N-Glycolyneuraminic Acid and N-Acetyleneuraminic Acid Define Feline Blood Group A and B Antigens

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Blood group incompatibility causes transfusion reactions and neonatal isoerythrolysis in cats. We investigated the molecular nature of the blood group antigens from cats that had blood type A, B, and AB erythrocytes. Naturally occurring anti-type B antibodies, *Triticum vulgaris* lectin, monoclonal antibody (MoAb) 32-27, and MoAb R-24 were used in agglutination tests, Western blots, and thin-layer chromatography (TLC) enzyme immunostaining. Type A erythrocytes had NeuGc-Galactose-Glucose-Ceramide ([NeuGc]2GD3) where NeuGc represents N-glycolyneuraminic acid, and NeuAc-NeuGc-GD3, where NeuAc represents N-acetyleneuraminic acid, and may have [NeuGc]2 disialylparagloboside and NeuAc-NeuGc-disialylparagloboside. Type B erythrocytes only had [NeuAc]2Gd3. Type AB erythrocytes had [NeuGc]2Gd3, NeuAc-NeuGc-GD3, and [NeuAc]2Gd3. Blood group antigens were also found on a 50-Kd membrane protein. We conclude that type B erythrocytes are characterized by [NeuAc]2Gd3 as the only form of this ganglioside and the presence of NeuAc on a 50-Kd membrane protein. NeuGc is the major determinant of the A antigen; specifically, [NeuGc]2Gd3 is the major glycolipid form. The A antigen is also present on a 50-Kd membrane protein.

**MATERIALS AND METHODS**

**Chemicals.** Diisopropyl fluorophosphate (DFP), polyoxyethylene sorbitan monolaurate (Twee 20), orcinol ferric chloride (Bial's reagent), biotinylated goat antimouse IgG, *T. vulgaris* lectin, and biotinylated *T. vulgaris* lectin were purchased from Sigma Chemical Co (St Louis, MO). Both lectins were reconstituted or diluted according to the manufacturer's directions. Cardboard diagnostic cards (PathoDx cards) were from Diagnostic Products Corp (Los Angeles, CA). PVDF membranes (Immobilon-P) were from Millipore Corp (Bedford, MA). Avidin biotinylated hors eradish peroxidase enzyme complex (ABC reagent), biotinylated goat antifeline IgG; and biotinylated goat antimouse IgM were from Vector Laboratories (Burlingame, CA). Aluminum-backed silica gel 60 TLC plates were from E. Merck (Darmstadt, Germany). Poly(isobutylmethacrylate) was from Aldrich Chemical Co Inc (Milwaukee, WI). MoAbs 32-27 (human IgM anti-[NeuGc]2GD3) results indicate that NeuGc is the major determinant of the blood group A antigen and, specifically, that NeuGc-NeuGc-Galactose-Glucose-Ceramide ([NeuGc]2GD3) is the major ganglioside representing the A antigen recognized by the cat. The A antigen is also present on a 50-Kd erythrocyte membrane protein. Type B erythrocytes are characterized by (NeuAc-NeuAc-Galactose-Glucose-Ceramide ([NeuAc]2GD3), the presence of NeuAc on a 50-Kd erythrocyte membrane protein, and lack of detectable NeuGc. Type AB erythrocytes are unique in having both [NeuGc]2GD3 and [NeuAc]2GD3 coexpressed on the membrane.

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Supported in part by Grant No. HL 01877 from the National Institutes of Health. Contribution no. 91-576-J from the Kansas Agricultural Experiment Station.

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and R-24 (murine IgG3 anti-[NeuAc]$_2$GD$_3$) were a generous gift from Dr. Kenneth O. Lloyd (Memorial Sloan-Kettering Cancer Center, New York, NY). [NeuAc]$_2$GD$_3$ (0.5 mg) was purchased from Biocarb Chemicals (Lund, Sweden).

**Feline blood typing.** Two blood type A and three blood type B cats maintained at the College of Veterinary Medicine, Kansas State University (KSU), were used as sources of typing sera. Two of the blood type B cats were obtained from Animal Blood Bank (Vacaville, CA). Erythrocytes of these two cats were typed by a commercial laboratory (Stormont Laboratories, woodland, CA). Erythrocytes of the remaining three cats were typed at KSU. Reagents used to type blood included naturally occurring anti-A antiserum collected from a type B cat (Animal Blood Bank), and anti-B antiserum collected from a type A cat immunized with type B erythrocytes. Blood types were confirmed by cross-matching red blood cells (RBCs) from each cat with the sera from the other four cats, as well as with known antiseria.

