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Binding Characteristics of M and L Isoantibodies to High and Low Potassium Sheep Red Cells

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Summary. Binding of highly purified ^{125}I labeled M and L antibodies, both belonging to the immunoglobulin G class, was studied in high potassium (HK) and low potassium (LK) sheep red cells. Anti-M and anti-L bound specifically to M and L antigen positive HK and LK red cells, respectively. Nonspecific binding was higher for anti-L to HK cells than for anti-M to LK cells. Once bound, the M and L antibodies were capable of inducing complement dependent immune hemolysis. Only 75–100 and 500–750 molecules of anti-M and anti-L immunoglobulins were required to hemolyze 50% of HK (MM) and LK (LL) red cells, respectively, suggesting that the M and L antigens may be clustered on the surfaces of these cells. Equilibrium binding studies revealed that the maximum number of M sites is $3-6\times10^3$ in HK (MM) and $1.5-4\times10^3$ in LK (LM) cells, respectively. In comparison, the number of L antigens is slightly lower in LK cells, about $1.2-1.8\times10^3$ in LL and less in LM (LK) red cells. The number of M and L antigens, therefore, is more than an order of magnitude larger than that of the Na^+K^+ pumps measured previously in these cells by ^3H-ouabain binding, thus precluding a quantitative correlation between M and L antigens and the Na^+K^+ pumps different in the three genetic types of sheep red cells. The binding affinities of both anti-M and anti-L could not be described by a single equilibrium dissociation constant indicating heterogeneous antibody populations and/or variability in the antigenic sets of individual HK or LK cells. The pronounced heterogeneity of antigens and/or antibodies in both the M and L systems was reflected in the antibody association kinetics which also exhibited a remarkable temperature dependence. The data suggest that the correlation between the M and L antigens and the Na^+K^+ pump molecules is more complex than that in goat red cells previously reported by others.

The M and L antigens are genetically associated with the cation polymorphism in sheep red cells [32, 31, 6, 42, 17]. High potassium red cells contain only the M-antigen (henceforth denoted as HK or MM cells) while red cells with a low potassium steady concentration possess the L antigen (henceforth denoted as LK or LL cells). Heterozygous red cells are both M and L antigen positive but due to the dominance of the LK gene [9] have a low intracellular potassium (K^+) and high sodium (Na^+) concentration (these cells will be called LM(LK) cells). HK red cells

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maintain their high potassium steady state concentration by a higher active Na^+K^+ pump and ATPase activity and by a lower passive K^+ permeability than LK red cells [39–41, 1]. The higher activity of the Na^+K^+ pump in HK red cells has been attributed to a greater number of pump sites [14] and to different kinetic properties of these pumps [40, 12].

The antigenic type of a given sheep red cell can be determined by complement dependent immune hemolysis of these cells incubated with the ovine iso-antibodies anti-M or anti-L, respectively [27, 19]. In the absence of complement, the L antibody exhibits an additional property by stimulating the active Na^+K^+ transport [6, 24, 28] and membrane ATPase [22, 2] in LL and LM (LK) red cells and their hemoglobin free membranes, respectively. This effect was found to be due to a kinetic change of the existing Na^+K^+ pumps in these cells, since a decrease in the inhibition of the pump by intracellular K^+ was observed [24, 10, 28] combined with an increased binding affinity for ouabain which inhibits the pump [14]. Similar observations have been made in red cells of goat [31] and cattle (J.C. Ellory and B.A. Rasmusen, *personal communication*). Since anti-M did not alter any of these parameters in MM or LM cells [23], the M antigenic substance appears not to be involved in the control of the K^+ steady state of sheep red cells [42].

In the light of the genetic structure activity correlation between the L antigens and the Na^+K^+ transport system it is important to know as to how closely the number of M and L antigenic sites agrees with the number of Na^+K^+ pumps in HK (about 120/cell) and LK red cells (about 50–70/cell) [14]. A stoichiometric relationship between antigens and pumps in these cells would indicate that these antigens indeed are structurally part of the molecules comprising the pump system. In fact, Kropp and Sachs have recently shown that the number of L-antigens compares well with that of the Na^+K^+ pumps in LK goat red cells [15]. Furthermore, it is of interest to study whether the number of M and L antigens in LM red cells would provide information regarding the subunit composition of the Na^+K^+ pumps in the heterozygous cells.

Here we report that the number of ^{125}I labeled M and L antibody molecules bound under equilibrium conditions is different in homozygous HK(MM) or LK (LL) red cells as compared to heterozygous LK(LM) cells. Assuming one binding site per antibody molecule, the number of M and L antigens determined exceeds that of the Na^+K^+ pumps by more than an order of magnitude. There is a considerable heterogeneity of M and L antibody binding to these cells, and variation in the maximum

number of antigenic sites occurs between different sheep of the same blood group type. Preliminary accounts of this work have been given elsewhere [25, 26].

Material and Methods

Red Blood Cells

Blood was obtained from healthy sheep by jugular venipuncture into heparinized vials (10 USP sodium-heparin per milliliter blood). The cells were usually washed in physiological saline buffered with 10 mM Tris/Cl pH 7.4 and their antigenic and cation types were determined by immune hemolysis [27, 19] and atomic absorption spectrophotometry [24], respectively. For immune hemolysis, M positive HK or LK red cells were incubated at 37 °C for 1 hr with 1/32 diluted anti-M serum (*see* below) and with 1/10 diluted guinea pig serum previously absorbed with sheep red cells as complement source [18]. For detection of the L antigen, L positive LL or LM (LK) red cells were incubated with 1/8 diluted anti-L plus 1/10 diluted GPS for 3 hr at 37 °C. Since it has been shown previously that ATP depletion of LK cells generally enhances their immune hemolytic response [20] and thus permits a clearer distinction between LL and LM type cells, hemolysis tests were also done on ATP depleted LK cells.

