LETTER TO THE EDITOR

Stimulation of Active K⁺ Transport by Anti-L Antibodies in Trypsin-Treated Low Potassium Sheep Erythrocytes

Dear Sir:

In this letter we attempt to resolve a discrepancy on the effect of trypsin on the sensitivity of low potassium (LK) sheep red blood cells to the anti-L antibody. We had reported the failure of trypsinization to abolish completely sensitivity to the antibody (1). More recently Lauf et al. published in this journal the observation that trypsin could indeed eliminate all sensitivity (10). We show here that red cells of LK sheep homozygous at the locus controlling the LK phenotype respond to trypsinization differently from the way cells from heterozygotes respond.

In red cells of the LK phenotype of sheep and goats, the rate of active Na/K transport is much lower than in red cells of high potassium (HK) animals (7, 11). LK red cells have a membrane-associated blood group antigen, L, which may be responsible for the low rate of transport; alloimmune anti-L serum raised in HK sheep stimulates active Na/K transport several-fold in LK red cells of sheep and goats (5, 6, 9). Lauf et al. (8) showed that, in LK sheep red cells pretreated with trypsin, the Na/K pumps were no longer stimulated by anti-L serum (trypsin had no other effect on cation transport), suggesting selective destruction of the L antigen by trypsin.

Anti-L serum also inhibits passive K transport in LK red cells (2, 5). Thus, there are two kinds of specificities in anti-L serum, one called anti-Lp which stimulates the pump, and another, anti-Ld, which inhibits passive transport (1). At the same time two antigen types on the cells can be distinguished; Lp, which is modified by trypsin, and Ld, to which anti-Ld binds, causing inhibition of passive K transport. The Ld antigen is unaffected by trypsin since anti-L serum inhibits passive K transport in trypsin-treated LK sheep cells fully as well as in untreated cells (1, 10).

It was suggested previously that treatment of LK sheep cells with trypsin did not completely abolish stimulation of the pump by anti-L serum (1). This observation was tentatively explained in terms of an action of anti-Ld on both pump and passive transport, and was consistent with the failure of extensive absorption of anti-L serum with LK goat cells to remove all stimulatory activity to the pumps of LK sheep cells (1, 7) (LK goat cells should remove anti-Lp much more effectively than anti-Ld).

Lauf et al. (10) reexamined the effect of trypsin on the response of LK sheep cells to anti-L, and reported complete abolition of stimulation of the pump in...
these cells. This finding, together with other considerations, was presented as "evidence against the concept of leak-pump interconversion," a concept proposed by us earlier (2, 4).

We have taken up the question of trypsin's action once again; we show here that in trypsin-treated cells from heterozygous LK (LM) sheep, significant stimulation of the Na/K pump by anti-L remains, while in cells from homozygous LK (LL) sheep little or no sensitivity to anti-L remains after trypsinization.

METHODS

The experiments were performed on red cells from Welsh Mountain or Clun Forest LK sheep. Their genotype (LL or LM) had been determined serologically as described before (12). Blood was drawn by jugular venipuncture into heparin; the red cells were washed four times (by centrifugation and resuspension) in "normal saline" (NaCl, 150 mM; Tris-HCl, 10 mM; glucose, 5 mM; pH 7.5).

Cells were exposed to trypsin (type III, Sigma Chemical Co., St. Louis, Mo.) in an incubation medium ("trypsin buffer") containing NaCl (125 mM), CaCl₂ (10 mM), Tris-HCl (20 mM) and glucose (5 mM), pH 8.0 (1, 8). The hematocrit of the suspension was 50%. To ensure the desired concentration of trypsin, cells were washed once in a trypsin solution prior to the incubation. The incubations were carried out at 37°C in Eppendorf 1.5-ml centrifuge tubes, and were terminated by extensive washing (eight times) of the cells in normal saline. Inhibitors of trypsin were not used after preliminary experiments with soy bean trypsin inhibitor (Sigma) resulting in no more effective termination of the effect of trypsin than centrifugation alone.

Antisera were raised as described before (1, 13) in HK sheep injected with washed, homogenized LK sheep cells suspended in Freund's complete adjuvant. Cells were exposed to immune reagents at 5% hematocrit for 30 min at 37°C, and then were washed four times in normal saline. Active (ouabain-inhibitable) unidirectional K influxes were determined as described before (3).

RESULTS

Heterozygous (LM) LK sheep red cells were exposed to trypsin at 2.5 mg/ml as described above (this is the same trypsin concentration used by Lauf et al. [10] in a similar experiment; see their Fig. 3). The time-course of the effect of trypsin on sensitivity to anti-L is shown in Fig. 1. As reported before (8), trypsin had no direct effect on active K influx ("control" points). Trypsin reduced subsequent stimulation of active K influx by anti-L from 2.5-fold in cells not exposed to trypsin to ~33% stimulation after 20 min in trypsin. At 60 min the stimulation of the pump over that in control cells by anti-L was 40%, and the curve for "stimulation" (difference between anti-L and control) does not appear to be approaching zero.

Fig. 2 shows the "dose/response" curve for the effect of various concentrations of trypsin (incubation time, 1 h) on sensitivity to anti-L in LM cells. Again, trypsin reduces, but does not abolish, the sensitivity of the Na/K pump to stimulation by anti-L.

