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Interaction of L Antibody with Low Potassium-Type Sheep Red Cells: Resolution of Two Separate Functional Antibodies

C.E. Smalley, E.M. Tucker, P.B. Dunham* and J.C. Ellory

ARC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, England, and
Physiological Laboratory, University of Cambridge, Cambridge, England

Summary. Antibodies of two specificities in alloimmune sheep anti-L sera, anti-L_P and anti-L_l, were separated by a new technique and characterized. Absorption of anti-L serum with trypsinized LK (*LL*) sheep red cells left anti-L_P antibodies; the absorbed anti-L_l antibodies were then eluted. Anti-L_P was only weakly lytic in the presence of complement; it had no effect on passive K influx, but stimulated active K influx. The stimulation could be reversed by eluting the antibody in glycine buffer at low pH. Stimulatory activity in the eluted cells could be restored by resensitization with anti-L_P. Anti-L_l was more strongly lytic than anti-L_P in the presence of complement; it had no effect on active K influx, but inhibited passive K influx. Pig anti-ruminant IgG conjugated to hemocyanin was used to visualize by electron microscopy the number of L_P and L_l antigen sites on *LL* sheep red cells sensitized with anti-L_P and anti-L_l. The values obtained were 590 L_P sites/cell and 847 L_l sites/cell.

Key words red cell membranes · potassium transport · alloimmune antiserum · high K–low K polymorphism · sheep red cells

Introduction

The M-L blood system in sheep is associated with the mechanism controlling transport of Na and K across the red cell membrane. Sheep of the low potassium type (LK) are homozygous (*LL*) or heterozygous (*ML*) for the gene controlling the expression of the L blood group antigen on the red cell membrane. Genetically high potassium type sheep (HK) are homozygous for the allelomorphic gene M (*MM*) (Rasmusen & Hall, 1966; Tucker & Ellory, 1970). It has been proposed that the L antigen inhibits the Na/K pump, and that this inhibitory effect can be alleviated by sensitizing LK cells with the alloantibody anti-L (e.g., *see* Ellory, 1977).

Anti-L has been shown to have two effects on potassium influx in LK cells, and it has been suggested that these two effects are brought about by antibodies of two different specificities. One antibody (anti-L_P) is only weakly lytic in the presence of complement; it combines with L_P antigen sites to stimulate the ouabain-sensitive active transport of Na and K (Ellory & Tucker, 1969; Lauf et al., 1970), but has no effect on passive transport (Dunham, 1976*a*). The other antibody, anti-L_l (previously called L_{LY}; Tucker & Ellory, 1970), is more strongly lytic (Ellory & Tucker, 1970) and combines with L_l antigen sites to inhibit the ouabain-insensitive passive K (but not Na) transport (Dunham, 1976*a*, *b*).

Anti-L reagents are prepared by immunizing HK sheep or goats with LK red cells, and the resulting immune antiserum, after appropriate absorption with pooled HK red cells, contains a mixture of anti-L_P and anti-L_l antibodies. Several attempts have been made to separate these two specificities. In a previous study we took advantage of the presence of L_l antigen and the absence of L_P antigen on fetal red cells of genetically LK lambs (Tucker et al., 1976). By absorbing an alloimmune anti-L serum with LK lamb red cells we attempted to remove anti-L_l antibodies but not anti-L_P. The use of such an anti-L_P reagent in electron microscopic studies enabled a tentative estimate of the number of L_P sites on mature *LL* type red cells to be made (Tucker et al., 1976). A disadvantage of this method of preparing anti-L_P is that, since lambs are born usually with a mixture of adult and fetal red cells as judged by hemoglobin studies (Drury & Tucker, 1963), one cannot be sure that some cells containing L_P are not present in the absorbing population of cells.

Attempts to separate the two specificities have also been made using cells from LK goats on the assumption that these cells have L_P antigen but not L_l. A partial separation was achieved, but it was not wholly satisfactory. The LK goats appear to have some L_l antigen and do not completely absorb anti-L_P (Ellory & Tucker, 1970; Dunham, 1976a).