Purification and ^{125}I-Labeling of Antibodies

Ovine iso-immune anti-M serum (S 31 B), and anti-L serum (S 39), previously freed of M/L-nonspecific antibodies by absorption with a panel of appropriate LK or HK sheep red cells, were obtained from Dr. B. A. Rasmusen, Dept. Animal Genetics, University of Illinois, Urbana. Previous tests had shown that in contrast to another anti-M serum (S 11) used in earlier work [27] the M antibody activity of this serum resided only in the immunoglobulin G (*IgG*) fraction and not in the macroglobulin protein fraction (*IgM*). The L antibody was previously identified as an IgG_1 protein belonging to the electrophoretically fast immunoglobulins of molecular weight 150–160,000 [36]. The bulk of the *IgG* proteins was precipitated in the cold with 1/3 volume of saturated ammonium sulfate, left overnight to complete precipitation, centrifuged and dissolved in deionized water. After a second precipitation at 33% ammonium sulfate, the final precipitate was collected by centrifugation and dissolved in and dialyzed against 0.02 M Tris/Phosphate buffer pH 8.0 until free of SO_4^{2-} ions. The protein retentate was then concentrated, again dialyzed and applied to a 2.5 × 45 cm DEAE-Sephadex A 50 column equilibrated with the dialysis buffer. As described elsewhere [36], the column was eluted with a linear TRIS/PO_4^{3-} buffer gradient (0.02–0.5 M, pH 8) until the well defined electrophoretically slow IgG_2 (about 20 relative %) and fast moving IgG_1 (about 80 relative %) appeared, of which the latter contained the M and L antibody activities.

The M or L antibody containing IgG_1 fraction was concentrated and dialyzed against 0.15 M NaCl buffered with 0.05 M PO_4^{2-}, pH 7.3 and iodinated with ^{125}I/KI using the lactoperoxidase method of Marchalonis [29] and Thorell and Johansson [38]. A usual preparation consisted of 10 separate test tubes each containing 300 µg IgG_1 protein in 50 µl phosphate buffered saline (*see* above), 5 µg milk lactoperoxidase (Sigma Chemical Company, St. Louis, Missouri, Lot #L 8503) in 10 µl buffer, 25 µl 0.1 mM KI plus 125 µC ^{125}I (New England Nuclear, Boston, Mass., carrier free) and 10 µl H_2O_2 (to give a final concentration of 5×10^{-4} M). The tubes were left at room temperature for 1 hr, the contents pooled and applied to a 0.9 × 54 cm Sephadex G 25 (fine) column equilibrated with 5×10^{-4} M NaN_3. The labeled protein was eluted with a buffer consisting of 10 mM Tris/Cl pH 7.4 plus 130 mM NaCl and

Table 1. Specific activities of purified antibody preparations

Antibody	Preparation	Specific activity		Number of experiment [a]
		$^{125}I/IgG$ (M/M)	10^{16} cpm/mole IgG	
Anti-M (S 31 B)	1	0.022	4.70	430
	2	0.013	2.55	433
	3	0.015	2.70	435, 436
Anti-L (S 39)	1	0.023	4.39	438, 439
	2	0.030	4.93	441
	3	0.013	2.28	448, 449

[a] see text.

10 mM KCl. The eluate was collected in small aliquots using a LKB fraction collector monitoring at 280 nm for protein, while radioactivity of each fraction was checked with a model CG 30 automatic gamma counter (Intertechnique). Free iodine appeared clearly separated at the end of the elution in the included volume. The labeled protein of the excluded (void) volume was vacuum concentrated in collodium bags (cutoff point: mol.wt. 25,000) and dialyzed against the elution buffer. The molar ratio of total iodine to IgG_1 was about 0.5. The molar $^{125}I/IgG$ ratio was computed from the molar concentration of IgG (assuming a mol.wt. of 150,000) and ^{125}I. The molar concentration of ^{125}I was obtained using Eq. (1): $N = dpm/\lambda$, where N is the number of ^{125}Iodine atoms and λ the decay constant of the isotope. The counting efficiency was 45–47%. The specific activity of each preparation then is given either as the computed $^{125}I/IgG$ ratio or directly as the cpm/mole protein (Table 1) and is used for the calculation of total, free and bound antibody (*see* below).

For final purification, the ^{125}I labeled IgG_1 protein was adsorbed for $2\frac{1}{2}$ hr at 37 °C to HK or LK cells (1 mg ^{125}I $IgG_1/10^{10}$ cells in 1 ml). The cells were then washed 7 times in Na^+K^+-Tris/Cl buffer (*see* above) at 4 °C and the final cell sediment hemolyzed with 10 mM Tris/Cl buffer pH 7.4. The ghosts were washed until hemoglobin free, diluted to about 0.5–1.0 mg/ml and acidified at 0 °C to pH 3 by adding 0.5 N HCl under stirring. The acidified suspension was centrifuged in a Sorvall RC 2B centrifuge at 15,000 rpm and 4 °C for 15 min, the supernatant removed and neutralized by adding 0.5 N NaOH and, after concentration, dialyzed against Na^+K^+-Tris/Cl buffer. The retentate containing the purified anti-M or anti-L was again centrifuged at 15,000 rpm at 4 °C for 15 min to remove any aggregated protein prior to the binding experiments. The degree of purification of anti-M or anti-L was tested by repeated absorption with free MM(HK) or LK(LL) red cells. It was found that up to 70% of the cpm of a given ^{125}I labeled anti-M eluate could be absorbed specifically by HK cells and up to 30% of ^{125}I anti-L eluate by LK red cells. Based on this information and on the calculation of the fraction of IgG_1 protein eluted from HK cells at least 0.13–0.15% and 0.04% of the crude IgG_1 protein constitutes M antibody and L antibody protein, respectively. Thus, in 1.0 ml of undiluted anti-M serum (S 31 B) containing about 59 mg total serum protein, there were 12.2% or 7.2 mg immunoglobulin and about 0.7 µg M antibody IgG, and in 1.0 ml undiluted L antiserum (S 39) (39 mg serum protein) 16.7% or 6 mg immunoglobulin and about 0.24 µg L antibody IgG_1. The effects of the iodination and elution procedures were tested on the hemolytic activity of anti-M and anti-L in presence of GPS. For example, in the standard hemolytic test (*see* above under Red Cells) 50% hemolysis of M positive HK red cells was achieved by 12.5 µg unlabeled IgG_1 protein, 20 µg ^{125}I labeled IgG_1 protein and 5 µg eluted ^{125}I labeled anti-M, respectively.

Binding Conditions

In all experiments shown, binding of ^{125}I antibodies was measured to spectrophotometrically quantitated M positive HK, and LK, or M negative LK red cells in presence of heat inactivated (56 °C for 0.5 hr) nonimmune serum dialyzed against Na^+K^+-Tris/Cl buffer containing 1.0 mM glucose. The presence of nonimmune serum considerably decreased nonspecific binding of ^{125}I IgG_1 to cells and adhesion to the glassware.

