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Enzymatic Modification of the L and M Antigens in LK and HK Sheep Erythrocytes and Their Membranes

The Action of Neuraminidase and Trypsin

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Summary. This paper reports on the effect of two hydrolytic enzymes, neuraminidas and trypsin, on the interaction of blood group L-positive low-potassium-type (LK) an blood group M-positive high-potassium-type (HK) sheep red cells with their respectiv isoimmune antisera. It was found that treatment of LK and HK red cells with neura minidase did not change the interaction of these cells with their homologous antibodie as measured by K^+-pump flux, complement-mediated immune hemolysis and absorp tion of antibody. Similarly, trypsin pretreatment of LK and HK red cells did not inter fere with the hemolytic action of anti-L and anti-M antibodies, respectively. In strikin contrast, however, it was observed that pretreatment of LK cells with trypsin rendere these cells insensitive to the K^+-pump stimulating antibody present in the anti-L serum

Previous work from our laboratory (Lauf, Rasmusen, Hoffman, Dun ham, Cook, Parmelee & Tosteson, 1969, 1970) and from the laborator of Ellory and Tucker (1969) has shown that an iso-immune antiserun (anti-L serum) prepared by immunization of high-potassium-type (HK blood-group M-positive sheep with blood from low-potassium-type (LK blood-group L-positive sheep contained one or more antibodies whicl stimulated K^+-pump influx four- to sixfold in LK red cells. This antiserun was also shown to stimulate the Na^+-K^+-dependent and ouabain-sensitive ATPase (S-ATPase) in hemoglobin-free membranes derived from LK sheep red cells (Ellory & Tucker, 1969; Lauf, Parmelee & Snyder, 1970). It wa demonstrated, in detail, that formation of a complex between the L-antigen and its antibody stimulates active K^+-transport in LK sheep red cells both by changing the kinetic properties of at least some of the pump sites and by increasing the number of pump sites (Lauf *et al.*, 1970). These ex- periments suggested that the L-antigen is intimately involved with the mole-

cules responsible for active transport of cations across the red cell membrane. If so, a study of the nature of the L-antigen in LK red cell membranes could give insight into the components comprising the Na^+-K^+ pump and thus provide further information concerning the mechanism by which this unique antigen-antibody reaction so profoundly affects active cation transport.

This paper reports on the effect of two hydrolytic enzymes, neuraminidase and trypsin, on the interaction of L-positive LK and M-positive HK sheep red cells with their respective isoimmune antisera. It was found that treatment of LK and HK red cells with neuraminidase did not change the interaction of these cells with their homologous antibodies as measured by K^+-pump flux, complement-mediated immune hemolysis and absorption of antibody. Similarly, trypsin pretreatment of LK and HK red cells did not interfere with the hemolytic action of anti-L and anti-M antibodies, respectively. In striking contrast, however, it was observed that pretreatment of LK cells with trypsin rendered these cells insensitive to the K^+-pump stimulating antibody present in the anti-L serum.

Materials and Methods

Cells, Antiserum and Complement

All experiments described were carried out with blood from homozygous HK and LK sheep freshly drawn into heparin (10 USP units sodium heparin/ml cell suspension). Red cells were separated from the plasma and buffy coat by centrifugation in a Sorvall RC2-B Centrifuge, washing in 153 mM NaCl buffered with 1 mM Tris-chloride, pH 7.4 (TBS), and suspended in the buffered solutions used in the experiments below. Hemoglobin-free membranes were prepared from LK red cells by osmotic lysis in 20 mosm Tris-chloride buffer, pH 7.6, and collected by centrifugation at $27{,}000 \times g$ at 4 °C for 15 min. The amount of membrane protein per ml suspension was determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin as standard. Iso-immune anti-M (S 11*) and anti-L antisera [anti-L (1) and anti-L (2) refer to the two anti-L antisera S 32* and S 39*] were generously provided by Dr. Ben A. Rasmusen (Dept. of Animal Science, University of Illinois, Urbana) and were specific anti-M and anti-L reagents (*e.g.*, L- and M-nonspecific antibodies were removed by absorption of the heat-inactivated serum). Non-immune serum (NI) from HK sheep were only heat inactivated at 56 °C for 30 min. Guinea pig serum obtained from clotted heart blood served as complement source. The fresh guinea pig serum was absorbed twice for 10 min at 4 °C with washed M/L-positive red cells from heterozygous LK sheep and stored in small quantities at −70 °C until used.

Hemolytic Assay, Antibody Absorption and K^+-Flux Measurements

The hemolytic assay system for quantitation of the degree of complement-mediated immune hemolysis by anti-M serum and assay for antibody absorption were used as described earlier (Lauf & Tosteson, 1969). The amount of antibody present in the anti-

serum was calculated in antibody units (AU) per ml final suspension, and the numbe of control and treated red cells binding 50% AU/ml was determined. Hemolysis of LI cells by anti-L before and after absorption with LK cells was tested in a similar manne Because of the low lytic activity of the anti-L antisera, no attempts were made to expre the amount of anti-L antibodies in AU/ml.

