Interaction between Complement Proteins C5b-7 and Erythrocyte Membrane Sialic Acid

By Patricia Marshall,* Akira Hasegawa,‖ Eugene A. Davidson,§ Victor Nussenzweig,¶ and Michael Whitlow*‡

*From the *Dermatology Service, Veterans Affairs Medical Center, New York 10016; ‡Ronald O. Perleman Department of Dermatology, and §Michael Heidelberger Division of Immunology, Department of Pathology, New York University Medical Center, New York 10016; ‖Department of Applied Bioorganic Chemistry, Gifu University, Gifu 50-11, Japan; and ¶Department of Biochemistry and Molecular Biology, Georgetown University Medical School, Washington, District of Columbia 20007*

Summary

The initial phase of membrane attack by complement is the interaction between C5b6, C7, and the cell membrane that leads to the insertion of C5b-7. Here we investigate the role of sialic acid residues in the assembly of C5b-7 intermediates on erythrocyte cell membranes. We find that C5b6 binds to glycophorin, whereas C5 or C6 does not bind, and desialylation of the glycophorin abolishes C5b6 binding. Complement lysis is inhibited by either masking glycophorin sialic acid with F_{ab} fragments of an mAb, or by removal of the sialylated region of glycophorin by mild trypsinization. Gangliosides inhibit C5b-7 deposition when added to the aqueous phase. Asialogangliosides and synthetic gangliosides lacking the carboxylic acid residue have no inhibitory activity. We conclude that C5b6 binds to sialylated molecules on the erythrocyte surface. We propose a new model of membrane attack in which C5b6 initially binds to membranes via ionic forces. C7 then binds to C5b6, disrupting the ionic interaction and leading to the exposure of hydrophobic domains. Sialic acid is known to inhibit complement activation. Thus, these findings reveal a paradoxical role for sialic acid in complement attack; the presence of sialic acid inhibits the generation of C5b6, but once the membrane attack pathway is initiated, sialic acid enhances complement lysis.

The membrane attack complex of the complement system consists of five proteins, C5, C6, C7, C8, and C9 (1–3). On activation, these proteins expose hydrophobic residues, insert into membranes, and form *trans*-membrane channels (2, 3). The process of channel formation can be divided into two phases: the initial interaction between the cell membrane, C5b6, and C7 to form a stably inserted C5b-7 complex, and the subsequent addition of C8 and multiple C9 molecules to form a *trans*-membrane channel (4, 5). The initial association of C5b-7 with the membrane is of central importance because it commits the C5b-9 complex to a particular site on the membrane at which the C5b-9 complex will assemble. We have previously shown that when erythrocytes are treated with C5b-9, the composition of the membrane that is shed as the cell vesiculates differs from the composition of the bulk plasma membrane, implying that the channels localize to certain membrane domains (reference 6 and Marshall, P.M., and M.B. Whitlow, unpublished observations). In addition, if the C5b-7 complex does not associate productively with the membrane, it can serve as an inhibitor of C5b-9 formation by binding to vitronectin and SP 40-40 to bind irreversibly C8 and C9, making them unavailable for channel formation (7, 8). Despite the important role of the membrane insertion of C5b-7, the precise mechanisms involved in this interaction are unknown. The current view is that after proteolytic activation of C5, the C5b fragment binds to C6. The C5b6 complex remains loosely bound to the cell surface, but the specific membrane molecules have not been identified (9, 10). Some evidence indicates that C5b6 may remain associated with the C3b subunit of the C5 convertase (9, 11). Other findings have suggested that ionic forces play an important role in C5b6 binding and C5b-7 deposition. Silversmith and Nelsestuen showed that binding of C5b6 to lipid vesicles is dependent on lipid composition; C5b6 bound to vesicles composed of either phosphatidic acid or phosphatidylglycerol, but did not bind to vesicles composed of phosphatidylcholine, phosphatidylserine, or phosphatidylinositol (12). In addition, polyanions such as heparin decrease membrane deposition of C5b-7 (13, 14). At physiological pH eukaryotic cells are negatively charged, thus the inhibitory activity of polyanions may represent a competition with the cell surface for binding (15).

Although these observations highlight the participation of anionic surface molecules in the assembly of C5b-7, the structure of these surface molecules has not been defined.

By virtue of their multiple sialic acid groups, glycophorin and gangliosides are the major polyanions of erythrocyte membranes. The present investigation is aimed at defining the role of these sialic acid moieties in C5b67 membrane assembly.

Materials and Methods

Buffers and Solutions. All buffers were prepared with glass-distilled water and ultrafiltered before use. PBS was composed of 150 mM NaCl, 5 mM sodium phosphate, pH 7.4. $DGVB^{2+}$ was composed of veronal-buffered saline containing 71 mM NaCl, 139 mM dextrose, 2.5 mM sodium veronal, (pH 7.4), 0.1% gelatin, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$. GVB^{2+} veronal buffered saline containing 142 mM NaCl, 2.5 mM sodium veronal (pH 7.4), 0.1% gelatin, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$.

Sialic Acid Determination. Erythrocytes were lysed and the membranes washed five times in 5 mM sodium phosphate buffer, pH 7.8. The membrane pellet was resuspended in an equal volume of phosphate buffer, followed by an equal volume of 0.2 M H_2SO_4 (0.1 M final concentration). The membranes were then hydrolyzed at 80°C for 60 min and the free sialic acid released assayed by an adaptation of the thiobarbituric acid method (16).

Erythrocytes and Complement Components. Guinea pig erythrocytes were stored in Alsever's solution until use, and washed three times with veronal-buffered saline containing 142 mM NaCl and 5 mM sodium veronal, pH 7.4. Human erythrocytes were collected by venipuncture from volunteers. Blood was initially collected in buffered EDTA solution, washed, and stored in $DGVB^{2+}$. Throughout this study, only human complement components were used. C5b6 was prepared from plasma according to Yamamoto and Gewurz (17) with minor modifications (18). C7, C8, and C9 were purchased from Quidel (San Diego, CA).

