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EFFECT OF ADENOSINE ON CONCANAVALIN A AGGLUTINATION OF HUMAN ERYTHROCYTES

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Summary

We have attempted to correlate the functional activity of protein 3 with its activity as a receptor for concanavalin A. The concanavalin A agglutination of human erythrocytes is enhanced by adenosine. It varies with time of storage of the blood and is dependent on the concentration of adenosine in the medium. Adenine and/or inosine, which increase cellular ATP, do not substitute for adenosine in enhancing agglutination, and adenosine enhances agglutination of fresh erythrocytes with normal levels of ATP. Thus, it appears that cellular ATP levels are not directly involved in modulation of concanavalin A agglutination by adenosine. Trypsin, which hydrolyzes most of the exposed proteins of the cell surface but does not alter protein 3, enhances concanavalin A agglutination without altering the relative response of the cell to adenosine.

Glucose, as well as the glucose transport inhibitors maltose and cellobiose, inhibits agglutination. High concentrations of adenosine reverse the inhibition by glucose and enhance agglutination in the presence of maltose and cellobiose.

Treatment of erythrocytes with 4,4′-diisothiocyanostilbene-2,2-disulfonic acid disodium salt, which selectively inhibits the anion transport function of protein 3, substantially inhibits adenosine-supported concanavalin A agglutination.

Treatment of erythrocytes with iodoacetate under conditions in which it selectively reacts with glyceraldehyde-3-phosphate dehydrogenase inhibits agglutination. Adenosine protects this dehydrogenase in erythrocytes from

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Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2-disulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine *N*′-2-propanesulfonic acid.

inactivation by iodoacetate, over the same concentration range in which it enhances agglutination.

Introduction

The major receptor for concanavalin A in the normal human erythrocyte is protein 3 [1], the most abundant membrane protein. This polypeptide has been implicated in anion [2] as well as glucose transport [3] and on the cytoplasmic surface, it interacts with a number of proteins such as glyceraldehyde-3-phosphate dehydrogenase, hemoglobin, the spectrin complex and aldolase [2].

We have previously reported the effect of metabolic alterations of the erythrocyte on phytohemagglutinin-P agglutination of the human erythrocyte [4,5] and in the present study, we have attempted to explore more definitively the functional activities of protein 3 with respect to its activity as a receptor of the lectin concanavalin A [6].

Materials and Methods

Concanavalin A (Type IV), adenosine (Sigma grade), adenine (Cryst., Sigma grade), inosine (yeast, Sigma grade), trypsin (Cryst., bovine pancreas, Type III), soybean trypsin inhibitor (Type I-S), glucose (anhydrous, Grade III), pyruvic acid (sodium salt, type II), lactic acid (sodium salt, 50%, L-isomer, DL-5), Hepes, glycogen (oyster), dibutyryl cyclic AMP (Grade II), ATP (sodium salt), and AMP were from Sigma (St. Louis, MO). Sodium arsenate (certified) and maltose (M-75) were from Fisher (Pittsburgh, PA). Iodoacetate (sodium salt, sodium iodide-free) was from Eastman Kodak (Rochester, NY). Phlorizin dihydrate and cellobiose were obtained from Pflatz and Bauer (Stamford, CT), and guanosine was from Schwarz (Orangeburg, NY). Aldrich (Milwaukee, WI) supplied the 6-methyladenosine (6-methylaminopurineriboside). 4,4′-Diisothiocyanostilbene-2,2-disulfonic acid disodium salt trihydrate (DIDS) was obtained from Pierce Chemical Co. (Rockford, IL). *p*-Nitrobenzylthioguanosine was generously provided by Dr. A.R.P. Paterson.

The general methods for preparation of washed erythrocytes, the trypsin treatment of erythrocytes and the agglutination assay have been given previously [6]. The concentrations of concanavalin A used were selected to be well above those found to influence agglutination levels. Blood was type A, Rh positive, except for the experiment reported in Table I, and was from donors of both sexes.

Studies of glyceraldehyde-3-phosphate dehydrogenase

Under the conditions employed, iodoacetate reacts almost exclusively with glyceraldehyde-3-phosphate dehydrogenase in the intact human erythrocyte [7].