For measuring the *association reaction* of anti-M or anti-L to HK and LK red cells, 100 µl aliquots of M or L antibody (6.9×10^{-10} or 10^{-10} M/liter) were added at $t=0$ to 10 ml aliquots of cell suspensions containing 10^8 cells/ml. Incubation was carried out at 0°, 20° and 37 °C, respectively, for up to 3 hr and one ml aliquots were taken at given time intervals. The cells were spun at 10,000 rpm and 0 °C and washed once in 9 ml ice cold buffer. The cell pellets were transferred into fresh tubes and counted. For determination of the *dissociation reactions* of anti-M, aliquots of 2×10^9 HK or LK cells per 1.8 ml nonimmune serum were mixed with 0.2 ml anti-M or anti-L (5×10^{-9} M/liter) and incubated for 2.5 hr at 37 °C. The sensitized cells were spun at 0 °C and washed 5 times each in 4 ml nonimmune serum. The washed cells were finally suspended in 600 ml Na^+K^+-Tris/Cl buffer prewarmed to 37 °C, and 30 ml samples were taken in duplicates at given time intervals, spun at 0 °C, resuspended in buffer and transferred to counting vials.

In the *equilibrium binding* experiments, 10 µl cell suspensions (in nonimmune serum) containing 10^7 cells were incubated for 2.5 hr at 37 °C with 50 µl anti-M diluted with nonimmune serum to the concentration indicated. Then the cells were diluted with 7.5 ml ice cold Na^+K^+-Tris/Cl buffer, transferred to plastic counting vials, centrifuged and washed 2 more times with each 7.5 ml of the buffer. The final cell pellet was counted and subsequently hemolyzed in 3.75 ml H_2O to determine directly at 414 nm the number of cells. From the optical density of the cell suspension at 414 nm ($OD^{414\,nm}$) the number of cells per milliliter was directly computed on the basis of the $OD^{414\,nm}$ for 1 ml fresh packed red cells. For computation of the number of IgG_1 protein molecules bound per cell, Eq. (2) was used:

$$\text{Molecules bound/Cell} = \frac{\text{cpm} \times N_A}{SA \times n}; \text{ where} \tag{2}$$

N_A = Avogadro's number
n = Number of cells
SA = Specific activity in cpm/mole.

For the Scatchard analysis [35] all values were corrected for "nonspecific" binding by subtracting the cpm bound nonspecifically to HK or LK control cells where studying specific binding of M or L antibody.

Results

Specificity of Radiolabeled Antibody Binding

The binding specificity of anti-M and anti-L to HK and LK sheep red cells was tested by measuring the uptake of the radiolabeled anti-M *IgG* to M positive or negative (L positive) sheep red cells in presence or absence of unlabeled (cold) M antibody. Fig. 1*A* compares the binding of ^{125}I labeled anti-M to three different LL(LK) red cells as function of

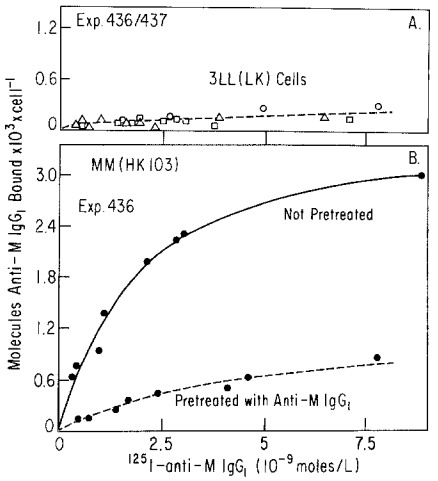

Fig. 1. Specificity of anti-M binding to M positive HK sheep red cells. (*A*) Nonspecific binding of ^{125}I anti-M to 3 LL (LK) red cells (○□△). (*B*) Specific binding of ^{125}I anti-M to MM (HK 103) cells (solid line) and to the same cells pretreated with 10^{-8} M unlabeled anti-M *IgG* (interrupted line)

antibody concentration. It can be seen that nonspecific absorption ranges from 20 at the lowest anti-M concentration (10^{-9} M) to 300 molecules anti-M per LL cell at the highest concentration (8×10^{-9} M) used. In contrast, binding of ^{125}I labeled anti-M to MM(HK) red cells steadily increased to more than 2,000 molecules per cell as the antibody concentration was raised (to 8×10^{-9} M) and was greatly reduced when the M antigenic sites on these cells were occupied by unlabeled (cold) M antibody molecules (M antibody concentrations at least 10^{-8} M, Fig. 1*B*).

The background binding of ^{125}I labeled anti-L to three MM(HK) red cells is shown in Fig. 2*A* and was found to be of similar magnitude as the uptake of ^{125}I labeled anti-L by LL(LK) red cells previously pretreated with saturating concentrations of unlabeled (cold) anti-L immunoglobulin (6×10^{-9} M). As with the M-antibody, binding of ^{125}I labeled anti-L may be expected to be even lower provided we would have had available a cold anti-L *IgG* preparation of a concentration higher than 10^{-8} M. In contrast, untreated LL red cells showed a specific uptake of labeled L antibody which reached a minimum of 1,000 molecules per cell at the highest anti-L concentrations employed (8×10^{-9} M).

In comparing the experiments shown in Figs. 1 and 2 it is apparent: (1) that there is specific binding of anti-M and anti-L to MM and LL cells, respectively; (2) that HK cells have more M antigenic sites than LK cells L antigens; and (3) that the nonspecific binding of the radiolabeled

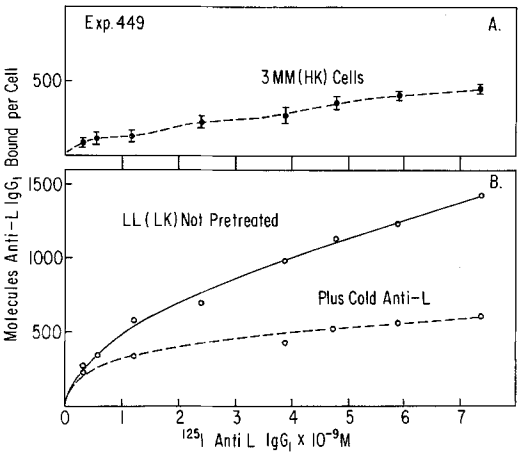

Fig. 2. Specificity of anti-L binding to L positive LK sheep red cells. (*A*) Nonspecific uptake of ^{125}I anti-L by 3 HK (MM) red cells. Bars indicate range of variation between the selected cells; (*B*) Specific binding of ^{125}I anti-L to LL (LK 126) red cells (solid line) and to the same cells pretreated with 6×10^{-9} M unlabeled (cold) anti-L *IgG* (interrupted line). Note that specific anti-L binding has not reached saturation levels seen in Fig. 9

anti-L preparation to HK cells is slightly higher than that of ^{125}I labeled anti-M to LK red cells.

