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Alterations in the Immunogenicity and Antigenicity of Mammalian Erythrocytes Following Treatment with Neuraminidase[1] (37770)

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The treatment of cells with *Vibrio cholera* neuraminidase results in the removal of 2–3 and 2–6 glycosidically linked sialic acid from mucopolysaccharides, glycoproteins and glycolipids on the cell surface (1–3). Removal of membrane associated sialic acid has been reported to increase the immunogenicity of tumor cells (4–8), lymphocytes (9–13), astrocytes (14), and possibly trophoblast (15, 16). Although there are numerous reports on the effects of neuraminidase on the antigenicity of nucleated cells, only a few studies have dealt with nonnucleated erythroid cells (17, 18). Since the immune response to erythrocyte antigens has been so extensively studied in experimental animals, especially the mouse, it was of interest to investigate the effect which neuraminidase had on the antigenicity and immunogenicity of erythrocytes derived from a variety of commonly used animals. In contrast to the increased antigenicity of nucleated cells, neuraminidase treatment markedly reduced the antigenicity of both sheep and horse RBC, and the immunogenicity of sheep RBC. On the other hand, no decrease was observed in either immunogenicity or antigenicity of goat and chicken erythrocytes. These findings suggest that membrane associated sialic acid may have an important function in determining the antigenicity and immunogenicity of erythrocytes of some mammalian species.

Materials and Methods. Animals. BALB/c mice weighing approximately 18–20 g were obtained from either the Jackson Memorial Laboratory, Bar Harbor, ME or Carworth Farms, New City, NY.

Neuraminidase treatment of erythrocytes. Neuraminidase, obtained from Behring Diagnostics, Woodbury, NY, was isolated from a culture filtrate of *V. cholera*. The enzyme preparation had an activity of 500 neuraminidase units/ml and contained no other glycolytic or proteolytic activity. It was dissolved in a 0.05 *M* sodium acetate buffer solution (pH 5.5) with the addition of 9 mg of NaCl and 1 mg of $CaCl_2$/ml. This was diluted with phosphate buffered saline (PBS) (pH 7.4) to the following concentrations: 250, 100, 10 or 1 unit/ml. Sheep erythrocytes (SRBC), always obtained from the same animal, and chicken erythrocytes (CRBC) were purchased from the Colorado Serum Co., Denver. Goat (GRBC) and horse (HRBC) erythrocytes were obtained from the Animal Care Unit, University of Kansas Medical Center. Cells were washed 3 times in PBS (pH 7.4) and then adjusted to a final concentration of 2%. Four milliliters of a 2% suspension of erythrocytes were centrifuged at 400*g* for 10 min, the supernatant was removed, the pellet was resuspended in 1 ml of various concentrations of neuraminidase, and incubated for 1 hr at 37° with intermittent shaking. The cells were then sedimented at 400*g* for 10 min, resuspended in PBS and washed three times following which the final volume was adjusted to 4 ml. In some experiments neuraminidase was inactivated by boiling for 30 min and then used as described above. Although this treatment resulted in denaturation of the protein, no precipitate was noted. Mice were immunized with 1 ml of a 2% suspension of either untreated or

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TABLE I. Effect of Neuraminidase Treatment on the Immunogenicity of Sheep Erythrocytes.

Neuraminidase[a] (units)		Splenic plaque forming cells[b]		Log_2 of reciprocal serum titer[b]	
		Total	PFC/10^6	HA	Hem
None		83,677 ± 8815	536 ± 32	7.0	6.6
250	Active	5431 ± 373	38 ± 2	5.0	4.8
100	Active	13,298 ± 1654	113 ± 12	4.0	5.3
	Inactive	52,416 ± 728	511 ± 6	5.7	6.3
10	Active	8485 ± 3393	69 ± 19	4.0	4.7
	Inactive	68,711 ± 5894	433 ± 26	6.0	5.3
1	Active	52,775 ± 2760	406 ± 10	5.3	5.3

[a]Neuraminidase was inactivated by heating at 100° for 30 min.

[b]Splenic PFC and serum antibody titers were determined 4 days following immunization with 4 × 10^8 SRBC ip.

neuraminidase treated erythrocytes (4 × 10^8 cells) administered intraperitoneally (ip) as indicated in Tables I–III.

Antibody detection. Splenic plaque forming cells (PFC) to sheep, horse, and goat erythrocytes were assessed 4 days following immunization by means of the hemolytic plaque technique as described by Jerne, Nordin, and Henry (19) and modified by Mishell and Dutton (20). The response to chicken RBC was determined 7 days following immunization using the same procedure. Target erythrocytes were always of the same species as those used for immunization. In some experiments the PFC response was assessed against both untreated and neuraminidase treated erythrocytes. Spleen cells (0.02–0.04 ml) and 0.04 ml of a 50% suspension of either untreated or neuraminidase treated erythrocytes were added to 0.5 ml of a 0.75% solution of agarose (L'Industrie Biologique Francaise, Gennevilliers, France), poured on glass slides, and incubated for 1 hr at 37° in an atmosphere containing 5% CO_2. The slides were then flooded with a 1:10 dilution of reconstituted guinea pig serum (Hyland Laboratories, Costa Mesa, CA) and incubated for an additional hour at 37°. Total splenic PFC and PFC/10^6 spleen cells were determined. Means and standard errors were computed from pooled data and levels of statistical significance were determined by Student's t test.