Saline-washed erythrocytes were washed and suspended in either Krebs-Ringer buffer (pH 7.4) or phosphate-buffered saline (pH 7.4) at room temperature. Freshly prepared iodoacetate in buffer was added to give the indicated

final concentrations. After various times, samples were cooled in ice and centrifuged, and the supernatant was removed. The pellets were then washed twice with phosphate-buffered saline using care to avoid loss of membrane and assayed for glyceraldehyde-3-phosphate dehydrogenase [8].

Determination of inhibition of concanavalin A-glycogen interaction

To determine the effect of potential lectin inhibitors in our system, the turbidometric assay for interaction of concanavalin A and glycogen [9] was used. To 1 ml of phosphate-buffered saline at 20°C, containing potential inhibitors, was added 0.2 ml of a solution comprising 170 μg of concanavalin A in phosphate-buffered saline. The reaction was initiated with 0.2 ml of a solution of 510 μg of oyster glycogen in phosphate-buffered saline and was assayed spectrophotometrically.

DIDS treatment of erythrocytes

Washed erythrocytes in isotonic saline 10 mM Hepes (pH 7.4) were incubated at 25% hematocrit with 10 mM DIDS for 30 min, at 37°C, washed twice in saline-Hepes, twice in saline-Hepes containing 0.5% bovine serum albumin and three times with phosphate-buffered saline.

Results

Storage of blood at 2—5°C in citrate/phosphate/dextrose anticoagulant for up to 3 days had a particularly pronounced effect on concanavalin A agglutination when short incubation times were used. Fresh erythrocytes did not agglutinate on short incubation times with lectin as well as did cells stored for 24 h at 2—4°C. This enhanced agglutination was gradually lost over the next 2 days storage. Further incubation for up to 8 days did not change agglutination appreciably.

Determination of agglutination of erythrocytes from six donors collected within 1 h indicated that there is considerable variability in concanavalin A

TABLE I

CONCANAVALIN A AGGLUTINATION OF ERYTHROCYTES FROM SEVERAL INDIVIDUALS

Blood was collected into citrate/phosphate/glucose anticoagulant within 1 h, stored for 26 h at 2—5°C, and assayed at equal erythrocyte concentration for concanavalin A agglutination under the conditions indicated. Each value is the average of triplicate determinations ± S.D. made without knowledge of the identity of the samples.

Individual	Presence of variant protein 3	Agglutination, no addition, 180 min, 37°C 270 μg concanavalin A/ml (%)	Agglutination, plus adenosine, 4 mg/ml, 160 min, 37°C, 270 μg concanavalin A/ml (%)
1	+	70 ± 3.2	76 ± 1.4
2	+	28 ± 4.6	46 ± 16.2
3	+	16 ± 3.7	46 ± 7.6
4	—	25 ± 7.0	33 ± 9.8
5	—	37 ± 3.6	50 ± 3.9
6	—	47 ± 3.1	41 ± 16.7

TABLE II

CONCANAVALIN A AGGLUTINATION OF ERYTHROCYTES TREATED WITH DIDS

Control erythrocytes were treated identically to the DIDS-treated erythrocytes, with the omission of DIDS from the reaction. Agglutination was in the presence of 15 mM adenosine.

Time of incubation of cells with concanavalin A (min)	Agglutination, DIDS-treated erythrocytes (%)	Agglutination, control erythrocytes (%)
65	—7.9	5.2
125	31.3	41.4
195	39.6	60.4

agglutinability of erythrocytes from different individuals (Table I), while confirming the reproducibility of the assay. The erythrocytes in samples 1, 2 and 3 contained a variant of protein 3 [10]. The results show that there is no detectable correlation between this variant protein and concanavalin A agglutinability.

Treatment of erythrocytes with DIDS caused a substantial inhibition of subsequent adenosine-supported concanavalin A agglutination (Table II).

