mM completely abolished the agglutination; even 1 mM α-methylglucose causes substantial inhibition. Similar results were obtained with either freshly drawn erythrocytes or with erythrocytes stored as long as 4 weeks under blood bank conditions.

It was necessary to incubate cells with concanavalin A while in the presence of adenosine to obtain maximum agglutination. Pre-incubation of the washed erythrocytes with adenosine for 35 min at 37 °C yielded only a slightly increased agglutination at short time intervals after the addition of concanavalin A, when the assay was run in the presence of adenosine, while pre-incubation had no effect if the agglutination was studied for longer time intervals after the addition of concanavalin A. Results of extension of the pre-incubation time to 3 h and investigation of subsequent lectin-induced agglutination after 60 min with concanavalin A, in both the presence

TABLE I

EFFECT OF PRE-INCUBATION OF ERYTHROCYTES AT 0 AND 37 °C, WITH AND WITHOUT ADENOSINE, ON SUBSEQUENT AGGLUTINATION BY CONCANAVALIN A, IN THE PRESENCE AND ABSENCE OF ADENOSINE

Washed erythrocytes were incubated at 25 % hematocrit for 3 h at the indicated pre-incubation temperature; adenosine, where indicated, was 4 mg/ml. 0.2 ml aliquots of pre-incubated erythrocytes were added to 1 ml aliquots of concanavalin A, 300 μg/ml; and incubated at 37 °C for 60 min and agglutination was determined as described under Methods.

Pre-incubation temperature (°C)	Adenosine in pre-incubation	Percentage agglutination by concanavalin A	
		Without adenosine	With adenosine
37	–	2.7	12.7
37	+	14.2	28.6
0	–	1.6	8.9
0	+	7.5	15.4

and the absence of adenosine, is shown in Table I. Pre-incubation of cells with adenosine at 37 °C resulted in increased agglutination while at a pre-incubation temperature of 0 °C the effect was not as marked. The presence of phosphate in the buffer had virtually no influence on agglutination by concanavalin A, in either the presence or the absence of adenosine.

Effect of metabolites in lysing and resealing media on subsequent agglutination of resealed membranes by concanavalin A

Resealed membranes showed no agglutination. When, however, the membranes were resealed in the presence of ATP a modest agglutination was obtained with concanavalin A, while the membranes resealed and agglutinated in the presence of adenosine showed substantial agglutination (Table II). Both ATP- and adenosine-supplemented resealed membranes incubated at room temperature for 18 h agglutinated about twice as well as samples maintained at 4 °C.

The incubation times with concanavalin A necessary to effect appreciable agglutination of resealed membranes is much longer that the time required for agglutination of membranes resealed in the presence of ATP with phytohemagglutinin-P [1].

TABLE II

EFFECT OF ATP OR ADENOSINE IN LYSING AND RESEALING MEDIA ON SUBSEQUENT AGGLUTINATION OF THE RESEALED MEMBRANES BY CONCANAVALIN A

0.2 ml aliquots of resealed membranes, prepared as described in Methods, were agglutinated with 0.333 mg concanavalin A under the conditions indicated.

Additive		Incubation with concanavalin A	Percentage of agglutination
In lysing and resealing medium	In concanavalin A solution		
–	–	160 min, 37 °C	0.2
ATP, 4 mM	–	160 min, 37 °C	1.5
Adenosine, 4 mg/ml	Adenosine, 4 mg/ml	160 min, 37 °C	1.0
–	–	215 min, 37 °C followed by overnight, 4 °C	3.1
ATP, 4 mM	–	215 min, 37 °C followed by overnight, 4 °C	5.8
Adenosine, 4 mg/ml	Adenosine, 4 mg/ml	215 min, 37 °C followed by overnight, 4 °C	23.3
–		215 min, 37 °C followed by overnight, room temp.	0.7
ATP, 4 mM	–	215 min, 37 °C followed by overnight, room temp.	14.6
Adenosine, 4 mg/ml	Adenosine, 4 mg/ml	215 min, 37 °C followed by overnight, room temp.	45.9

Effect of vinblastine and cytochalasin B

Neither cytochalasin B at 40 μg/ml nor vinblastine sulfate at 0.2 mM had much influence on the agglutination of erythrocytes by concanavalin A in the presence or absence of adenosine. There was an apparent small enhancement by both drugs of the agglutination when adenosine was present.