Fig. 3 shows the effects of various trypsin concentrations (incubation, 1 h) on the sensitivity to anti-L in cells from a homozygous LK sheep (LL). Here
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FIGURE 1. Time-course of the reduction by trypsinization of the sensitivity of heterozygous (LM) LK sheep cells to anti-L antibody. All of the cells were incubated at 37°C for 65 min in the trypsin buffer with or without trypsin. The various times of exposure to trypsin were achieved by removing aliquots of cells at desired times, separating them from their suspending medium by centrifugation, and washing them once in the buffer with trypsin before the final addition of trypsin. At the end of the incubation all aliquots of cells were removed and washed eight times in normal saline. Each aliquot was divided into two; one of each pair of aliquots was then incubated with anti-L serum for 30 min while the other was incubated with normal saline. Then unidirectional K influxes were determined ([K]₀ = 2.5 mM) ± ouabain (0.05 mM). The active (ouabain-inhibitable) K influxes are shown. The upper curve (○) shows the influxes in cells incubated with anti-L after trypsinization for various times; the middle curve (▲) is for control cells after trypsin; the lower curve (△) shows the difference between the upper two curves, or the magnitude of stimulation by anti-L after the various times of trypsinization. The error bars for the upper two curves are SEM (n = 3). The bars for the stimulation show the SE of the difference.

the sensitivity was much more nearly completely abolished than in the cells from a heterozygous sheep (Figs. 1 and 2). The highest trypsin concentration was 4 mg/ml here and 8 mg/ml for the LM cells in Fig. 2.

The same results as shown in the above figures were obtained with red cells from two other homozygous sheep and three other heterozygotes.
FIGURE 2. The effect of trypsin concentration on sensitivity of heterozygous (LM) LK sheep cells to stimulation by anti-L. The experiment was carried out the same way as the one in Fig. 1, except that the incubations were at different trypsin concentrations, and were all for 1 h. The symbols all have the same meanings as in Fig. 1.

FIGURE 3. The effect of trypsin concentration on sensitivity of homozygous (LL) LK sheep cells to stimulation by anti-L. The experiment was carried out the same as the one in Fig. 2. The symbols have the same meanings as in Figs. 1 and 2.
DISCUSSION

The results show that trypsinization of red cells from a homozygous LK sheep abolishes sensitivity of the cells to anti-L, whereas the sensitivity cannot be completely abolished in cells from heterozygous sheep. This resolves the discrepancy between the findings of Dunham (1) and of Lauf et al. (10). All of the sheep in which Lauf et al. observed complete elimination of sensitivity to anti-L by trypsin treatment were homozygous. The sheep used in the work of Dunham (1) and Ellory and Tucker (7) were apparently heterozygotes.

The discrepancy between the two sets of findings is not likely to be due to the use of trypsin from two different sources (Sigma Chemical Co., Ref. 1; Worthington Biochemical Corp., Ref. 10). Sigma trypsin was as potent as Worthington trypsin by one criterion: Lauf et al. (10) achieved 80–90% of the maximal effect of the Worthington enzyme at 0.6 mg/ml (see their Fig. 2); the maximal effect was measured at 5 mg/ml. In the present work 0.5 mg/ml of Sigma enzyme gave 89% of the effect obtained at 8 mg/ml.

An obvious difference between LL and LM cells, which might account for the different results, is the presence or absence of the M antigen. M antigen may protect some of the L antigen sites against trypsin on LM cells. In one type of preliminary experiment that we carried out, there was no protection apparent; the sensitivity of LM cells to stimulation by anti-L was unaltered by pretreatment with anti-M serum. But this result does not exclude the possibility that the M antigen, bound or not to anti-M antibody, affects a subpopulation of L antigens, modifying their sensitivity to trypsin. M antigen might also affect either the interaction of L antigens with adjacent pumps, or their binding to anti-L antibodies. Moreover, any modification of the M antigen by anti-M might have been reversed by trypsin.

The residual sensitivity to anti-L in trypsinized cells was offered as being consistent with the hypothesis of the interconvertibility of membrane loci mediating active and passive K transport (1). But the residual activity can be demonstrated only on LM cells, so it is not strong evidence for interconvertibility. Since anti-L stimulates the pump in trypsinized LM cells, obviously trypsin does not abolish binding of anti-L antibodies to LM cells as it does anti-L\textsubscript{p} to LL cells (10). (Trypsinization abolishes binding of anti-L\textsubscript{p} to LL cells, but not anti-L\textsubscript{t} as mentioned above [1, 10].) It has not yet been shown if anti-L\textsubscript{p} can bind to trypsinized LM cells. If it can, then the residual sensitivity to anti-L is probably due to a difference in L\textsubscript{p} antigens between LL and LM cells, perhaps a function of the M antigen as suggested above. If anti-L\textsubscript{p} cannot bind to trypsinized LM cells, then the residual sensitivity may be stimulation of the pumps on LM cells by anti-L\textsubscript{t} antibody, as suggested earlier (1).

Any variation in the responses of L\textsubscript{p} and L\textsubscript{t} antigens to trypsinization must be considered in relation to the different responses of L\textsubscript{p} antigens on red cells of sheep and goats. Trypsin abolishes antibody binding to L\textsubscript{p} of sheep with no apparent effect on the inhibitory action by the antigen on the pump. In contrast, we recently found that trypsin treatment of LK goat cells actually results in stimulation of the pump through an action on the L antigen (3).
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Dr. Dunham’s permanent address is Department of Biology, Syracuse University, Syracuse, New York 13210; Dr. Tucker’s permanent address is Agriculture Research Council, Institute of Animal Physiology, Babraham, Cambridge, England.

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