Preincubation of LK red cells with anti-L antiserum and K^+-flux measuremen were generally done in the media (5 mM KCl, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ 5 mM glucose and 10 mM Tris-chloride, pH 7.6) and under the conditions described i the previous paper (Lauf *et al.*, 1970). The sampling, however, was modified slightly Duplicate samples (10 ml of a 0.25% cell suspension) were divided into 5-ml portion to which either 0.25 ml of medium or 0.25 ml of medium containing ouabain (10 mM was added and placed into a 37 °C waterbath shaker (Metabolyte, New Brunswic Scientific). A few minutes was allowed for equilibration of the samples. At $t=0$, ^{42}K was added (high specific activity, Cambridge Nuclear) to each test flash and the K^+-flu experiment was stopped after 2 hr by placing the samples into an ice bath. All sample were then centrifuged at 4 °C for 1 min at 10,000 rpm to separate cells from super natants. Analysis for hemoglobin of the cells washed in 0.12 M $MgCl_2$, as well as fo total K^+ and ^{42}K in cells and supernatants was done essentially as described earlier Although this simplified flux method does not compare with the linearity of the two point flux used in the previous report, the rapid action of ouabain (within a minute) an the simultaneous sampling of all flasks permitted a satisfactory estimation of K^+-pum flux and its stimulation in anti-L antibody-sensitized red cells. This one-point flu procedure is described elsewhere in a more detailed form (Kepner & Tosteson, *in preparation*).

In the experiments designed to measure absorption of the K^+-pump flux stimulatin anti-L serum, equal volumes of red cell suspensions containing 10^8 to 3×10^{10} cells/m and undiluted anti-L antiserum were mixed and incubated for 1 hr at 32 °C. The sample were then centrifuged at 10,000 rpm at 4 °C for 5 min and the supernatants recovered These cell-free supernatants were subsequently tested for remaining antibody activity by measuring their stimulating effect on K^+-pump flux of fresh LK cells. Simultaneously K^+-pump flux stimulation was measured as a function of decreasing concentration of anti-L serum. In Fig. 1, the values (in duplicates) of K^+-pump flux ($^iM_K^P$) found in two

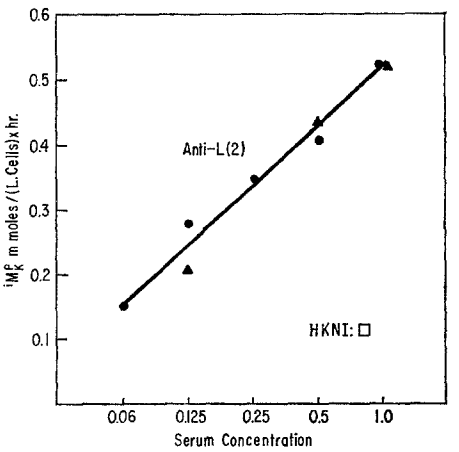

Fig. 1. Stimulation of active K^+-pump influx in LK 188 (LL) red cells at decreasing concentrations of anti-L (2) serum. Circles and triangles represent two different experiments performed in duplicates on different days

independent experiments are plotted semilogarithmically as a function of the relative antiserum concentration. Using this graph, the anti-L concentration remaining in a previously absorbed sample could be determined for any concentration of antiserum between 1.0 and 0.06.

Enzyme Treatment of LK and HK Red Cells and Assays for Released Membrane Fragments

In all experiments described below, the red cells were washed at least six times in 20 volumes of TBS per wash prior to enzyme treatment. It was found that less washing had an adverse effect on the reproducibility of the results, probably because of the presence of plasma glycoproteins absorbed onto the red cell surface.

Neuraminidase

One volume of packed HK or LK red cells was incubated with four volumes of a buffered solution (153 mM NaCl, 5 mM $CaCl_2$ and 1 mM Tris-chloride, pH 7.4) with or without 10, 20 or 50 units of neuraminidase (Lots 966S and 668S, pure from *Vibrio cholerae* Behringwerke, Marburg, Lahn, Germany) at 37 °C for 1 hr in a waterbath shaker. After incubation the samples were centrifuged at 10,000 rpm and 4 °C for 5 min, and cells and supernatants were carefully separated. The cells were then washed three times in 20 volumes of TBS per wash and divided in four aliquots each for control and treated cells. The cells of three pairs of aliquots were then analyzed respectively for (a) complement-mediated hemolysis by anti-M and anti-L antisera, (b) their capacity to absorb lytic anti-M and anti-L antibodies from the respective antisera, and (c) for $^{i}M_K^P$ in presence and absence of anti-L serum. The fourth pair of aliquots was lyzed by osmotic hemolysis and membranes were prepared as described above. Equal volumes of these membranes were then mixed with 0.3 N H_2SO_4 and hydrolyzed at 80 °C for 1 hr. The hydrolysates were precipitated with 10% ice cold trichloroacetic acid (TCA) and centrifuged for separation of TCA-insoluble membrane components. The TCA supernatants were filtered through Whatman No. 1 filter paper and subsequently assayed for neuraminic acid by the methods of Aminoff [(1963) (TBA method)] and of Boehm, Dauber and Baumeister [(1954) (BIAL method)] using three-times crystallized N-acetyl-neuraminic acid as standard (Lot No. 4401, Seikagaku Kogyo Co., Ltd., Tokyo, Japan). The amount of neuraminic acid found in membranes before and after treatment with neuraminidase was calculated as μmoles sialic acid per mg membrane protein as determined by the Lowry method.