In this paper we describe a new method for the preparation of anti-L_P and L_l reagents. It is based

* *Permanent address:* Department of Biology, Syracuse University, Syracuse, New York

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upon the observation that treatment of LK sheep red cells with trypsin destroys L_P so that subsequent sensitization with anti-L_P no longer simulates the Na/K pump (Lauf, Parmelee, Snyder & Tosteson, 1971; Dunham, 1976*a*; Lauf, Stiehl & Joiner, 1977). Trypsinization apparently does not destroy L_l since anti-L still inhibits passive K transport in cells so treated (Dunham, 1976*a*; Lauf et al., 1977). Therefore, absorption of anti-L serum with trypsinized LK cells should remove anti-L_l but not anti-L_p, giving an anti-L_P reagent. Elution of the absorbed anti-L_l from the trypsinized cells should yield a specific anti-L_l reagent. This paper describes the preparation and properties of reagents prepared in this manner. Although the effects of anti-L on K transport in LK cells have been studied in some detail, it is still not known how the antibody activates the Na/K pump. Anti-L binds to the outside surface of LK cells, but kinetic experiments have shown it to exert its principal effect on the apparent K affinity at the *inside* loading sites of the Na/K pump (Lauf et al., 1970; Sachs et al., 1974; Cavieres & Ellory, 1977). Such a transmembrane effect implies an allosteric interaction between the antibody, antigen, and pump, and it is therefore of interest to see whether pump activation by the antibody is reversible. The present paper demonstrates this reversibility by using a mild acid elution procedure to remove the antibody from intact LK cells.

Material and Methods

Serology

Anti-L (absorbed with HK cells) and anti-M (absorbed with LK cells) reagents were prepared and tested serologically as described previously (Tucker & Ellory, 1970).

Separation of L_P and L_l Antisera

An *LL* type sheep was bled into heparin; the cells were washed three times in saline, then once in a "trypsinization" buffer of 125 mM NaCl, 10 mM $CaCl_2$, 20 mM Tris-HCl, pH 8.0 (Lauf et al., 1971). One volume of packed red cells was mixed with 1 volume of 10 mg/ml trypsin (Sigma Chemical Co., prepared from bovine pancreas, Type III, Lot 68 C – 00142, cat. no. T8253) in the above buffer. The cells were incubated for 30 min at 37 °C. Control cells in trypsinization buffer with no trypsin were also incubated. The cells were washed twice in saline, then once in saline containing 80 mg/ml of trypsin inhibitor (Sigma, prepared from soy beans, type I–S), then twice more in saline. One volume of packed trypsinized red cells was added to two volumes of an anti-L antiserum. After 15 min at room temperature the cells were removed by centrifugation and anti-L_l was eluted from them as described below. The supernatant serum was absorbed four more times with fresh aliquots (one volume each) of trypsinized cells. This antiserum, which had been absorbed five times with trypsinized cells, was used as the anti-L_P reagent.

Elution

Antibody was eluted by treatment with glycine-HCl buffer (275 mosmol/liter) at pH 3.0 according to the method of Rekvig and Hannestad (1977). The cells had been trypsinized as described above, sensitized with anti-L, and then washed 3 times. A 50% (vol/vol) saline suspension of these antibody-coated cells was mixed at 4 °C with the glycine-HCl buffer for 1 min and then centrifuged in a high speed Eppendorf centrifuge for 30 sec. The supernatant solution was removed and immediately neutralized by adding 0.02 ml of 0.5 M Tris base per 1 ml eluate; bovine serum albumin was added to give a final concentration of 10 mg/ml. This was used as the anti-L_l reagent. The anti-L_l and anti-L_P reagents were stored at −20 °C.

Potassium Influx

Unidirectional potassium influxes were measured using ^{86}Rb as a tracer for K after the method of Dunham and Ellory (1980). Passive K influx was taken as that measured in the presence of ouabain (0.1 mM). Active K influx was taken as the ouabain-inhibitable flux (the difference between total and ouabain-insensitive influxes). To test anti-L reagents on passive K influx, LK cells osmotically swollen to 15% above their normal volume were used, thereby maximizing the passive flux (Dunham & Ellory, 1981). Fluxes are given as μmol per liter of packed cells per hour. Mean values are based on three determinations; errors are given as SEM.

Electron Microscopy

Pig anti-ruminant immunoglobulin G conjugated to hemocyanin from the marine whelk *Busycon caniculatum* (PARI-Hcy) was used to label L antigen sites on monolayers of red cells, as described previously (Ostrand-Rosenberg, 1975; Tucker et al., 1976). Replicas of sensitized cells were examined in a transmission electron microscope. The total number of hemocyanin-labeled sites seen over the cell surface exposed on electron micrographs at 35,000× magnification was counted ("sites per cell face"). Area of the "cell face" was measured so that results could also be expressed as sites per μm^2. Control *LL* cells were treated with anti-M, followed by PARI-Hcy to measure nonspecific binding of hemocyanin conjugate. Background counts (about six sites per face) were subtracted from the total site counted on sensitized cells. All values given in results were corrected for background.

