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ANTI-L SERUM

TWO POPULATIONS OF ANTIBODIES AFFECTING CATION TRANSPORT IN LK ERYTHROCYTES OF SHEEP AND GOATS

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SUMMARY

Isoimmune sheep anti-L serum was fractionated, yielding two antibodies with different specificities of action on potassium transport in LK red cells of sheep and goats: anti-L_p, which stimulates active transport, and anti-L_i, which inhibits passive transport.

INTRODUCTION

Populations of sheep are dimorphic with respect to the cation concentrations of their red blood cells [1, 2]. HK sheep have red cells with high K^+ and low Na^+ concentrations, while LK cells have low K^+ and high Na^+ concentrations. HK cells have high active Na^+ and K^+ fluxes, low passive cation fluxes, and high numbers of Na^+, K^+ pump sites per cell compared to LK cells [3, 4]. This dimorphism, which is controlled by a single genetic locus with two alleles [5, 6], is associated with the M-L blood group antigen system in that HK cells have only M antigen whereas LK cells possess the L antigen [7]. Goat red cells have similar HK-LK and M-L dimorphisms [8]. Isoimmune anti-L serum raised in HK sheep immunized with LK sheep cells stimulates the Na^+, K^+ pump in LK cells of sheep [9, 10] and goats [11, 12]. Passive K^+ transport in LK sheep cells is reduced by some preparations of anti-L serum [13, 14] but not by others [15, 16]. (Passive K^+ influx in LK sheep cells is a saturable function of external K^+ concentration [4, 14], and therefore is not simple diffusion.) For a recent review of the immunology of the HK-LK dimorphism, see Lauf [17].

Two types of results have been offered as evidence that anti-L serum contains two populations of antibodies with different specificities, one population affecting the pump and the other causing hemolysis. In the first type of experiment, LK sheep cells pretreated with trypsin remained susceptible to complement-dependent lysis mediated by anti-L [16]. However, the trypsin treatment prevented anti-L-mediated stimulation of active transport. In another type of experiment, anti-L serum absorbed with LK goat red cells did not stimulate active transport in goat red cells but was fully hemolytic to LK sheep cells [11]. (LK goat cells are not hemolyzed by unabsorbed anti-L antiserum). The absorbed serum had some stimulatory activity to the pump in sheep

cells. Adsorbed antibodies eluted from the goat cells stimulated active transport in both sheep and goat LK cells, but were only weakly lytic to sheep cells. These two lines of evidence suggest one population of anti-L antibody (anti-L_l) which is lytic for sheep cells and another population (anti-L_p) which is stimulatory to the pump in both types of LK cells. LK goat cells absorb anti-L_p but not anti-L_l. Finally there is stimulatory activity for active transport in sheep cells coincident with the hemolytic activity. (The term anti-L_p has the same meaning here as previously assigned [11, 16]. Anti-L_l has the same meanings as anti-L [11] and anti-L_{LY} [16] regarding hemolytic activity.)

The experiments in this report were undertaken to determine the relationship of the reduction of passive K^+ transport by anti-L serum to the lytic and pump-stimulating activities of the antiserum. The main conclusion to be drawn is that two populations of antibodies affect K^+ transport: anti-L_p stimulates the pump and has no effect on passive transport; anti-L_l inhibits passive transport in both sheep and goat cells; in addition anti-L_l has some stimulatory action to the pump in LK sheep cells.

METHODS

General. Blood was drawn into heparin from Dorset sheep and Nubian goats. The red cells were washed by centrifugation in isotonic saline (150 mM NaCl; 5 mM glucose; 10 mM Tris · HCl; pH 7.5). Some of the experiments required that the cells be used the day after they were drawn, which accounts for the low active and high passive K^+ fluxes observed. Isoimmune anti-L serum was raised in HK sheep immunized with LK cells [18]. LK cells were washed three times in isotonic saline. Packed cells were mixed 1 : 1 with Freund's complete adjuvant, and injections of 5 ml each of the mixture were made into the four upper limbs of the HK sheep. After 1 month booster injections were made of cells mixed with incomplete adjuvant. One source of anti-L serum was used for all of the experiments. Prior to use sera were heated at 56 °C for 20 min to inactivate complement.

