Biochimica et Biophysica Acta, 373 (1974) 313–326

BBA 76792

THE INTERACTION OF CONCANAVALIN A WITH SHEEP ERYTHROCYTES

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(Received June 11th, 1974)

SUMMARY

Concanavalin A and ^{125}I-labeled concanavalin A were used as probes for comparison of sheep low-potassium (LK) and high-potassium (HK) erythrocytes with respect to their agglutinability and membrane properties. Under conditions of equivalent concanavalin A binding, freshly isolated sheep HK cells agglutinated to a much greater extent than the LK cells at 23 or 35 °C; agglutination was progressive over periods of 1–4 h, but the differential between LK and HK cells persisted. On additional standing overnight in the cold, both LK and HK cells were extensively agglutinated. After preincubation in a salt-buffer solution (without lectin) for 4 h at 35 °C followed by standing overnight at 4 °C, rapid and extensive agglutination but not increased binding of concanavalin A occurred in both sheep HK and LK cells. Cells preincubated in the presence of an energy source and/or bovine plasma albumin also agglutinated rapidly. The rate of agglutination of freshly isolated cells of either kind was not decreased by the presence of an energy supply. Agglutination of sheep LK and HK erythrocytes by concanavalin A appears to proceed by two steps: the first, a relatively rapid and temperature-independent binding of lectin which is very similar if not identical in the LK and HK cells; and the second, agglutination of the cells, which is slow by comparison with dog and rabbit erythrocytes under the same conditions. The second step depends upon a change in the cells that occurs relatively slowly after their isolation whether or not concanavalin A or an energy supply is present. Possibly this change occurs more rapidly in HK cells and thus causes the differential in agglutinability of LK and HK cells.

INTRODUCTION

Erythrocytes of sheep, goats, cattle, and buffalo are polymorphic in their sodium and potassium content, with the low-potassium (LK) (low-K^+, high-Na^+) animals being more prevalent than the high-potassium (HK) (high-K^+, low-Na^+) ones [1]. This variation is associated with and probably results from differences in membranes in permeability, in ion transport, in (Na^+–K^+)-activated ATPases, and at least in

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

sheep in content of specific antigens called L and M [1–9]. The HK cells are more active in $(Na^+–K^+)$-ATPase [3–5, 9] and those of sheep contain antigen M that is also present in heterozygous LK cells [2, 7, 8]. All sheep and goat LK but not HK erythrocytes contain antigen L [2, 3, 7] of which a component, on binding anti-L antibody, produces stimulation of the $(Na^+–K^+)$-ATPase and Na^+ and K^+ transport [4, 6–8]. These differences in red cells apparently are determined by a single genetic locus with two alleles [10].

In this investigation, concanavalin A and ^{125}I-labeled concanavalin A were used as probes of erythrocyte membrane structure and properties, particularly in the sheep LK and HK erythrocytes. In a variety of other cell systems lectins have been valuable for detection and characterization of differences in surface properties and structure, particularly those associated with the regulation of cell growth and with cell transformation [11–14]. Other evidence indicates that concanavalin A can alter Na^+ and K^+ transport in Ehrlich ascites tumor cells [15], that contact inhibition of growth and changes in concanavalin A binding sites affect specific activities of the $(Na^+–K^+)$-ATPase and adenyl cyclase in mouse 3T3 cells [16], that concanavalin A fragments stimulate these enzymes in SV40-transformed 3T3 cells [16], and that concanavalin A stimulates $(Na^+–K^+)$-independent ATPase in rat lymphocyte microsomes and ATPase of rat brain microsomes [17]. Thus a number of concanavalin A effects on cells are analogous to those of anti-L antibody of the IgG fraction of ovine isoimmune antiserum, which stimulates active potassium transport [8] and $(Na^+–K^+)$-activated ATPase in LK erythrocytes of sheep and goats [4, 6,7]. Therefore, our studies on the binding of concanavalin A and agglutination of LK and HK erythrocytes relate also to the functional role of concanavalin A binding sites in membranes and to the mechanism of concanavalin A agglutination.

MATERIALS AND METHODS

Preparation of red blood cells

Red blood cells were prepared from freshly drawn heparinized blood (10 mg heparin/100 ml) from unfasted sheep, humans, cows, dog, and rabbits. The main focus of the investigation was on sheep cells, the other species being used mainly for comparative purposes and evaluation of our lectin preparations. 22 different Hampshire sheep were bled and six were bled again after an interval of several months. These sheep included 12 HK and 10 LK animals as judged by atomic absorption analysis of the sodium and potassium content of the red cells. The cow red cells were LK. The group of LK sheep undoubtedly included both homozygous and heterozygous animals, but at least three animals were known to be heterozygous for LK from tests of parents and their progeny.

After removal of plasma and buffy coat, the cells were washed with 10 vol. of isotonic saline, or phosphate-buffered saline, or HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–Hanks solution that contained no glucose. Many experiments were carried out with three times washed cells, but the later ones were done with one time washed cells since there was no significant difference in the sheep erythrocyte agglutination tests.

In those experiments involving trypsinized cells, washed cells were divided into a control sample (no trypsin) and a sample that was pretreated with trypsin (10 μg/ml

in a 2 % cell suspension) for 20 min at 23 °C. The trypsin was removed by washing the cells before the agglutination and concanavalin A binding experiments.

