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THE SOLUBILIZATION OF THE L AND M ANTIGENS FROM SHEEP RED CELL MEMBRANES

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The L_p, L_l and M antigens from sheep red cells were solubilized using the non-ionic detergent Triton X-100 in the presence of dithiothreitol. Recovery rates were improved when membranes were sonicated at 4°C in the presence of the detergent; values in the range 16–25% (M) and 9–17% (L_p and L_l) were achieved for recovery.

Sheep red blood cells show a dimorphism with respect to their potassium concentrations, the M and L blood group antigens being associated with these differences [1,2]. The M antigen is found on homozygous high potassium (HK) red cells (MM type), the L on homozygous low potassium (LK) cells (LL type), and both M and L are found on heterozygous cells (ML type). Sensitization of HK cells with the antibody anti-M causes lysis in the presence of complement but has no other known effect on HK cells. The antibody anti-L, in addition to causing complement lysis of LK cells, stimulates the ouabain-sensitive Na^+/K^+ pump in LK but not HK erythrocytes [3]. More recently, the lytic and the pump-stimulating effects of the L antibody have been separated [4,5] and shown to be associated with two distinct antigens in the cell membrane (L_l and L_p, respectively). It is postulated that L_p acts by inhibiting active K^+ transport via the sodium pump by modifying the transport system kinetically [6].

Much work has been directed towards understanding the mechanism of the inhibitory effect of the L_p antigen on the K^+ transport system but surprisingly little is known about the physical nature of the antigen [6,7]. Little more is known about the M antigen. Some success has been achieved in solubilizing M in deoxycholate and Triton X-100 [8,9], and there is evidence that it requires the presence of phospholipid and cholesterol for full expression of its antigen activity [10]. Antigen L has to date been totally refractory to any solubilization procedures. We now report the recovery of L_l, L_p and M activities from membranes after solubilization in Triton X-100. In common with other workers [11] we have considered all material remaining in the supernatant after centrifugation at 150000 × *g* for 60 min to be in solution.

Membranes were solubilized by resuspending white ghosts (prepared essentially as described by Dodge et al. [12]) to their packed cell equivalent in 1% (w/v) Triton X-100 in 50 mM Tris-HCl buffer pH 7.2 containing 0.3 mM dithiothreitol. The suspension was either stirred by magnetic stirrer at 4°C for one hour or sonicated at the same temperature for 5 min at 40 kHz using a Pulsatron 55 (Kerry Ultrasonics Ltd., U.K.). Triton X-100 was removed from membrane extracts by absorption with Bio-Beads SM-2 (20–50 mesh)

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(Bio-Rad Laboratories, Richmond, CA). The Bio-Beads were precycled and used as described by Holloway [13]. Agitation of 1 ml of a 1% detergent solution with 0.4 g of damp Bio-Beads at 4°C overnight was effective in removing detergent down to a non-lytic concentration, and in contrast with previous reports adsorbed no more than 18% of the protein in the sample [9,14].

Extraction of whole membranes with 1% Triton X-100 resulted in solubilization of both L_1 and M antigen activities estimated by an immune haemolysis inhibition test [15], using alloimmune anti-L and anti-M reagents [5]. However, the recoveries of activity in the membrane extracts were low (varying from 5% up to, at best, only 10% of the starting inhibition activities). Control experiments confirmed that no inhibitory M antigen activity was present in soluble extracts from M-negative membranes and no L activity was present in extracts from L-negative membranes. Raising the Triton X-100 concentration to 2 or 3%, doubling the membrane concentration, or increasing the extraction temperature from 4°C to 37°C did not increase the recovery of either antigen.

Membranes sonicated for 5 min in the presence of 1% Triton X-100 showed a significant amount of L_1 and M antigen in solution as judged by inhibition of haemolysis (Fig. 1a and b). The L_1 antigen inhibition concentration in the undiluted soluble extract was close to that given by a 1/8 dilution of whole LL type membranes i.e. approximately 12% recovery. The M antigen concentration lay between 1/8 and 1/4 of whole membrane strength i.e. 16–25% recovery.

The Triton extracts of sonicated L and M membranes were separated by isoelectric focusing on thin agarose gels over the pH range 3.5 to 10. No differences in band patterns between the membranes were apparent but the large number of bands present gave an indication of the high degree of solubilization achieved.

L_p activity was measured by the ability of solubilized membrane preparations to inhibit the stimulatory effect of anti-L_p on active K^+ influx in LL type red cells [16] (Fig. 2). In two experiments the apparent L_p antigen concentration in the undiluted solubilized extract was equivalent to 9% and 17% of that for untreated membranes, respectively. In contrast, soluble extracts prepared from L-ve