The supernatants of the enzyme-treated cells were likewise precipitated with equal volumes of 10% ice-cold TCA and filtered through Whatman filter paper No. 1 and analyzed for sialic acid. In the case of a time study, red cells were incubated with neuraminidase and samples were taken at various time intervals, quickly cooled in an icebath and immediately centrifuged at 10,000 rpm and 4 °C for 1 min to recover the supernatants.

Trypsin

Treatment of LK and HK Red Cells. Equal volumes of six-times washed packed red cells and a buffer (125 mM NaCl, 10 mM $CaCl_2$, and 20 mM Tris-chloride, pH 8.0) with or without 0.1, 1.0, 6.0 or 10.0 mg trypsin/ml final cell suspension (50%) were mixed and incubated for 1 hr at 37 °C. The trypsin (three times recrystallized by supplier) was purchased from Worthington Biochemical Corporation (Freehold, N. J.) and contained 190 units/mg. After incubation, the samples were precooled in an icebath and centrifuged at 10,000 rpm and 4 °C for 2 min. Cells and supernatants were carefully separated.

The cells were washed thoroughly in TBS and subsequently divided into three aliquo for measuring immune hemolysis, antibody absorption and ${}^{i}M_K^P$. Because of the slig hemolysis (<1%) during preincubation with trypsin, the extent of the enzyme catalyze hydrolysis of LK or HK red cells was ascertained by analysis of the cell-free supe natants for protein-bound sialic acid rather than for protein by the Lowry metho Trypsin did not cause release of free sialic acid. Sialic acid in these samples was measure either using the BIAL-method without prehydrolysis or the TBA method for the aci hydrolyzed (final concentration 0.1 N H_2SO_4) and in 5% TCA-soluble supernatan material. The amount of sialic acid detected was found to be identical as measured b either method, indicating that sialic acid is bound to macromolecules (glycopeptides? soluble in 5% TCA.

When the release of peptide-bound sialic acid was measured as a function of time incubation was started at 37 °C, and samples were taken at various time intervals an immediately centrifuged. The cell-free supernatants were transferred to cold test tube containing equal volumes of 10% TCA and analyzed for sialic acid as described above When, however, the effect of trypsin on ${}^{i}M_K^P$ in LK cells sensitized with anti-L wa studied as a function of time, samples were removed from the trypsin-containing flask at various time intervals and transferred into test tubes containing an amount of eg white trypsin inhibitor (ovomucoid, Mann Research Laboratories, New York, N.Y. equimolar to the trypsin present. The cells were then washed as usual with TBS an analyzed for ${}^{i}M_K^P$ in the presence and absence of anti-L antiserum.

Treatment of LK Membranes. Hemoglobin-free membranes were prepared from LK red cells as described above. In order to assess the time course of protein release by trypsin, membranes (final concentration 4 to 7 mg/ml) were incubated at 37 °C up to 120 min with 0.1 or 1.0 mg trypsin/ml membrane suspension in a buffer containing 50 mM Tris-chloride, pH 8.2, and 10 mM $CaCl_2$. Samples were taken at various time intervals and the reaction was stopped by adding equimolar amounts of ovomucoid. The samples were then centrifuged at 15,000 rpm for 15 min. The clear supernatants were carefully removed and analyzed for protein content by the Lowry method. The values obtained were corrected for trypsin and ovomucoid present in the samples.