Serum hemagglutinin and hemolysin titers were determined by the microtiter method as previously described (21) using either untreated or neuraminidase treated erythrocytes.

Results. Effect of neuraminidase treatment on the immunogenicity of sheep erythrocytes. Sheep RBC which had been treated with 250, 100 or 10 units of neuraminidase were significantly less immunogenic than untreated cells (Table I). Mice immunized with neuraminidase treated RBC had 5000–13,000 PFC compared to 84,000 in mice immunized with nontreated cells ($P < 0.01$). Heat inactivation of neuraminidase completely destroyed its ability to reduce the immunogenicity of erythrocytes indicating that the effect was specifically dependent upon enzyme activity. Ten units of neuraminidase effectively reduced the immunogenicity of 16 × 10^8 SRBC while 1 unit did not. Serum hemagglutinin and hemolysin titers were correspondingly reduced by 2–3 log_2 units in mice which had been immunized with neuraminidase treated cells.

Effect of neuraminidase treatment on the immunogenicity and antigenicity of sheep, horse, goat and chicken erythrocytes. The antibody response of mice immunized with either untreated or neuraminidase treated sheep, horse, goat and chicken erythrocytes was assessed independently against untreated and treated target RBC. Mice immunized with SRBC had a maximum response of 42, 000 PFC when assessed against untreated SRBC and 9300 when assessed against neuraminidase treated cells (Table II). Mice immunized with neuraminidase treated SRBC had 14,000 PFC against untreated and only

TABLE II. Effect of Neuraminidase Treatment on the Immunogenicity and Antigenicity of Sheep, Horse, Goat and Chicken Erythrocytes.

Immunizing[a] erythrocyte	Test erythrocytes	Splenic plaque forming cells[b]		Log$_2$ of reciprocal[c] serum titer	
		Total	PFC/10^6	HA	Hem
SRBC	SRBC	41,841 ± 3986	367 ± 24	6.5	7.0
	NA-SRBC	9275 ± 272	82 ± 24		
NA-SRBC	SRBC	14,016 ± 1038	145 ± 8	3.8	5.0
	NA-SRBC	4958 ± 514	51 ± 4		
HRBC	HRBC	20,955 ± 1790	173 ± 11	5.7	6.0
	NA-HRBC	20,575 ± 1301	168 ± 6		
NA-HRBC	HRBC	1087 ± 160	11 ± 2	2.5	3.0
	NA-HRBC	990 ± 188	10 ± 2		
GRBC	GRBC	26,544 ± 2400	216 ± 20	Nil	Nil
	NA-GRBC	57,873 ± 4593	471 ± 39	Nil (4.0)	Nil (4.0)
NA-GRBC	GRBC	23,910 ± 4829	215 ± 42	Nil	Nil
	NA-GRBC	50,283 ± 6607	499 ± 119	Nil (3.0)	Nil (3.0)
CRBC	CRBC	1000 ± 35	103 ± 2	5.9	6.0
NA-CRBC	CRBC	950 ± 45	102 ± 2	5.6	6.0

[a]Either untreated or neuraminidase treated (100 units) sheep (SRBC), horse (HRBC), goat (GRBC) or chicken (CRBC) erythrocytes were used for immunization.

[b]Splenic PFC and serum antibody titers were determined 4 days following immunization in all experiments except the response to chicken RBC which was studied at 7 days. PFC were assessed against both untreated (RBC) and neuraminidase treated erythrocytes (NA-RBC).

[c]Serum antibody titers routinely were determined against untreated erythrocytes. The serologic response to goat RBC also was assessed against neuraminidase treated cells and these values are included in parenthesis.

5000 against neuraminidase treated SRBC thus indicating a reduction in both immunogenicity and antigenicity of enzyme treated cells. Neuraminidase treatment of horse RBC resulted in a profound reduction in their immunogenicity (21,000 PFC decreased to 1000) but not in their antigenicity since there was no reduction in the number of PFC detected when assessed against either treated or untreated RBC. In contrast to these findings, there was an increase in the antigenicity (or sensitivity to hemolytic antibody and complement) of neuraminidase treated goat RBC. There was a doubling of the number of PFC detected (25,000 increased to 50,000) if neuraminidase treated RBC were used for assessment. Serum hemagglutinin and hemolysin titers were nil against untreated GRBC and ranged from 3 to 4 log$_2$ units when neuraminidase treated GRBC were used. Neuraminidase treatment of chicken erythrocytes had no effect on their immunogenicity although the maximum response was low (1000 PFC).