Fractionation of serum. Immune reagents were prepared from anti-L serum by absorption with red cells of LK goats or sheep and subsequent elution of the adsorbed antibodies.

Two separate preparations, designated G-1 and G-3, were made using LK goat cells to absorb the antiserum. In preparation S-2, sheep cells were used. Each step in the absorption procedures was carried out at room temperature for 1 h. In preparation G-1, one volume of serum was absorbed with one volume of cells. In preparation G-3, 1 : 1 and 1 : 2 absorptions (v : v, serum to cells) were made in sequence, giving an aggregate absorption of 1 : 3. The two aliquots of cells were from different LK goats. In preparation S-2, using sheep cells, two absorption steps, 1 : 1, were carried out in sequence.

The antibodies adsorbed to the red cells were eluted using the procedure of Snyder et al. [19]. In preparations involving two absorption steps, the eluates were made from cells pooled from both steps. The cells were lysed in 10 volumes of hypotonic buffer (10 mM Tris · HCl, pH 7.6) and washed free of hemoglobin by centrifugation in the same solution. The membranes were suspended in distilled water at 0 °C at a volume 7.5 times the original volume of serum. To this mixture 0.75 volumes of cold *n*-butanol were added. After intermittent mixing for 1 h at −1 °C, the aqueous phase

was separated by centrifugation and lyophilized. The material was suspended in a volume of isotonic saline equal to the original volume of serum against which the cells had been absorbed.

Sera and eluates were dialyzed 18 h in the cold against 50 volumes of isotonic saline and cleared by centrifugation.

Trypsin treatment. Pretreatment of cells with trypsin before exposure to antiserum was carried out according to the method of Lauf et al. [16]. Cells were first washed eight times in isotonic saline and then were suspended at 50 % hematocrit in a solution containing 125 mM NaCl; 10 mM $CaCl_2$; 20 mM Tris · HCl, pH 8.0. This medium contained either no trypsin or trypsin at 2 or 10 mg/ml (Sigma Chem. Co.). The suspensions were incubated at 37 °C for 1 h and then washed thoroughly by centrifugation in the cold.

Potassium influx. Before measuring fluxes cells were exposed to undiluted immune reagents (sera or eluates) at 5–10 % hematocrit for 30 min at 32 °C and then washed three times by centrifugation in isotonic saline. Active and passive unidirectional influxes of K^+ were measured as described previously [12, 20]. The K^+ concentration in the media during the fluxes was 5 mM. Active, or pump, and passive fluxes were taken as the ouabain-sensitive and ouabain-insensitive fluxes, respectively, measured after exposure of aliquots of cells to 10^{-3} M ouabain for 10 min at 37 °C in K^+-free medium. During the flux the ouabain concentration was 10^{-4} M.

In the initial experiments on the effects of anti-L serum on transport, some aliquots of cells were pretreated with serum from an HK sheep which had not been immunized. The fluxes in these cells were always the same as in cells incubated at the same time in isotonic saline. In earlier studies isoimmune sheep antiserum directed against another, but similar, specificity, the M antigen, was shown to have no effect on K^+ transport in either LK cells [9] or HK cells [17]. For these reasons it was deemed unnecessary to use routinely, in experiments on transport, cells incubated in non-immune serum as a control for cells incubated in anti-L serum. However, cells treated with non-immune serum were used as controls in this study in the experiments on antibody binding and effects of trypsin treatment.