Incorporation of Gangliosides into Erythrocytes. For these experiments ganglioside GT_{1b} (Sigma Chemical Co., St. Louis, MO), at two concentrations, 7.6 μg/ml and 3.8 μg/ml, was incubated with guinea pig erythrocytes (8.7×10^8/ml) for 2 h at 37°C. Cells were then washed three times in PBS containing 0.25% BSA. An aliquot of cells was then further washed and analyzed for sialic acid. To calculated ganglioside incorporation, we measured sialic acid, which is 37.9% by weight of ganglioside GT_{1b}. Cells were then used in hemolytic assays.

Hemolytic Assays. Hemolytic assays were performed as in (18). Briefly, 0.1 ml of 1.5×10^8 erythrocytes were incubated with various dilutions of C5b6 at 30°C for 30 min. C7 was then added and the mixture incubated at 30°C for an additional 15 min. C8 and C9 were then added and the cells incubated at 37°C for 60 min. Cells were then pelleted and hemolysis estimated by the absorbance of hemoglobin at 412 nm. In experiments in which gangliosides were added as complement inhibitors, the molecules of interest were added immediately before C5b6 addition. In several experiments gangliosides or glycophorin were added with C8 and C9. In these experiments, the cells were washed after the incubation with C5b6 and C7. Cells were then resuspended, the inhibitor added, C8 and C9 added, and the cells incubated at 37°C for 60 min. C5 assays were carried out using GPE[1] and C5-deficient human serum (C5DS) (Quidel). 0.1 cc of guinea pig erythrocytes (GPE) ($1.5 + 10^8$/ml) in GVB^{2+} were incubated with 0.2 ml C5DS supplemented with 4 μg Clq (Quidel). 0.1 ml of the column fractions was added, and the cells incubated at 37°C for 60 min and hemolysis measured spectrophotometrically. C6 was assayed in a similar manner using C6-deficient serum (Quidel).

Treatment of Human Erythrocytes with Trypsin. Human erythrocytes (2.5 ml, 2×10^9/ml) were incubated with 100 μl of L-(tosylamido 2-phenyl)ethyl chloromethyl ketone-treated TPCK-trypsin (Sigma) in 50 mM sodium phosphate, pH 7.5, at 37°C for 45 min. The reaction was stopped by the addition of 750 μg soybean trypsin inhibitor (Sigma) and allowed to incubate at room temperature for 5 min. Cells were washed four times in PBS and stored in $DGVB^{2+}$. To assess the degree of glycophorin cleavage, the cell membranes were run on SDS PAGE, and Western blotting was performed using monoclonal antiglycophorin antibody (Sigma). This antibody binds to glycophorins A and B. Cleavage of glycophorin was estimated by the disappearance of the glycophorin A dimer at 85 kD. The degree of cleavage was estimated visually.

Glycophorin-Sepharose and Asialoglycophorin-Sepharose Columns. Cyanogen bromide–activated Sepharose (CNBr-Sepharose) was purchased from Pharmacia Biotech Inc. (Piscataway, NJ), and was used according to the manufacturer's instructions. Briefly, the gel was washed with 1 mM HCl, pH 3.0. Human glycophorin (Sigma) was resuspended in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.36) and added to the activated CNBr-Sepharose with mixing overnight at 4°C. 7 mg glycophorin was coupled to 7.0 ml CNBr-Sepharose. Coupling efficiency was 83%. The column was then blocked with 0.1 M Tris, pH 8.0, for 4 h at room temperature. Beads were washed in PBS. An affinity column (3.0 × 1.5 cm) was prepared, and the column was stored at 4°C in PBS-azide until use. To desialylate the glycophorin, the glycophorin-sepharose column was loaded with *Vibrio cholerae* sialidase. 1 U sialidase in 2.5 ml was added to the column and incubated at room temperature for 60 min. The eluate was monitored for free sialic acid, and 100% of the sialic acid was released by this procedure. For the affinity column experiments, 37 μg of C5, 37 μg of C6, or 37 μg of C5b6 was dialyzed against 10 mM sodium phosphate, pH 7.5. The complement component of interest was loaded onto the column, and the column washed with two column volumes of 10 mM sodium phosphate, pH 7.5. Bound material was eluted with two-column volume gradient to 10 mM sodium phosphate/100 mM NaCl, pH 7.4. Fractions were tested for hemolytic activity and conductivity measured at 22°C.

Generation and Use of F_{ab} Fragments of Antiglycophorin Antibodies. Monclonal antiglycophorin antibodies 3H2 and 2B10 were generated as in reference 19. Both are mouse IgG_1, K. The antibody was purified from ascites using an anti–mouse IgG_1-agarose column (Sigma). F_{ab} fragments were produced by the method of Mishell and Shiigi (18a). Briefly, antibody was dialyzed against 0.1 M phosphate 4×10^{-3} M EDTA, pH 7.0. The antibodies were incubated with mercuripapain (Sigma) (2% wt/wt), 10^{-3} M cysteine, 4×10^{-3} M EDTA, for 4 h at 37°C. Reaction was stopped by addition of 50 μl of 0.3 M iodoacetamide, pH 8.0. The mixture was held on ice for 30 min and dialyzed against 0.1 M Tris, pH 8. F_{ab} fragments were purified by absorption of undigested antibodies and F_c fragments to proteins G beads (Pharmacia), and purity of the F_{ab} fragments confirmed by SDS-PAGE. For the experiments using the F_{ab}, 100 μl of human erythrocytes (1.5×10^9/ml) was incubated with various dilutions of F_{ab} fragments for 30 min at 37°C. Cells were washed twice in GVB^{2+} and used in a hemolytic assay.

[1] *Abbreviations used in this paper:* CNBr-Sepharose, cyanogen bromide–activated Sepharose; GPE, guinea pig erythrocytes; IC_{50}, concentration of ganglioside that inhibited lysis by 50%; SCR, short consensus repeat; TS1, thrombospondin type 1.