Analysis for L-Antigen in Material Released by Trypsin from LK Red Cells and Their Membranes

Supernatants (10 ml) derived from 10 ml of packed and trypsinized LK red cells or HK red cells (L-negative control) were divided into three aliquots; one was lyophilized and two were kept frozen until used. These aliquots were treated as follows. (*1*) The lyophilized aliquot (from 3 ml supernatant) was resuspended in 1 ml deionized water and desalted on a Sephadex G 10 column (dimensions using water as eluent: 1.5 × 80 cm). Two major peaks emerged. The peak appearing in the void volume showed a descending portion with several spikes indicating heterogeneity (*e.g.*, trypsin, ovomucoid, membrane fragments, hemoglobin). This material was lyophilized and was dissolved directly in 0.6 ml of anti-L antiserum which then was tested in the K^+-pump flux experiment. The second peak appearing in the included volume contained most of the salt but also showed a sharp UV-absorption maximum at 276 nm. This second peak was also lyophilized, but then redissolved in deionized water and the solute concentration reconstituted to 300 mosm with distilled water. One volume of this solution was then incubated with one volume of anti-L serum, and the mixture was assayed for its effect on ${}^{i}M_K^P$ in LK red cells. (*2*) The second aliquot (frozen 4 ml) was mixed with 90% redistilled phenol and centrifuged at room temperature for 15 min. The clear water phase was removed, lyophilized and, after resolubilization in deionized water, desalted on a Sephadex G 10 column as described above. A small peak emerged in the void volume whereas the second

peak contained salt only. The material of the first peak was lyophilized, dissolved in anti-L antiserum and the mixture tested for its effect on ${}^{i}M_{K}^{P}$. The material of the phenol phase was recovered by pouring the phenol phase into 10 volumes of ice-cold methanol containing 3% sodium acetate (Lauf & Kickhoefen, *in preparation*). After the mixture stood for a few hours, a fine precipitate was visible which was recovered by centrifugation at 13,000 rpm and 4 °C for 10 min. This precipitate was washed three times in ice-cold pure methanol, resuspended in slightly alkalinized water and then lyophilized. The lyophilized material was then added to the anti-L serum and the mixture tested for its effect on K^+ flux in LK sheep red cells. (*3*) The third aliquot (3 ml) was mixed directly with anti-L antiserum, incubated for 1 hr at 32 °C and then assayed for its K^+-pump stimulating activity.

All assays for K^+-pump stimulating activity were done in the presence of ovomucoid which was added to the samples in amounts equimolar to the trypsin concentration present.

When the effect of trypsin on the L-antigen in membranes was studied, a sample of these membranes was incubated with 1 mg trypsin/ml for 90 min at 37 °C under the conditions described, and the reaction was stopped with ovomucoid. The sample was divided into two aliquots. One aliquot was added to an equal volume of anti-L serum, and the mixture was incubated at 37 °C for 1 hr. The other aliquot was separated by centrifugation at $30{,}000 \times g$ for 15 min into a membrane residue and supernatant. Both residue and supernatant were incubated separately with anti-L serum. The antisera preabsorbed in this way were then tested for K^+-pump flux stimulation in LK cells. Untreated LK membranes served as controls in the absorption experiments.

Results

The Effect of Neuraminidase Treatment on the Immune Reaction of LK and HK Sheep Red Cells with Anti-L and Anti-M Antibodies

Amount of Sialic Acid in LK and HK Sheep Red Cells. Maximum release of sialic acid from either LK or HK sheep red cells was obtained after 60-min incubation at 37 °C using 20 units neuraminidase per ml cell suspension. Table 1 compares the amount of sialic acid released by neuraminidase from packed LK or HK sheep red cells with the amount of sialic acid released by acid hydrolysis from white membranes and from similar membranes prepared from red cells which had been exposed to the enzyme. It is evident that neither enzymatic cleavage of sialic acid from whole cells nor acid hydrolysis of white membranes revealed a difference in the sialic acid content of the two genetic types of sheep red cells. The average amount of acid-hydrolyzed sialic acid was found to be 0.047 μmole/ml protein for membranes from untreated LK and HK control cells and 0.0045 μmole sialic acid/mg protein in membranes obtained from red cells pretreated with the enzyme. Thus, assuming that this difference between the two types of membranes developed during the exposure of intact red cells to neuraminidase, it can be estimated that such treatment removed 90% of the membrane sialic acid. Assuming that 1 ml packed red cells contains roughly

Table 1. *Amount of sialic acid released from HK and LK sheep red cells*

Sheep		Red cells (μmoles sialic acid per ml packed cells)[a]	Membranes (μmoles sialic acid per mg protein[b]	
Cation-type	Antigen-type		Control	Sialidase pretreatment
LK 103	LL	0.295	0.051	0.005
LK 104	LL	0.295	0.041	0.004
LK 107	LM	0.340	0.045	0.004
LK 109	LM	0.318	0.052	0.005
HK 4146	MM	0.330	0.045	0.005
HK 180	MM	0.286	0.050	0.004

[a] Released by 20 units of neuraminidase.
[b] After hydrolysis of membranes in 0.1 N H_2SO_4 for 1 hr at 80 °C.

8 mg membrane protein and that each mg of membrane protein is associated with 0.051 μmole sialic acid (Table 1), 0.038 μmole or 82% of the total sialic acid was found in the supernatant of enzyme-treated packed red cells. Thus, both methods of calculation indicate that 80 to 90% of the sialic acid present in HK and LK red cells is removed by treatment with neuraminidase.