Effect of adenosine or related compounds

Concanavalin A agglutination of intact human erythrocytes was stimulated by adenosine at 15 or 1.5 mM. However, adenine, inosine and exogenous ATP and AMP had no effect on the agglutination. Dibutyryl cyclic AMP at 3 mM caused a modest inhibition of agglutination. Guanosine caused a modest enhancement of agglutination although adenosine and guanosine in combina-

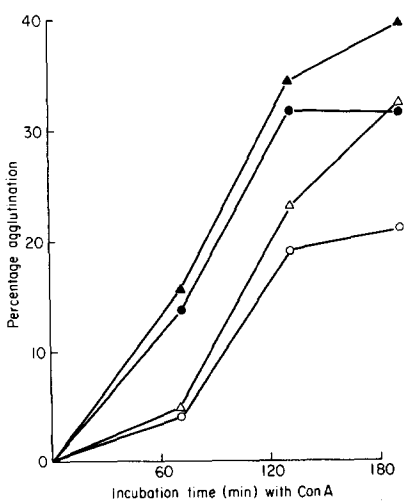

Fig. 1. Concanavalin A (Con A) agglutination in the presence of adenosine and/or guanosine. Washed erythrocytes, with a hematocrit of 40, in phosphate-buffered saline, were diluted with 10 vols. of phosphate-buffered saline containing 0 or 300 μg concanavalin A/ml in phosphate-buffered saline (○), 1.5 mM adenosine (▲), 1.5 mM guanosine (△), or 1.5 mM adenosine plus 1.5 mM guanosine (●). Agglutination was determined after the indicated times of incubation with concanavalin A.

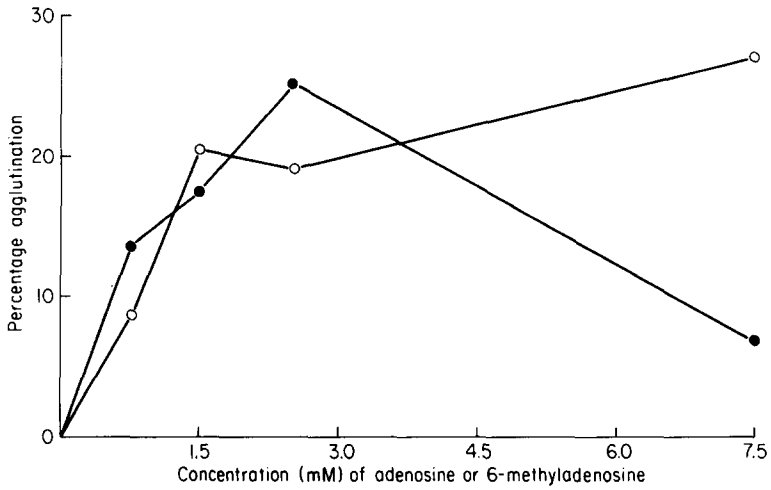

Fig. 2. Concanavalin A agglutination in the presence of adenosine or 6-methyladenosine. Washed erythrocytes, 40% in phosphate-buffered saline, were incubated with 10 vols. of phosphate-buffered saline with or without concanavalin A, 300 μg/ml, containing either adenosine (○) or 6-methyladenosine (●) at the indicated concentrations for 175 min.

tion were slightly less effective than the adenosine alone (Fig. 1). Agglutination was supported by 6-methyladenosine, as well as by adenosine at lower concentrations, but less effectively at higher concentrations (Fig. 2).

The agglutination at low concentrations of adenosine is shown in Fig. 3.