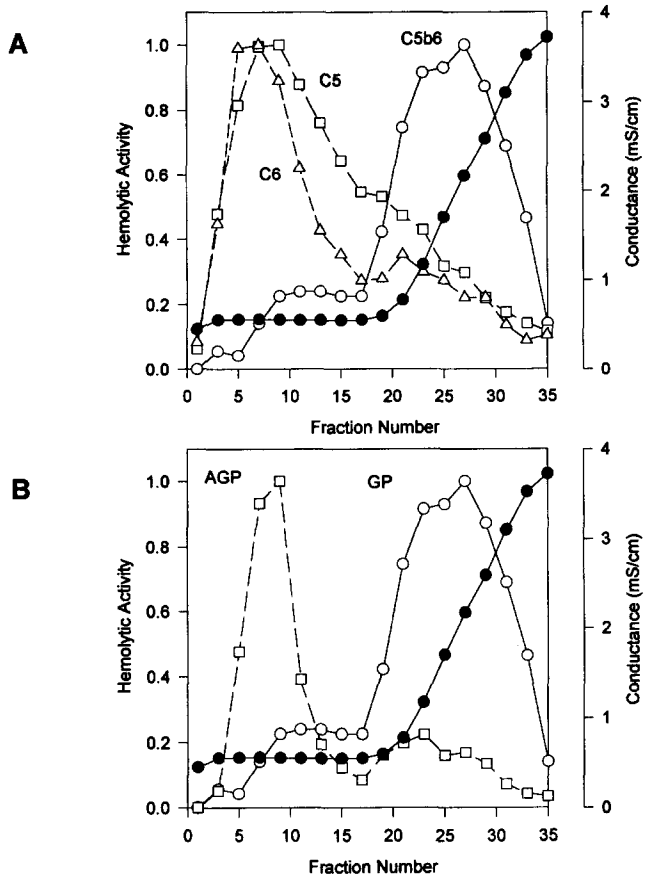

Figure 1. C5b6 binds to glycophorin-Sepharose but not to asialoglycophorin-Sepharose. (*A*) C5, C6, or C5b6 was loaded onto a glycophorin-Sepharose column in 10 mM sodium phosphate, pH 7.5. After washing, the column was eluted with a gradient from 0 to 0.1 M NaCl in 10 mM sodium phosphate, pH 7.5. Shown are C5 (□), C6 (△), C5b6 (○), and conductance (●). (*B*) Experiments were conducted on the same column as *A*, before and after neuraminidase treatment. C5b6 was loaded onto the column, and the chromatography performed as outlined in *A*. The elution profiles from asialoglycophorin (□), glycophorin (○), and conductance (●) are shown.

Gangliosides. (R = -CH_2CH(NH-CO-$C_{17}H_{35}$)-CHOH-CH=CH-$C_{13}H_{27}$, Cer, ceramide); asialo GM_1, galactose-ceramide (GalCer); GM_1, GM_2, GD_{1b}, and GT_{1b} were purchased from Sigma. Monosialoganglioside GM_3, Neu5Ac(α2-3)DGal(β1-4) DGlc-O-R_1, GSC-17 (20); Neu5Ac(α2-6)DGal(β1-4)DGlc-O-R_1, GSC-61 (21); 7-deoxy GM_3, GSC76 (22); 4-methoxy GM_3, GSC78 (23); 8-deoxy GM_3, GSC153 (24); Neu5Ac(α2-3)DGal-Cer, GSC26 (25); Neu5(OH)(α2-3)DGal(β1-4)DGlc-Cer, GSC198 (26); Neu5(OH)(α2-3)DGal-Cer, GSC113 (27); O-95-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)-(2-3)-O-β-D-glyucopyranosyl-(1-1)-(2S,3R,4E)-2-octadecanamido-4-octadecene-1,3-diol, GSC80 (28).

Results

Affinity Chromatography on Gluycophorin-Sepharose and Asialoglycophorin-Sepharose. To demonstrate binding to glycophorin, C5, C6, or C5b6 was chromatographed on glycophorin-agarose. Fig. 1*A* shows that C5b6 is retained, while C5 or C6 alone is eluted in the breakthrough. Fig. 1 *B* shows that C5b6 binding requires sialylated glycophorin, since treatment with sialidase abolishes the interaction.

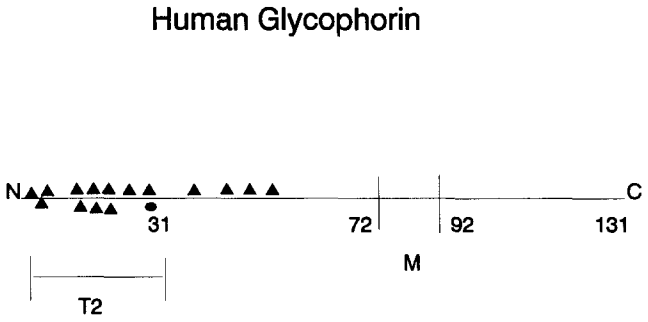

Figure 2. Structure of glycophorin. The O-glycosidically linked tetrasaccharides (▲) and the asparagine-linked oligosaccharide (●) are indicated. T2, fragment released by trypsin; membrane-spanning domain.

Effect of Antiglycophorin mAbs on C5b-9–mediated Lysis. To assess the functional consequences of C5b6 binding to membrane glycophorin, we performed blocking experiments using F_{ab} fragments of the monoclonal antiglycophorin antibodies, 2B10 and 3H2. Fig. 2 is a diagram of human glycophorin. It consists of 131 amino acids in three domains: an extracellular domain (amino acids 1–72), a transmembrane domain (amino acids 73–91), and an intracellular domain (amino acids 92–131). Glycophorin is readily cleaved by trypsin on the COOH-terminal side of the arginine residue at amino acid 31 to release the T2 fragment. T2 contains 11 of the 15 O-glycosidically linked tetrasaccharides (each tetrasaccharide contains two sialic acid residues). mAB 2B10 binds to sialic acid residues on the T2 fragment and 3H2 binds in close proximity to the membrane (amino acids 55–70) (19). On the basis of our binding data, we reasoned that 2B10, but not 3H2, might block

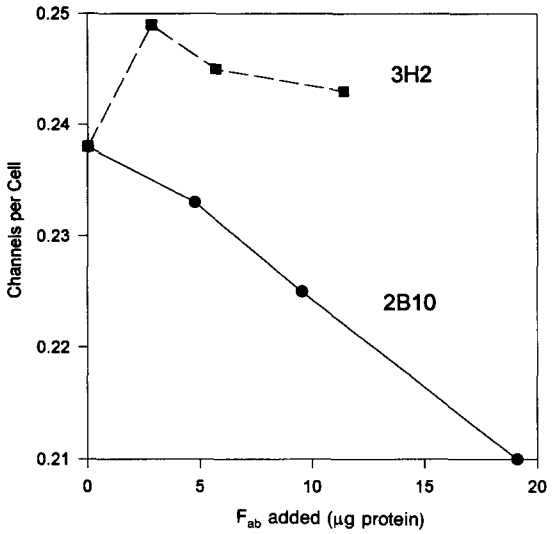

Figure 3. Complement-mediated lysis of erythrocytes treated with antiglycophorin mAb. Human erythrocytes were pretreated with F_{ab} fragments of 2B10 (●) and 3H2 (■). Cells were washed, C5b-9 added, and hemolysis assessed.