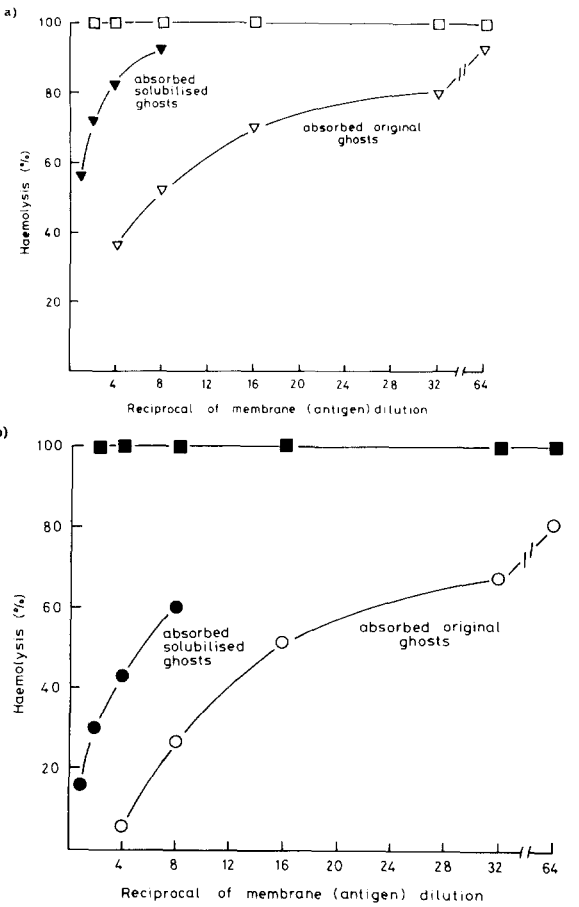

Fig. 1. The effect of solubilized, sonicated LL and MM membranes on haemolysis by anti-L and anti-M reagents. (a): Tested against dilutions of anti-L. ▽———▽, LL whole membranes (normal); ▼———▼, LL solubilized membranes; □———□, MM solubilized membranes. (b) Tested against dilutions of anti-M. ○———○, MM whole membranes (normal); ●———●, MM solubilized membranes; ■———■ = LL solubilized membranes. One volume of an appropriate antibody dilution was incubated with 1 volume of isotonic packed cell equivalent membrane preparation for 30 min at room temperature in isotonic buffered saline pH 7.2. One volume of 2% (v/v) sheep red cell suspension (LL or MM cells) was then added and incubated for a further 30 min. The cells were washed once in 4 volumes of saline, resuspended in 1 volume of saline, 1 volume of undiluted rabbit complement added, and incubated for 4 h at 32°C. Haemolysis was judged by eye, using a score of 0 (no lysis) to 5 (100% lysis) for each antibody dilution. Membrane antigen concentrations at different dilutions were measured by testing against serial dilutions of antibody (anti-L or M) and summing the haemolysis readings for each dilution to obtain a 'haemolysis score'. The degree of inhibition of haemolysis by L and M ghost preparations was expressed as a percentage of the haemolysis score (100% haemolysis) obtained for control M and L-negative membranes, respectively.

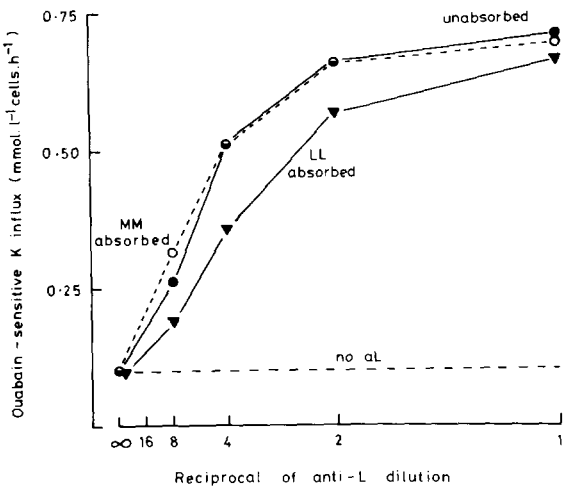

Fig. 2. The effect of solubilized, sonicated LL and MM membranes on anti-L_p stimulated K^+ influx in sheep red cells. ▼——▼, anti-L absorbed LL solubilized membranes; ●——●, anti-L absorbed MM solubilized membranes; ○——○, unabsorbed anti-L. Serial dilutions of anti-L reagent were prepared and 1 volume of each dilution mixed with 1 volume of packed cell equivalent membrane preparation for 1 h at room temperature. The mixtures were then put at −20°C until used for flux measurements. LL type red cells were washed three times by centrifugation (5 min 4000 × *g*) in a medium containing 150 mM NaCl, 10 mM glucose, 15 mM Tris pH 7.5 at 20°C. Aliquots of cells (0.02 ml) were then incubated at 32°C for 30 min with 1 ml aliquots of the preabsorbed anti-L sera. The tubes were centrifuged (10000 × *g*, 15 s) and the supernatant removed by aspiration. Ouabain-sensitive K^+ influx was measured at 37°C over 30 min using tracer ^{86}Rb, in a medium containing 145 mM NaCl, 5 mM KCl, 5 mM glucose, 15 mM Tris pH 7.5 at 37°C ± 0.1 mM ouabain with a rapid washing technique (5 spins, 4 washes, 10000 × *g*, 15 s, see Dunham and Ellory [16]).

membranes were not inhibitory. We therefore conclude that sonication of LL type membranes in the presence of Triton-X-100 solubilizes L_p as well as L_1 activity, to about the same extent.

In the present experiments, estimates of the proportions of total antigen activities recoverable after solubilization were made by measuring their ability to inhibit or absorb antibody. Reynolds [17] discusses the difficulty of comparing solubilized with native antigen systems because of possible changes in the stoichiometry of interaction between antigen and antibody. The absolute amount of antigen in solution is therefore difficult to quantify accurately. However, for most practical purposes it is necessary only to make comparative estimates of antigen concentrations. In our study we have obtained recoveries of approximately 12% for L_1, 13% for L_p and 20% for M antigen as defined by the inhibitory activity remaining in a 150000 × *g* supernatant.

Isolation of human red cell membrane antigens associated with the Rh blood groups, like the sheep L and M antigens, has proved very difficult, largely because of the small quantities of antigen in the membrane and their integral membrane nature requiring lipid for optimum expression. Recently, however, a successful technique has been described for the isolation of the Rh antigens employing ^{125}I-labelled membranes dissolved in 1% Triton X-100 [18]. Since L and M antigens are soluble in this detergent, we anticipate that this same technique could be used for the isolation and characterization of these antigens.

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