**Feline erythrocytes.** Blood used for agglutination tests was obtained by venipuncture with EDTA as an anticoagulant. Blood samples were obtained from five cats maintained at KSU, from the veterinary clinical pathology laboratory at KSU, and from Professional Animal Laboratory (Irving, CA). Blood used to prepare RBC membrane ghosts was obtained by venipuncture into EDTA tubes, washed three times with 0.02 mol/L phosphate-buffered saline (PBS) pH 7.4, and processed immediately.

**Agglutination tests.** Agglutination tests were performed on PathoDx cards. Whole blood (50 mL) was placed in one well of the card. Typing reagent (50 mL) was then added and mixed with the blood. Typing reagents were used at the following concentrations: *T. vulgaris* (100 µg/mL); feline antiserum (undiluted); MoAb R-24 (80 µg/mL); MoAb 32-27 (1:2 dilution of hybridoma supernatant).

**Erythrocyte membranes.** Hemoglobin-free erythrocyte ghosts were prepared according to the method of Dodge et al. Washed erythrocytes were lysed with 10 mmol/L phosphate buffer containing 1 mmol/L DFP. Protein concentration of purified membranes was determined by bichinoninic acid protein assay (Pierce Chemical Co, Rockford, IL) with bovine serum albumin (BSA) standards.

**SDS-PAGE.** Erythrocyte membrane proteins were separated by electrophoresis in 7.5% polyacrylamide slab gels containing SDS using a discontinuous buffer system. Erythrocyte membrane proteins were electrophoretically transferred (blotted) to a PVDF membrane and washed with 0.05 mol/L Tris-buffered saline (TBS) pH 7.4 (TBS).

**Lectin immunoblotting.** Blots were blocked for 1 hour in TBS containing 0.1% Tween 20, and incubated with biotinylated *T. vulgaris* lectin (10 µg/mL in TBS-Tween 20) for 3 hours. Blots were then washed three times in TBS-Tween 20. Blots were incubated with ABC reagent (prepared according to the manufacturer’s directions in TBS-Tween 20) for 1 hour before a final washing in TBS and development with 4-chloro-napthol substrate solution.

**Western blotting with feline anti-A antiserum.** Blots were blocked in TBS containing 0.1% Tween 20 and 1% nonfat dry milk (Blotto) for 1 hour. Blots were incubated with feline anti-A antiserum (diluted 1:10) in Blotto for 3 hours. Blots were then washed three times with TBS-Tween 20 and incubated with biotinylated goat antifeline IgG (5 µg/mL) in Blotto for 1 hour. Blots were then washed, incubated with ABC reagent, washed with TBS, and developed as described above.

**Lipid extraction and thin-layer chromatography (TLC).** Lipids were extracted from RBC ghosts by a simple chloroform:methanol extraction procedure. RBC ghosts (400 µL) were placed in 5 mL of chloroform:methanol (1:1 by volume) and heated to 60°C for 5 minutes. Insoluble material was separated by centrifugation at 1,000g for 15 minutes. Dissolved material was dried under a stream of nitrogen and redissolved in 250 µL of chloroform:methanol (CM) 1:1 for spotting (25 to 30 µL per spot) on aluminum-backed silica gel 60 TLC plates. The ganglioside standard ([NeuAc]$_2$GD$_3$) was dissolved in 2 mL of CM 50:50 before being spotting on TLC plates. Each lane was spotted with 20 to 25 µL. The plates were developed with CM:5N NH$_4$OH (60:40:9 by volume) until the solvent was close to the top of the plate, removed from the chamber, and dried at room temperature. Glycolipids were visualized with Bial’s reagent and heating at 100°C until bands were visible.

