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ACTIVE POTASSIUM TRANSPORT AND THE L AND M ANTIGENS OF SHEEP AND GOAT RED CELLS

J. C. ELLORY AND ELIZABETH M. TUCKER

Institute of Animal Physiology, Agricultural Research Council, Babraham, Cambridge (England)

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SUMMARY

1. Active K^+ transport and the distribution of the L and M antigens have been investigated in sheep and goat red cells.

2. Low K^+ (LK) type goat red cells have a significantly lower rate of active K^+ uptake than high K^+ (HK) type cells. Active K^+ transport and the associated (Na^+-K^+)-activated ATPase activity in LK goat red cells was stimulated 1.5–8-fold by sensitization with sheep anti-L, even though the presence of this antibody could not be detected by complement lysis.

3. Ether and acid eluates of anti-L from sensitized goat and sheep red cells haemolysed sheep but not goat red cells and stimulated active K^+ transport in both goat and sheep red cells. There was no apparent correlation between the presence of the M antigen and K^+ types in goats as there is in sheep.

4. It is suggested that there may be two specificities of L substance, one associated specifically with the K^+ pump in sheep and goat red cells and not readily detectable serologically, and the other, found only on sheep red cells which may or may not be associated with the pump but which is easily detected by haemolytic tests.

INTRODUCTION

Sheep[1], goats[2], buffaloes[3], cattle[4] and possums[5] all show a discontinuous distribution in their red cell K^+ concentrations, and in general, individuals of these species can be classified into high (HK) or low (LK) types. The HK type red cells maintain their higher internal K^+ level by an increased rate of K^+ transport and transport ATPase activity[4–6]. It has recently been shown[7–9] that in sheep, the M-L blood group system is associated with the K type, in that homozygous LK sheep red cells have the L antigen (originally called m) present, whereas homozygous HK red cells have the M antigen present; heterozygous LK cells have both the L and M antigens. Sensitization of LK sheep red cells with anti-L results in a 3-8-fold stimulation of active K^+ uptake and of (Na^+-K^+)-activated ATPase activity in these cells[8]. Treatment with anti-M has no effect. The present work sets out to investigate the effect of the L and M antibodies on the red cells of goats, since it is known that there are some serological similarities between sheep and goats.

METHODS

Blood samples were collected by jugular venepuncture into heparinised containers.

Preparation of anti-sera

The M reagent was an antiserum made by immunizing a homozygous LK type sheep with HK type red cells, and the L reagent by immunizing an HK type sheep (R502) with LK type red cells[9].

Normal haemolytic test

This was a simple 3-drop test using 2 % red cell suspension, reagent and rabbit complement[10].

Weak antibody test

This test for weak antibodies employs guinea pig complement diluted 1 in 4 with saline[11].

The complements were prepared from pooled rabbit or guinea pig sera previously absorbed in the cold with sheep and goat red cells. They were stored at −70° until just before use.

Haemolysis score

Red cells were tested against 'doubling-up' dilutions of reagent from 1 in 1 to 1 in 512. The degree of haemolysis in each tube was recorded as 0–5, 5 being complete haemolysis. The score was obtained by summing the degree of haemolysis recorded for each dilution.

Immunoelectrophoresis

The method of HIRSCHFELD[12] was used.

2-Mercaptoethanol treatment

The method of ADINOLFI *et al.*[13] was used.

Absorptions

Washed packed red cells were mixed with serum at a ratio of 1:1.5, respectively, and after 30 min at room temperature the absorbed serum was separated by centrifugation. When more than one absorption was carried out, the serum was transferred to a further volume of packed red cells and the process repeated.

Elution

The red cells or stroma were sensitized for 1 h at 32° with an equal volume of antiserum and washed at least 3 times. Heat elution was carried out at 56°, ether elution by shaking 1 vol. of sensitized red cells or stroma with 2 vol. of diethyl ether for 30 min at room temperature[14] and acid elution by adjusting to pH 3.5 at 20° with 0.2 M HCl (ref. 15).