Abbreviations

Tris: Tris (hydroxymethyl) amino methane; MOPS: Morpholinosulfonic acid; PARI-Hcy: Porcine anti-ruminant IgG, conjugated to hemocyanin.

Results

Properties of Anti-L_P and Anti-L_l Reagents

K influx was measured in *LL* cells sensitized with unfractionated anti-L serum and with the two purified reagents prepared as described in Methods. The results are shown in Table 1. Unfractionated anti-L serum stimulated active K transport fivefold and reduced passive transport 2.5-fold. The antibody eluted from the trypsinized cells (anti-L_l) reduced passive K influx about twofold, but had no significant effect

Table 1. Active and passive potassium influx measured in LK red cells taken from a homozygous (*LL*) sheep

Treatment	K influx ($\mu mol \cdot l^{-1} \cdot hr^{-1}$)	
	Active	Passive
Control	93 ± 2	143 ± 3
Anti-L_l	107 ± 11	83 ± 3
Anti-L_P	414 ± 21	131 ± 3
Anti-L (unfrac.)	486 ± 11	56 ± 4

The cells were treated with the purified immune reagents anti-L_P and anti-L_l, or with unfractionated anti-L serum. The purified reagents were made using trypsinized red cells from an adult LK sheep (*see* Methods). $[K]_o$ = 7.5 mM. Results are means ± SEM (n = 4). Similar results were obtained in four other experiments.

on the pump. The antibody remaining in the anti-L serum after absorption with trypsinized cells (anti-L_P) stimulated the pump nearly fivefold but had no effect on passive influx. Thus the two functions of anti-L in affecting passive and active K influx selectively were separated successfully using this procedure.

Table 2 shows the serological properties of two anti-L_P reagents prepared from anti-L sera from two different HK (*MM*) type sheep, and of an anti-L_l reagent prepared from one of them. The reagents were used in hemolytic tests against red cells from 5 *LL* and 10 *ML* sheep in the presence of rabbit complement. The two anti-L_P reagents showed partial, weak reactions with *LL* cells but failed to hemolyse *ML* cells. In contrast, anti-L_l hemolysed both types of cells up to a dilution of 1 in 16.

The effect of diluting the reagents on the activation of the Na/K pump (Fig. 1) and the numbers of sites seen by PARI-Hcy labeling (Fig. 2) was determined. Figure 1 shows the dose-response curve for activation of the Na/K pump by anti-L_P reagent. Maximal pump stimulation occurred when 8 µl packed cells were sensitized with 70–80 µl of antiserum. The results also show that anti-L_l had no effect

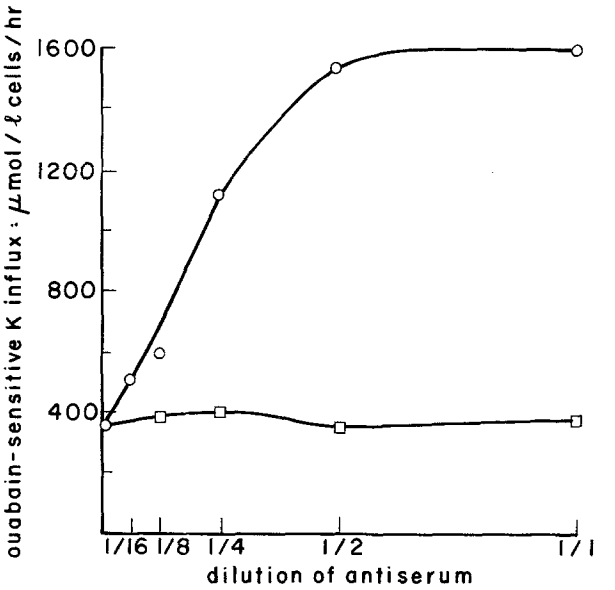

Fig. 1. Activation of the Na/K pump in *LL* sheep red cells by different dilutions of anti-L_P and L_l reagents. An aliquot of 0.2 ml of a 5% red cell suspension was centrifuged and the supernatant carefully removed; 0.2 ml of undiluted or serially diluted anti-L_P (○) or L_l (□) reagent was added and the tube mixed and incubated at 37 °C for 15 min. The tubes were recentrifuged, the antibody supernatant was aspirated, and 0.5 ml of solution of 10 mM K (containing ^{86}Rb), 140 mM NaCl, 15 mM MOPS, 5 mM glucose ± 0.1 mM ouabain added. After 30 min the cells were washed by centrifugation and processed for scintillation counting

on the pump at any concentration tested. The pump flux in Fig. 1 without anti-L is unusually high for LK sheep cells, but not uniquely so (*cf.* Lauf et al., 1970).