Correlation Between Antibody Binding and Immune Hemolysis

In presence of serum complement anti-M and anti-L sera specifically hemolyze M and L positive sheep red cells, respectively, provided all the M and L nonspecific antibodies are removed by prior absorption [27, 19]. Hence measurement of the complement dependent immune hemolysis of HK and LK red cells previously sensitized with increasing amounts of M and L antibody, respectively, not only provides further proof for the specificity of the antigen-antibody interaction, but constitutes also an important test for the physiologic integrity of the purified antibody preparation after enzymatic labeling with ^{125}I and acid-elution from the cells.

Fig. 3*A* shows a sigmoidal relationship between percent immune hemolysis and the number of M antibody molecules bound per M positive HK(MM) or LK(LM) red cell. Evidently only 100 and 75 anti-M molecules per cell are required to cause 50% hemolysis of MM and LM cells, respectively. No hemolysis was observed in M negative (LL)LK red cells, nor occurred when LL cells were exposed to ^{125}I-labeled anti-M and

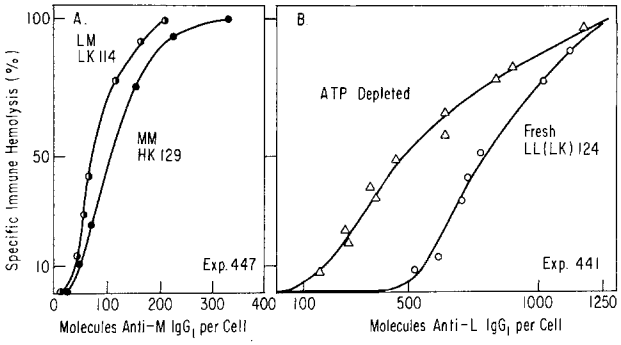

Fig. 3. Correlation between complement mediated immune hemolysis and specific binding of (*A*) anti-M to MM and LM cells and of (*B*) anti-L to fresh and ATP depleted LL (LK 124) cells

complement. Fig. 3*B* shows also that the immune hemolysis mediated by anti-L is a sigmoidal function of the number of L antibody molecules specifically bound per *fresh* LK(LL) red cell. However, in contrast to the M/anti-M system almost ten times more L antibody molecules per cell are necessary to produce 50% immune hemolysis. Thus there is a clear difference in the number of M and L antibody molecules required to hemolyze their respective target cells.

In a previous report [20] we have shown that the magnitude of the immune hemolysis of LK(LL) red cells by anti-L is determined by the metabolic state of these cells: LK cells depleted of ATP (by starvation in absence of glucose or presence of 2-deoxy-D-glucose or by treatment with iodacetamide) exhibited a greater response to immune hemolysis by anti-L and complement than fresh control cells with normal ATP levels [20]. This effect could not be explained in terms of an increase of L antigenic sites since, compared to fresh cells, ATP depleted LK red cells absorbed identical quantities of L antibody [20]. It was therefore of interest to study the degree of immune hemolysis as a function of the number of L antibody molecules bound per ATP depleted LK red cell. Fig. 3*B* demonstrates that the curve relating immune hemolysis to anti-L binding is shifted to the left, and that under these conditions only some 500 molecules of anti-L are required to produce 50% immune hemolysis. These data confirm our previous observation that ATP depleted LK red cells exhibit a higher susceptibility to immune hemolysis by anti-L and complement. As shown further below, ATP-depleted LL cells did not bind more L antibody molecules than fresh, ATP-containing control cells.

Association and Dissociation Behavior of Anti-M and Anti-L

Previous work from this laboratory suggested that M and L antibodies differ to some degree in their binding affinities to the M and L antigens on HK and LK red cells, but that the temperature requirements for binding are similar [27, 16]. Figs. 4 and 5 compare the association reactions of radiolabeled anti-M and anti-L with HK and LK sheep red cells at three different temperatures.

In the experiment shown in Fig. 4*A*, *B* the concentration of anti-M initially present (1.5×10^{-10} M) was lower than that of the M-antigenic sites in HK(MM) cells (5×10^{-10} M) and LK(LM) cells (3×10^{-10} M) as estimated from equilibrium binding studies shown below. The initial binding rates of anti-M to M positive HK and LK red cells was rapid and fastest at 37 °C while significantly and markedly reduced at 25° and 0 °C, respectively. As time progressed and most of the free M antibody was bound by the two types of red cells, the binding curves leveled off to less than one-third of the maximum binding at 37 °C and to even lower levels at the two lower temperatures. Note that at all temperatures the maximum binding obtained in this experiment is higher for HK (Fig. 4*A*) than for LK cells (Fig. 4*B*) and that nonspecific binding to LL(LK) red cells is negligible, remaining constant over the whole time period studied.

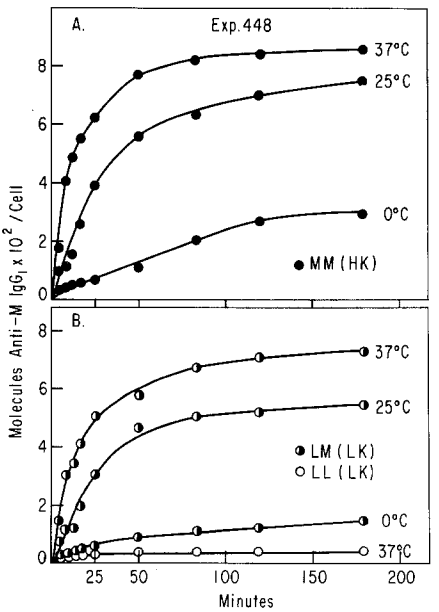

Fig. 4. Association kinetics of ^{125}I anti-M with (*A*) MM and (*B*) LM sheep red cells at different temperatures. Nonspecific binding of anti-M to LL (LK) red cells at 37 °C is shown in *B* (○)