Preparation of agglutinins

Concanavalin A from Miles Laboratories (Elkhart, Indiana) was dissolved shortly before use in agglutination assays to give 1 mg/ml in a solution containing 0.3 M NaCl, 0.001 M Ca^{2+}, 0.001 M Mn^{2+} and 0.01 M Tris–HCl buffer at pH 7.2. Concanavalin A (10 mg/ml in a solution of 0.3 M NaCl and 0.01 M Tris–HCl at pH 7.3) was iodinated by the Na ^{125}I–H_2O_2–lactoperoxidase procedure essentially as described by Marchalonis [18]. The reaction mixture was then applied to a Sephadex G-50 column (0.9 cm × 40 cm) equilibrated with 0.15 M NaCl at 10 °C. The column was washed with about 50 ml 0.15 M NaCl to remove unbound protein and unreacted ^{125}I. About 95 % of the total protein ^{125}I-labeled concanavalin A was bound to the Sephadex and was eluted with a solution containing 0.1 M glucose and 0.15 M NaCl. Glucose was removed from the ^{125}I-labeled concanavalin A by ultrafiltration. Two preparations of ^{125}I-labeled concanavalin A with specific radioactivities of 6 and 14 mCi/g, respectively, were used. Both preparations were indistinguishable from the starting lectin in disc electrophoresis at pH 8.3 in 7.5 % polyacrylamide gel. Both preparations gave essentially the same results in binding experiments with sheep HK and LK cells. Wheat germ agglutinin was obtained from Miles Laboratories.

Agglutination assay

The extent of agglutination was assessed in three ways: by visual observation of the test mixture, by observation with a microscope of a drop of the test mixture, and by counting free cells in a hemocytometer. The assay was done usually in 35-mm plastic Petri dishes but sometimes in spot dishes. Cells at $2 \cdot 10^8$–$3 \cdot 10^8$/ml in a total volume of 1.0 or 1.5 ml were incubated with gentle gyratory mixing under conditions of agglutinin concentration, medium composition, temperature and incubation time noted in Results. For comparison of different kinds of cells, assays with and without concanavalin A were done simultaneously under identical conditions.

Assay of concanavalin A binding to cells

Cells at $2 \cdot 10^8$–$3 \cdot 10^8$/ml in a total volume of 1.5 ml in the plastic Petri dishes were incubated for 15, 30, or 60 min intervals at 24 or 35 °C with ^{125}I-labeled concanavalin A at 5, 10, 20, or 40 μg/ml as noted in Results. Controls for assessment of non-specific binding contained 0.1 M methyl-α-D-mannopyranoside. For determination of bound ^{125}I-labeled concanavalin A, 1.0 ml was taken for washing and counting of the ^{125}I. The remainder was used for assessing the extent of agglutination. The concentration of cells in the reaction mixture was determined with the hemocytometer and the recovery after washing was determined by the method of Lowry et al. [19] for protein determination. For the latter, the cells in the counting vial were dissolved in a known volume of 0.1 M NaOH and appropriate aliquots were taken for analysis (after determination of the radioactivity).

RESULTS

Differential agglutination in sheep LK and HK erythrocytes

At several different concanavalin A concentrations in the range 1–25 μg/ml,

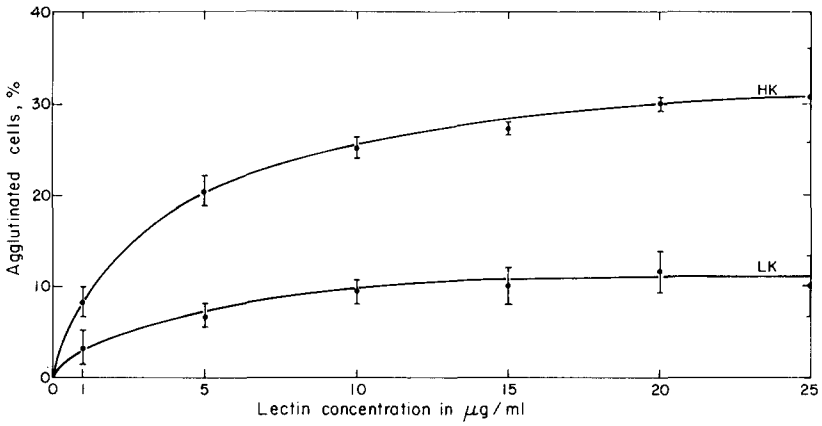

Fig. 1. Differential agglutination by concanavalin A of freshly isolated sheep LK and HK erythrocytes in isotonic saline after 1 h at 23 °C.

the extent of agglutination at 23 and 35 °C was less for the sheep LK cells than that for HK cells at hourly intervals up to 4 h (Figs 1 and 2). The freshly prepared LK cells varied in the extent of agglutination from almost none to a small amount and only the HK cells gave considerable agglutination during the 4-h incubation. During the first 4 h after addition of concanavalin A, agglutination of sheep LK and HK cells was more extensive at 35 °C than at 23 °C; there was no agglutination after 4 h at 4 °C. Both kinds of sheep cells were extensively agglutinated by concanavalin A after 4 h at 23 or 35 °C followed by standing overnight at 4 °C (Fig. 2). In both LK and HK cells preincubated in HEPES–Hanks solution (no glucose) for 4 h at 35 °C and kept overnight at 4 °C, addition of concanavalin A was followed by very rapid agglutination, much faster than in either freshly prepared HK or LK cells (Fig. 3). Inclusion of an energy source (0.002 M adenosine + 0.002 M inosine + 0.005 M glucose) and/or bovine plasma albumin (2.5 %) in the preincubation and incubation media did not prevent the effect of this prolonged preincubation (Fig. 3).