Figure 2 shows the number of L_P and L_l sites at the different dilutions of the reagents. For anti-L_l, saturation occurred at a dilution of 1 in 2 and for anti-L_P at a dilution of 1 in 4. A dilution of 1 in 2 was therefore used routinely for both reagents. The two methods of characterizing the reagents (flux measurement and hemocyanin-labeling) are not directly

Table 2. Serological reactions of anti-L_P and anti-L_l reagents prepared using trypsinized red cells from an LK sheep

Reagent	Cells	Dilutions of antisera								Hemolysis score
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
Anti-L_P (W14)	*LL*	2	2	2	1	0	0	0	0	7
	ML	0	0	0	0	0	0	0	0	0
Anti-L_P (V134)	*LL*	2	2	2	1	0	0	0	0	7
	ML	0	0	0	0	0	0	0	0	0
Anti-L_l (W14)	*LL*	5	5	5	5	5	4	2	0	31
	ML	5	5	5	5	5	4	2	0	31

The degree of hemolysis at each dilution was recorded as 0–5, 5 being complete lysis and 0, no lysis. The scores represented means from determinations on cells from 5 *LL* sheep and 10 *ML* sheep.

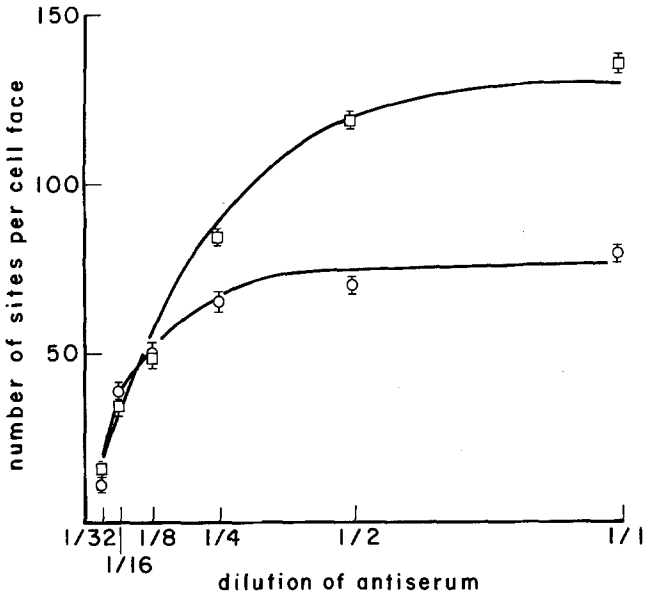

Fig. 2. The number of antigen sites seen on *LL* sheep red cells at different dilutions of L_P and L_l reagents. Cells were prepared and labeled with PARI-Hcy as described in Methods. Anti-L_P (○) and anti-L_l (□) were serially diluted down to 1/32 as shown. 10 cells were counted at each dilution. Bars indicate one SEM

Table 3. The number of L_P sites on red cells from 5 different *LL* type sheep

Sheep no.	*n*	Sites/cell "face"	Area per cell "face" (μm^2)	Sites/μm^2
1	10	90± 3	12.1±0.3	7.8±0.3
2	9	80±13	9.4±0.4	9.0±1.1
3	12	66± 5	7.6±0.3	8.6±0.4
4	11	74± 7	9.4±0.3	7.8±0.6
5	12	76± 5	7.2±0.3	10.6±0.6
Means		78± 3	9.0±0.3	8.8±0.3

Cells were sensitized with anti-L_P, diluted 1 in 2, and then labeled with PARI-Hcy as described in Methods. The area of the exposed cell "face" is approximately 1/7 of the total area if 67 μm^2 is taken as the total surface area of a sheep red cell (Ponder, 1952). Values are means ± SEM; *n* is the number of cells analyzed.

Table 4. Experiment to determine if the presence of either anti-L_P or L_l influences the ability of the other to bind to *LL* cells

Treatment	*n*	Sites/cell "face"	Cell "face" area	Sites/μm^2
A) Anti-L_P	20	88± 3	9.8±0.5	9.3±0.3
B) Anti-L_l	20	132± 6	10.7±0.3	12.7±0.6
C) Anti-L_P, then $-L_l$	20	176±10	10.6±0.4	16.8±0.8
D) Anti-L_l, then $-L_P$	19	200±13	11.1±0.4	18.0±0.8

Monolayers of cells were treated with anti-L_P alone (A), anti-L_l alone (B) or the two reagents in sequence (C) and (D). Values are means ± SEM; *n* is number of cells counted.

comparable since the ways in which the cells were sensitized and washed were so different (in the one case in a test tube and in the other case as a monolayer on a coverslip). However, since known volumes of cells and antiserum were applied to the coverslip, it was possible to estimate the ratio of volume of anti-L reagent/volume packed cells treated for the hemocyanin method. In this case the hemocyanin method used 100–200 µl reagent/µl packed cells, a 5- to 10-fold excess over the minimal concentration required to give maximal pump stimulation in Fig. 1.