**TLC immunostaining with MoAbs 32-27 and R-24.** TLC immunostaining was modified from the procedure described by Buehler and Macher. TLC plates were spotted, developed, and dried as previously described. The plates were then immersed in 0.3% poly(isobutylmethacrylate) dissolved in hexane for 5 minutes, removed, and air dried. Plates were blocked with PBS containing 1% BSA and 0.1% Tween 20 for 1 hour, then incubated for 2 hours with either MoAb 32-27 (1:10 dilution of hybridoma supernatant) or MoAb R-24 (10 µg/mL) in PBS-Tween 20 and washed three times with PBS-Tween 20. The plates were incubated for 2 hours in biotinylated goat antihuman IgM (1 drop/10 mL) or biotinylated goat antimouse IgG (10 µg/mL) in PBS-Tween 20 and washed three times with PBS-Tween 20. Plates were incubated in horseradish peroxidase ABC reagent (prepared in PBS-Tween 20) for 1 hour and washed three times with PBS. The plates were developed with a substrate solution consisting of 400 µg/mL O-phenylenediamine in 80 mmol/L citrate-phosphate buffer, pH 5.0, containing 0.03% H$_2$O$_2$.

**TLC immunostaining with feline antiserum.** TLC plates were spotted, developed, and plastic coated as described above. Plates were blocked for 2 hours in PBS containing 0.1% Tween 20 and 1% nonfat dry milk (blocking solution). The plates were incubated in feline anti-A antiserum (diluted 1:50 in blocking solution) and incubated for 4 hours. Plates were washed three times with PBS Tween 20 for 0.5 hours. The plates were incubated with biotinylated goat antiacet IgG (1.5 µg/mL) in blocking solution for 3 hours and plates washed three times in PBS-Tween 20 for 0.5 hours. The plates were incubated in ABC reagent for 1.5 hours, followed by the final three washes in PBS, and substrate development, as described above.

**RESULTS**

Card agglutination tests were performed with feline anti-A antiserum and *T. vulgaris* lectin on a total of 391 feline blood samples. Most (370) were agglutinated by feline anti-A antiserum only, 15 were agglutinated by *T. vulgaris* lectin only, and 6 were agglutinated by both reagents. Therefore, these samples were typed as A, B, and AB, respectively. Agglutination tests were then performed with MoAb R-24 and MoAb 32-27 on 25 of these blood typed samples (10 A, 10 B, and 5 AB) (Table 1). Type A samples were agglutinated by MoAb 32-27 only, type B samples were agglutinated by MoAb R-24 only, and type AB samples were agglutinated by both reagents.

| Table 1. Positive Agglutination Test Results of 25 Blood-typed Feline Erythrocyte Samples With MoAb R-24 and MoAb 32-27 |
|---------------------------------------------------------------|-----------------|-----------------|
| Type A | Type B | Type AB |
| MoAb R-24 | 0 | 10 | 5 |
| MoAb 32-27 | 10 | 0 | 5 |
TLC of feline erythrocyte membrane glycolipids from blood typed cats (Fig 1) shows that type B erythrocytes have a band that comigrates with the [NeuAc]$_2$G$_{D3}$ standard (Rf 0.43) (Rf = mobility from the origin relative to that of the solvent front). This band is absent from the type A glycolipid TLC profile. The type A TLC profile has a darkly staining band, which is absent from the type B profile (Rf 0.31). This band is [NeuGc]$_2$G$_{D3}$, the major feline erythrocyte glycolipid. The TLC profile of type AB shows two bands that migrate to positions intermediate between Rf 0.31 and the [NeuAc]$_2$G$_{D3}$ standard and two bands that migrate to positions slightly above the [NeuAc]$_2$G$_{D3}$ standard.

TLC immunostaining of feline erythrocyte membrane glycolipids from blood-typed cats with MoAb 32-27 (human IgM anti-[NeuGc]$_2$G$_{D3}$) (Fig 2) shows strong reactivity with four bands of the blood type A sample and three bands of the type AB sample. The bands with Rf values of 0.31 and 0.41 are [NeuGc]$_2$G$_{D3}$ and NeuAc-NeuGc-G$_{D3}$, respectively. The identity of the bands at Rf 0.08 and 0.14 of the type A cat and Rf 0.25 of the type AB cat are unknown. MoAb 32-27 showed no reactivity with type B erythrocyte glycolipids or with the [NeuAc]$_2$G$_{D3}$ standard.

MoAb R-24 reacted strongly with the [NeuAc]$_2$G$_{D3}$ standard and one band from the type B erythrocytes, which comigrated with the [NeuAc]$_2$G$_{D3}$ standard (Fig 3). MoAb R-24 reacted faintly with one band from the type AB erythrocytes and was nonreactive with the type A erythrocyte glycolipids.