Wheat germ agglutinin at 5, 10 and 20 μg/ml agglutinated freshly prepared sheep LK and HK cells in isotonic saline or HEPES–Hanks (no glucose) solution equally well and very extensively within 1 h at 23 °C (Fig. 4). Three LK and three HK sheep were tested. It also agglutinated the washed cells that had been preincubated overnight before addition of the lectin.

Agglutination of erythrocytes of different species

Under our experimental conditions, rabbit and dog erythrocytes were agglutinated extensively within less than 1 h by low concentrations (1–5 μg/ml) of concanavalin A as reported by Sumner et al. [20]. These investigators reported also that human and cow erythrocytes were not agglutinated with concanavalin A and sheep, goat and pig cells were agglutinated very little if at all. Even after incubation with concanavalin A for 4 h at 35 °C followed by 16 h at 4 °C, our cow cells did not agglutinate at all at concanavalin A concentration of 5, 20, and 40 μg/ml. A small amount of agglutination by concanavalin A after 1 h at 35 °C was occasionally seen on microscopic examination of fresh human erythrocytes, and the extent of agglutination was increased in types A and B cells at least after prolonged incubation.

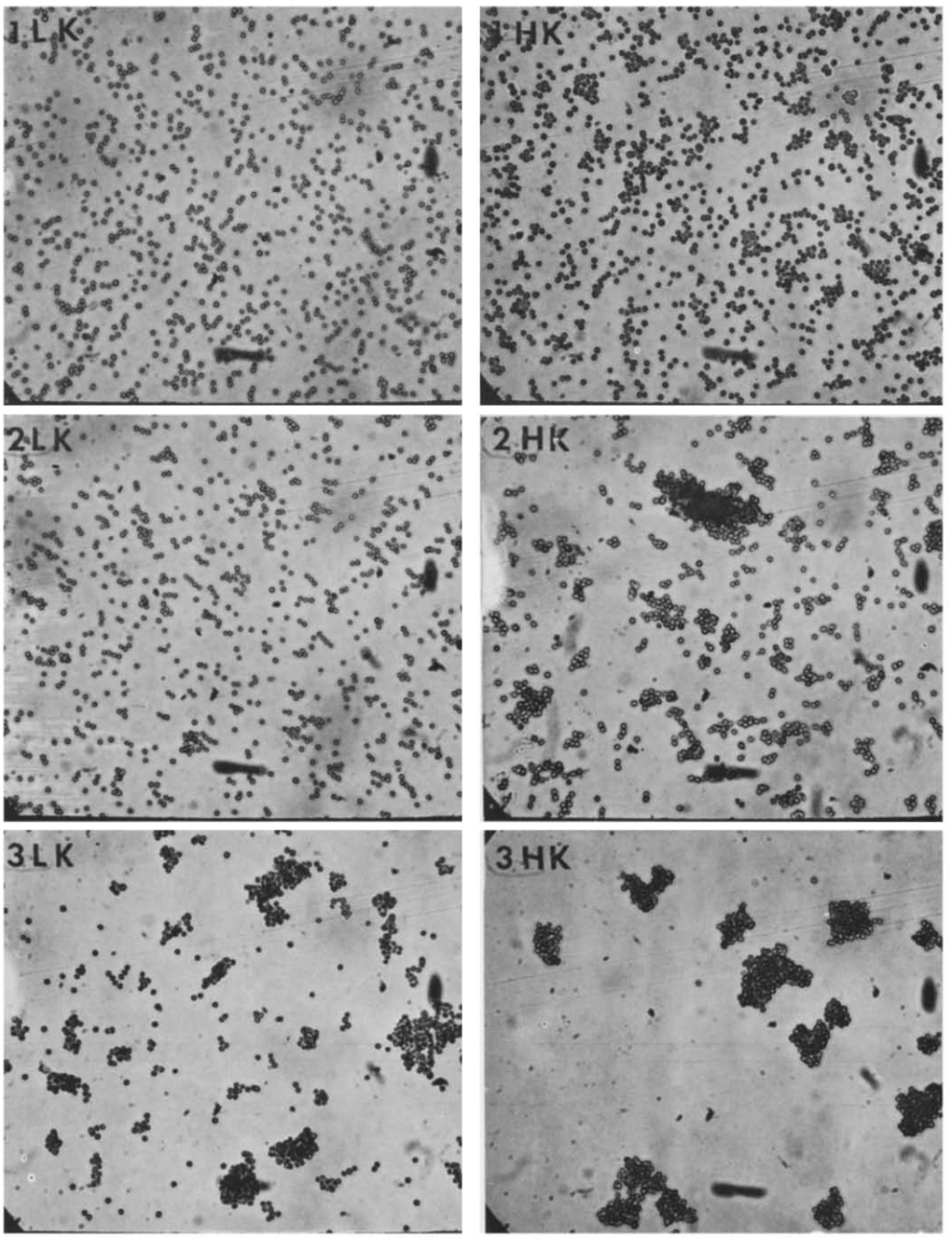

Fig. 2. Agglutination by concanavalin A (10 μg/ml) of sheep LK and HK erythrocytes in HEPES–Hanks (no glucose) solution. 1–3 LK, 1–3 HK:LK and HK cells after 1 h at 35 °C, 4 h at 35 °C, and 4 h at 35 °C plus 16 h at 4 °C, respectively.