$^{42}K^+$ uptake

Washed red cells were incubated at a haematocrit of 0.05 in a solution of 10 mM KCl (containing ^{42}KCl), 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose,

10 mM Tris (pH 7.4). Ouabain was added to half the duplicate samples, to a final concentration of 50 μM. Samples (1 ml) were taken at time intervals from 15 to 120 min, washed 3 times by centrifugation in 25 ml of cold unlabelled medium, haemolysed in 7 ml of distilled water and counted in a Packard γ-scintillation spectrometer.

(Na^+-K^+)-activated ATPase

Red cell ghosts were prepared by the method of DODGE *et al.*[16], and assayed for ATPase activity as previously described[17].

Ouabain binding

Labelled ouabain was obtained from New England Nuclear Corporation (1.3 C per mmole), and its purity assessed by isotope dilution, and by chromatography in *n*-butanol–water (85:15, by vol.). After preliminary experiments to establish the time-course of ouabain binding to the K^+ pump, simultaneously measuring inhibition of ^{42}KCl uptake and amount of [^{3}H]ouabain bound, 4 h was chosen as a time when the pump was 95 % inhibited, at an ouabain concentration of 6 nM, but the apparent non-specific binding component was small. Therefore washed red cells were incubated for 4 h at a haematocrit of 0.05 in a solution containing 125 mM NaCl, 25 mM CsCl, 5 mM glucose, 5 mM Tris (pH 7.4), 6 nM [^{3}H]ouabain (*cf.* HOFFMAN[18]). Aliquots (2 ml) were washed 3 times by centrifugation in 50 ml of cold unlabelled medium, and the packed cells extracted with 15 ml of Bray's solution. After 6 h the samples were centrifuged, and the supernatant solutions counted in a Packard β-scintillation spectrometer, using automatic external standardisation for quench correction.

K^+ determinations

Washed red cells were haemolysed with 750 vol. of distilled water and analysed for K^+ in a Zeiss PMQ II or EEL Series A flame spectrophotometer. Where samples were taken in the field and a delay of up to 24 h occurred before analysis, whole blood was diluted for K^+ analysis, and the haematocrit used to calculate red cell K^+ concentrations.

RESULTS

Red cell K^+ concentrations in the 246 goats tested ranged from 25 to 110 mM, and the distribution was consistent with there being 2 K^+ types, the HK type having K^+ concentrations greater than 65 mM and the LK type less than 60 mM. Only 19 LK type goats were found. Cells from 132 individuals, including the 19 LK type animals were also tested for the presence of the L and M antigens using the normal haemolytic test (Fig. 1). 116 of the goats had M positive red cells; these included both HK and LK types. None of the red cells were haemolysed by the L reagent. Unlike the situation in sheep, there was therefore, no obvious correlation between the distribution of K^+ types and the occurrence of the L and M antigens. Of these goats 10 HK and 9 LK types were selected for more detailed observations on their red cells.

Serological tests for the L antigen

Goat No. B395 was chosen because its red cells had a low K^+ concentration (33 mM) and were M negative. Both the normal haemolytic and the weak antibody

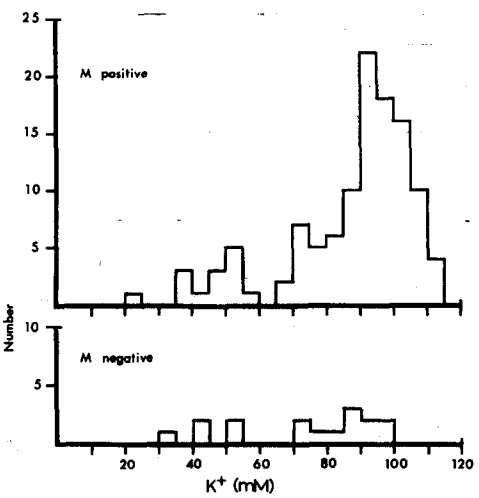
tests failed to identify any substance of L specificity on these red cells. Also it was not possible to absorb out the anti-L activity, for even after 8 absorptions with Goat No. B395 red cells the L reagent still gave the usual haemolysis score against sheep red cells. Similar absorptions using red cells from 4 other LK type goats were also unsuccessful. There was therefore no direct serological evidence that L substance was present on LK goat red cells.