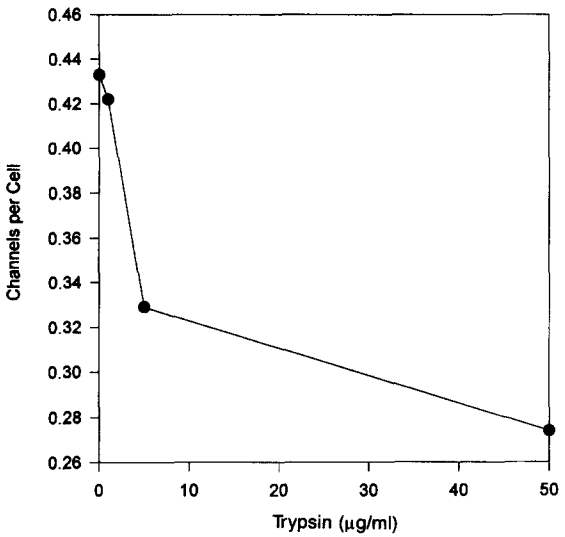

Figure 4. Treatment of human erythrocytes with trypsin decreases susceptibility to reactive lysis. Human erythrocytes were trypsinized, washed, and treated with C5b-9.

the binding of C5b6 to red cells. As can be seen in Fig. 3, F_{ab} fragments of 2B10 inhibit lysis, while the F_{ab} fragments of 3H2 do not. FACS® analysis (not shown) demonstrates binding of both F_{ab} fragments to the erythrocyte.

Effect of Trypsin Treatment on Lysis. To confirm the data obtained with mAbs, we subjected human erythrocyte glycophorin to trypsin digestion to release the T2 fragment (19, 29). After trypsin treatment, samples of red cell membranes were run on SDS PAGE and glycophorin cleavage was assayed by Western blot using monoclonal antiglycophorin antibody. 5 μg/ml trypsin cleaved ~50% of the glycophorin, and 50 μg/ml trypsin cleaved 80% of the glycophorin (not shown). Fig. 4 shows the relationship between the concentration of trypsin used to treat the erythrocytes and subsequent lysis in a hemolytic assay. Cleavage of the glycophorin is associated with a significant decrease in lytic susceptibility. Taken together with the mAb-blocking experiments, we conclude that the sialylated portion of glycophorin acts as a productive acceptor for C5b6, i.e., it increases the efficiency of channel formation by the terminal complement components.

Effect of Exogenous Gangliosides on Lysis. The major sources of erythrocyte sialic acid are glycophorin and gangliosides. To determine whether gangliosides can also act as acceptors for C5b6, we performed competition experiments, in which gangliosides were present during the incubation of C5b6 and C7 with the erythrocytes. These experiments were performed with guinea pig erythrocytes, as they contain gangliosides and demonstrate greater sensitivity to human C5b-9. Fig. 5 shows that sialylated gangliosides inhibit lysis significantly, while the asialo GM_1 does not. The disialoganglioside (GD_{1b}) shows greater inhibition than the monosialogangliosides, consistent with the hypothesis that C5b6 interacts with the sialic acid residues. When gangliosides were present only during incubation of cells with C8 and C9, there was an effect on lysis (not shown).

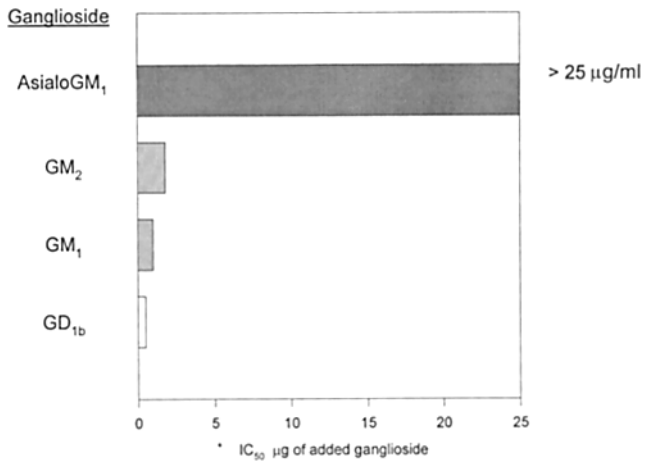

Figure 5. Inhibition of hemolysis by exogenous gangliosides. Gangliosides were incubated with GPE and human C5b-7. Cells were washed, C8 and C9 added, and hemolysis measured after 60 min.

Effect of Ganglioside Incorporation on Lysis. The data shown in Fig. 5 relate to the inhibition of lysis by gangliosides added to the fluid phase. The assess the role of membrane gangliosides, we incorporated the trisialoganglioside, GT_{1b}, into erythrocytes and determined their susceptibility to lysis. To quantitate levels of ganglioside incorporation, we measured the increase in sialic acid in the membrane. As can be seen in Fig. 6, the presence of the trisialoganglioside results in a significant increase in lysis by C5b-9. We performed similar experiments with the monosialogangliosides GM_1 and GM_3, but there was no measurable uptake of ganglioside or change in hemolysis of the treated erythrocytes.