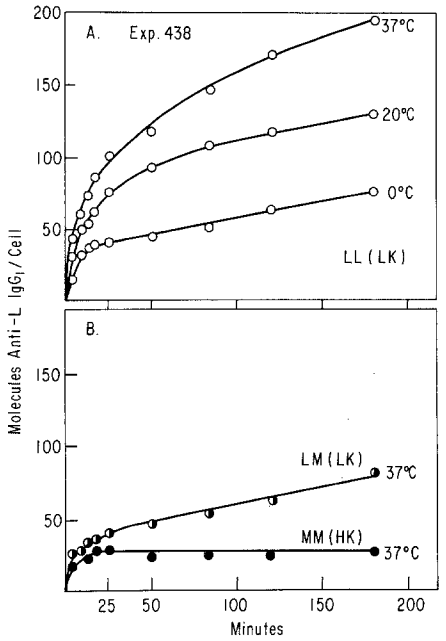

Fig. 5. Association kinetics of ^{125}I anti-L with (*A*) LL and (*B*) LM low potassium sheep red cells at different temperatures. Nonspecific binding of anti-L to MM (HK) red cells at 37 °C is shown in *B* (●)

Similar association characteristics can be shown for the binding of the L antibody to LL and LM red cells. As shown in the experiment of Fig. 5*A*, *B*, the initial rate of L antibody binding to homo- and heterozygous LK red cells appears to be slower than that of the M antibody to M positive cells. Maximum adsorption levels of anti-L (initial concentration: 10^{-10} M) are not achieved even after 3 hr of incubation at 37 °C, and again a striking temperature dependence can be observed for LL red cells. The binding rate of anti-L to LM red cells at 37 °C was comparable with that to LL red cells at 0 °C and therefore only 2 to 3-fold greater than the nonspecific uptake by L negative HK red cells (Fig. 5*B*).

The association reactions of the antibodies with their respective antigens could not be adequately described in terms of a simple first or second order process. On the basis of total antigen concentrations estimated from the equilibrium binding studies discussed below, the binding data for the two antibodies at progressing times neither yielded exponential curves nor provided useful information applying second order rate equations.

Analysis of the dissociation of anti-M and anti-L from HK and LK red cells suggests a similar complexity. The dissociation data at 37 °C for

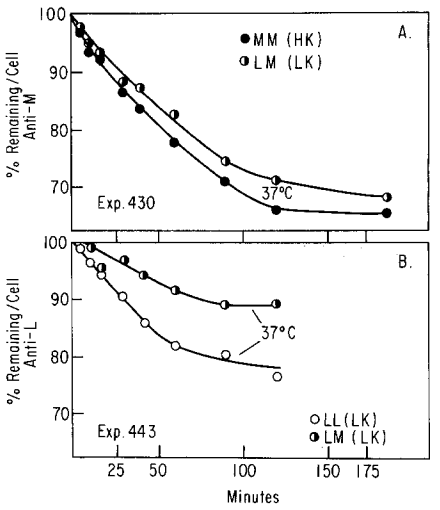

Fig. 6. Dissociation kinetics of (*A*) ^{125}I anti-M from MM and LM red cells and (*B*) of ^{125}I anti-L from LL and LM red cells

anti-M using M positive HK and LK red cells (Fig. 6*A*) and for anti-L with LK red cells (Fig. 6*B*) cannot be easily fitted into an exponential first order curve. However, the data obtained suggest a rather slow dissociation of the two antibodies which appeared to be less temperature dependent than the forward reaction. The M antibody dissociates from HK(MM) and LK(LM) cells at similar rates (Fig. 6*A*), while the dissociation of the L antibody appears to be somewhat slower from heterozygous LK(LM) red cells (Fig. 6*B*).

Binding of M and L Antibody at High Concentrations

Saturation binding of anti-M and anti-L to the three genetic types of sheep red cells was studied in equilibrium binding experiments in which the antibody concentration exceeded the concentration of the M and L antigens by up to twentyfold. Under these conditions the experiments with anti-M always gave better results than those with anti-L because the nonspecific binding of anti-M to LK(LL) red cells was always lower as compared to that of anti-L to HK red cells, and the exponential uptake of radiolabeled antibody occurred with anti-M at higher *IgG* concentrations than with anti-L.

Fig. 7 shows a typical binding experiment with anti-M. Saturation binding levels are seen around 10^{-8} M/liter anti-M; at higher M antibody

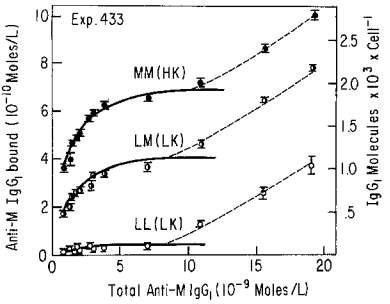

Fig. 7. Equilibrium binding of ^{125}I anti-M to MM (HK 103) and LM (LK 112) red cells. Also shown is nonspecific binding of anti-M to LL (LK) red cells

concentrations we consistently observed a rapid but proportional uptake of radiolabeled antibody which may be interpreted as a Langmuir type adsorption of proteins onto the cell surfaces of all three genotypes studied. Clearly, LM(LK) cells bind less M antibody at saturation. At equilibrium the binding of anti-M, considered here as a monovalent ligand, may be described by Eq. 3:

$$[\mathrm{Ag}\cdot\mathrm{Ab}] = K([\mathrm{Ag}]_i - [\mathrm{Ag}\cdot\mathrm{Ab}]) \times ([\mathrm{Ab}]_i - [\mathrm{Ag}\cdot\mathrm{Ab}]) \qquad (3)$$

where $[\mathrm{Ag}\cdot\mathrm{Ab}]$ is the concentration of bound anti-M or M antigen (Bound), $[\mathrm{Ag}]_i$ and $[\mathrm{Ab}]_i$ are the initial M antigen and M antibody concentrations and K the equilibrium constant. As $[\mathrm{Ab}]_i$ is in excess and thus the free antibody concentration (Free) approximates $[\mathrm{Ab}]_i$, a Scatchard plot [35] of bound/free anti-M versus bound anti-M (Eq. 4)

$$\mathrm{Bound/Free} = K\cdot[\mathrm{Ag}]_i - K\cdot[\mathrm{Bound}] \qquad (4)$$

should provide a linear relationship from which at infinitely high free M antibody concentration the concentration of anti-M bound can be found by extrapolation to the x-axis. Using this plot the data of experiment 433 (Fig. 7) corrected for nonspecific binding are shown in Fig. 8*A*. Extrapolation of the linear regression line to the x-axis yields 4×10^{-10} and 8×10^{-10} moles/liter of anti-M bound for LM 112 and MM 103 cells, respectively. Since in these experiments 1.67×10^{11} cells/liter were used, the maximum number of M antigens/cell were calculated as 3×10^3 for the MM(HK) and 1.5×10^3 for the LM(LK) cell, respectively. It should be pointed out, however, that the ratio of bound/free anti-M never exceeded 0.4–0.5. Thus, the apparent dissociation constant (K_D) of 10^{-9} M/liter calculated from the slope of the regression line does not encompass M antibody binding with higher affinities than 10^{-9} M. In order to obtain