K^+-Pump Flux Stimulating Activity. Pretreatment of LK or HK red cells with neuraminidase did not result in any significant change of K^+-pump and leak fluxes in these cells. In addition, preincubation of enzyme-treated LK red cells with two different anti-L antisera [*e.g.*, anti-L (1) and (2)] produced about the same K^+-pump flux stimulation as observed with the controls. Leak fluxes were also found to be identical for both treated and control red cells (Table 2). Since it has been shown that the K^+-pump flux

Table 2. *K^+-pump flux stimulation of neuraminidase-treated and control LK cells by anti-L serum*

Cells LK 188 (LL)	Serum	K^+ Influx [mmoles K^+/(liter cells) × hr]	
		Pump	Leak
Control cells	HK NI	0.13	0.17
	Anti-L (1)[a]	0.39	0.23
	Anti-L (2)	0.41	0.21
Neuraminidase-treated cells	HK NI	0.09	0.17
	Anti-L (1)	0.35	0.22
	Anti-L (2)	0.38	0.20

[a] (1) and (2) represent two different anti-L antisera.

Table 3. *Absorption of K^+-pump stimulating anti-L antibody by neuraminidase-treated and control LK red cells*

Treatment	Serum	K^+-Influx [mmoles K^+/(liter cells) × hr]	
		Pump	Leak
Unabsorbed	HK NI	0.10	0.10
	Anti-L (1)	0.48	0.07
	Anti-L (2)	0.40	0.18
Absorbed with control cells	HK NI	0.12	0.11
	Anti-L (1)	0.09	0.26
	Anti-L (2)	0.09	0.17
Absorbed with treated cells	HK NI	0.08	0.12
	Anti-L (1)	0.07	0.23
	Anti-L (2)	0.05	0.18

stimulating antibody(ies) present in anti-L serum can be absorbed by LK red cells but not by HK red cells, it was of interest to investigate if neuraminidase-treated LK cells are still capable of absorbing this antibody. Table 3 shows the results of such an absorption experiment. Anti-L (1) and anti-L (2) sera were incubated with control or neuraminidase-treated red cells. After absorption, the cells were centrifuged and the supernatants assayed for remaining K^+-pump stimulating effect in LK test red cells. The results in Table 3 clearly indicate that the K^+-pump flux stimulating activity of both antisera was removed by absorption with either cells. These experiments permit the conclusion that the removal of about 90% of sialic acid from LK red cells by treatment with neuraminidase did not change the K^+-pump stimulation brought about by the immune reaction of LK red cells with anti-L antibody(ies) nor did it interfere with the binding of that antibody to these cells.

Lytic Activity. We confirmed earlier observations of Arquilla, Hamlin, Hamashige and Miller (1964) that neuraminidase-treated sheep red cells lyze rapidly in the presence of guinea pig complement. This high susceptibility to lysis by complement alone was seen with either LK or HK sheep red cells which had been pretreated with the enzyme. Therefore, hemolysis was not a suitable test to ascertain changes in the amount and affinities for their respective iso-antibodies of the L and M antigen of enzyme-treated LK and HK red cells. Instead we chose the absorption assay. Pretreatment of LK cells with neuraminidase did not inactivate their capacity to remove

Table 4. *Absorption of the lytic anti-L activity from two anti-L sera by LK red cells treated with neuraminidase or trypsin*[a]

Treatment	Hemolysis observed[b] (%)	
	Anti-L (1)	Anti-L (2)
Unabsorbed	100	100
Absorbed with control LK cells	7	5
Sialidase-treated LK cells	20	10
Trypsin-treated LK cells	19	6

[a] Antiserum was absorbed with equal volume of packed LK cells.
[b] Hemolysis of fresh LK cells in presence of 1/5 diluted guinea pig serum.

Table 5. *Binding of anti-M antibody by HK red cells treated with neuraminidase or trypsin*

Sheep	Treatment	Number of red cells binding 50% AU/ml[a]
HK 2562 (MM) (exp. 72)	Control cells	3.1×10^8
	Sialidase-treated cells	3.6×10^8
HK S (MM) (exp. 143)	Control cells	3.0×10^8
	Trypsin-treated cells	4.9×10^8

[a] 100% = 5.7×10^8 antibody units (AU)/ml.

the lytic antibody from the antiserum as seen in Table 4. The number of neuraminidase-treated HK (MM) cells necessary to absorb 50% of the number of AU present in the serum tested did not vary greatly from that of the control cells (Table 5). Binding of the lytic anti-L or anti-M activity to LK or HK red cells, therefore, appears not to be significantly altered when these cells are pretreated with neuraminidase.

The Effect of Trypsin Treatment on the Immune Reaction of LK and HK Red Cells with Anti-L and Anti-M Antisera

Amount of Protein-Bound Sialic Acid Released by Trypsin from LK and HK Sheep Red Cells. Fig. 2 (left-hand scale) shows the time course of release of protein-bound sialic acid as a measure of trypsin action on HK and LK sheep red cells. No further release of protein-bound sialic acid was discovered after 60-min incubation with 1 mg trypsin/ml 50% cell suspension.