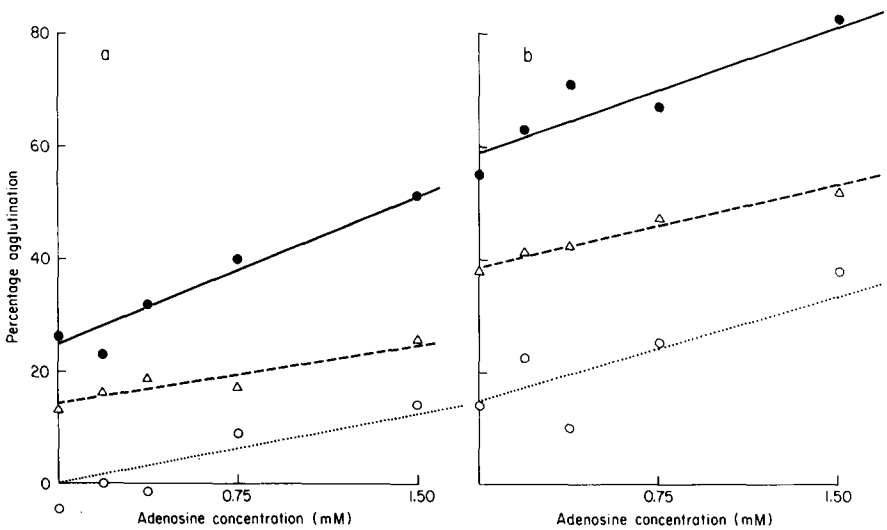

Fig. 3. Concanavalin A agglutination of intact and trypsinized erythrocytes in the presence of varying concentrations of adenosine. To a 40% hematocrit of washed erythrocytes suspended in phosphate-buffered saline was added 0.2 vol. trypsin in phosphate-buffered saline, to give a final trypsin concentration of 0 (○), 5 (△) and 20 (●) μg/ml; after 40 min at 37°C, a 5-fold excess of soybean trypsin inhibitor was added. Cells were washed three times with phosphate-buffered saline and resuspended at 40% hematocrit. Aliquots of these samples were added to phosphate-buffered saline with or without concanavalin A, 300 μg/ml containing the indicated concentrations of adenosine. Concanavalin A agglutination was determined after 80 min (a) and 220 min (b) incubation.

Agglutination was linearly dependent on adenosine concentration in the 0—1.5 mM range. Trypsin-treated red cells retain protein 3 intact but other proteins on the outside of the cell are hydrolyzed. Trypsin-treated cells agglutinate readily and agglutination is also dependent upon adenosine concentration (Fig. 3a). At long times of incubation (Fig. 3b), similar dependence on adenosine concentration was obtained for all the samples, although the extent of agglutination was greater with increased trypsinization.

A specific inhibitor of facilitated nucleoside transport, *p*-nitrobenzylthioguanosine [11], caused only a modest inhibition of adenosine-activated agglutination at a concentration sufficient to inhibit both transport of adenosine and the release of ammonia from adenosine, as shown in Table III.

Effect of glucose and compounds related to its metabolism on agglutination

Glucose at 15 mM strongly depressed agglutination. However, this inhibition was abolished by adenosine as shown in Fig. 4.

Various glucose transport inhibitors used were tested for inhibition of the concanavalin A lectin activity by a turbidometric assay [9]. With this method, concentrations of the glucose analogs which did not substantially inhibit the lectin were selected for agglutination studies. Phlorizin, at 125 μg/ml, strongly inhibited agglutination in the presence of adenosine (not shown). Maltose, on the other hand, exhibited a mild inhibition of the agglutination at low adenosine concentrations (0—3 mM), but at 15 mM adenosine, maltose actually enhanced the agglutination (Fig. 5). 200 μg/ml 2-deoxyglucose and 500 μg/ml cellobiose, also inhibitors of glucose transport, showed a similar pattern of moderate inhibition of agglutination at lower concentrations and enhancement at higher concentrations of adenosine (Fig. 5).

0.5 mM ouabain, 4.5 mM arsenate, 15 mM pyruvate or 15 mM lactate had little effect on concanavalin A agglutination with or without adenosine and/or glucose.

TABLE III

EFFECT OF 15 μM *p*-NITROBENZYLTHIOGUANOSINE, A NUCLEOSIDE TRANSPORT INHIBITOR, ON THE CONCANAVALIN A AGGLUTINATION OF ERYTHROCYTES IN THE PRESENCE OF VARYING CONCENTRATIONS OF ADENOSINE

Cells were incubated for 165 min.

