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## Carbohydrate interference of complement-dependent cell lysis

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*Summary.* The antibody-mediated cytotoxicity of three autoreactive sera, an allogeneic hyperimmune serum and a xenogeneic hyperimmune serum was abrogated by the presence of either glucosamine, galactosamine, lactulose or lactose. This inhibition could be overcome in a dose-dependent fashion by increasing the amount of complement in the cytotoxicity assay, but not by increasing the amount of antibody. Furthermore, the inhibition was specific for these sugars in that isomers and N-acetylated derivatives were not inhibitory. The results suggest that these sugars directly blocked events of the complement cascade.
*Key words.* Complement; antibody; carbohydrate; cell-lysis.

Carbohydrates have been shown to inhibit a variety of biological phenomena such as antibody-mediated lysis[1], antigen-induced lymphocyte proliferation[2], natural killer cell reactions[3], mixed-lymphocyte reactivity[4], tumoricidal lymphocytes[5] and adhesion of macrophages[6]. I have reported the existence of autoreactive antibodies in the sera of tumor-bearing, genetically autoimmune and alloimmunized mice[7,8], which were detected by their cytotoxicity against neuraminidase-treated syngeneic spleen cells. Together, these observations motivated attempts to identify putative carbohydrate-like antigens at the surface of neuraminidase-treated cells by blocking techniques using carbohydrates in aqueous solution. However, during the course of the experiments, it became apparent that certain carbohydrates specifically blocked antibody-mediated lysis in a fashion that suggested carbohydrate-mediated block of the complement (C) cascade.

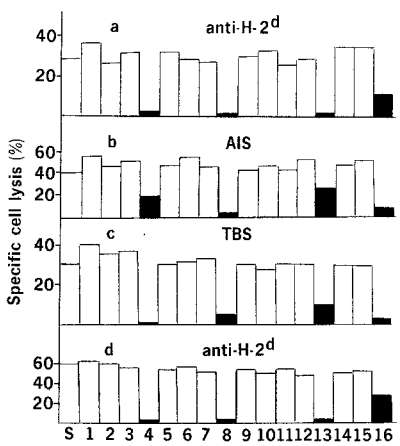


Figure 1. Percent specific cytotoxicity of antisera for murine spleen cells, in the presence of either saline or cabohydrates. *a–c* Three autoreactive sera incubated with neuraminidase (VCN)-treated spleen cells. Anti-H-$2^d$ serum was a polyclonal, hyperimmune C57BL/6 anti-DBA/2. Autoimmune serum (AIS) was from NZBWF1 mice greater than 8 months of age. Tumor-bearing serum (TBS) was from L1210 lymphoma-bearing B6D2F1 mice. These 3 sera have complement-dependent cytolytic antibodies against VCN-treated syngeneic cells. This cytotoxicity was blocked by 75 mM solutions of carbohydrates 4, 8, 13 and 16. *d* Inhibition of cytotoxicity of the anti-H-$2^d$ serum by the same carbohydrates as above, when the sera was reacted with normal DBA/2 spleen cells. Code for carbohydrate solution: Control, isotonic saline; 1 = D-(+)-glucose, 2 = βD-(+)-glucose, 3 = 2-deoxy-D-glucose, 4 = D-(+)-glucosamine, 5 = α-Methyl-D-glucoside, 6 = N-acetyl-D-glucosamine, 7 = D-(+)-galactose, 8 = D-(+)-galactosamine, 9 = α-Methyl-D-galactoside, 10 = N-Acetyl-D-galactosamine, 11 = α-D-(+)-fucose, 12 = α-L-(−)-fucose, 13 = β-D-lactulose, 14 = α-Methyl-D-mannoside, 15-N-Acetyl-β-D-mannosamine, 16 = α-lactose.

*Materials and methods. Preparation of serum.* The three autoreactive sera used in this study have been described[7,8]. Tumor-bearing serum (TBS) was defined as the sera obtained from B6D2F1 mice on day 5 following an injection of L1210 leukemia cells. Autoimmune sera (AIS) was obtained from NZBWF1 mice of greater than 8 months of age and known to contain autoreactive antibodies. Anti-H-$2^d$ sera was prepared by 4 monthly injections of DBA/2 liver and spleen cells (H-$2^d$) into the peritoneal cavity of histoincompatible C57BL/6 mice (H-$2^d$). Sera were collected from clotted blood after bleeding the tail ventral artery into ice-cold plastic tubes. These sera were cytotoxic to a variety of neuraminidase-treated (*Vibrio cholera*, Grand Island Biological Co.) cells at a maximum titer of 1:8 with greater than 90% cell kill. Rabbit anti-mouse sera (Cappel Laboratories) was used at a 1:20 dilution (40–70% cytotoxicity against murine spleen cells). All mice, except for the NZBWF1 (ORU Biomedical Research Center breeding facilities) were obtained from the Jackson Laboratories.