Role of Free Sialic Acid on Reactive Lysis. Fig. 7 shows that GM_3 effectively inhibits C5b-9–mediated hemolysis, whereas

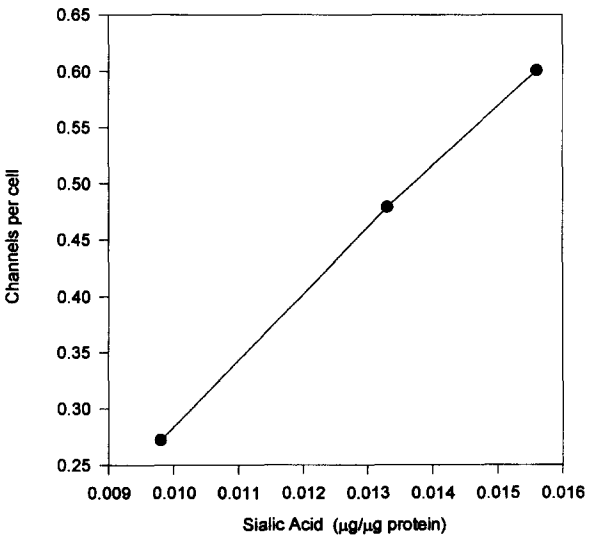

Figure 6. Membrane-incorporated trisialogangliosides increase C5b-9–mediated lysis. Guinea pig erythrocytes were incubated with ganglioside GT_{1b}, washed, and treated with C5b-9. GT_{1b} incorporation was estimated by measurement of membrane sialic acid.

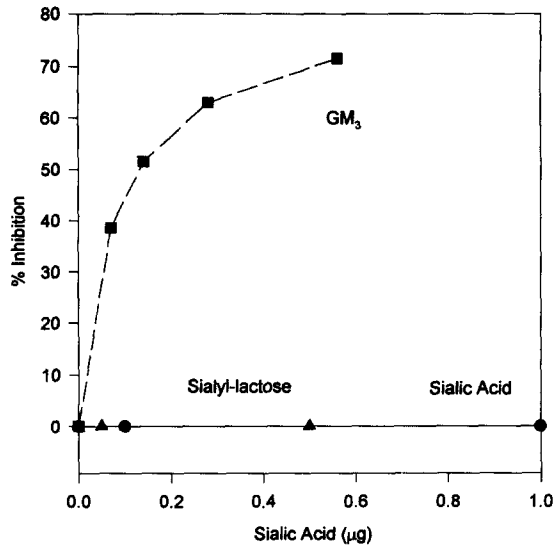

Figure 7. Inhibition of C5b-9–mediated lysis by sialic acid, sialyl-lactose, and GM_3. Sialic acid (●), sialyl-lactose (▲), or ganglioside GM_3 (■) were incubated with GPE and human C5b-7. Cells were washed, C8 and C9 added, and hemolysis measured after 60 min.

free sialic acid and sialyl-lactose do not. Therefore the sialic acid must be part of a larger molecule, or must be multivalent to inhibit C5b-7 deposition on the membrane.

Structure–Function Mapping of Ganglioside GM_3. We next used competition experiments to study the effect of structural changes in the sialic (neuraminic) acid residue of GM_3 on the interaction with C5b67. Native GM_3 is Neu5Ac(α2-3)Gal(β1-4)Glc-Cer (GSC17). Inhibitory activity of native GM_3 and some synthetic GM_3 analogues are shown in Fig. 8. GSC80 has an alcohol substituted for a carboxylic acid on the 1 carbon of sialic acid, and has no inhibitory activity. GSC23 lacks the exocyclic side chain (consisting of carbons 8 and 9) and shows diminished activity. The analogue GSC198, which substitutes a hydroxyl for the *N*-acetyl group on carbon 5 of sialic acid, also shows reduced activity. The absence of carbon 9 (GSC23) has no significant effect. In data not shown, removal of the glucose residue from GM_3 (Neu5Ac[α2-3]Gal-Cer) increased the IC_{50} from 1 μg to 7 μg. Thus, structural features in addition to carboxylic acid are involved in C5b6 recognition. Other modifications without significant effect are: lack of a hydroxyl moiety on carbon 4, carbon 7, carbon 8, or carbon 9; methylation of the 4-OH group; and changing the linkage between the sialic and galactose to (β2-1).

Discussion

Membrane deposition of the C5b-7 complex is enhanced by the presence of the two sialylated molecules of the erythrocyte membrane, gangliosides and glycophorin. In this work we demonstrate direct binding of C5b6 to glycophorin, and show that this binding is dependent on the presence of sialic acid. The net effect of C5b6 binding to glycophorin in the membrane is to increase C5b-7 uptake as determined by mAb studies and trypsinization experiments. Gangliosides, the other major source of erythrocyte sialic acid, also enhance the efficiency of C5b-7 deposition. Gangliosides added to the fluid phase compete with the erythrocyte membrane for C5b67 uptake, and this inhibition is dependent on ganglioside sialic acid. The inhibition of lysis by sialic acid requires that the sialic acid be part of a macromolecule such as glycophorin or a ganglioside; free sialic acid or sialyl-lactose has no activity. Using synthetic ganglioside analogues, we demonstrate the structural features important for the interaction with C5b-7. The carboxylic acid residue is essential for the interaction, and modification of sialic acid, either by the removal of the exocyclic side chain, or by the removal of the *N*-acetyl group, also decreases the effectiveness of ganglioside–complement interaction.

A number of membrane proteins have been shown to be inhibitors of complement activation. The studies reported here show that membrane molecules, specifically gangliosides and glycophorin, can serve to enhance complement attack. These studies highlight a paradox, since sialylated membrane molecules inhibit human complement activation. Sialic acid enhances the affinity of factor H for C3b. This binding has two important consequences: (*a*) factor B

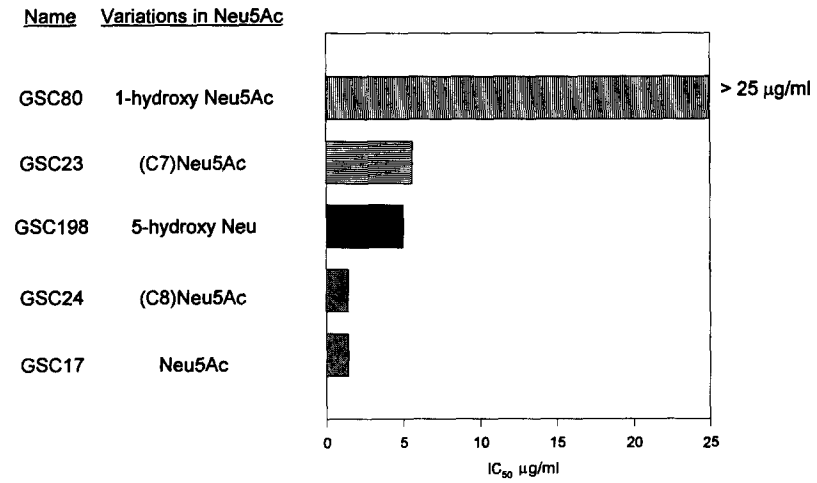

Figure 8. Inhibition of lysis by exogenous synthetic GM_3 analogue. Various synthetic analogues of GM_3 were titrated with GPE and C5b-7, washed, and C8 and C9 added.