Antibody binding. Binding of antibodies to LK sheep cells from anti-L serum was measured by the Coombs indirect antiglobulin test for agglutination [21]. Two drops of a cell suspension (2 % hematocrit) were incubated for 1 h at 32 °C with two drops of serial (2-fold) dilutions of immune reagent, using isotonic saline as diluent. Serum from a non-immunized HK sheep was used as a control. After incubation with these reagents (triplicate samples at each dilution) the cells were washed thoroughly by centrifugation. To the cell pellets were added four drops of a cross-linking agent, rabbit anti-sheep IgG serum (Pentex, Miles Laboratories), diluted 1 : 20 with isotonic saline. Agglutination was then judged macroscopically. The agglutination titer was taken as the highest dilution of reagent to which cells were exposed which caused agglutination after addition of antiglobulin.

Hemolytic tests were made using the procedure of Tucker [22]. Fresh rabbit serum was used as a source of complement; the serum was first absorbed in the cold with mixed sheep red cells to remove Forssman antibodies.

It should be noted that, in the absence of complement, the residual hemolysis was negligible after incubation of cells in any of the immune reagents, and was no greater than that observed for cells incubated in isotonic saline alone.

RESULTS

Serum fractions and potassium transport

Sheep anti-L serum, absorbed antiserum, and eluted antibodies were tested for their effects on active and passive influxes of K^+ in LK red cells of sheep and goats. Table I shows the effects of three separate preparations of these immune reagents: in preparations G-1 and G-3, LK goat cells were used to absorb the antiserum, and in preparation S-2, LK sheep cells were used. The results are given as the ratio of K^+ influx in cells treated with immune reagents to the influx in control, or untreated cells.

TABLE I

EFFECTS OF ANTI-L SERUM AND SERUM FRACTIONS ON ACTIVE AND PASSIVE K^+ TRANSPORT IN LK RED CELLS OF SHEEP AND GOATS

The influxes are expressed as the ratio of the influx in reagent-treated cells to the influx in control cells. The values are means of two determinations. Similar results were obtained in two other experiments with each of the preparations of reagents. The mean fluxes in cells not pretreated with immune reagents were (mmol/l cells per h): sheep, active, 0.06; passive, 0.41; goats, active, 0.21; passive, 0.14. The effects of preparations G-3 and S-2 on fluxes in sheep cells were determined at the same time, thus the values for unabsorbed serum are the same. In all of these experiments, 36 fluxes in duplicate were determined. The mean of differences between pairs of duplicate determinations, as a percent of each mean, was 4.2 %.

Reagent	K influx					
	Active			Passive		
	Preparation*			Preparation		
	G-1	G-3	S-2	G-1	G-3	S-2
LK goat red cells						
Unabsorbed serum	2.9	2.5	–	0.82	0.77	–
Absorbed serum	2.1	1.0	–	0.83	0.83	–
Eluted antibodies	2.7	1.4	–	1.1	1.0	–
LK sheep red cells						
Unabsorbed serum	2.6	8.4	8.4	0.43	0.40	0.40
Absorbed serum	1.9	4.0	0.92	0.45	0.45	0.68
Eluted antibodies	2.4	8.4	3.4	1.0	0.71	0.76

* Designations G-1, G-3, and S-2 indicate preparations made by absorptions with LK goat cells, 1 or 3 volumes (G-1; G-3), or with LK sheep cells, 2 volumes (S-2).

In LK goat cells (upper part of Table I) both preparations of unabsorbed serum stimulated the pump more than 2-fold (cf. refs. 11–13). In a finding not reported previously for goat cells, the passive K^+ influx was reduced by anti-L (cf. ref. 13). The reduction is small, 20–30 %, but reproducible. Absorption of the serum with goat cells reduced the pump-stimulating activity; it was reduced altogether in serum absorbed with three volumes of cells (preparation G-3). In contrast absorption had no effect on the inhibition of passive transport by the serum. The eluted antibodies stimulated the pump but did not inhibit passive transport. These results show separation of anti-L_p, the pump-stimulating antibody, from anti-L_l, and further that anti-L_l reduces passive K^+ transport in goat cells. Anti-L_l was not absorbed by LK goat cells

in a procedure which absorbed anti-L_p. Nevertheless anti-L_l inhibited passive transport in goat cells. A likely explanation for this observation is that anti-L_l is present in much higher concentration in the serum than anti-L_p, as will be shown below in Table III.