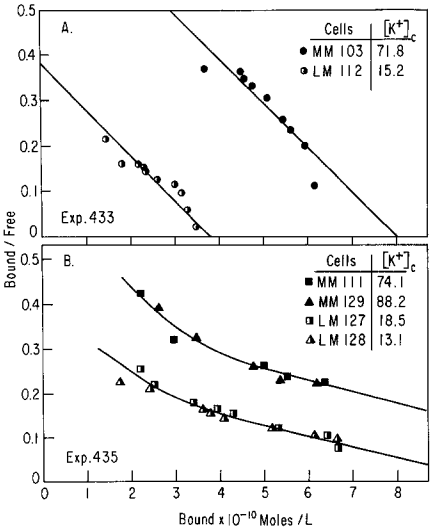

Fig. 8. Scatchard plot of equilibrium binding of ^{125}I anti-M to 3 MM and 3 LM red cells. (*A*) Replot from Fig. 7 (experiment 433); (*B*) Additional experiment (435) with 2 MM and 2 LM cells. $[K^+]_c$ = mM/L cells

an estimate of the binding sites with greater affinities than 10^{-9} M, experiments would be required with an antibody preparation of much higher specific activity. Nevertheless, the data are suggesting that the number of M antigens may be about 50% lower in LM(LK) 112 than in the MM(HK 103) cells.

While the experiment shown in Figs. 7 and 8*A* permitted a reasonable approximation of the number of M antibody molecules bound with K_D values near 10^{-9} M, another experiment on four additional HK and LK sheep red cells displayed a much greater heterogeneity as apparent from the Scatchard plot in Fig. 8*B*. The relationship between bound/free versus bound anti-M is curvilinear, indicating a range of K_D values from at least 10^{-9} M to much lower affinities. Again, as already pointed out in experiment 433 shown in Fig. 8*A*, the low specific activity of anti-M prohibited measurement of K_D values higher than 10^{-9} M. In spite of these ambiguities, the two LM red cells (LM 127 and 128) seem to have a lower number of M antigenic sites than the two HK cells (MM 111 and 129) since there occurs a significant parallel upward shift of the binding curve for HK cells. Linear extrapolation from the last experimental points, however, would yield even higher numbers of M antigens on these four cells than on those shown in Fig. 8*A*.

The difficulties in demonstrating specific saturation binding as well as specific binding at very low antibody concentrations are even more

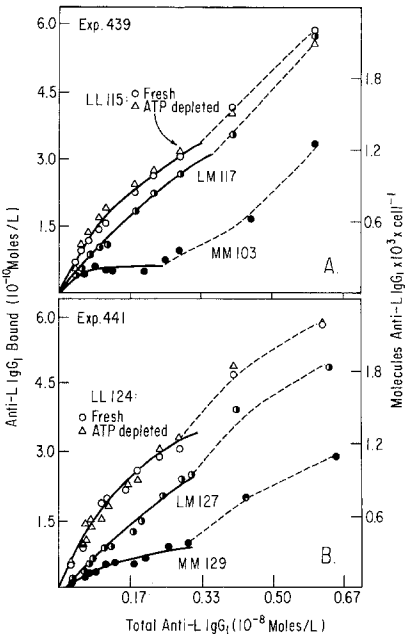

Fig. 9. Equilibrium binding of ^{125}I anti-L to 2 LL and 2 LM potassium sheep red cells. (*A*) Experiment 439. (*B*) Experiment 441. Binding of anti-L is also shown to LL (LK 115 and 124) red cells ATP depleted by starvation [20]. Incubation time 2.5 hr. Longer incubation times did not improve the binding curves

pronounced in our L antibody experiments. Fig. 9*A*, *B* show two binding experiments with two homozygous (LL) and two heterozygous (LM) LK red cells. First, the rapid nonspecific binding of radiolabeled anti-L occurring beyond about 0.3×10^{-8} M anti-L excluded the use of higher concentration ratios of L antibody to LK cells (L antigen) than those used in this experiment. Second, even at low anti-L concentrations, nonspecific uptake by HK(MM) cells is a considerable fraction of the total anti-L bound, particularly to LM red cells, which makes it extremely difficult to correct the data obtained with these cells for a reasonable presentation in the Scatchard plot of Fig. 10*A*, *B*. The data points for LL(LK 124) cells (Fig. 10*A*), however, permitted computation of a linear regression line with a correlation coefficient of 0.9 and an intercept on the *y*-axis (bound/free) higher than that shown previously in the M antibody experiments. From the slope of the line a K_D of 3×10^{-10} M can be estimated and extrapolation to the *x*-axis yielded some 1.8×10^3 L antibody molecules bound per cell. Thus, provided the L antibody binds with one valency to LK red cells, the minimum number of L antigenic sites is

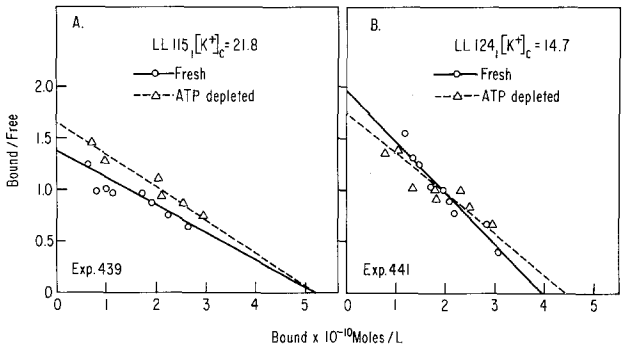

Fig. 10. Scatchard plot of equilibrium binding of ^{125}I anti-L to 2 LL (LK) red cells. (*A*) and (*B*) are replots of the experiments shown in Fig. 9. Data for LM cells were omitted (*see* text). $[K^+]_c$ = mM/L cells

greater than 10^3 in LL red cells and appears to be somewhat smaller in LM red cells. A similar conclusion can be reached from experiment 439 shown in Fig. 10*B*. Again, the data obtained from the LM cells do not permit a useful replotting in the Scatchard diagram.