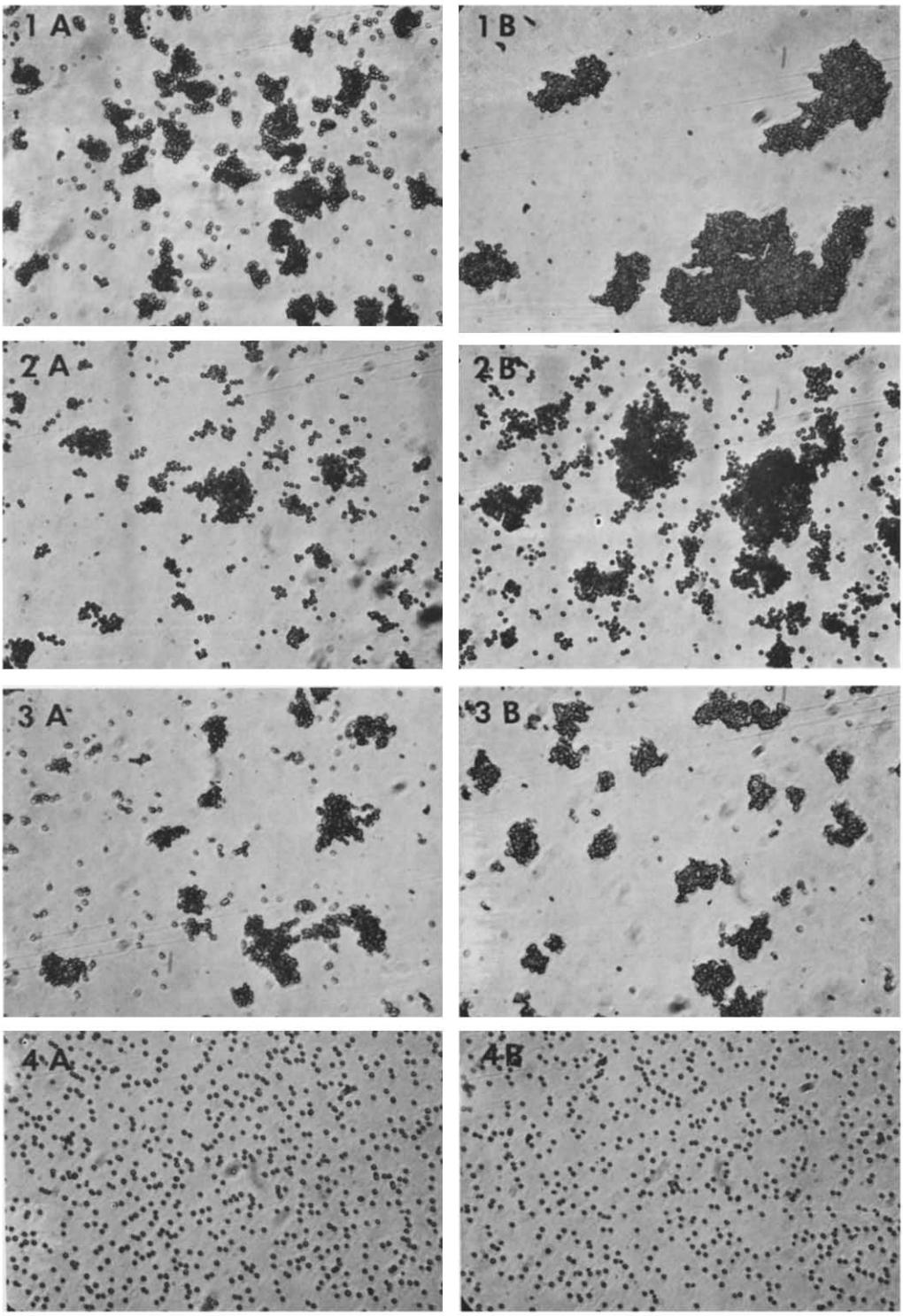

Fig. 3. Effect of preincubation on agglutination of washed erythrocytes of sheep. Cells in HEPES–Hanks solution (no glucose) were preincubated with and without an energy source (adenosine