is dissociated from the C3bBb complex, thus inhibiting the alternative pathway C3 convertase; and (*b*) factor H acts as a cofactor to promote the cleavage and inactivation of C3b by factor I in both the classic and alternative pathways. Thus the presence of sialylated membrane molecules inhibits both pathways of complement activation, although the effect on the alternative pathway is more significant (30). If the complement system is able to overcome this inhibition, the presence of sialic acid on adjacent membrane molecules enhances C5b-9–mediated lysis. Whether sialic acid leads to a net increase or decrease in lysis depends on the mode of complement activation (classic or alternative pathway) and the magnitude of sialic acid inhibition of activation versus enhancement of lysis. Consequently, microorganisms must evolve more than one mechanism to evade complement attack efficiently.

The structural features of sialic acid required for inhibition of the activation pathways and enhancement of the membrane attack pathway show some interesting similarities. Specifically, both pathways require the presence of a carboxylic acid on carbon 1 (31). The exocyclic side chain consisting of carbons 8 and 9 is important in both factor H and C5b6 binding, although the requirements differ. Binding of sialic acid to factor H requires the presence of carbon 9 (31). Binding to C5b6 is enhanced by the presence of carbons 8 and 9 of the exocyclic side chain; however, it is not an absolute requirement, and removal of only carbon 9 has no effect on activity. The roles of the glucose residue and the *N*-acetyl group, which enhance C5b6 binding, have not been investigated in the case of C3b degradation. The reason for these similarities is not known, but may be due to the fact that factor H and C6 both share short consensus repeat (SCR) protein modules. Factor H consists of 20 SCRs in linear array, and the SCR that is the probable anion-binding site has been identified (32).

In addition to the SCR modules, the terminal complement proteins share several structural domains that are found in other proteins such as the thrombospondin type 1 motif (TS1), the low density lipoprotein receptor motif, the epidermal growth factor motif, and the factor I module (33). C6 has three and C7 has two TS1 repeat domains. One C6 thrombospondin motif shows close homology to human thrombospondin; the other two TS1 sequences in C6 and both of the C7 TS1 domains are less conserved (34). TS1 domains are present in a number of molecules, including thrombospondin, antistasin (an inhibitor of coagulation and metastasis), the malarial coat protein from several plasmodia species, and the complement protein properdin (35, 36). This sequence of amino acids binds to negatively charged molecules such as heparin, heparan sulfate proteoglycan, sulfatides, and cholesterol sulfate (36). C5b6 binds to polyanions such as heparin and sulfatides (Marshall, P.M., and M.B. Whitlow, unpublished observations), and C6 binds to heparin-Sepharose columns (37). TS1 domains contain grouped positive charges making them likely candidates to bind to anions such as sialic acid, but the precise binding site or sites for sialic acid on C5b6 remain to be elucidated.

We propose the following mechanism for the interaction of the terminal complement proteins with glycophorin and gangliosides. C5 and C6 do not have affinity for sialylated molecules. C5 is cleaved by the classic or alternative pathway C5 convertase and subsequently binds to C6 to form C5b6. This complex binds to the sialic acid residues of glycophorin or gangliosides via ionic forces. As C6 contains one highly conserved TS1 domain, we postulate that this is the site of interaction between anions and C5b6. Binding of C7 to C5b6 disrupts the ionic interaction between TS1 and the membrane, and additionally leads to the exposure of hydrophobic residues on C5, C6, and C7. C7 has two domains of grouped anionic amino acids, the low density lipoprotein domain, and the epidermal growth factor domain (38). In our model, one of these domains binds to the TS1 domain of C6, and disrupts C5b6–membrane protein interaction. In addition, binding of C7 also induces the exposure of hydrophobic domains on C5b, C6, and C7, leading to membrane insertion (4, 10, 39). Our model is novel as it emphasizes the importance of the ionic interaction between C5b6 and membranes, and postulates that the addition of C7 to the C5b6 complex not only leads to the exposure of hydrophobic domains, but also disrupts the ionic binding of C5b6 to membrane anions. This model predicts that if the C5b6–membrane anion binding were strong enough, C5b-7 formation would be inhibited. Interestingly, this question was addressed by Silversmith and Nelestuen (12). They examined membrane binding of the terminal complement proteins on vesicles made from phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and phosphatidic acid. They found that vesicles made from the most anionic lipid, phosphatidic acid, bound more C5b6 than vesicles made from the other lipids. However, this membrane-bound C5b6 could not interact with C7 to form a C5b-7 complex. These results are consistent with our prediction that high affinity binding between membrane molecules and C5b6 will inhibit C5b-7 formation.

When gangliosides or glycophorin are present in the fluid phase, they bind to C5b6, and compete with the membrane for C5b6 uptake. Thus our data do not contradict previous studies showing that glycophorin added to the fluid phase inhibits complement deposition on erythrocytes (40).

The requirement for multivalency for oligosaccharide–protein interaction, as seen in Fig. 7, has been previously described in selectins (41). The mechanism is not known, but has been postulated to be either due to summation of a large number of weak interactions, or a specific secondary structure of the oligosaccharide residue that must be recognized (41).

The observation that GT_{1b} incorporated into the membrane increases lysis (Fig. 6) raises the possibility that some of the gangliosides used in the inhibition experiments (Fig. 5) incorporated into the erythrocyte membrane. However, during the competition experiments, the gangliosides were in contact with the cells for much shorter times and at lower temperatures (40 min at 30°C, compared with 120

min at 37°C) than those used in the incorporation experiments. As the amount of lipid uptake increases as a function of time and temperature, any ganglioside incorporation in the competition experiments will be minimized. In addition, the concentrations of ganglioside used in the incorporation experiments were significantly higher than in the competition experiments. Thus any effect of incorporated gangliosides in these experiments should be negligible, although the true concentration of ganglioside that inhibited lysis by 50% (IC_{50}) of the gangliosides may be slightly lower than the values shown.