Adenosine concentration (mM)	Percent concanavalin A agglutination	
	p-Nitrobenzylthioguanosine present	*p*-Nitrobenzylguanosine absent
15	16	17
3.8	41	37
2.3	36	24
1.5	22	20
1.1	20	19
0.8	22	13
0.4	13	8
0	12	10

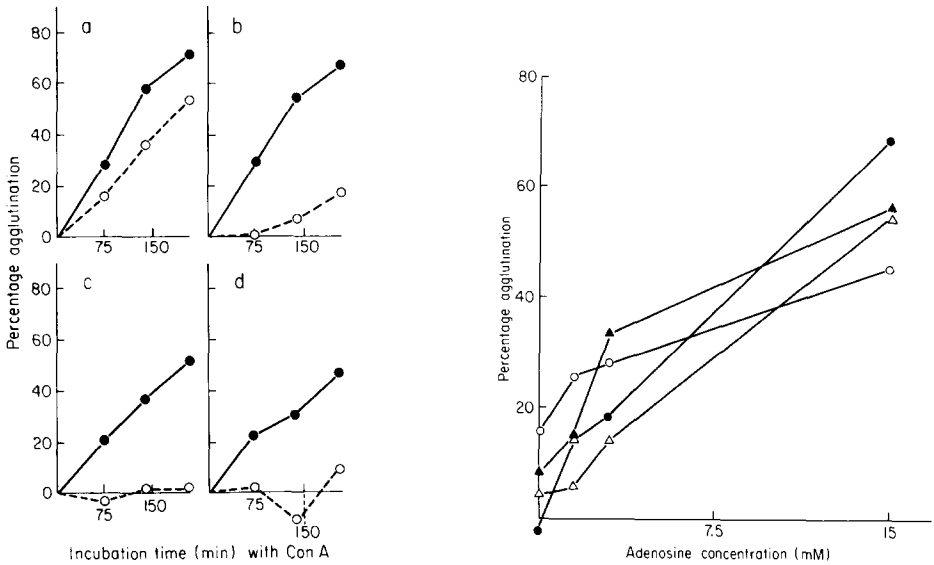

Fig. 4. Influence of glucose on concanavalin A (con A) agglutination in the presence of varying concentrations of adenosine. A final 4% hematocrit of washed erythrocytes was incubated with concanavalin A, 300 μg/ml, containing no glucose (•) or 15 mM (○) glucose. Agglutination was determined after the indicated times of incubation with concanavalin A in the presence of 7.5 mM (a), 1.5 mM (b), 0.3 mM (c) or 0 mM (d) adenosine.

Fig. 5. Effect of maltose, 2-deoxyglucose, or cellobiose on the concanavalin A agglutination of erythrocytes in the presence of varying concentrations of adenosine. Washed erythrocytes, 40%, were added to 10 vols. of phosphate-buffered saline with or without concanavalin A, 300 μg/ml, containing the indicated concentrations of adenosine and either 180 μg maltose/ml (•), 500 μg 2-deoxyglucose/ml (△), 500 μg cellobiose/ml (▲), or no further addition (○). Agglutination was determined after 180 min incubation with concanavalin A.

Glyceraldehyde-3-phosphate dehydrogenase

Erythrocytes were treated with varying concentrations of iodoacetate and the inactivation of glyceraldehyde-3-phosphate dehydrogenase was followed. About 90% of the enzyme was inhibited before any inhibition of agglutination could be observed. Glucose had no effect on the iodoacetate inhibition, nor did the iodoacetate treatment alter the inhibition of agglutination by glucose.

However, treatment of intact erythrocytes with adenosine did decrease the inactivation of the dehydrogenase by iodoacetate. Comparison of the response to varying adenosine concentrations of both the concanavalin A agglutination and the inactivation of glyceraldehyde-3-phosphate dehydrogenase by iodoacetate showed a similar dependence of both on adenosine concentration (Fig. 6).