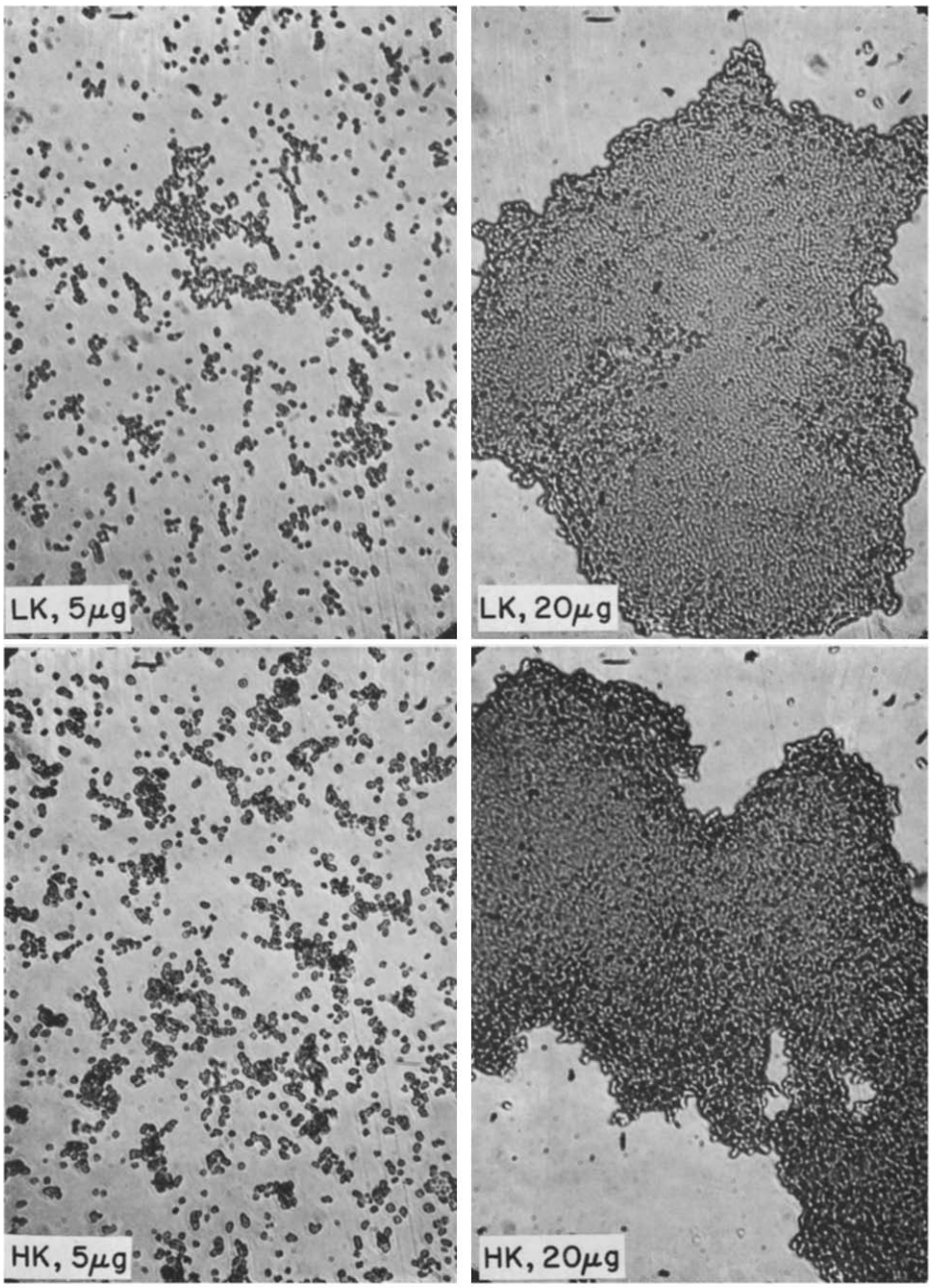

Fig. 4. Agglutination of freshly prepared sheep LK and HK erythrocytes in HEPES–Hanks (no glucose) solution by wheat germ agglutinin at 5 and 20 μg/ml. Incubation was 30 min at 23 °C.

inosine + glucose) and/or 2.5 % bovine plasma albumin for 4 h at 35 °C followed by 16 h at 4 °C before addition of concanavalin A to a final concentration of 10 μg/ml. Pictures were taken at 1 h after concanavalin A addition. 1A, 1B: LK and HK cells, respectively; 2A, 2B; LK and HK cells − energy source; 3A, 3B: LK and HK cells + energy + albumin; 4A, 4B: preincubated LK and HK cells without concanavalin A.

Binding of concanavalin A to erythrocytes

Removal of concanavalin A initially present at 1, 5, or 10 μg/ml from the suspending medium at 15 min, before any agglutination was evident on microscopic examination, did not markedly change the time course or the final extent of agglutination in sheep LK and HK cells. Even when cells were exposed to concanavalin A only at 4 °C before removal of unbound concanavalin A, the agglutination proceeded when the cells were warmed to 23 or 35 °C. Thus, the differential agglutination between sheep LK and HK cells seemed not to be related to a difference in the rate of binding of concanavalin A to the cells, and in neither case was concanavalin A binding a rate-determining factor in the slow process of agglutination.

Radiolabeled lectin was used to quantitate concanavalin A binding in sheep and cow erythrocytes. In these experiments the extent of agglutination was also assessed. At an initial lectin concentration of 10 μg/ml, 9–12 % of the radioiodine was bound to sheep cells; at 1 μg/ml, approx. 40 % was bound during 1 h incubation at 23 °C. Even though their course of agglutination differed (as noted above with unlabeled concanavalin A), sheep LK and HK cells were very similar in amount of bound lectin at all concanavalin A concentrations tested, including 1 μg/ml. At 23 °C, binding within the first 15 min of incubation of cells was about 20 % greater with 20 μg/ml ^{125}I-labeled concanavalin A than with 5 μg/ml (Table I). At 10 μg ^{125}I-labeled

TABLE I

^{125}I-LABELED CONCANAVALIN A BINDING TO FRESHLY ISOLATED ERYTHROCYTES IN HEPES–HANKS SOLUTION (NO GLUCOSE)

Bound lectin was determined after 15 min at 23 °C.