Effect of pH and sialic acid on neuraminidase activity. Neuraminidase in acetate buffer (pH 5.5) had greater activity than neuraminidase in phosphate buffer (7.4) correlating with the pH optimum of the enzyme (4, 5). Mice immunized with sheep RBC which had been treated with neuraminidase in acetate buffer had 3400 PFC compared to 11,300 in PBS (Table III). The addition of 1 or 2 mg of sialic acid to the erythrocyte suspension substantially decreased its effectiveness indicating that enzyme activity could be product inhibited. Furthermore, heating the enzyme at 100° for 30 min com-

TABLE III. Effects of pH and Sialic Acid on Neuraminidase Treatment of Sheep Erythrocytes.

Neuraminidase (units)	Treatment of erythrocytes	Splenic plaque forming cells[a]		Log_2 of reciprocal serum titer	
		Total	PFC/10^6	HA	Hem
100	Phosphate buffer (pH 7.4)	11,283 ± 174	116 ± 2	4.0	4.5
100	Acetate buffer (pH 5.5)	3377 ± 493	40 ± 5	3.0	3.3
100	Inactivated	52,416 ± 728	511 ± 6	7.0	7.7
100	1 mg Sialic acid	21,741 ± 1955	199 ± 14	4.5	5.0
100	2 mg Sialic acid	19,283 ± 603	175 ± 4	5.3	6.0
None	Phosphate buffer (pH 7.4)	41,841 ± 3986	367 ± 24	6.5	7.0

[a]Splenic PFC and serum antibody titers were determined 4 days following immunization with 4 × 10^8 SRBC ip.

pletely destroyed its ability to reduce the immunogenicity of sheep RBC.

Discussion. Treatment of sheep RBC with *V. cholera* neuraminidase profoundly reduced their immunogenicity in BALB/c mice and decreased their antigenicity *in vitro*. A reduction in the *in vivo* immunogenicity of horse RBC was noted although there was no corresponding decrease in their antigenicity. In contrast to these findings, no change in either immunogenicity or antigenicity was noted following neuraminidase treatment of chicken erythrocytes. The decreased immunogenicity and antigenicity of sheep erythrocytes was clearly related to the removal of membrane associated sialic acid since the effect could be abrogated either by the addition of sialic acid or by heat inactivation of the enzyme.

There are a number of possible explanations for the reduction in immunogenicity and antigenicity which we have observed following neuraminidase treatment of sheep and horse RBC. The removal of sialic acid from glycoprotein isolated from sheep plasma has been reported to produce conformational changes and a loss of biological activity (22). Although these changes were not associated with an alteration in immunogenicity of the plasma glycoprotein (22), it has been reported that neuraminidase treatment of human erythrocytes removed M and N substance (a glycoprotein) from the cell surface (23). Since sialic acid plays such a dominant role in the biosynthesis of blood group antigens (24), its removal also might result in antigenic modification of the plasma membrane. Others have shown that neuraminidase treatment of sheep erythrocytes can produce a marked reduction in electrokinetic charge and electrophoretic mobility (25, 26). Such treatment also resulted in increased phagocytosis of erythrocytes (27) and this has been associated with a concomitant decrease in their immunogenicity (28).

Under other experimental conditions neuraminidase treatment of sheep erthrocytes has been reported to produce an increase in their immunogenicity and antigenicity (29). In these studies 500 units of enzyme were routinely employed. If smaller quantities were used, then results similar to our own were obtained (30). The observation that neuraminidase treatment may also decrease the immunogenicity of cells is of particular importance to clinicians contemplating immunotherapy protocols employing neuraminidase treated tumor cells. It may explain the paradoxical observation that under some circumstances neuraminidase treatment actually may cause enhanced tumor growth (30). It should be apparent from both our own findings and those of Schmidtke and Simmons (29) that neuraminidase treatment can result in an increase, decrease or no change in the immunogenicity and antigenicity of erythrocytes and no simple generalizations can be made.

Summary. The immunogenicity and anti-

genicity of sheep, horse, goat and chicken erythrocytes (RBC) were studied following their exposure to *Vibrio cholera* neuraminidase. BALB/c mice immunized with neuraminidase treated sheep or horse RBC had approximately a 15–20-fold decrease in the number of splenic plaque forming cells and corresponding reductions in serum hemagglutinin and hemolysin titers. When neuraminidase treated sheep erythrocytes were used as target cells in the hemolytic plaque assay a fourfold reduction in PFC was noted. These data suggest that sialic acid is an important constituent of glycoprotein antigens of sheep and horse erythrocyte membranes. In contrast to these findings, there was no alteration in either immunogenicity or antigenicity of chicken erythrocytes following their treatment with neuraminidase.

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