These studies clearly show the importance of ionic interactions in the process of membrane attack by the terminal complement proteins. In addition, the present findings provide a strategy for the design of soluble and membrane inhibitors of the terminal complement proteins. Akami et al. (42), and Dong and Tomlinson (unpublished observations) have shown that recombinant CD59 is more effective as an inhibitor of C5b-9 when it lacks the asparagine-linked oligosaccharide which contains sialic acid (42, 43). We would predict that any recombinant inhibitor of the membrane attack complex should lack sialic acid for maximal activity. This observation may prove to be important in the design of genetically engineered organs to circumvent hyperacute xenograft rejection (20).

This work also provides a strategy for the design of soluble complement inhibitors. A soluble form of complement receptor 1 (CR1, CD35) has been shown to decrease tissue damage in an animal model of myocardial infarction (41). However, soluble inhibitors of the terminal complement proteins have not been studied in vivo. Heparin is an effective in vitro inhibitor of C5b-7 deposition, but heparin's anticoagulant activity limits its usefulness. The data presented in this study show that sialic acid derivatives inhibit deposition of the terminal complement proteins. Native gangliosides or glycophorin obviously cannot be used as inhibitors clinically; however, soluble molecules such as fetuin or synthetic fetuin derivatives might be possible C5b-9 inhibitors. In our studies fetuin effectively inhibited C5b-9 lysis (not shown). In addition, the presence of sialylated molecules in the aqueous phase inhibition, the presence of sialylated molecules in the aqueous phase inhibits the membrane attack pathway at the step of C5b-7 binding. This inhibition should be additive with CD59 which inhibits lysis at the C8 and C9 stage of membrane attack. Recent studies have focused on the use of transgenic pigs expressing CD59 and DAF to avoid hyperacute xenograft rejection (44). Thus an optimal strategy to circumvent hyperacute graft rejection may involve tissue expression of DAF and CD59 as well as the presence of soluble sialic acid derivatives or other anions.

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Address correspondence to Dr. Michael B. Whitlow, The Ronald O. Perelman Department of Dermatology, 550 First Avenue, New York, NY 10016.

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References

1. Mayer, M. 1978. Complement, past and present. *Harvey Lect.* 72:139–193.
2. Mayer, M.M., D.W. Michaels, L.E. Ramm, M.B. Whitlow, J.B. Willoughby, and M.L. Shin. 1981. Membrane damage by complement. *Crit. Rev. Immunol.* 2:133–165.
3. Esser, A.F. 1994. The membrane attack complex of complement. Assembly, structure and cytotoxic activity. *Toxicology* 87:229–247.
4. Hammer, C.H., A. Nicholson, and M.M. Mayer. 1975. On the mechanism of cytolysis by complement: evidence on insertion of C5b and C7 subunits of the C5b,6,7 complex into phospholipid bilayers of erythrocyte membranes. *Proc. Natl. Acad. Sci. USA.* 72:5076–5080.
5. Hammer, C.H., M.L. Shin, A.S. Abramovitz, and M.M. Mayer. 1977. On the mechanism of cell membrane damage by complement: evidence on insertion of polypeptide chains from C8 and C9 into the lipid bilayer of erythrocytes. *J. Immunol.* 119:1–8.
6. Iida, K., M.B. Whitlow, and V. Nussenzweig. 1991. Membrane vesiculation protects erythrocytes from destruction by complement. *J. Immunol.* 147:2638–2642.
7. Preissner, K.T. 1989. The role of vitronectin as multifunctional regulator in the hemostatic and immune systems. *Blut.* 59:419–431.
8. Jenne, D.E. and J. Tschopp. 1992. Clusterin: the intriguing guises of a widely expressed glycoprotein. *Trends Biochem. Sci.* 17:154–159.
9. Goldlust, M.B., H.S. Shin, C.H. Hammer, and M.M. Mayer. 1974. Studies of complement complex C5b,6 eluted from EAC1-6: reaction of C5b,6 with EAC4b,3b and evidence on the role of C2a and C3b in the activation of C5. *J. Immunol.* 113:998–1007.
10. Hu, V.W., A.F. Esser, E.R. Podack, and B.J. Wisnieski. 1981. The membrane attack mechanism of complement: photolabeling reveals insertion of terminal proteins into target membrane. *J. Immunol.* 127:380–386.
11. Hammer, C.H., A.S. Abramovitz, and M.M. Mayer. 1976. A new activity of complement component C3: cell-bound C3b potentiates lysis of erythrocytes by C5b,6 and terminal components. *J. Immunol.* 117:830–834.