Numbers of L_P Antigen Sites

To determine if the sensitization time influenced the number of L_P sites measured, *LL* cells were sensitized with anti-L_P for 10, 30 and 120 min before PARI-Hcy treatment. Mean [± SEM(*n*)] sites per "cell face" were, respectively 72±5 (7), 74±7 (6), and 83±10 (6). Statistical tests showed that these three groups were not significantly different from each other. Ten minutes was therefore used as a standard time for all subsequent sensitizations. To determine variability between individuals, monolayers of red cells from five different *LL* sheep were tested for L_P sites using anti-L_P (Table 3). Variation, ranging from 66 to 90 sites per "cell face," was found between individuals, the mean value being 78±3 for the five animals examined. It is also apparent from Table 3 that surface area per cell face showed some variation between individuals from 7 to 12 μm^2 face, as did the density of sites (8 to 11 sites/μm^2).

Relationship between L_P and L_l Antigen Sites

A simple binding assay using sequential sensitization with anti-L_P and anti-L_l reagents was performed to determine if the two antibodies affect one anothers' binding. The combined results of two separate experiments are shown in Table 4. There was no significant difference between the total numbers of sites seen whether anti-L_P was applied before or after anti-L_l (Table 4, *C* and *D*). This experiment also showed that the mean number of L_l sites per "cell face" (Table 4, *B*) was 132±6 (20), compared with 88±3 (20) L_P sites. Figure 3 is an electron-micrograph showing L_P and L_l sites on red cells from this experiment.

Reversibility of Anti-L Stimulation

Experiments were carried out eluting anti-L with glycine in attempts to answer three questions: (i) when anti-L_P is eluted from cells, does the pump remain stimulated? (ii) If the stimulation is reversible by this criterion, can the cells be stimulated a second time?

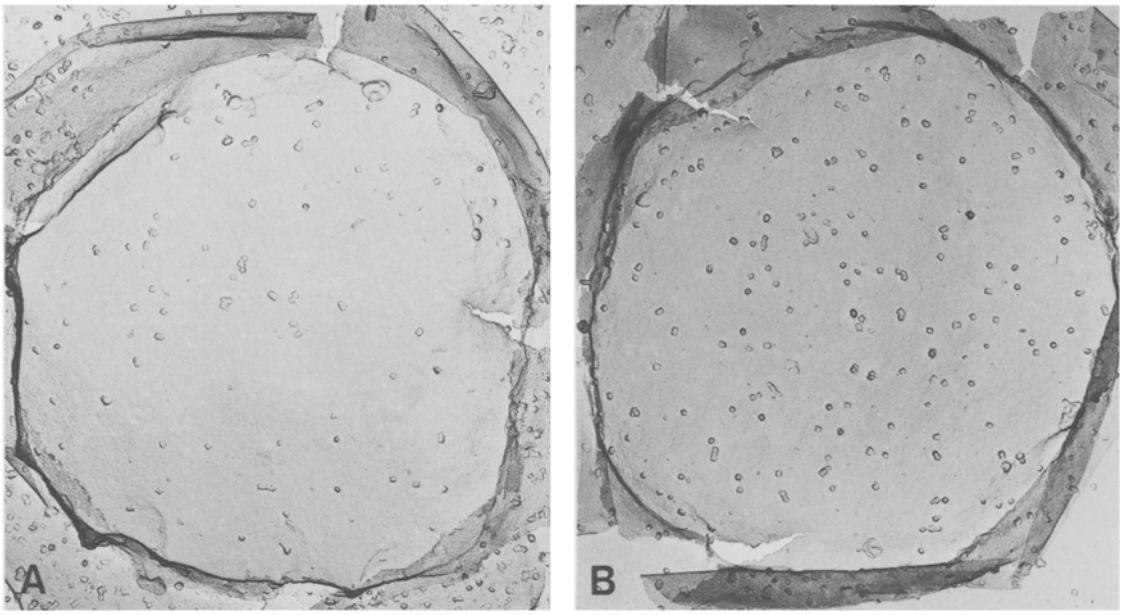

Fig. 3. Electron micrographs of (*a*) L_P and (*b*) L_I sites on red cells from an *LL* sheep. The cells were incubated for 10 min at room temperature with the antiserum diluted 1 in 2 and then treated with PARI-Hcy. Magnification ×20,000