It was shown above (Fig. 3*B*) that ATP depletion reduced the number of L antibody molecules required to cause 50% immune hemolysis of LL cells in presence of complement. There appears to be no gross difference in the binding of anti-L to ATP depleted and fresh LL red cells (Fig. 9*A*, *B*) and the data points for ATP depleted cells fall on an almost identical regression line in the Scatchard plot (Fig. 10*A*, *B*). These findings are evidence that metabolic manipulation of LL cells does not unmask new L antigenic sites but rather renders the cells more susceptible to the combined hemolytic action of anti-L *and* complement [20].

The studies presented in Figs. 7–9 revealed that the number of M antigenic sites exceeds that of L antigen sites, that homozygous red cells always seem to have a greater number of M or L antigen sites than heterozygous LK red cells, and that the binding affinity of neither anti-M nor anti-L can be easily described by a single dissociation constant. In other experiments, not shown here, we have found that ouabain (10^{-4} M) was without any significant effect on the binding parameters of the two antibodies, and that anti-L, when bound to LM(LK) red cells prior to labelling with anti-M, did not alter the binding characteristics of the M antibody. Inherent to all experiments was that we were not able at present to define M and L antibody binding and affinities at very low antibody concentrations.

Discussion

The purpose of this study was to characterize the binding of the M and L antibodies to HK and LK sheep red cells. The following findings warrant special emphasis: (1) Specific binding of anti-M and anti-L to their respective M and L antigenic HK and LK sheep red cells was demonstrated. (2) The integrity of both M and L antibodies, ^{125}I labeled by the peroxidase method and acid eluted from the red cell membrane, was documented by their ability to initiate the complement dependent immune hemolysis. (3) The interaction of M and L antibodies with their respective target cells revealed antigen and/or antibody heterogeneity. (4) The number of M antigens in HK sheep red cells was at least 3,000 in one blood studied and exceeding this number in two additional HK red cells. In general, M antigen positive LK(LM) sheep red cells appear to have fewer sites, but only one of the three LM cells tested had 50% of the number of M antigens found in HK red cells. (5) The number of L antigens appears to be greater than 1,000 per cell and was found to be higher in homozygous than in heterozygous LK red cells. Nonspecific background binding of anti-L, however, precluded establishment of a reproducible LL/LM ratio for the L antigen.

The preparation of ^{125}I labeled anti-M and anti-L involved several steps and the calculation of our data necessitated several assumptions relevant to the final analysis of the data. The two antibodies constitute only minute fractions of the serum immunoglobulins. It is assumed that the ^{125}I labeling of *IgG* is random, i.e. that the specific activity of the acid eluted anti-M or anti-L antibodies are identical to that of the M and L nonspecific immunoglobulins which are removed by the washing procedure of the cells prior to acid elution. The elution process itself may be selective and the iodination procedures harmful to the integrity of the antibody molecule [11]. However, the intactness of the eluted M and L antibody molecules labeled by the lactoperoxidase method [29–37] was demonstrated by the observation that both antibodies specifically bound to their respective target cells and induced complement dependent immune hemolysis (Figs. 1–3). Furthermore, purified anti-L, labeled with iodine by this method and subsequently eluted at low pH from LK membranes, was capable of stimulating active K^+ influx in LK cells similar to native L antibody preparations. (In preliminary experiments a fivefold stimulation of K^+ pump influx was obtained in LK red cells to which some 1,000 molecules of ^{125}I anti-L derived from S 39 serum were bound. Considering that at least about half of these molecules may be bound "nonspecifically",

these data indicate that there must be about 6–8 times more L antibody molecules bound per LK cell than there are Na^+K^+ pumps; *see* ref. 26 and 14).

The apparent heterogeneity of the antibodies and/or antigens studied here and the number of M and L antigens estimated on HK and LK cells are the most important results of this paper and must be considered in the light of the genetic association of the M/L antigen system with the cation polymorphism in sheep red cells. All estimations rest on the assumption made with other blood group antigen systems [13, 30] that, when studied under equilibrium binding conditions, the actually bivalent antibody molecules [5, 36] bind to the M and L antigenic determinants only through one valency. This treatment, however, neglects the alternative possibility that these antibodies bind bivalently to two adjacent M or L determinants. This may well be the case in our association kinetic studies where the concentrations of the reactants were within the same order of magnitude.

The number of M antigens appears to be in the order of $3\text{–}6 \times 10^3$ in HK (MM) and $1.5\text{–}4 \times 10^3$ in LK (LM) red cells, and the equilibrium dissociation constants (K_D) vary around 10^{-9} M for the M antibody (Figs. 7–9). In homozygous LK (LL) cells the number of L antigenic sites approached $1.2\text{–}1.8 \times 10^3$/cell but seems to be lower in the heterozygous LM (LK) cells. Here, the K_D value in one experiment was found to be around 10^{-10} M. Quantitative evaluation of the association and dissociation rate coefficients was obviated by two persistent difficulties: both antigenic systems demonstrated considerable heterogeneity of antigens and/or antibodies; in addition, the low antibody concentration required for association rate studies favors bivalent binding of antibody which hinders the kinetic analysis (Figs. 4–5). Thus the K_D values can only be estimated from the Scatchard plot (Figs. 8 and 10). It is not unreasonable to assume that much higher K_D values might be found provided the ratio of bound to free antibody concentrations were greater than 2.0. No tentative conclusion could be reached concerning the enthalphy of the binding processes of anti-M and anti-L, both of which showed a remarkable temperature dependence in HK and LK red cells. It is conceivable that also at low temperatures the accessibility of the antigenic determinants is reduced.