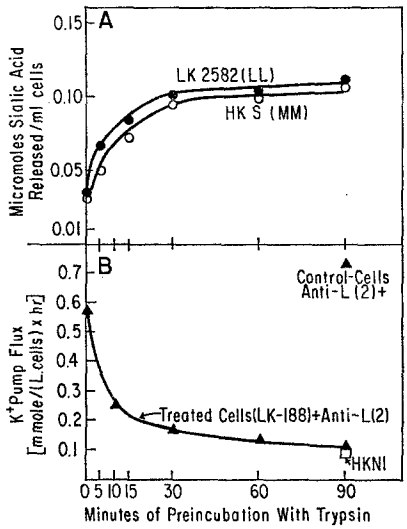

Fig. 2A and B. Time course of release of "bound" sialic acid (A) and inactivation of the response of LK red cells to K^+-pump stimulation by anti-L (2) serum (B)

Table 6. *Amount of protein-bound sialic acid released from HK and LK sheep red cells by trypsin*

Cation type	Antigen type	μmoles sialic acid per 3×10^{10} red cells
LK 103	LL	0.13
LK 104	LL	0.12
LK 107	LM	0.13
LK 109	LM	0.14
HK 4146	MM	0.14
HK 180	MM	0.13

The amount of bound sialic acid liberated by trypsin was roughly 34% of the total sialic acid determined by acid hydrolysis in these membranes. There was no significant difference between HK and LK sheep red cells as borne out in Table 6 which compares the amount of sialic acid found in the trypsin supernatants of two HK and four LK sheep red cells.

K^+-Pump Stimulating Activity. Treatment of LK and HK sheep red cells with trypsin did not impair K^+-pump and leak fluxes in these cells. When, however, trypsinized LK cells were incubated with anti-L serum, the K^+-pump flux stimulation characteristic of untreated but anti-L sensitized LK cells was lost. Table 7 compares two experiments using red cells from two

Table 7. *Effect of trypsin treatment of LK cells on K^+-pump flux stimulation by anti-L antiserum*

Sheep	Cells	Serum	K^+ Influx [mmoles K^+/(liter cells) × hr	
			Pump	Leak
LK 188 (LL) (exp. 143)	Control cells	HK NI	0.08	0.19
		Anti-L (1)	0.39	0.22
		Anti-L (2)	0.42	0.21
	Treated cells	HK NI	0.09	0.18
		Anti-L (1)	0.12	0.18
		Anti-L (2)	0.10	0.19
LK 103 (LL) (exp. 146)[a]	Control cells	HK NI	0.12	0.18
		Anti-L (2)	0.41	0.27
	Treated cells	HK NI	0.13	0.17
		Anti-L (2)	0.15	0.26

[a] In exp. 146, preincubation of the control cells with anti-L was performed in the presence of 0.3% trypsin and 0.4% ovomucoid.

animals and two sources of anti-L serum. It can be clearly seen that ${}^iM_K^P$ in the trypsin-treated LK cells sensitized with anti-L (1) or anti-L (2) sera is not greater than that of the HK NI control. The leak fluxes were found to be unaltered. It should be noted that neither trypsin alone nor trypsin with its inhibitor ovomucoid showed any inactivation of the anti-L serum with respect to its capacity to stimulate untreated LK red cells. This inactivating effect of trypsin was followed further in a time course study. Fig. 2B shows that the time course of inactivation of the K^+-pump stimulating action of anti-L coincides quite well with the time course of release of membrane sialic acid (Fig. 2A). Although anti-L serum failed to stimulate ${}^iM_K^P$ in trypsinized LK red cells, these cells did still absorb K^+-pump stimulating activity from the anti-L serum as shown in Table 8. The values given for the unabsorbed antiserum were obtained from Fig. 1. The K^+-pump stimulation by anti-L serum absorbed with various number of cells is shown in columns 3 and 4 of Table 8. Experiment 154 reveals that absorption with 3×10^{10} control cells reduced K^+-pump flux stimulation by anti-L almost to the value obtained in presence of HK NI serum [0.10 mmole K/(liter cells) × hr], whereas absorption with trypsin-treated LK cells left still some pump-stimulating activity in the serum. When, however, the antiserum was absorbed twice with 3×10^{10} trypsinized LK cells (exp. 155), all K^+-pump stimulating activity was removed. Membranes treated with trypsin for 90 min at 37 °C (1.5 mg membrane protein equi-

Table 8. *K^+-pump flux stimulation of LK cells by anti-L serum before and after absorption with trypsinized LK red cells and membranes and with untreated controls*

Exp. no. [LK 188 (LL) cells]	K^+-Pump flux [mmoles K^+/(liter cells) × hr] in presence of anti-L serum			Number of cell membranes or mg protein used in absorption
	Unabsorbed	Absorbed with		
		Control	Treatment	
154	0.38	0.34	0.37	10^8
	0.38	0.31	0.38	10^9
	0.42	0.16	0.25	10^{10}
	0.41	0.13	0.25	3×10^{10}
155	0.51	0.10	0.07	6×10^{10}[a]
S 43	0.43	0.10	0.10	1.5 mg[b]
	0.43	0.10	0.31	1.5 mg[c]

[a] Absorption done in two steps with each 3×10^{10} cell.
[b] Addition of ovomucoid immediately after exposure of membranes to trypsin.
[c] Ovomucoid added 90 min after exposure to trypsin.