Table 5. Reversibility of anti-L stimulation of active K influx in red cells from a homozygous (*LL*) sheep

Treatment	Active K influx ($\mu mol \cdot liter^{-1} \cdot hr^{-1}$)		
	Anti-L		Nonimmune serum
	7 vols	4 vols	
1st serum incubation	494 ± 16	312 ± 12	104 ± 13
1st glycine elution	126 ± 1	–	104 ± 10
2nd glycine elution	115 ± 7	149 ± 12	76 ± 14
2nd incubation (anti-L)	465 ± 4	356 ± 23	370 ± 12

The cells were first sensitized for 30 min at 32 °C, with an anti-L serum known to contain L_P and L_I activity, at a ratio of 1 vol cells to 7 vols antiserum or 1 vol cells to 4 vols antiserum. The cells were washed and the adsorbed antibodies were eluted twice (in sequence) with glycine (pH 3.0) as described in Methods. Control aliquots of cells which had been incubated with autologous (nonimmune) serum were also subjected to the glycine treatment. Cells which had been eluted twice with glycine were reacted a second time with fresh anti-L serum to determine if sensitivity to stimulation remained. Cells treated with nonimmune serum in the first incubation were reacted with anti-L in the second incubation. Active K influxes were measured in aliquots of cells taken after each treatment. Fluxes are means ± SEM ($n = 4$).

(iii) Does the eluted antibody retain activity? Table 5 shows that, even after the first elution, influx had returned almost to the rate seen in the control cells (nonimmune serum). Further, the K influx in the cells after the second glycine treatment was capable of being restimulated on resensitization with anti-L to a level similar to that seen originally.

Table 6. Na/K pump stimulation and serological reaction of glycine-eluates from anti-L sensitized homozygous (*LL*) sheep red cells

Initial serum treatment	Fractional pump stimulation		Hemolysis score	
	1st eluate	2nd eluate	1st eluate	2nd eluate
Anti-L (7 vols)	2.62	1.54	24	17
Anti-L (4 vols)	2.04	1.32	17	18
Autologous (nonimmune)	1.04	1.10	0	0

The two eluates were tested for their ability to lyse and to stimulate active K influx in fresh *LL* cells. The effects of the eluates were compared with those of autologous serum. The active K influxes are expressed as fractional stimulation, i.e., the ratio of the pump in serum-treated cells to control cells.

All eluates from the above experiment were tested serologically and for their ability to stimulate K influx. The results in Table 6 show that both the first and second glycine eluates were hemolytic in the presence of complement and possessed substantial pump stimulating activity against fresh normal *LL* cells.

A Possible Role of M Antigen

Although trypsin treatment destroys L_P sites on *LL* cells (Lauf et al., 1971; 1977), we showed recently that trypsin does not completely eliminate binding of anti-L_P to *ML* cells (Dunham, Tucker, Simonsen & Ellory, 1980). We speculated that an association

Table 7. Effect of anti-M serum on active K influx in red cells from a heterozygous (*ML*) sheep

Treatments			Active K influx ($\mu mol \cdot liter^{-1} \cdot hr^{-1}$)			
I	II	III	Expt. 1	Stimulation by anti-L	Expt. 2	Stimulation by anti-L
A) Anti-M	Trypsin	Anti-L	304±12	115	129±10	19
B) Anti-M	Trypsin	Saline	189± 7		110± 4	
C) Anti-M	Control	Anti-L	537± 7	315	512±15	380
D) Anti-M	Control	Saline	222±11		132± 6	
E) None	Trypsin	Anti-L	316±11	85	106± 4	21
F) None	Trypsin	Saline	231± 7		85± 5	
G) None	Control	Anti-L	555± 3	336	514± 6	413
H) None	Control	Saline	219± 8		101± 4	

Aliquots of cells which had been reacted with anti-M serum (treatment of I), as well as untreated cells, were trypsinized (2.5 mg/ml, 60 min., 37 °C, treatment II) or kept as controls. Aliquots of each type of cells were then reacted with anti-L serum or saline (treatment III) and active K influxes were measured ($[K]_o = 7.5$ mM). Results are shown from two separate experiments on cells from two different sheep. Fluxes are means ± SEM ($n = 4$).