*Neuraminidase treatment.* Murine spleen cells were washed twice in RPMI 1640 medium containing 1% heat-inactivated fetal calf serum (1% medium) and treated with VCN as previously described[7]. VCN treatment was 1 unit/$10^6$ cells, pH 5.6, 1 h, 37 °C.

*Cytotoxicity assay.* Washed, untreated or VCN-treated cells were labeled with $^{51}Cr$ (100 μCi per $10^7$ cells, 3 h, 37 °C) and triplicate determinations of the complement-dependent cytotoxicity of the sera were made as follows: 50 μl of labeled cells were added to equal volumes of antisera (diluted 1:5) which had been preincubated with saline (positive control) or a panel of isotonic carbohydrate solutions used at a final concentration of 75 mM.

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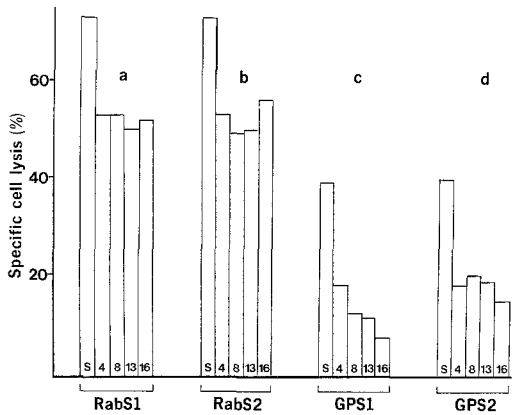


Figures 2. Inhibition of rabbit anti-mouse serum cytotoxicity toward DBA/2 cells by D-glucosamine, D-galactosamine, $\beta$-D-lactulose and $\alpha$-lactose. The cytotoxicity (and inhibition) assay was performed as described in fig. 1 using 4 different sources of complement; two normal rabbit whole sera (RabS) and Lot No. 41307 and 41090 of guinea pig whole sera (GPS) from M.A. Bioproducts. *a, b* two different rabbit sera; *c, d* two different guinea pig sera. S = Saline, 4 = D-glucosamine, 8 = D-galactosamine, 13 = $\beta$-D-lactulose, 16 = $\alpha$-lactose. Each bar represents the mean of triplicate determinations with a standard deviation of < 5%. Note that the rabbit sera results in greater cell lysis when used as a source of complement for the rabbit anti-mouse antibodies than the use of guinea pig sera.

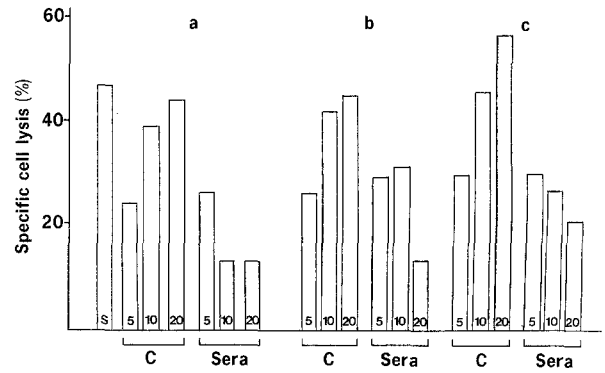


Figure 3. Reversal of carbohydrate-inhibition of cytotoxicity of rabbit anti-mouse serum toward DBA/2 cells by increasing the amount of complement, not serum. The cytotoxicity (and inhibition) assay was performed as described in fig. 1. *a* inhibiting carbohydrate was 75 mM D-glucosamine, *b* inhibition by 75 mM D-galactosamine; *c* inhibition by 75 mM $\alpha$-lactose. Percent specific cytotoxicity is shown from triplicate samples of cells incubated with saline alone (S) plus 5 µl C; and left to right for each panel, 5 µl C+sugar, 10 µl C+sugar, 20 µl C+sugar, 5 µl C+additional 5 µl serum, 5 µl C+additional 10 µl serum, 5 µl C+additional 20 µl serum. Standard deviations were all < 5%. Control samples of rabbit anti-mouse serum plus 10 µl and 20 µl C did not result in greater cytotoxicity than 5 µl C shown in the figure. The noticeable decrease in cell kill using increased levels of rabbit anti-mouse serum is attributed to high-serum prosone effects frequently noted in high-titer serum studies.