Table 9. *Effect of trypsin on K^+-pump flux of LK red cells pretreated with anti-L serum*

Sheep	Pretreatment		K^+-Pump flux [mmole/(liter cells) × hr]
	First	Second	
LK 103 (LL) (exp. 152b)	None	Anti-L (1)	0.25
	Trypsin	Anti-L (1)	0.09
	Anti-L (1)	Trypsin	0.24
	None	HK NI	0.08
LK 188 (LL) (exp. 152c)	None	Anti-L (2)	0.34
	Trypsin	Anti-L (2)	0.14
	Anti-L (2)	Trypsin	0.34
	None	HK NI	0.12

valent to about 10^{10} LK red cells) did bind some of the K^+-pump stimulating antibody as seen in the last value of column 3 and compared to the effect on ${}^iM_K^P$ of anti-L absorbed with control membranes. These data suggest that trypsin-treated LK cells and their membranes have lost only part of their capacity to absorb the K^+-pump flux stimulating activity from anti-L serum. Apparently, modification of the L-antigen system by trypsin results in disappearance of the unique effect of the anti-L antiserum on ${}^iM_K^P$ in LK sheep red cells. This effect of trypsin, however, could be prevented when LK cells were pretreated with anti-L serum prior to trypsin treatment. Table 9 shows an experiment using LK red cells from two different animals.

Samples were first incubated for 1 hr at 32 °C in enzyme buffer with or without trypsin or with anti-L serum. All samples were then washed thoroughly in medium, incubated further with anti-L, trypsin or HK NI serum as indicated in the second column of the table and, after washing, assayed for K^+ flux. The results (Table 9, third column) indicate that preincubation of LK cells with anti-L serum protects the ability of LK cells to respond to K^+-pump stimulating activity of the anti-L serum, whereas trypsinized control cells did not reveal K^+-pump stimulation by anti-L serum. The comparatively lower stimulation of ${}^iM_K^p$ found in all samples may be ascribed to the two washing steps after the first and second pretreatment.

Attempts were made to identify trypsin-released membrane components which would inhibit the K^+-pump stimulating activity of anti-L serum. Incubation of equal volumes of supernatant obtained from trypsinized LK or HK red cells or the trypsin supernatant of LK membranes recovered after centrifugation of the trypsinized membranes at $27{,}000 \times g$ with anti-L antiserum did not reduce the K^+-pump stimulating activity of the antiserum even if as much as 8 mg protein/ml was tested in the assay. There was no inhibition of the pump-stimulating activity when anti-L serum was preincubated with the salt-free fractions nor with the organic material (aromatic peptides?) seen in the salt peaks subsequent to gel filtration of the trypsin supernatants on Sephadex G10. Furthermore, no inhibition of the pump-stimulating activity was observed when anti-L serum was exposed to trypsin-released protein extracted by the biphasic phenol-water system.

Lytic Activity. Since trypsin treatment of LK cells resulted in disappearance of their ability to respond to anti-L serum with an increased K^+-pump rate and the capacity of these cells to absorb the pump stimulating activity was reduced, it was of interest to study the effect of trypsin on the reaction of LK red cells with the lytic anti-L serum activity in the presence and absence of complement. Trypsinized and control LK cells were incubated for 90 min at 37 °C with decreasing concentrations of the two different antisera, anti-L (1) and (2) and complement. It was found that trypsin-treated red cells were more sensitive to hemolysis by anti-L serum than were untreated cells. Fig. 3 shows the ratio of the hemolysis found with trypsinized cells to the hemolysis with control cells plotted against the relative concentration of the two different anti-L sera. It can be seen that lysis of trypsinized cells was greater than that observed in control cells at higher concentrations of the anti-L sera. The slopes of the ratio for both antisera