In one experiment the cells were tested for complement lysis at the end of treatment II. On a scale of 0–5 (5 = complete lysis), the cells in sample A scored 3, sample C, 5-, and samples E and G, 0 (no lysis).

between M and L antigens in the membranes of *ML* cells may confer resistance to trypsin to some of the L sites, and further, that pretreatment of the cells with anti-M might modify that resistance. To test these possibilities, *ML* cells were sensitized with anti-M serum and treated as described in Table 7. The results were compared with those of control *ML* cells not sensitized with anti-M. As found in previous experiments (Dunham et al., 1980), residual sensitivity to stimulation by anti-L remained after trypsinization (Table 7, samples *A* and *E*). Secondly, sensitizing cells with anti-M prior to trypsinization did not reduce the apparent resistance of some of the L_P sites to trypsin. For example in experiment 1, active K influx in anti-M-trypsinized cells was stimulated by anti-L ($115\ \mu mol \cdot l^{-1} \cdot hr^{-1}$). Trypsinized cells not pretreated with anti-M were stimulated by $85\ \mu mol \cdot liter^{-1} \cdot hr^{-1}$, not significantly different from 115. In experiment 2 the corresponding stimulations were 19 and $21\ \mu mol \cdot liter^{-1} \cdot hr^{-1}$.

Two other conclusions may be drawn from the data in Table 7. First, anti-M had no direct effect on active K influx either in otherwise untreated cells (*compare* samples D and H), in cells subsequently treated with anti-L (*compare* C and G), trypsin (B and F), or both trypsin and anti-L (A and E). Furthermore, anti-L stimulated anti-M-sensitized cells nearly as well as it did untreated cells (C and G).

The failure of anti-M to alter the sensitivity to trypsin of some of the L_P sites might have been due to an action of trypsin on the bound anti-M antibody (Davies, Barrett & Hembry, 1978). Therefore, experiments were carried out to determine whether or not anti-M could be demonstrated bound to the cells after trypsinization. Two tests were employed: complement lysis and PARI-Hcy binding. The results of both tests showed that anti-M remained on the cells after trypsin treatment. The amounts of anti-M were reduced somewhat as judged by complement lysis (Table 7: hemolysis score of 3 as compared with 5- for the control), but were not reduced as judged by binding of PARI-Hcy (results not shown).

The Effect of Trypsinization on Bound Anti-L

The results on anti-M described in the previous section raise the question of the effects of trysinization of cells sensitized with anti-L. The results of an experiment carried out to test this are shown in Table 8. The design of the experiment was similar to that in Table 7. Cells from an heterozygous (*ML*) sheep were incubated either with anti-L (samples D through H) or autologous (nonimmune) serum (A–D). Half of each of these samples was then either treated with trypsin or kept as a control (treatment II). Each of these samples was, in turn, separated into two aliquots and sensitized with anti-L (some for the second time: E and G) or kept as a control (treatment III). Complement lysis was determined on aliquots of cells not sensitized with anti-L in treatment III (these all would have been fully hemolyzed with a score of 5).

The primary object of this experiment was the effect of anti-L bound to the cells on subsequent trypsinization and the effect of trypsin of the bound anti-L. A comparison of samples F and H shows that trypsin treatment removed some, but not all, of the bound anti-L. This is also shown by the hemolysis scores of these two samples. (Similar results were ob-

Table 8. The effect of trypsin on anti-L antibody bound to *ML* cells: anti-L-stimulated active K influx and complement lysis

Treatments			Active K influx ($\mu mol \cdot liter^{-1} \cdot hr^{-1}$)		Hemolysis score
I	II	III		stimulation by anti-L	
A) Nonimmune serum	Saline	Anti-L	593 ± 4	505	–
B) Nonimmune serum	Saline	Saline	88 ± 9		0
C) Nonimmune serum	Trypsin	Anti-L	126 ± 5	19	–
D) Nonimmune serum	Trypsin	Saline	107 ± 9		0
E) Anti-L serum	Saline	Anti-L	647 ± 9	184	–
F) Anti-L serum	Saline	Saline	463 ± 10		5
G) Anti-L serum	Trypsin	Anti-L	532 ± 15	255	–
H) Anti-L serum	Trypsin	Saline	277 ± 25		3

Aliquots of cells which had been reacted with either anti-L serum or autologous (nonimmune) serum (treatment I) were trypsinized (*see* Table 7) or kept as controls (treatment II). Aliquots of each group were then reacted with anti-L serum or saline (treatment III) and active K influxes were measured. Samples were also tested for complement lysis after treatment II and the hemolysis reading recorded as detailed in the legend to Table 7.

tained with trypsin on bound anti-M; *see* above.) In addition there was evidence for protection by anti-L against trypsin. Sensitization with anti-L of sample G in treatment III stimulated the pump by 255 $\mu mol \cdot 1\ liter^{-1} \cdot hr^{-1}$ (compare G and H), while in the corresponding control there was stimulation by only 19 $\mu mol \cdot liter^{-1} \cdot hr^{-1}$ (*compare* C and D).