The lower portion of Table I shows influxes in LK sheep cells measured after treatment with the same preparations of reagents, and with preparation S-2 as well. Unabsorbed serum stimulated the pump and inhibited passive transport (cf. refs. 9, 10, 13 and 14). As with goat cells, absorption of the antiserum reduced the pump-stimulating activity. Inhibition of passive transport was not affected by absorption of serum with goat cells. The eluted antibodies caused substantial stimulation of the pump, but little or no inhibition of passive transport.

Absorption of the serum with three volumes of goat cells (preparation G-3) completely removed pump-stimulating activity to goat cells but after the same absorption, activity remained to pumps of sheep cells. It had previously been shown that serum exhaustively absorbed with goat cells remained stimulatory to the pump in LK sheep cells (eight successive absorptions, 1 : 1.5, v : v, serum to cells, ref. 11). These observations are probably not attributable to failure of the goat cells to absorb all of anti-L_p. More likely, anti-L_l also stimulates the pump in sheep cells. Serum absorbed with sheep cells did not stimulate the pump in sheep cells (preparation S-2), but the ability of this reagent to inhibit passive K^+ transport was less than that of unabsorbed serum. Therefore the failure of preparation S-2 to stimulate the pump in sheep cells is due to the prior absorption by LK sheep cells of anti-L_l antibodies as well as anti-L_p. Further evidence will be presented in connection with Table II in support of the argument that both anti-L_p and anti-L_l stimulate the pumps of LK sheep cells.

TABLE II

THE EFFECT OF PRETREATMENT OF LK SHEEP RED CELLS WITH TRYPSIN ON ANTI-L-MEDIATED CHANGES IN K^+ TRANSPORT

Cells were treated with trypsin as described under Methods washed and incubated at 5 % hematocrit with anti-L serum or, as a control, with serum from a non-immunized HK sheep. Then the active and passive fluxes were measured. Fluxes in cells treated with non-immune serum were unaffected by pre-exposure to trypsin. The mean fluxes in these cells were (mmol/l cells per h): active, 0.04; passive, 0.56. The results are given as the ratio of the influx in anti-L-treated cells (with and without trypsin pretreatment) to the influx in the control cells. 12 fluxes in duplicate were determined. The mean of differences between pairs of duplicate determinations, as a percent of each mean, was 4.8 %.

Pretreatment	K^+ influx	
	Active	Passive
No trypsin	10.9	0.56
Trypsin (2 mg/ml)	2.2	0.53
Trypsin (10 mg/ml)	2.4	0.50

Trypsin pretreatment

As mentioned above pretreatment of LK sheep cells with trypsin rendered the K^+ pumps insensitive to anti-L, but had no effect on sensitivity to lysis by anti-L [16]. Table II shows results of an experiment comparing the effects of trypsin treatment on

the actions of anti-L on active and passive K^+ influxes. There was no effect of trypsin on active or passive transport in control cells (incubated with non-immune serum after pretreatment with trypsin). Trypsin pretreatment did reduce the pump-stimulating activity of anti-L, as observed previously [16]. Nevertheless the stimulation of the pump was not abolished by trypsin, and remained greater than 2-fold as the trypsin concentration was increased from 2 to 10 mg/ml in the preincubation medium.

At the same time trypsin pretreatment was without effect on the inhibition of passive transport by anti-L, about 2-fold in all three samples of cells, indicating that the action of anti-L_i is not affected by trypsin. The results on the effect of trypsin on pump stimulation are consistent with the view that trypsin prevents the action of anti-L_p, and that the remaining stimulation of the pump is due to anti-L_i.