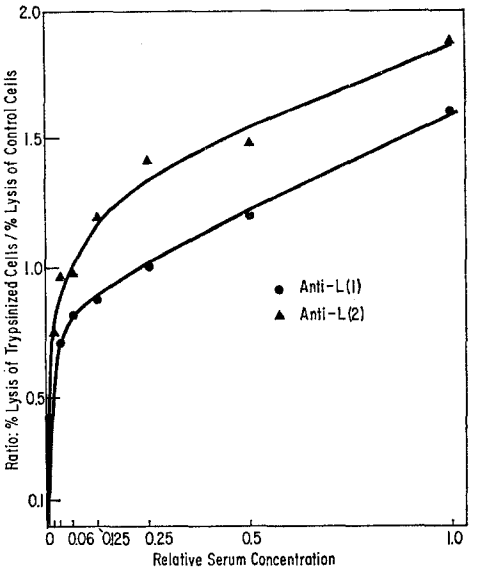

Fig. 3. Lytic reaction of trypsinized and control LK red cells with two different anti-L sera and guinea pig complement. The results are expressed as ratio of lysis (%) of trypsinized to lysis (%) of untreated control LK cells

in the concentration range from 1.0 to 0.25 are similar. This result is not consistent with the hypothesis that trypsin treatment makes LK cells more susceptible to lysis by anti-L serum by exposing more antigenic sites. If this were the case, the ratio plotted in Fig. 3 would be greater than unity, even at still lower antiserum concentrations. Table 4 contains the data of the experiment in which the absorption of the lytic anti-L activity by LK cells was studied. It can be seen that absorption of anti-L serum with trypsinized LK red cells lowered its property to lyze fresh test cells in the presence of complement to less than 10%. These experiments, therefore, demonstrated that the part of the L-antigen which reacts with the components in anti-L serum responsible for its lytic activity in the presence of complement is not altered by trypsin.

For the M-antigen in HK red cells, it was found that trypsin treatment did not impair immune lysis of HK (MM) red cells by anti-M, although enzyme-treated HK red cells again tended to lyze slightly better than their untreated control cells. Table 5 shows that the number of trypsinized red cells necessary to absorb 50% of the anti-M antibody is not significantly different from the number of control cells. Thus, the M antigen in HK red cells does not appear to be very susceptible to trypsin treatment under the conditions chosen. Since these experiments seem to preclude the possi-

bility of trypsin-released M-antigenic sites, no further attempts have bee made to demonstrate inhibition of anti-M antiserum with fragments release by trypsin from HK red cells.

Discussion

Neuraminic acid (sialic acid) is known to occur in terminal positio on the surface of all mammalian red cells α-0-glycosidically linked t protein-bound oligosaccharide side chains. It participates in a number o antigenic specificities such as the MN blood group antigens and the myxo virus receptor substances in red cell glycoproteins of man (reviewed b Springer, 1970), and in the infectious mononucleosis receptor substanc in sheep red cells (Springer & Rapoport, 1957). The negative charge of th red cells is mainly due to the carboxyl groups of sialic acid which ar ionized under physiological conditions. In sheep red cells, neuramini acid was found to be present in about equal amounts in the N-acetyl an N-glycolyl forms totaling about 7.6×10^6 molecules sialic acid per cel surface (Eylar, Madoff, Brody & Oncley, 1962). Our studies did not disclos any significant differences in the sialic acid content of HK and LK red cells and the total amount of sialic acid found per cell (7.4×10^6 molecules) does agree well with the findings of Eylar *et al.* (1962).

Our experiments show clearly that treatment of LK cells with neuramini dase did not affect their response to the K^+-pump stimulating activity in anti-L serum nor did it alter their capacity to absorb this activity from the anti-L serum. In addition, neuraminidase-treated LK cells also absorbed the lytic activity from the anti-L serum. Treatment of M-positive HK red cells with neuraminidase likewise did not inactivate the M-antigen on these cells as measured by their ability to absorb lytic activity from anti-M serum. In the presence of complement, trypsin-treated LK and HK red cells were still hemolyzed by anti-L and anti-M antisera, respectively. Trypsin also did not alter the capacity of LK and HK red cells to absorb the lytic activity from the anti-L and anti-M antisera.

In marked contrast, however, is the observation that trypsin treatment rendered LK cells incapable of responding to the K^+-pump flux stimulating activity present in anti-L serum, and also reduced their capacity to absorb this activity. This effect of trypsin, however, was prevented when LK cells were sensitized with anti-L serum prior to enzyme treatment.

The results are consistent with but do not prove the hypothesis that anti-L serum contains antibodies to two different L antigens. One antigen (L_{1y}) reacts with its antibody to produce lysis in the presence of complement.

Reaction of the other antigen (L_p) with its antibody leads to increased active K^+ transport. Trypsin affects neither the reactivity of the L_{1y} antigen with its antibody nor the lytic action of the antigen-antibody complex in the presence of complement. On the other hand, trypsin greatly reduced the reactivity of the L_p antigen with its antibody as expressed in the capacity of LL sheep red cells to bind and to respond to the K^+-pump stimulating activity in anti-L serum. This effect of trypsin could be due to either removal or modification (or both) of the L_p-antigenic sites. Experiments designed to test this tentative hypothesis are in progress in our laboratory.

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