Other conclusions may also be drawn from the results in Table 8. A comparison of A and B shows the normal stimulation of the pump by anti-L. Comparison of C *vs.* D confirms the residual sensitivity of the pump in *ML* cells to stimulation by anti-L after trypsin (*c.f.* Table 7; Dunham et al., 1980). The pump flux in E is higher than in F, showing the elution of some anti-L from F during treatment II.

Discussion

The anti-L_P and L_l reagents prepared by absorption with and elution from trypsinized cells behaved entirely as predicted, and we can conclude that this is a satisfactory method of separating the two specificities from a mixed antiserum. The only other method described for preparing anti-L_P, apart from absorbing with newborn lamb cells, is by elution from LK goat red cell ghosts sensitized with anti-L (Ellory and Tucker, 1970). Reagents prepared in this way are not entirely satisfactory. Although LK goat red cells have an antigen which behaves more like sheep L_P than sheep L_l the exact interrelationship of the sheep and goat antigens has not been established and they are most likely not identical (Tucker, Ellory & Kilgour, 1979).

The potassium flux/elution experiments provide evidence for the first time that the stimulatory effect of anti-L_P is a reversible phenomenon and also show that it is possible to restimulate the same cells by further sensitization with anti-L_P. This finding supports the concept that anti-L has an allosteric effect by bringing about a reversible (and subtle) conformational change in the membrane-bound antigen.

The observation that presensitization with anti-L protects the antigen against trypsin confirms the previous experiments of Lauf et al. (1971). However, since we have shown in the present work that activation of the pump by anti-L is fully reversible, this result can now be unequivocally interpreted in terms of a direct protective action of the anti-L antibody on the L_P antigen, rather than via an irreversible activating effect of the antibody on the LK Na/K pump. Since pump activity is still stimulated following trypsinization of anti-L-sensitized cells, and there is also complement lysis of both anti-M and anti-L treated cells after trypsin, we can conclude that, at least functionally by these two criteria, most of the bound antibody survives the proteolytic enzyme. However, clearly some do not (*compare* F and H, Table 8), so there is some uncertainty regarding the mechanism of protection.

In our previous paper using a hemocyanin conjugate to count antigen sites and testing red cells from only one sheep, we put forward a tentative estimate of about 350 L_P sites on *LL* cells (Tucker et al., 1976). This estimate was based on the unverified assumption that the surface area of a sheep red cell is 67 μm^2 (Ponder, 1952). No account was taken of differences in cell size between individuals which occur, for example, between lambs and adults or between sheep of different hemoglobin types (Blunt, 1975). Because of this variability we have in the present paper expressed

the results as sites (hemocyanin molecules) per "cell face" as seen on the electron micrograph. This value can then be related to sites per 1.0 μm^2 on the individual cell examined. The mean value for the one *LL* sheep examined (Table 3) was 8.8 sites per 1.0 μm^2, which multiplied by the arbitrary figure of 67 gives 590 L_P sites per cell. The latter number is close to that obtained from the *LL* cells used in the experiment shown in Table 4 ($67 \times 9.3 = 623$ sites per cell). Mean L_l sites per cell calculated for that experiment are $67 \times 12.7 = 851$, making a total of 1,474 $L_P + L_l$ sites per cell. This value is appreciably higher than that obtained in the same experiment when cells were treated first with one reagent and then with the other (anti-L_P then anti-L_l, $67 \times 16.8 = 1{,}126$ sites per cell; anti-L_l then anti-L_P, $67 \times 18 = 1{,}206$ sites per cell). One possible explanation for this is that L_P and L_l antigen sites are sufficiently close together so that when the red cell is labeled with both reagents, binding of the large hemocyanin conjugate is interfered with due to steric hindrance. However, the lower estimate of 1,100–1,200 total sites per cell is still higher than we reported previously, using an antiserum which contained both anti-L_P and L_l specificities (Tucker et al., 1976), but is close to that for L sites given by Lauf and Sun (1976) using ^{125}I-labeled antibody (unfractionated). These estimated values for the number of L sites per cell are, however, still an order of magnitude or so higher than the upper limit for numbers of sodium pumps estimated on LK cells by ^{3}H-ouabain binding (50 sites/cell, Lauf et al., 1977). This large excess of antigen molecules precludes the theory of a simple stoichiometry of L with the Na/K pump. Coupled with the observation that trypsinization fails to destroy L_P on heterozygous *ML* cells (Dunham et al., 1980), it is clear that the precise details of the associations between M, L_P, and L_l, and the Na/K pump in sheep red cells are complex and await further investigation.

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