Active K^+ uptake and the L substance

The mean ouabain-sensitive K^+ uptake in HK and LK type goat red cells was 0.733 ± 0.067 and 0.311 ± 0.035 mmole/h per l packed cells ±S.E., for results from 9 and 10 individuals, respectively. Thus the increased rate of active K^+ transport was associated with a higher internal K^+ concentration (87.8 ± 4.1 and 41.4 ± 3.4 mmoles/l packed cells ± S.E.)[6]. When LK type goat red cells were treated with anti-L, there was a 1.5–8-fold increase in their active K^+ uptake (Table I). The antibody had no effect on HK red cells, and sera from non-immunized HK type sheep and goats had no effect on LK goat red cells. Therefore, although no activity was detectable serologically, the sheep anti-L enhanced active K^+ transport in LK goat red cells in a manner similar to that shown for sheep red cells. To assess the sensitivity of this effect, a dose–response curve was constructed (Fig. 2) to compare the stimulation of active K^+ transport in goat and sheep red cells by serial dilutions of anti-L. Although the net stimulation was higher in the sheep than in the goat, the concentration dependence of the response was about the same, anti-L at a dilution of 1:16 having a significant effect in both the goat and sheep erythrocyte.

Ouabain binding and (Na^+-K^+)-activated ATPase activity

To confirm that this stimulation of ouabain-sensitive K^+ uptake by anti-L was directly connected with the active transport mechanism, two other parameters of



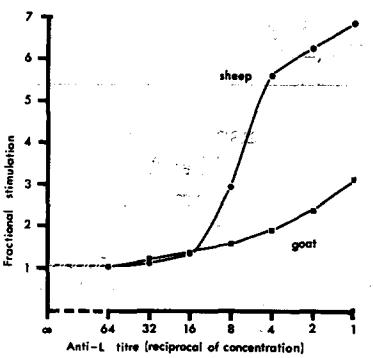


Fig. 1. The M antigen and the distribution of red cell K^+ levels in goats. K^+ concentrations are expressed in mmoles/l packed cells, calculated from analysis of whole blood.

Fig. 2. Dose–response curve for the stimulation of active K^+ transport in sheep and goat LK erythrocytes by anti-L. Fractional stimulation = (ouabain-sensitive K^+ uptake after anti-L treatment) / (ouabain-sensitive K^+ uptake in control).

TABLE I

THE EFFECT OF ANTI-L ON THE ACTIVE K^+ UPTAKE IN GOAT RED CELLS

Washed red cells were incubated with either anti-L or a control HK sheep serum, which had previously been heated to 56° for 20 min. After 1 h at 32° the cells were washed once by centrifugation, and incubated in the $^{42}K^+$ solution.

Goat No.	*Red cell K^+ concn. (mmoles/l packed cells)*	*Ouabain-sensitive K^+ uptake (mmoles/l packed cells per h)*	
		Control	*Anti-L treated*
B395	33	0.309	0.705
H20	25	0.213	1.00
HDAV	35	0.318	0.697
HM118	38	0.218	0.758
CL133	53	0.472	0.847
CA548	54	0.180	0.599
CL361	53	0.251	1.341
CL347	40	0.444	0.643
CA 508	42	0.394	0.961
H62	74	0.541	0.542
CA158	89	0.745	0.805
CB26	95	0.846	0.862
CB2	93	0.798	0.763

TABLE II

THE EFFECT OF ANTI-L ON THE (Na^+-K^+)-ACTIVATED ATPase ACTIVITY AND OUABAIN-BINDING CAPACITY OF GOAT RED CELLS

Red cell ghosts (approx. 2 mg) were incubated for 30 min at 32° with either anti-L or a control HK sheep serum, washed by centrifugation (25000 × *g*, 10 min), and assayed for ATPase activity. Washed red cells were incubated with anti-L or a control HK sheep serum for 1 h at 32°, washed by centrifugation, and incubated with [^{3}H]ouabain. Results are the mean of duplicates in two separate experiments.