Antibody binding

As stated above, a part of the earlier evidence for two populations of antibodies came from studies on complement-dependent hemolytic activity of serum and serum fractions [11]. These results were confirmed by hemolytic tests performed in the same manner. Hemolytic activity to LK sheep cells of serum absorbed with LK goat cells was not significantly reduced compared with the activity of unabsorbed serum. Activity was reduced considerably by absorption with LK sheep cells. Eluates from either the sheep or goat cells used to absorb were only slightly hemolytic.

To extend these results, antibody binding was measured by the Coombs indirect antiglobulin test for agglutination [21]. This method has the advantage over the hemolytic test in that it is more sensitive, does not depend on complement, and can detect incomplete antibodies.

The binding of antibodies from the various reagents is shown in Table III. The binding was the same for unabsorbed and absorbed serum. There was much less

TABLE III

BINDING OF ANTIBODIES FROM ANTI-L SERUM AND SERUM FRACTIONS TO LK SHEEP RED CELLS

Binding was measured by an indirect antiglobulin test for agglutination [21]. The immune reagents were of preparation G-3. Details are given in the text under Methods. Agglutination titer is the highest dilution of reagent which caused agglutination after addition of antiglobulin.

Immune reagent	Agglutination titer
Unabsorbed serum	1 : 80
Serum absorbed (1 : 3)	1 : 80
Eluted antibodies	1 : 10
Non-immune serum	< 1 : 10

binding from the eluted antibodies, and none from non-immune serum diluted 1 : 10, the lowest dilution tested. These results confirm the findings obtained using the hemolytic test. The failure of anti-L_p (in the eluate) to cause substantial hemolysis could indicate a failure of anti-L_p to bind complement, but the results of the Coombs test indicate rather that the concentration of anti-L_p in anti-L serum is much lower than the concentration of anti-L_i.

DISCUSSION

The results of this study indicate anti-L serum contains two populations of antibodies with different actions on K^+ transport in LK cells. Anti-L_p has as its sole action stimulation of the K^+ pump in LK red cells of both sheep and goats. Anti-L_t reduces passive K^+ transport in both sheep and goat cells, and also has some stimulatory effect on the pump in sheep cells.

The antibodies inhibiting passive transport and promoting hemolysis are apparently the same, i.e. anti-L_t, since absorption by LK goat cells and pretreatment of the cells with trypsin are both without effect on either activity.

Anti-L_t stimulates the pump in sheep cells but not in goat cells. It follows that the antibody which is not absorbed by goat cells and stimulates the pump in sheep cells is the same antibody which stimulates the pump after trypsin treatment. Nevertheless, absorbed serum may prove to contain more than one population of anti-L antibody, and the activities ascribed to anti-L_t on the basis of the evidence summarized above may in fact not reside in a single antibody.

It has been proposed that anti-L acts on LK sheep red cells by converting cation transport sites from a passive to an active mode of operation [4, 14]. In terms of the results of this study anti-L_p may act only on pump sites which are already active in untreated LK cells, increasing active transport by altering the relative affinities of the pump for intracellular Na^+ and K^+ [10, 12, 23]. In this scheme anti-L_t antibody may act on active sites, but would also have an affinity for inactive pump sites, converting their mode of K^+ transport from passive to active. The two antibodies in anti-L serum affecting K^+ transport may reflect differences in the antigenic determinants of active and inactive pump sites which result from the process of differentiation, the inactivation of pump sites [20] determined by the LK gene.

The effects of anti-L antibodies on membrane function in red cells may relate to other types of interactions between antibodies and membranes. For example, antibodies specific for cell surface antigens stimulate blast transformation in lymphocytes [24] and growth of tumor cells [25]. Stimulation of membrane transport may be a correlate of these antigen-antibody interactions [26–28].

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