Effect of trypsinization

Mild trypsinization of erythrocytes caused enhanced agglutination by concanavalin A in the presence of adenosine as shown in Fig. 1.

At a relatively low concanavalin A concentration (55 μg/ml), little agglutination was shown by untreated erythrocytes (Fig. 2a), and only moderate agglutination by erythrocytes previously treated with 5 μg trypsin/ml (Fig. 2b). At higher trypsin concentrations (Fig. 2c), there was a significant increase in the agglutination by concanavalin A in the presence of adenosine, while cells which were not supplemented with adenosine showed little enhancement of agglutination. When erythrocytes were treated with 50 μg trypsin/ml (Fig. 2d), agglutination of both control and sample are increased, and there is still a marked difference between samples with and without adenosine. More extensive treatment with trypsin at 150 μg/ml (Fig. 2e) resulted in

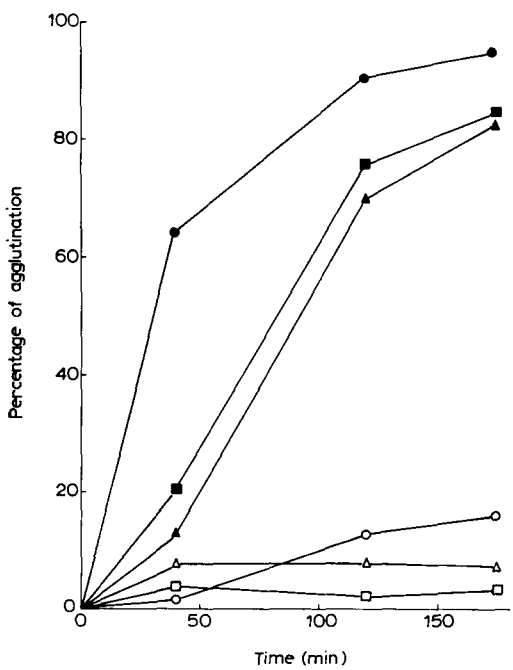

Fig. 1. Effect of mild trypsin treatment of erythrocytes on their subsequent agglutination by concanavalin A in the absence and presence of adenosine. Cells were treated for 40 min at 37 °C with 0, 0.3 or 5 μg trypsin/ml, as described under Methods. The washed, trypsin-treated erythrocytes were agglutinated at a final concanavalin A concentration of 45 μg/ml. Adenosine when present was at a final concentration of 3.6 mg/ml. 0 μg trypsin/ml, without adenosine, △; 0 μg trypsin/ml, with adenosine, ▲; 0.3 μg trypsin/ml, without adenosine, □; 0.3 μg trypsin/ml, with adenosine, ■; 5 μg trypsin/ml, without adenosine, ○; 5 μg trypsin/ml, with adenosine, ●.

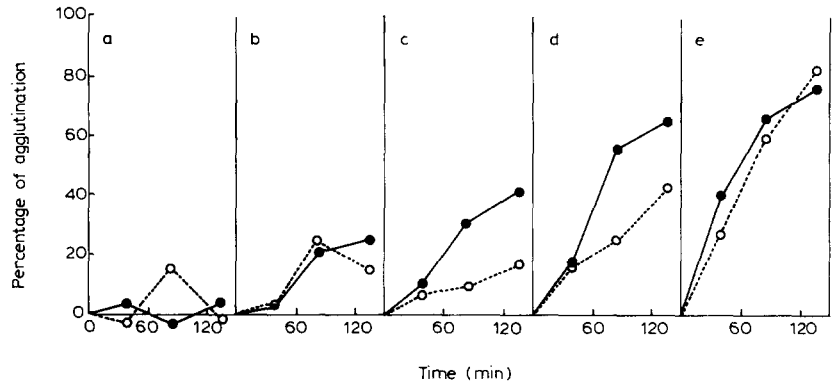

Fig. 2. Effect of trypsin treatment of erythrocytes on their subsequent agglutination by concanavalin A in the absence and presence of adenosine. Erythrocytes were treated for 40 min at 37 °C with the indicated concentrations of trypsin, as described under Methods. The washed, treated erythrocytes were agglutinated at a final concanavalin A concentration of 55 μg/ml. Adenosine, when present, was at a final concentration of 3.6 mg/ml. Agglutination in the presence of adenosine, ●; agglutination in the absence of adenosine, ○. (a) 0 μg trypsin/ml; (b) 5 μg trypsin/ml; (c) 15 μg trypsin/ml; (d) 50 μg trypsin/ml; (e) 150 μg trypsin/ml.