Genetically, the observed variability in M and L antigenic sites is not unexpected considering that there may be several M and L alleles, as evident from some variability of the M specific reagents thus far obtained by isoimmunization [33] and from the K^+ pump stimulating action of anti-L in sheep and cattle red cells. On the *cellular level* heterogeneity of

the antibody binding affinity may reflect differences in the red cell populations analyzed, i.e., the affinity of anti-M or anti-L to young red cells is different from that to old red cells. In support of this possibility are studies from our laboratory as well as from Ellory and Tucker, that the M and L antigens are weakly expressed in red cells of newborn lambs [7]. The *physiological* implication of our findings is that the number of M and L antigens is clearly greater by more than one order of magnitude than the number of Na^+K^+ pumps estimated for these cells from ^{3}H-ouabain binding experiments in this laboratory [14]. This conclusion is also born out by our preliminary data on the number of L antibody molecules/cell required for a full stimulation of K^+ pump influx in LK red cells (*see* above).

The discrepancy between the number of M antigens and Na^+K^+ pumps in HK red cells is not surprising in the light of Tucker's statement [42] that it is not the M but the L substance that controls the cation steady state composition of sheep red cells. The difference in the total number of M antigens as well as the unequivocal heterogeneity in the binding affinity of anti-M make it difficult to relate a certain fraction of the M antigens directly to the membrane constituents comprising the Na^+K^+ pump system in these cells. Furthermore, ouabain and anti-L did not interfere with M antibody binding to LM cells thus supporting our previous hypothesis of a lack of an obvious structural correlation between the M antigens and the Na^+K^+ pumps [27]. That anti-L did not influence M antibody binding in LM red cells may be interpreted to mean that either the M and L antigenic determinants are relatively far apart, or that anti-L does not sterically affect anti-M binding to sites nearby.

There are about 70 and 50 Na^+K^+ pumps per LL and LM(LK) sheep red cell, respectively [14]. Kropp and Sachs have shown that some 70 L antibody molecules are required to achieve full K^+ pump stimulation of LK goat red cells which have about 60 Na^+K^+ pumps [15]. However, our present studies show that LK sheep red cells bind about 1,000 and more L antibody molecules, which suggests a similar number of L antigenic sites. Hence an explanation for the difference between our findings and those of Kropp and Sachs [15], and for the lack of stoichiometry between L antigens and Na^+K^+ pumps in LK sheep red cells has to be sought. These explanations, of course, rest on the assumption made earlier for our purified ^{125}I labeled L antibody preparation.

It is known that in LK sheep red cells anti-L stimulates the Na^+K^+ pump or in presence of complement hemolyzes these cells [6, 24]. The finding of our laboratory [21] and subsequent confirmation by Dunham

on goat red cells [4] that trypsinization abolishes the K^+ pump stimulation but not the hemolytic action of anti-L suggested the existence of two types of L antigenic sites, one associated with the Na^+K^+ pump (L_p sites) and the other independent of it (L_{1y} sites). Furthermore, Ellory and Tucker [8] demonstrated that goat red cells of LK type absorb ovine L antibody stimulating the pump (anti-L_p) but not the hemolytic anti-L_{1y} [8]. Thus LK goat red cells may possess the smallest possible number of L antigens (L_p antigens) i.e., only the L_p antigens through which anti-L alters the K^+ pump activity. The bulk of these previous findings is consistent with our results presented in this paper, namely that the LK sheep red cell has more L antigenic sites than there are Na^+K^+ pumps. Some of these antigens may be closely associated with the active cation transport system and are involved in binding L_p antibody molecules; others may be unrelated to the membrane constituents comprising the Na^+K^+ pump but mediate complement dependent immune hemolysis by associating with anti-L_{1y}. Alternatively, all antigens may be structurally close to or part of the Na^+K^+ pump but differ in their determinants sufficiently enough to distinguish anti-L_p and anti-L_{1y}. In this case antigenic differences may reflect different stages of biochemical completion of the final determinants (precursor molecules?) either on the same cell as known for other blood group antigens [37] or on different cells. Thus, molecular differentiation of antigens must affect their number on the cell surface as well as the affinity with which antibodies bind. Other explanations, of course, are possible.

Finally, the observation that ouabain did not affect equilibrium binding of anti-L to LK sheep red cells (as in the M antigen/antibody system) raises the question as to how the various membrane receptors, e.g., ouabain binding site, and the M and L determinants, relate to each other on the membrane molecular and topographical level. In particular, one would like to know whether a quantitative correlation of the number of M and L antigens in heterozygous LM (LK) red cells with the total number of M and L antigens in HK (MM) and homozygous LK (LL) red cells may provide any clue as to the subunit composition of the Na^+K^+ pump in these cells, if indeed a fraction or all antigens are molecularly part of the Na^+K^+ pump constituents. As previously discussed, the M antigen seems to be an improbable candidate in such a search, and we now have presented evidence that the L antigen does not represent the Na^+K^+ pumps in LK sheep red cells. Moreover, no clear cut L antigenic dose response could be seen in our studies with ^{125}I labeled anti-L between LM and LL (LK) red cells although previous studies from this laboratory

revealed a threefold difference using the immune hemolytic assay and K^+ pump stimulation [19, 16]. Apparently other factors such as differences in complement reactivity or selection of complement insensitive immunoglobulins introduce complexities into the immune hemolytic assay which preclude a direct quantitative comparison between the two results but do not reduce the validity of our earlier discovery of a qualitative difference between homo- and heterozygous LK red cells [16].

Our studies then revealed the extraordinary difficulties inherent in reaching an approximative understanding of the relation of the M and L antigens to the Na^+K^+ pumps and the structural coordination between these antigens and the Na^+K^+ pump subunits on LM sheep red cells. Although on the basis of gross antigenic analysis and physiological effects of the L antibody, there is an unequivocal genetic association between cation polymorphism and membrane surface antigens, the membrane antigenic expression of the M and L genes exceeds quantitatively that of the Na^+K^+ transport sites. With respect to the membrane topographical distribution of the M and L antigens, our observation that only some 75–100 M antibody and some 500–750 L antibody molecules per cell are required for 50% immune hemolysis of suspensions of HK and LK sheep red cells, respectively, deserves final emphasis: from other blood group antibody systems it is known that several hundred *IgG* antibody molecules are required to statistically generate *IgG* doublets essential for activation of the first component of the hemolytic complement system [3]. Thus, it seems not unlikely that particularly the M antigens and maybe also the L antigens are clustered and not randomly distributed at the sheep red cell surface. Perhaps clustering of L antigens is enhanced when LK cells are metabolically depleted, an interesting aspect with respect to possible lateral mobility of these important antigens (and their pumps?) within the cell surface.

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