Red cell type	*Red cell K^+ concn. (mmoles/l packed cells)*	*(Na^+-K^+)-activated ATPase activity (μmole P_i/mg protein per h)*		*Bound ouabain (molecules/cell)*	
		Control	*Anti-L*	*Control*	*Anti-L*
LK (B395)	33	0.020	0.043	16.1	26.8
HK (B167)	93	0.067	0.069	31.2	—

active transport were measured. Table II shows the (Na^+-K^+)-activated ATPase activity of LK and HK goat red cell ghosts incubated with either anti-L or control HK sheep serum. There was a definite specific stimulation of ATPase activity by anti-L. Because of the potentially large contribution from non-specific binding when using [^{3}H]ouabain to measure pump sites on LK type red cells, it is the present intention to use this technique comparatively, to show that the binding of [^{3}H]-ouabain to LK goat red cells was specifically stimulated by treatment with anti-L, as was in fact demonstrated (Table II).

Absorption experiments

Preabsorption of the L reagent with either LK goat or LK sheep red cells abolished its ability to stimulate active K^+ transport in goat red cells. However,

absorption with LK goat red cells still left a significant stimulation for sheep red cells (Table III). Even absorbing 8 times with LK goat red cells failed to completely abolish this effect. It would seem therefore that there is a fraction of the L activity which is specific for sheep red cells.

Elution experiments

Tests were carried out to see if anti-L could be eluted from sheep and goat red cells which had been treated with anti-L and then washed thoroughly in saline. Various methods of elution were tried, both from intact red cells and from ghosts. The eluted fractions were tested serologically for the presence of antibody to LK type sheep and goat red cells, and also for their ability to stimulate active K^+ transport, (Table IV). It was found that an antibody of L specificity, which reacted serologically

TABLE III

THE EFFECT OF ABSORPTION WITH HK OR LK CELLS ON THE HAEMOLYSIS SCORE AND THE STIMULATION OF ACTIVE K^+ TRANSPORT BY ANTI-L

Fractional stimulation of active K^+ uptake = (ouabain-sensitive K^+ uptake after anti-L treatment)/ (ouabain-sensitive K^+ uptake in control). K^+ uptake results are the mean, with the number of observations in brackets afterwards, $\pm$ S.E. for four or more determinations.

Anti-L absorbed twice with	*Fractional stimulation of active K^+ transport*		*Haemolysis score*	
	LK sheep (R53)	*LK goat (B395)*	*LK sheep (R53)*	*LK goat (B395)*
HK sheep cells	4.41 (2)	2.17 (1)	38	0
LK sheep cells	1.01 (3)	1.03 (2)	0	0
HK goat cells	4.59 $\pm$ 0.31 (4)	2.27 $\pm$ 0.21 (4)	38	0
LK goat cells	2.95 $\pm$ 0.34 (5)	1.16 $\pm$ 0.09 (6)	33	0

TABLE IV

THE HAEMOLYSIS AND FRACTIONAL STIMULATION OF ACTIVE K^+ TRANSPORT IN SHEEP AND GOAT RED CELLS BY ELUATES OF ANTI-L

Haemolytic tests scored non-parametrically. N.T. = not tested. Fractional stimulation is expressed as the mean, with the number of observations in brackets.

Elution method	*Eluted from*	*Haemolytic test*		*Fractional stimulation*	
		Sheep LK	*Goat LK*	*Sheep LK*	*Goat LK*
56°, intact red cells	Goat LK	+	—	N.T.	N.T.
	Sheep LK	+	—	N.T.	N.T.
Ether, intact red cells	Goat LK	+	—	N.T.	N.T.
	Sheep LK	+	—	N.T.	N.T.
Ether, stroma	Goat LK	+	—	4.19 (4)	2.13 (3)
	Sheep LK	+	—	3.16 (2)	2.06 (2)
Acid, stroma	Goat LK	+	—	2.49 (4)	1.86 (2)
	Sheep LK	+	—	2.61 (4)	1.97 (3)
Ether, stroma	Goat HK	—	N.T.	1.00 (1)	0.96 (1)
	Sheep HK	—	N.T.	0.99 (1)	1.02 (1)
Acid, stroma	Goat HK	—	N.T.	1.03 (1)	1.01 (1)
	Sheep HK	—	N.T.	N.T.	N.T.

with sheep but not with goat erythrocytes, and which stimulated K^+ transport in sheep and goat red cells, could be eluted off both sheep and goat red cells which had been treated with anti-L. The eluate from sheep cells haemolysed sheep red cells as avidly as the original L reagent, but the eluate from goat red cells was only weakly lytic for sheep red cells.