greater increase in agglutination, which is more pronounced for samples without adenosine, thus giving little difference between samples with and without adenosine.

The effect of temperature on agglutination of trypsin treated cells is shown in Fig. 3. Erythrocytes moderately trypsin treated (Fig. 3A) did not agglutinate well at 0 or 22 °C, but agglutinated at 37 °C with some response to adenosine. More exten-

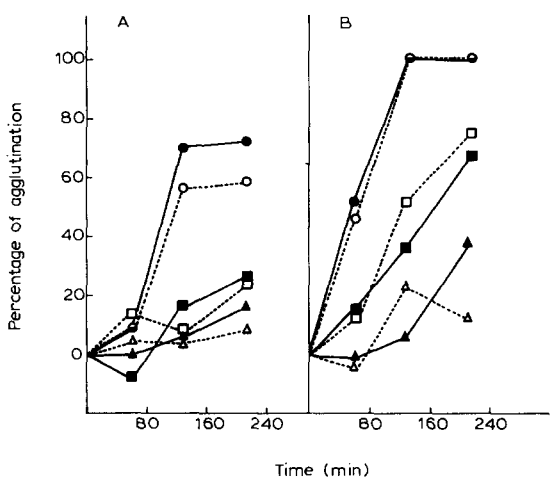

Fig. 3. Effect of temperature on the agglutination of trypsin-treated erythrocytes by concanavalin A in the absence and presence of adenosine. Erythrocytes were treated with 40 or 160 μg trypsin/ml, for 40 min, at 37 °C, as described under Methods. The washed, treated erythrocytes were agglutinated at a final concanavalin A concentration of 55 μg/ml. Adenosine, when present, was at a final concentration of 3.6 mg/ml. 37 °C, without adenosine, ○; 37 °C, with adenosine, ●; 22 °C, without adenosine, □; 22 °C, with adenosine, ■; 0° C, without adenosine, △; 0 °C, with adenosine, ▲. (A) 40 μg trypsin/ml; (B) 160 μg trypsin/ml.

sively treated cells (Fig. 3B) agglutinated very markedly at 37 °C, and gave better agglutination at 22 and 0 °C than did the cells treated less vigorously with the protease, but they did not respond to adenosine at any temperature.

Untreated erythrocytes, in the presence of 3.6 mg adenosine/ml and 0.227 mg concanavalin A/ml, which agglutinate very well at 37 °C, showed virtually no agglutination at 0 °C over 3 h.

DISCUSSION

It has been reported that intact human erythrocytes are not agglutinated by concanavalin A [4] despite the fact that they bind concanavalin A [5]. The present study has shown that human erythrocytes are much more readily agglutinated by concanavalin A if adenosine is also present. There is some variability in the exact magnitude of the effect, some of which might be anticipated from the use of a variety of donors and periods of storage before use of the blood. However, the various aspects of the adenosine-related effects noted are quite reproducible.

The effect of adenosine on the agglutination probably involves a metabolic conversion of the adenosine. Adenosine is frequently employed in maintenance of the viability of stored blood [6]. The fact that pre-incubation of erythrocytes with adenosine results in greater agglutination by concanavalin A and that the pre-incubation is more effective at 37 than at 0 °C suggest that metabolic conversion is involved. The necessity for the presence of adenosine during incubation of the erythrocytes with concanavalin A in order to produce a very large enhancement of the agglutination further indicates that the metabolic changes can be rapidly reversed and thus the process may involve a rapidly reversed process. However, the possibility that adenosine itself is mediating some transient state in the erythrocyte which favors agglutination cannot be ruled out. In any event, the concanavalin A agglutination of erythrocytes in the presence of adenosine requires the specific carbohydrate-binding site of the lectin; it does not appear to reflect large differences in binding of concanavalin A, as determination of relative amounts of concanavalin A bound to erythrocytes in the presence and absence of adenosine revealed little difference in these values.