Cells	Concanavalin A (μg/ml)	Bound concanavalin A	
		μg/mg protein	molecules/cell
Sheep LK	5	0.118	10 500
Sheep LK	20	0.146	13 000
Sheep HK	5	0.116	10 400
Sheep HK	20	0.143	12 700
Cow LK	5	0.079	
Cow LK	20	0.102	

concanavalin A/ml the binding to cells was not significantly different at 23 °C from that at 35 °C, and extension of the incubation period for 15 min to 1 h increased the amount of bound ^{125}I-labeled concanavalin A by about 10–15 % in both LK and HK cells of sheep (Table II). In all of these experiments with ^{125}I-labeled concanavalin A (except the controls containing methyl-α-D-mannopyranoside, which completely inhibited agglutination), the cells were extensively agglutinated after 4 h at 23 or 35 °C followed by 16 h at 4 °C even though the unbound lectin was removed at 15, 30 or 60 min. At essentially equal levels of bound ^{125}I-labeled concanavalin A, a differential existed among LK and HK sheep cells in extent of agglutination at shorter incubation intervals, i.e. up to 4 h.

TABLE II

^{125}I-LABELED CONCANAVALIN A BINDING TO SHEEP LK AND HK ERYTHROCYTES

Cells at approx. $2 \cdot 10^8$/ml in HEPES–Hanks solution without glucose were incubated with 10 μg lectin/ml for 15–60 min at 23 or 35 °C or for 4 h at 35 °C plus 16 h at 4 °C (20 h).

Cells	Bound concanavalin A (μg/mg cell protein)			
	15 min	30 min	60 min	20 h
LK, 23 °C	0.133	0.176	0.167	
LK, 35 °C	0.163	0.173	0.184	
LK, 35 °C + 4 °C			0.170	0.179
HK, 23 °C	0.140	0.170	0.180	
HK, 35 °C	0.165	0.177	0.176	
HK, 35 °C – 4 °C			0.167	0.183

The number of binding sites is not increased on prolonged incubation of sheep erythrocytes in HEPES–Hanks solution (no glucose) with or without concanavalin A. Freshly washed cells and cells that had been preincubated for 4 h at 35 °C followed by 16 h at 4 °C bound essentially the same amount of ^{125}I-labeled concanavalin A within 1 h after addition of lectin (Table III); the preincubated cells, both LK and HK, but not the freshly prepared cells, were extensively agglutinated at 1 h. In a separate experiment in which ^{125}I-labeled concanavalin A was added to freshly prepared cells, the amount of bound lectin was about the same after incubation in the presence of 10 μg lectin/ml for 4 h at 35 °C followed by 16 h at 4 °C as that bound after 1 h at 35 °C (Table II). In this experiment, there was also extensive agglutination after the 20-h incubation but not after 1 h.

The effect of trypsin pretreatment on the binding of ^{125}I-labeled concanavalin A was also investigated in freshly prepared sheep and cow LK erythrocytes, because we had found that trypsinization increased the agglutination rate of sheep HK and LK

TABLE III

EFFECT OF METABOLIC STATE ON ^{125}I-LABELED CONCANAVALIN A BINDING TO ERYTHROCYTES IN HEPES-HANKS (NO GLUCOSE) SOLUTION

Bound lectin was determined after 1 h exposure to 10 μg concanavalin A/ml at 23 °C.

Cells	Condition of cells	Bound concanavalin A (μg/mg)
HK	Freshly prepared	0.178
HK	Freshly prepared + energy source	0.168
HK	Preincubated 4 h at 35 °C + 16 h at 4 °C	0.180
LK	Freshly prepared	0.169
LK	Freshly prepared + energy source	0.160
LK	Preincubated 4 h at 35 °C + 16 h at 4 °C	0.182

TABLE IV

EFFECT OF TRYPSIN PRETREATMENT OF ERYTHROCYTES ON ^{125}I-LABELED CONCANAVALIN A BINDING IN PHOSPHATE-BUFFERED SALINE DURING 1 h AT 23 °C

Cells	Concanavalin A (μg/ml)	Bound concanavalin A	
		μg/mg protein	molecules/cell
Sheep LK	20	0.158	14 000
Sheep LK + trypsin	20	0.146	13 000
Sheep LK	40	0.162	14 400
Sheep LK + trypsin	40	0.157	14 000
Cow LK	20	0.099	
Cow LK + trypsin	20	0.096	
Cow LK	40	0.108	
Cow LK + trypsin	40	0.103	