Discussion

The effect of adenosine on the concanavalin A agglutination of human erythrocytes reported earlier [6] has now been shown to be dependent on the concentration of adenosine for both intact and trypsinized erythrocytes. Specificity for adenosine is not absolute, as either guanosine or 6-methyladenosine affords some stimulation of the agglutination. The fact that adenine

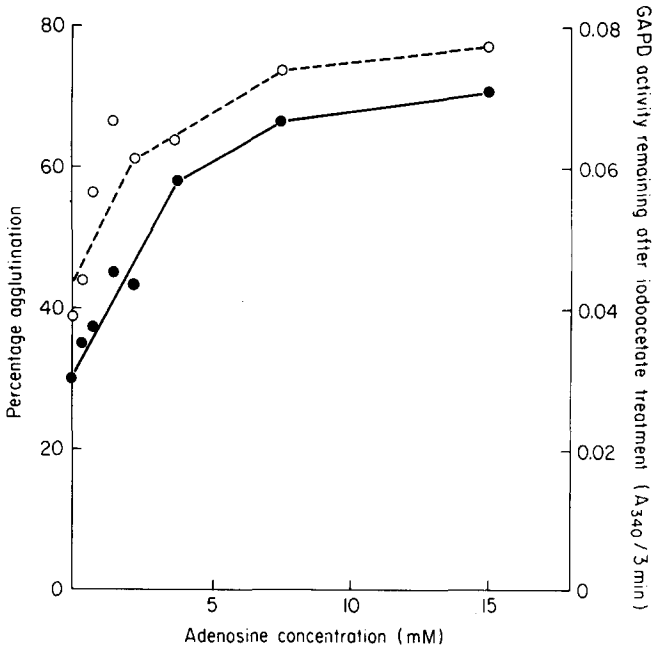

Fig. 6. Effect of adenosine on concanavalin A agglutination and on iodoacetate inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPD) in erythrocytes. Washed erythrocytes, with a hematocrit of 40% were added to 6.7 vols. of adenosine at the indicated concentrations, in phosphate-buffered saline with or without concanavalin A, 400 μg/ml, and incubated at 37°C. After 90 min, aliquots of samples without concanavalin A were allowed to come to room temperature and were treated for exactly 15 min with 0.12 vols. of 0.10 mM sodium iodoacetate in phosphate-buffered saline. Pellets were washed twice with cold phosphate-buffered saline, and their glyceraldehyde-3-phosphate dehydrogenase activity (○) was determined. After 190 min at 37°C, agglutination (●) was determined.

and inosine cannot substitute for adenosine in the stimulation of the agglutination provides evidence that cellular ATP levels are not involved. Erythrocytes from freshly drawn blood, which have high levels of ATP, also respond to adenosine in the concanavalin A agglutination, lending further support to this conclusion.

Trypsinized erythrocytes responded more readily, and to a greater extent, to adenosine than untreated erythrocytes. The mechanism by which trypsin treatment enhances concanavalin A agglutinability may be due to the fact that trypsin does not modify protein 3, the major 90 000 dalton protein in the erythrocyte membrane [12], but it does hydrolyze the other major exposed proteins of the red cell, the sialoglycoproteins. Thus, accessibility of the concanavalin A to its major binding site, protein 3, may be increased by trypsinization [13].

Specific nucleoside transport inhibitors have been shown to protect adenosine from the erythrocyte's adenosine deaminase, which appears to be proximal to the transport site [11]. We found only slight inhibition of adenosine-supported concanavalin A agglutination by an inhibitor of nucleotide transport (*p*-nitrobenzylthioguanosine). This effect may be contrasted with the sizeable enhancement by the same transport inhibitor of phytohemagglutinin-P agglutination of metabolically depleted erythrocytes incubated with adenosine [5].

NH_4^+ has been shown [14] to increase the flexibility of stored erythrocytes and NH_3 released by deamination of adenosine might be expected to play a role in the effect of adenosine on concanavalin A agglutination. However, in the presence of *p*-nitrobenzylthioguanosine, which inhibits deamination of adenosine in the intact erythrocyte [11], adenosine still enhances concanavalin A agglutination. Thus, it would appear that NH_4^+ derived by deamination of adenosine plays no role in concanavalin A agglutination.

The effect of adenosine on concanavalin A agglutination of erythrocytes, after storage of the blood in citrate/phosphate/dextrose anticoagulant, shows that storage has a readily demonstrable effect, especially pronounced with short times of incubation with the lectin in the absence of adenosine. Comparison of erythrocytes from several donors indicates that concanavalin A agglutinability of erythrocytes can vary considerably from individual to individual. These effects of storage and of individual variability on concanavalin A agglutinability may contribute to the discrepancies in the literature between those who have reported agglutination [15—17], and those who have not [18, 19]. However, the sensitivity of the assay method is also particularly important, since the concanavalin A-induced aggregates are relatively small.