ATP is known to be readily generated by the human erythrocyte from adenosine [6]. However, the low concanavalin A agglutinability of very fresh erythrocytes and only moderate response to prior incubation of erythrocytes with adenosine at 37 °C or to resealing ATP within the membrane, all argue against a direct relation of ATP concentration to concanavalin A agglutinability of human erythrocytes, although a specific pool of ATP could be the direct metabolic mediator of the activation of agglutination. Direct resealing of ATP within the membrane did yield some enhancement of the concanavalin A agglutination of the resealed membranes, but resealing with adenosine and incubation with adenosine and the lectin gave a more pronounced effect.

Mild trypsin treatment of human erythrocytes causes a greater sensitivity to adenosine activation of concanavalin A agglutination. The mechanism by which protease treatment in general elicits high lectin agglutinability is not yet understood. However, it is known [7] that trypsin does not act on band 3, which contains major concanavalin A receptors [3]. Since trypsin acts on glycoprotein, the changes in concanavalin A agglutination may involve accessibility of the lectin to receptor or

increased mobility of receptor. In the extensively trypsin-treated erythrocyte, concanavalin A agglutinability is very high, with a resulting decrease in the effect of adenosine.

The effect of the lower temperatures on concanavalin A agglutination is less pronounced in extensively trypsin-treated erythrocytes than in mildly treated cells. This finding is in apparent contradiction to the general observation [4] that temperature sensitivity of agglutination by several lectins was not altered by trypsin treatment. However, quantitative evaluation of the agglutination was not made in these studies, only a single set of conditions was investigated and, in the case of concanavalin A, agglutination was seen only in the trypsin-treated erythrocytes. These apparent differences may reflect only the method of determining agglutination and the different conditions employed.

These results and those from the earlier study [1] and investigation of cultured cells [8] demonstrate that the metabolic state of a cell can have profound effects on its lectin agglutinability. It is also becoming apparent that the nature of metabolic modulation of lectin agglutinability of cells can vary greatly, both for modulation of agglutinability of different cells by the same lectin and for modulation by the same cell of agglutinability by different lectins. In particular, a comparison of the agglutination of human erythrocytes by concanavalin A and by phytohemagglutinin-P [1], which have receptors that are at least partially distinct in the human erythrocyte, shows many contrasts. Modulation of agglutinability of erythrocytes by both lectins may be achieved by metabolic manipulations. However, even extensively metabolically depleted erythrocytes have considerable phytohemagglutinin-P agglutinability, and show a significant enhancement of this agglutinability on pre-incubation of the depleted erythrocytes with adenosine on the day preceding the agglutination experiment. Additions of adenosine to fresh erythrocytes in the presence of phytohemagglutinin-P does not enhance their agglutination. On the other hand, fresh erythrocytes show little agglutination with concanavalin A unless incubated with adenosine. Pre-incubation with adenosine just prior to addition of the lectin causes only modest increases in concanavalin A agglutination, while very large increases in agglutination are achieved by incubating the erythrocytes with adenosine in the presence of concanavalin A.

Resealed membranes containing ATP show a large immediate enhancement of phytohemagglutinin-P agglutination, but only a small improvement in the concanavalin A agglutination. A much larger increase in concanavalin A agglutination of resealed membranes is effected by presence of adenosine in the lysing and resealing medium, and in the lectin solution.

Neither phytohemagglutinin-P nor concanavalin A agglutination is influenced by cytochalasin B. Vinblastine mimics adenosine in causing metabolically depleted erythrocytes to agglutinate with phytohemagglutinin-P, and when the depleted erythrocytes are pre-incubated with adenosine, no response to vinblastine is obtained. On the other hand, concanavalin A agglutination is not substantially affected by vinblastine under any conditions investigated.

The possibility that variations in the metabolic state of cells or in the nature of their metabolic modulation of lectin agglutinability may often govern the frequently large differences of agglutinability encountered in the same cell in different biological states seems worthy of consideration.

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