cells (particularly LK cells) and also allowed agglutiunation of cow cells by unlabeled concanavalin A. Pretreatment with trypsin (10 μg/ml for 20 min) did not significantly change the quantity of ^{125}I-labeled concanavalin A bound by either sheep or cow LK cells (Table IV); however, ^{125}I-labeled concanavalin A caused extensive agglutination of trypsinized sheep LK cells, but not untreated cells, after 1 h at 23 °C (Fig. 5). Binding to cow cells was investigated at 23 °C at 5, 20, and 40 μg ^{125}I-labeled concanavalin A/ml; these concentrations gave no agglutination of untrypsinized cow erythrocytes at 23 or 35 °C even after incubation for 4 h at 35 °C followed by 1–2 days at 4 °C. The amounts bound to cow cells after 15 min at 20 and 40 μg lectin/ml were essentially the same as at 1 h and considerably greater than at 5 μg/ml. Although the amount of concanavalin A bound per mg protein is less in cow erythrocytes than in sheep erythrocytes (Tables I and IV) the latter cells are smaller. Thus, cow and sheep cells contain comparable numbers of concanavalin A binding sites. The number of concanavalin A binding sites on untrypsinized cow cells seems to be adequate, but other factors do not favor agglutination, at least in these LK cells. The trypsinized cow cells were agglutinated at all three levels of ^{125}I-labeled concanavalin A (Fig. 5).

Effect of composition of medium on agglutination

With some lectins, agglutination of cells is promoted by polyvinylpyrrolidone or serum albumin [21]. Polyvinylpyrrolidone was necessary also for hemagglutination of sheep erythrocytes by antibody globulins [22]. Polyvinylpyrrolidone (mol. wt 360 000) at 1 % promoted agglutination by concanavalin A of the sheep HK cells and particularly the LK cells of the sheep and cow. Extensive agglutination by 5 and 10 μg concanavalin A/ml was seen after 1 h at 23 °C. Bovine serum albumin (2.5 %) did not affect the agglutination of sheep or cow cells by concanavalin A at either short or long incubation times.

Regardless of the medium in which the cells were washed and agglutinated by concanavalin A (isotonic NaCl, phosphate-buffered saline or HEPES–Hanks (no glucose) solution, all at pH 7.2–7.3), the qualitative differences and extent of agglutination for human, cow, and sheep LK and HK cells were as reported above and in

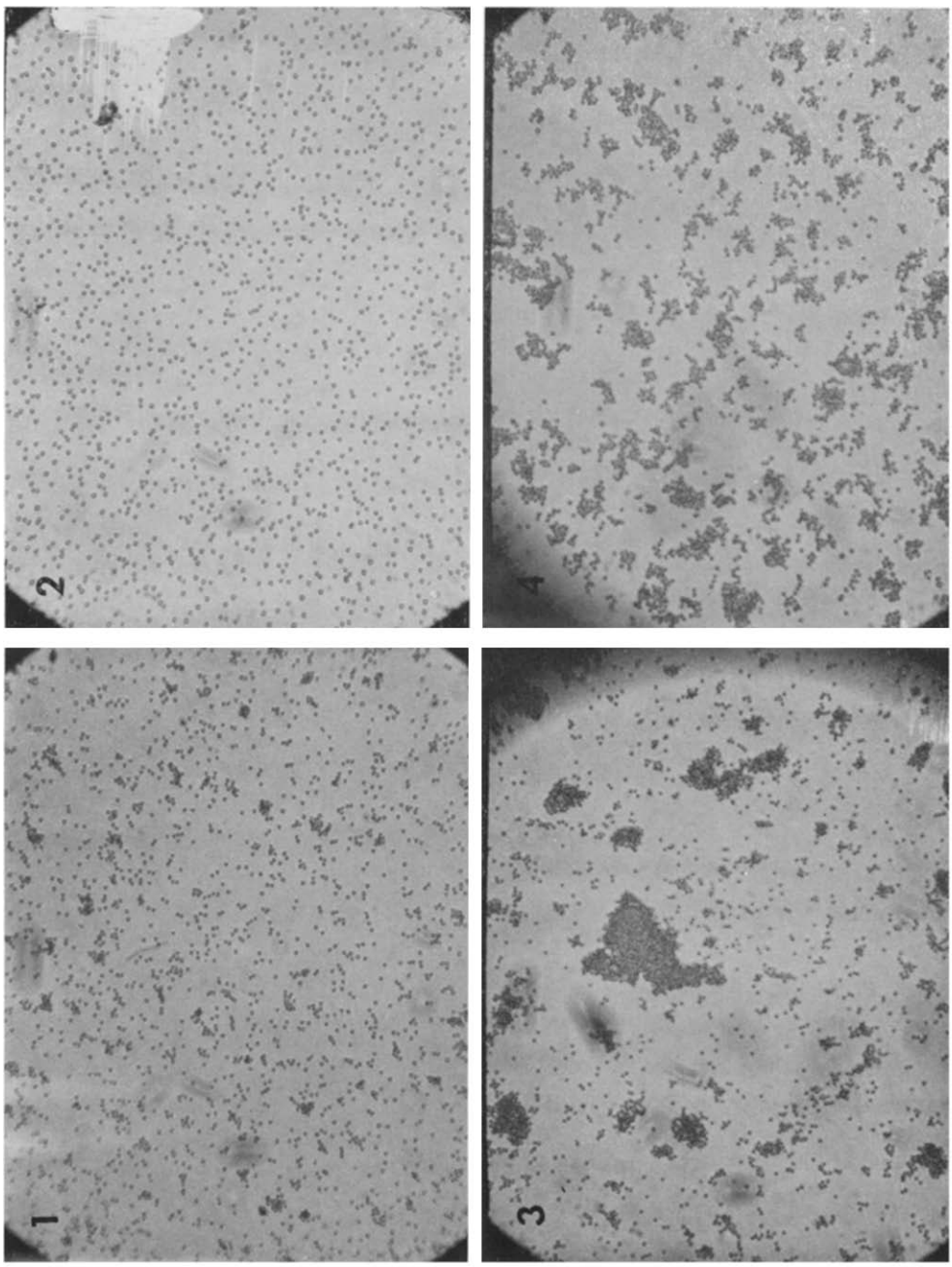

Fig. 5. Effect of trypsin pretreatment on agglutination of sheep and cow LK erythrocytes by concanavalin A at 23 °C. 1, 2: sheep and cow cells, respectively, no trypsin; 3, 4: sheep and cow cells + trypsin.