The inhibition of the concanavalin A agglutination by glucose is greatly influenced by adenosine concentration. Glucose does not appear to be acting as a haptene inhibitor of the lectin, since high concanavalin A agglutination is observed when sufficient adenosine is included in the assay mixture.

Several findings indicate that the metabolism of glucose is not involved in the mediation of its effect on agglutination. Exogenous pyruvate and lactate, to which the erythrocyte is permeable [20], do not influence the agglutination, whether in the presence or absence of glucose. The complete inhibition of agglutination by glucose in the absence of adenosine is not relieved by prior iodoacetate treatment. The results with arsenate and ouabain also do not support a role for the enzymes, phosphoglycerate kinase or $(Na^+ + K^+)$-ATPase, in the glucose modulation of agglutination.

The effect of the various specific inhibitors of glucose transport on the agglutination, on the other hand, does indicate that a glucose transport site is involved in the effect on concanavalin A agglutination of erythrocytes. Maltose, cellobiose and 2-deoxyglucose all cause moderate inhibition of agglutination at low adenosine concentrations, but actually enhance agglutination in the presence of high (15 mM) concentrations of adenosine. Phlorizin is the only glucose transport inhibitor tested which failed to show increased agglutination with adenosine, causing instead an inhibition of agglutination. Phlorizin differs from the other inhibitors tested in binding to the transport system on the inside of the cell, while cellobiose and maltose bind to the transport system only at the outer surface of the cell and 2-deoxyglucose binds to the system on both sides [21]. This may indicate that binding of a glucose analog to the transport site at either membrane face will inhibit concanavalin A agglutination, but only inhibition elicited by binding to the transport system at the outer surface can be modulated by adenosine. This may have some relevance to the possible involvement of protein 3 with glucose transport [3].

The anion-transport function of protein 3 is well established to be inhibited by treatment of the erythrocyte with DIDS, which reacts selectively with pro-

tein 3 [22]. The substantial inhibition of concanavalin A agglutination by prior DIDS treatment of the erythrocyte suggests that DIDS modification of protein 3 not only blocks anion transport but significantly alters the state of protein 3 in the membrane. This interpretation is in accord with the effect on the phospholipid state detected calorimetrically in erythrocytes with DIDS-modified protein 3 [22].

The inhibition of concanavalin A agglutination produced by treatment with iodoacetate, under conditions in which glyceraldehyde-3-phosphate dehydrogenase is selectively inactivated [7], does not seem to be due to the loss of catalytic function of this glycolytic enzyme, as the degree of inhibition of agglutination by iodoacetate is not altered by the presence of glucose in the agglutination assay. The state of glyceraldehyde-3-phosphate dehydrogenase with respect to iodoacetate susceptibility is altered by adenosine. This effect does not appear to be attributable to ATP concentration in the cell, although ATP does promote dissociation of the dehydrogenase from the membrane [23], since adenosine has an effect on the dehydrogenase's iodoacetate susceptibility even in fresh erythrocytes which have high levels of ATP. The concentration range over which adenosine modulates the agglutination and protects the dehydrogenase from iodoacetate suggests that the adenosine effect on concanavalin A agglutination could be mediated through an influence on the dehydrogenase-concanavalin A receptor interaction.

In summary, the expression of the concanavalin A receptor of the human erythrocyte depends in part on its interaction with the glucose and anion transport systems and with glyceraldehyde-3-phosphate dehydrogenase. The agglutinability by concanavalin A is strongly modulated by adenosine but does not appear to involve ATP. The effects of adenosine on surface properties of the erythrocyte are quite pronounced, and may be compared with a number of effects which adenosine has on various other cells and tissues [24]. These observations raise the question of a possible role of adenosine, a known vasoactive agent which is released endogenously [25], in influencing circulation through its influence on the erythrocyte surface, as well as on the vascular bed.

Acknowledgements

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