The sera was left to react with the cells for 30 min, followed by the addition of 5 µl complement (whole guinea pig serum, M.A. Bioproducts) and the release of $^{51}Cr$ from lysed cells was measured 2 h later. Spontaneous $^{51}Cr$ release was less than 2% of total counts per min and addition of complement alone resulted in the release of less than 5% of the total counts per min contained in the cells. Standard deviation of triplicate measurements was less than 5%. Percent specific cytotoxicity was computed by measuring the cpm of (cells plus serum plus complement, C) minus (cells plus C) divided by (total cell cpm minus background) times 100.

*Results.* As shown in figure 1 *a–c*, the antibody-mediated lysis of VCN-treated murine cells by three autoreactive sera was inhibited in an identical manner by D-(+)-glucosamine, D-(+)-galactosamine, $\beta$-lactulose and $\alpha$-lactose (all lytic assays required the presence of complement). It seemed plausible that these carbohydrates might have inhibited lytic events resulting from the C-cascade, rather than block antibody-binding to putative carbohydrate-containing antigens. Hence, additional experiments using polyclonal allogeneic and xenogeneic antisera were designed to test this hypothesis. The results shown in figure 1*d* unexpectedly confirmed that addition of the same four carbohydrates to the cytotoxic assay blocked lysis of untreated DBA/2 cells by hyperimmune anti-H-$2^d$ serum. Furthermore, in all of the studies described here, the sugars merely needed to be present at the time C was added to the assay. The same results were obtained whether sugars were combined with either antisera or cells prior to the addition of C (data not shown). The inhibition was probably due to low-affinity binding, since at least 50–75 mM carbohydrate was required in the assay, and inhibition was abolished at lower concentrations.

These same carbohydrates also blocked the lysis of DBA/2 cells by a potent rabbit anti-mouse serum (RAMS), using 4 different sources of complement (fig. 2). The most convincing argument that the carbohydrates interfered with C-mediated events is shown in figure 3. DBA/2 cells were incubated with either increasing amounts of C, or additional amounts of RAMS, in the presence of 75 mM sugar. Specific cell lysis was about 45% for cells treated with RAMS, C and saline, compared to 24, 26 and 30% when either glucosamine, galactosamine or lactose was added to the C, respectively. This inhibition was easily overcome, in a dose-dependent fashion, by increasing the amount of C, but not antibody. Control lysis using C alone was less than 5% cell kill.

*Discussion.* Several features of these results argue that these carbohydrates interfered with the C-cascade. First, it seems highly unlikely that 5 different, polyclonal antisera could have complement-fixing antibodies directed against carbohydrate antigens only; indeed,it is virtually impossible for allogeneic and xenogeneic antisera[9,10]. In addition, possible osmotic effects of these sugar solutions on C-mediated lysis[11] seems improbable in that all the tested sugar solutions were of the same ionic strength. As shown above, the carbohydrate interference could be quickly abrogated by increasing the amount of C, but not antisera.

Similar results to those presented here have been reported by Lambre et al.[12] who observed that mannose, glucose and galactose abrogated the cytolytic activity of autologous sera against neuraminidase-treated guinea pig erthrocytes. These authors concluded it was possible that the autoantibodies were directed against carbohydrate-containing epitopes, in contrast to anti-complement activity associated with the carbohydrates. In addition, galactose and N-acetylgalactosamine inhibited the cytolysis of human neuraminidase-treated erythrocytes by autologous sera, whereas glucose and mannose had no effect. The proof that such inhibition was not anti-complementary rested in the observation that the hemolytic activity of the sera against sensitized sheep erythrocytes was not affected by carbohydrates. However, this observation does not exclude the possibility that early events of the complement cascade were inhibited by the carbohydrates, since sensitized cells may be lysed by activation of the alternate complement pathway[15].

Indeed, the results reported here support the more direct studies by Mussel et al.[14] who showed that the c3b complement receptor of human erythrocytes may involve carbohydrate, since the binding of c3b was destroyed by periodate treatment of the cells, and specifically inhibited by galactose and glucose.