Figs 1–3. When KCl was substituted for NaCl to give 0.07 M K^+ rather than 0.006 M in the HEPES–Hanks solution, the extent of agglutination observed in sheep LK and HK cells was decreased somewhat at 1–4 h, but the differential between LK and HK cells was qualitatively the same as in solutions low in K^+. In this experiment, the cells were washed with the high potassium solution and preincubated for 30 min at 35 °C before addition of concanavalin A.

Similarly, when freshly washed cells were preincubated in HEPES–Hanks solution containing an energy supply (0.002 M adenosine + 0.002 M inosine + 0.005 M glucose) for 1 h at 35 °C and then agglutinated in the same solution, the qualitative differences in agglutination between LK and HK sheep cells were unaltered and cow cells still were not agglutinated. However, the rates of agglutination were increased in both LK and HK cells, although not to a striking extent. The quantitative binding of ^{125}I-labeled concanavalin A by sheep LK and HK cells was not significantly changed by the presence of the energy supply (Table III).

DISCUSSION

The differential agglutination of erythrocytes by concanavalin A could result from differences in the kinds of binding sites, in the number of binding sites per cell, in the distribution of binding sites on the cell surface, or in steric hindrance from groups vicinal to the concanavalin A binding sites. Agglutinability of other cells by concanavalin A has been found to be sensitive to metabolic inhibitors [23], to be a function of the ATP content of the cells [24], and to involve mobility of concanavalin A binding sites [12–14, 24] and membrane fluidity [12]. Agglutination by concanavalin A is inhibited at low temperatures [14, 23] and promoted by protease treatment of normal cells [25].

In sheep LK and HK erythrocytes, the differential in agglutination probably is not due to differences in the concanavalin A binding sites. These erythrocytes bind concanavalin A similarly in terms of quantity bound per cell and the rate of binding. They probably have the same kinds of binding sites and their distribution on the surface may also be the same, because their membranes are very similar in composition as indicated by similar disc electrophoresis patterns of the proteins (Shore, V., unpublished) and lipid analyses [26, 27]. The L and M antigens and the pump sites [28], the major known differences, probably are not binding sites, since the cells do not differ significantly in the quantity of concanavalin A bound per cell.

Our data indicate that agglutination of both sheep LK and HK erythrocytes and also human erythrocytes is promoted by a change in the cell surface that occurs on incubation of washed cells irrespective of whether an energy supply or concanavalin A is present. This change in the cells occurs more slowly when heparinized blood is aged in the refrigerator. Possibly the differential between sheep LK and HK erythrocytes in agglutination occurs because of a faster rate of this change in the HK cells. This change that promotes agglutinability did not involve sphering or echinocytosis of the cells as far as we could tell from their microscopic appearance.

Whether or not the changes in sheep cells that promote agglutination by concanavalin A are the same as those that promote immune-hemolysis of sheep LK erythrocytes by ovine anti-L serum and rabbit serum complement [29] is not clear. Comparison of the effects of prolonged preincubation of washed cells on concanavalin

A agglutination with the effects of metabolic depletion in promoting the immune-hemolysis suggests that the mechanisms in the two different processes may be similar to some extent. However, it was possible to prevent the increased lysis by including glucose in the incubation medium [29], but we could not similarly prevent the increased agglutination. It is also not clear whether there is any parallel between the differential agglutination of sheep LK and HK cells and that between normal and trypsinized cells [25] or between normal and transformed cells [14], although in each instance the differential appears not to depend upon differences in concanavalin A binding.

The agglutination of sheep erythrocytes by concanavalin A is a two-step process, as is hemagglutination by immunoglobulins [30]. The first step involves a relatively rapid and temperature-insensitive binding of concanavalin A that is quantitatively the same in LK and HK cells. The second, agglutination of the cells, is relatively slow and accounts for the differential agglutination of sheep LK and HK cells. However, the second step appears not be a simple process of interaction of erythrocytes containing bound concanavalin A (i.e. a simple reaction with a high energy of activation); instead, the process of agglutination appears to depend upon changes in the cells that occur relatively slowly after their isolation from the sheep, whether or not concanavalin A is present. The altered cell surface is more favorable for cell agglutination mediated by concanavalin A but does not affect agglutinability by wheat germ agglutinin.

ACKNOWLEDGEMENTS

The authors express their appreciation to Mr Anthony Silva for his technical assistance and to Dr Valeska Evertsbusch for her suggestions on the manuscript preparation. This work was supported by the U.S. Atomic Energy Commission. Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the U.S. Atomic Energy Commission to the exclusion of others that may be suitable.

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