Immunoelectrophoretic analysis showed that the eluates contained both IgG1 + IgG2 components. However, some of the eluates which had been prepared from ghosts which had been sensitized as stroma and not as intact red cells, gave weak albumin and α- and β-globulin precipitation lines. This indicated that in spite of four washes in 25 volumes, some of the sensitizing serum may still have been trapped and could have accounted for the anti-L activity. Tests in which the eluates were prepared from stroma which were made from well-washed sensitized red cells were clear of contaminating serum proteins and yet had anti-L activity. Both acid and ether elution methods from stroma were very successful in extracting concentrations of anti-L of reasonable titre, but it was found that in the K^+ studies there was an increased ouabain-insensitive leak component in red cells which had been incubated with the acid eluates. This effect was also found with control elutions from HK cells. Ether eluates were entirely satisfactory and it was concluded that the best method of elution was to extract with ether from stroma which had been sensitized as intact red cells and then thoroughly washed. Tests with M reagent indicated that this antibody could also be successfully eluted from sheep red cells.

After mercaptoethanol treatment there was no decrease in the serological activity of anti-L, consistent with the antibody being of the IgG class. Recent chemical purification of anti-L by Mr. M. HOBART of this Institute, using a modification of the method of SOBER AND PETERSON[19], indicates that both the serological and pump-stimulating activities reside in the IgG1 fraction. The IgG2 fraction had no activity.

To compare different batches of L reagent

Five different collections of anti-L were made over the course of the last year from sheep R502, after booster doses of red cells from the same donor had been given. When the sera were tested for antibody titre and for ability to stimulate active K^+ transport in LK sheep red cells (Table V) there was no correlation between haemolysis score and fractional stimulation, Batch 1 being the most effective in stimulating active K^+ transport, and yet the weakest serologically.

TABLE V

COMPARISON OF HAEMOLYSIS SCORE AND FRACTIONAL STIMULATION OF ACTIVE K^+ UPTAKE WITH DIFFERENT BATCHES OF ANTI-L

Batch of anti-L	*Fractional stimulation of K^+ uptake*	*Haemolysis score*
1	5.52	25
2	3.33	36
3	4.17	44
4	2.45	45
5	3.12	41

DISCUSSION

RASMUSEN AND HALL[20] came to the conclusion that there is an association between the M antigen and K^+ types in goats as well as in sheep. At that time L antigen had not been identified and their tests were done only with the M reagent. They found that out of 84 goats tested 34 had K^+ levels of 60 mM or less and these they called LK type. All the M negative individuals (13) except one were contained in the LK group. The present results also show that the dividing line between LK and HK types is not so clearly defined in goats as in sheep, but is somewhere around the 65-mM point. However, no correlation was obvious between the M antigen distribution and K^+ types, M negative cells being found amongst the HK types as well as the LK types.

The disparity between the K^+ transport studies and the serological results is puzzling. It would appear that L substance is present on LK goat red cells, because not only does treatment with anti-L alter the transport properties, but this antibody can be eluted off LK goat red cells which have been treated with anti-L. In general the results are consistent with a hypothesis that there are two antibody specificities present. One, (anti-L) readily haemolyses LK sheep but not LK goat red cells and may or may not affect the K^+ pump. The other (anti-Lp), is also not lytic for goat red cells and only weakly lytic for sheep cells, but affects the K^+ pump of both sheep and goat red cells. This idea is supported by the results of the elution and absorption experiments, and further indirect evidence is provided by the fact that, while the serological titre of anti-L has increased over the course of several immunizations, its fractional stimulation of K^+ transport (anti-Lp) has not increased at all.

Our original hypothesis[8] that the L substance acts in sheep red cells by inhibiting pump sites, which are unmasked by sensitizing with the L antibody, was partially substantiated by ouabain binding experiments and kinetic studies[21], and experiments following the development of LK type red cells in lambs[22], and the present results indicate that a similar mechanism seems to be operating in LK goat red cells.

It is therefore suggested that an LK sheep red cell has the specificities L and Lp, and the LK goat red cell only the specificity Lp, the latter antigen being specifically involved with the transport ATPase system. Experiments are in progress to determine if a specific Lp reagent can be made by immunizing HK type goats and sheep with LK type goat red cells. If such an antibody is found then the present hypothesis would be further substantiated.

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