Complement-mediated lysis of mammalian cells involves the sequential activation of cell-bound, serum-derived proteins[15]; and the nature of C receptors is not entirely known, but they do involve glycoproteins[16]. How the carbohydrates used in the

present study blocked C-cascade events normally resulting in cell lysis is quite unclear. Several mechanisms should be considered. Perhaps the carbohydrates bind to complement components themselves, rendering them unable to bind cell membrane receptors. Related to this suggestion would be the possibility that carbohydrate-binding to certain complement molecules may inhibit enzymatic events needed to complete the sequences of the cascade necessary for cell lysis. However, it seems equally reasonable that the carbohydrates may bind to cell membrane receptors for complement in a fashion that either blocked complement attachment or impeded further activation events at the cell surface. This latter mechanism suggests that components of the C-cascade and carbohydrates may compete for common binding sites at the cell membrane, an attractive possibility in light of other studies which demonstrated that C-fragments bind to conglutinin by carbohydrate moieties found on the complement molecule, and the direct lytic action of melittin, a hydrophobic peptide thought to disrupt membranes in an analogous manner to the C-cascade, was inhibited in a competitive fashion by galactosamine and glucosamine[17]. No matter what mechanism, the interference was specific with regard to both the availability of a reactive C-2 group on the sugars, and the observations that neither isomers, such as mannose, nor related molecules, such as N-acetylated carbohydrates blocked C-mediated lysis.

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## Human specific immunoglobulin protects against infection with common *Staphylococcus* in mice

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*Summary.* Mice infected with non-capsulated *Staphylococcus aureus* strains highly resistant to methicillin survived after the administration of specific immunoglobulin extracted from pooled human sera by using homologous capsular type strains, but no protective effect was shown with a conventional immunoglobulin preparation and methicillin, even with high doses.
*Key words.* Immunoglobulin; *Staphylococcus aureus;* methicillin.

Despite the fact that therapeutic effects of conventional immunoglobulin preparations vary considerably, they have been widely applied for infectious diseases[1]. Recently, we extracted specific immunoglobulin from pooled human sera against several species of capsulated bacterial strains including *Staphylococcus aureus,* and significant prophylactic effects of the preparation were observed in mice[2]. For the practical use of immunoglobulin preparations against *S. aureus,* however, the preparation would also be required to overcome antibiotic-resistant non-capsulated strains, since the majority of causative organisms are of this type. The experiments were designed to observe whether the specific immunoglobulin preparations were effective against infection with common *S. aureus* strains.

Four *S. aureus* strains were used for challenge infection in mice. The minimal inhibitory concentration of methicillin for these strains was more than 100 μg per ml, tested by the method described in the Manual of Clinical Microbiology[3]. Although they were non-capsulated as determined by the criteria proposed by Yoshida and Minegishi[4], the strains MRSA-198 and MRSA-580 both had a capsular type antigen A and B, while both strains MRSA-121 and MRSA-125 were of the bivalent capsular type, A plus B, tested by the method of Yoshida et al.[5]. For the extraction of the specific immunoglobulin, eluate containing specific immunoglobulins was obtained from an antigen-antibody complex using propionic acid in the presence of 5% sucrose by a method noted elsewhere[2]. Antigen used for elution was Smith surface antigen[6] (SSA), the protection-inducing antigen of the Smith strain, capsular type A, and whole cells of the ATCC-21734 strain, capsular type B, both of which strains were capsulated and methicillin-sensitive. The capsular types were determined by the method of Yoshida[7]. Protein and immunoglobulin levels in the eluate were measured by the method of Lowry et al.[8] and by using immunoplates (Hyland).

Mice infected with MRSA-198 strain (capsular type A) were completely protected from lethal effects by treatment with an amount of eluate from SSA containing 0.06 mg protein. The amount was the same doses as that for the Smith strain in mice. With challenge by the MRSA-580 strain (capsular type B) plus treatment with an eluate obtained using the ATCC-21734 strain, containing 0.12 mg protein, all animals survived. Eluate containing double the amount of protein content was required for complete protection against ATCC-21734 strain. For the challenge infection with strains MRSA-121 or MRSA-125 (bivalent capsular type) the animals were primarily treated with eluates obtained using the SSA or ATCC-21734 strain; however, the animals succumbed even when given high doses of those eluates. Minimum protein amounts of the eluates capable of protecting against challenge with the single capsular type of the capsulated strains A and B were combined and administered. Animals survived otherwise lethal infections with strains MRSA-121 or MRSA-125 with these doses (table 1). In these experiments, no effect was shown even with 2.36 mg of Venoglobulin I (Midori-Juji Pharmaceutical Co. Ltd., Tokyo), a preparation that includes biological properties of IgG and does not contain isolated