12. Silversmith, R.E., and G.L. Nelsestuen. 1986. Interaction of complement proteins C5b-6 and C5b-7 with phospholipid vesicles: effects of phospholipid structural features. *Biochemistry*. 25:7717–7725.
13. Tschopp, J., and D. Masson. 1987. Inhibition of the lytic activity of perforin (cytolysin) and of late complement components by proteoglycans. *Mol. Immunol.* 24:907–913.
14. Baker, P.J., T.F. Lint, B.C. McLeod, C.L. Behrends, and H. Gewurz. 1975. Studies on the inhibition of C56-induced lysis (reactive lysis). VI. Modulation of C56-induced lysis by polyanions and polycations. *J. Immunol.* 114:554–558.
15. Vargas, F.F. 1994. Electrical surface phenomenon of endothelial cells. *In* Cell Electrophoresis. J. Bauer, editor. CRC Press, Boca Raton, FL. 241–253.
16. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* 234:1971–1975.
17. Yamamoto, K., and H. Gewurz. 1978. The complex of C5b and C6: isolation, characterization and identification of a modified form of C5b consisting of three polypeptide chains. *J. Immunol.* 120:2008–2015.
18. Whitlow, M.B., I. Stefanova, A. Bernard, and V. Nussenzweig. 1990. H19, a surface membrane molecule involved in T-cell activation, inhibits channel formation by human complement. *Cell. Immunol.* 133:3235–3241.
18a. Mishell, B.B., and S.M. Shiigi. 1980. Preparation of active antibody fragments. *In* Selected Methods in Cellular Immunology. B.B. Mishell and S.M. Shiigi, editors. W H Freeman and Company. 284–286.
19. Su, S., R.S. Ashok, I. Ekwere, and E.A. Davidson. 1993. A monoclonal antibody capable of blocking the binding of PF200(MSA-1) to human erythrocytes and inhibiting the invasion of *Plasmodium falciparum* merozoites into human erythrocytes. *J. Immunol.* 151:2309–2317.
20. Murase, T., H. Ishida, and A. Hasegawa. 1989. A facile, regio- and stereo-selective synthesis of ganglioside GM_3. *Carbohydr. Res.* 188:71–80.
21. Hasegawa, A., H. Ogawa, and M. Kiso. 1992. Synthesis of a ganglioside GM_3 position isomer, *N*-acetylneuraminosyl(α2-6)lactosyl(β1-1)ceramide. *Biosci. Biotech. Biochem.* 56:535–536.
22. Hasegawa, A., K. Adachi, M. Yoshida, and M. Kiso. 1992. Synthesis of ganglioside GM_3 analogs containing the chemically modified sialic acids. *J. Carbohydr. Chem.* 11:95–116.
23. Hasegawa, A., K. Adachi, M. Toshida, and M. Kiso. 1992. Synthesis of a series of ganglioside GM_3 analogs containing a deoxy-*N*-acetylneuraminic acid residue. *J. Carbohydr. Chem.* 230:273–288.
24. Yoshida, M., H. Ishida, M. Kiso, and A. Hasegawa. 1996. Synthesis of *N*-acetyl-4-8-dideoxyneuraminic acid-containing ganglioside GM3. *Carbohydr. Res.* 280:331–338.
25. Murase, T., A. Kameyama, K.P.R. Kartha, H. Ishida, M. Kiso, and A. Hasegawa. 1989. Synthetic studies on sialoglycoconjugates. V. A facile, regio and stereoselective synthesis of ganglioside GM4 and its position isomer. *J. Carbohydr. Chem.* 8:265–283.
26. Hasegawa, A., N. Suzuki, F. Kozawa, H. Ishida, and M. Kiso. 1996. *J. Carbohydr. Chem.* In press.
27. Terada, T., M. Kiso, and A. Hasegawa. 1993. Synthetic studies on sialoglycoconjugates. 44. Synthesis of KDN-gangliosides GM4 and GM3. *J. Carbohydr. Chem.* 12:425–440.
28. Hasegawa, A., K. Adachi, M. Yoshida, and M. Kiso. 1992. Synthetic studies on siloglycoconjugates. 32. Synthesis of a ganglioside GM3 analog containing a hydroxymethyl group in place of the carboxyl group in the *N*-acetylneuraminic acid unit. *Biosci. Biotech. Biochem.* 56:445–447.
29. Perkins, M. 1981. Inhibitory effects of erythrocyte membrane proteins on the in vitro invasion of the human malarial parasite (*Plasmodium falciparum*) into its host cell. *J. Cell Biol.* 90: 563–567.
30. Ollert, M.W., K. David, R. Bredehorst, and C.-W. Vogel. 1995. Classical complement pathway activation on nucleated cells. Role of factor H in the control of deposited C3b. *J. Immunol.* 155:4955–4962.
31. Michalek, M. T., C. Mold, and E. G. Bremer. 1988. Inhibition of the alternative pathway of human complement by structural analogues of sialic acid. *J. Immunol.* 140:1588–1594.
32. Pangburn, M. K., M. A. Atkinson, and S. Meri. 1991. Localization of the heparin-binding site on complement factor H. *J. Biol. Chem.* 266:16847–16853.
33. Haefliger, J., J. Tschopp, N. Vial, and D.E. Jenne. 1989. Complete primary structure and functional characterization of the sixth component of the human complement system. Identification of the C5b-binding domain in complement C6. *J. Biol. Chem.* 264:18041–18051.
34. Smith, K. F., K. F. Nolan, K. B. M. Reid, and S. J. Perkins. 1991. Neutron and X-ray scattering studies on the human complement protein properdin provide an analysis of the thrombospondin repeat. *Biochemistry*. 30:8000–8008.
35. Holt, G.D., M.K. Pangburn, and V. Ginsburg. 1990. Properdin binds to sulfatide and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J. Biol. Chem.* 265:2852–2855.
36. Holt, G.D., H.C. Krivan, G.J. Gasic, and V. Ginsburg. 1989. Antistasin, an inhibitor of coagulation and metastasis, binds to sulfatide and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J. Biol. Chem.* 264:12138–12140.
37. Sahu, A., and M. K. Pangburn. 1993. Identification of multiple sites of interaction between heparin and the complement system. *Molec. Immunol.* 30:679–684.
38. DiScipio, R. G., D. N. Chakavarti, H. J. Muller-Eberhard, and G. H. Fey. 1988. The structure of human complement component C7 and the C5b-7 complex. *J. Biol. Chem.* 263: 549–560.
39. Podack, E.R., W. Stoffel, A.F. Esser, and H.J. Muller-Eberhard. 1981. Membrane attack complex of complement: distribution of subunits between the hydrocarbon phase of target membranes and water. *Proc. Natl. Acad. Sci. USA.* 78: 4544–4548.
40. Tomita, A., E. Radike, and C. J. Parker. 1995. Isolation of erythrocyte membrane inhibitor of reactive lysis type II. Identification as glycophorin A. *J. Immunol.* 151:3308–3323.
41. Varki, A. 1994. Selectin ligands. *Proc. Natl. Acad. Sci. USA.* 91:7390–7397.
42. Akami, T., M. Arakawa, M. Okamoto, K. Akioka, I. Fujiwara, I. Nakai, M. Mitsuo, R. Sawada, M. Naruto, and T. Oka. 1994. Enhancement of the complement regulatory function of CD59 by site-directed mutagenesis at the N-glycosylation site. *Transplant. Proc.* 26:1256–1258.
43. Ninomiya, H., B.H. Stewart, S.A. Rollins, J. Zhao, A.L.M. Bothwell, and P. Sims. 1992. Contribution of the N-linked carbohydrate of erythrocyte antigen CD59 to its complement-inhibitory activity. *J. Biol. Chem.* 267:8401–8404.
44. Magee, J. C., and J.L. Platt. 1994. Xenograft rejection : molecular mechanisms and therapeutic implications. *Ther. Immunol.* 1:45–58.