TLC immunostaining with feline anti-A antiserum (Fig 4) shows strong reactivity with one band from the type A and faint staining of one comigrating band from type AB erythrocyte glycolipids. The Rf value of 0.31 identifies this band as [NeuGc]$_2$G$_{D3}$, as in Figs 1 and 2. Feline anti-A antiserum also faintly stains at least two other glycolipid bands of the type A, type AB, and type B erythrocytes.
Flg 3. TLC immunostain of feline erythrocyte membrane glycolipids from blood-typed cats with MoAb R-24. Lane 1, type A; lane 2, type AB; lane 3, [NeuAc]₂GD₃ standard; lane 4, type B. Equal amounts of glycolipids extracted from feline erythrocyte membranes were spotted in each lane. The number on the left indicates the Rf value.

Tvulgaris lectin immunoblotting of SDS-PAGE preparations of feline erythrocyte membrane proteins from blood-typed samples shows strong staining of a 50-Kd protein from a type B cat and faint staining of the same molecular weight protein from a type AB cat (Fig 5). There is no reactivity with proteins from the type A cat. Western blotting of SDS-PAGE preparations of feline erythrocyte membrane proteins from blood-typed samples with feline anti-A antiserum shows staining of a 50-Kd protein from a type A cat, which is not detected in the type B or AB cats (Fig 6).

**DISCUSSION**

It is well established that the major glycolipids of cat erythrocyte membranes are [NeuGc]₂GD₃ and NeuAc-NeuGc-GD₃. The data indicate that these two gangliosides are present only on cat erythrocytes expressing the blood group A antigen (type A and type AB). The fact that researchers use these gangliosides isolated from cat erythrocytes as authentic standards is not surprising, considering the high incidence of blood type A in the general cat population, particularly in the United States. A study in Japan found that 2 of 207 cat sera tested contained an IgM isoagglutinin that reacted with the erythrocytes of the other 205 cats. The agglutination was inhibited by NeuGchematoside. Anti-A isoagglutinin in pooled sera from two type B cats was found to be both IgM and IgG. The blood group A antigen is expressed on lymphocytes of blood type A and AB cats, but the B antigen does not appear to be expressed on the lymphocytes of type B cats.
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The TLC immunostain of type A erythrocyte glycolipids with MoAb 32-27 (Fig 2) is similar to that reported by others using similar TLC procedures. In that report, the investigators again confirmed that the two main gangliosides of feline erythrocytes were NeuGc-GD3 and NeuAc-NeuGc-GD3. However, we found staining of four bands of the type A cat erythrocyte glycolipids with MoAb 32-27. Based on the TLC mobility of the glycolipids and binding specificity of MoAb 32-27, we propose that type A cat erythrocytes also contain NeuGc disialylparagloboside and NeuAc-NeuGc-disialylparagloboside. These gangliosides have not been reported previously from feline erythrocytes.

The data indicate that only feline erythrocytes expressing the B antigen (type B and type AB) have NeuAc-GD3. NeuAc-GD3 has been reported in erythrocyte membranes from two individual Persian cats, which had no detectable NeuGc-containing gangliosides. Approximately 25% of Persian cats are blood type B.

T. vulgatus lectin agglutinates only feline type B and type AB erythrocytes and reacts with a broad 50-Kd glycoprotein band on the type B erythrocytes (Fig 5). Lectin immunoblotting with succinylated T. vulgatus lectin showed no reactivity with any erythrocyte membrane proteins from A, B, or AB cats (data not shown). T. vulgatus lectin binds sialoglycoproteins containing NeuAc but not those containing NeuGc. Succinylated T. vulgatus binds N-acetylgalactosamine but does not bind glycoconjugates containing NeuAc. Based on these carbohydrate-binding specificities, we conclude that the 50-Kd glycoprotein of type B cats has a terminal NeuAc and, therefore, is a sialoglycoprotein. Conversely, it seems reasonable to hypothesize that the 50-Kd protein on the type A erythrocytes detected by Western blotting with feline typing serum (Fig 6) may have a terminal NeuGc.

The finding of several type AB samples is interesting for several reasons. Type AB is a very rare blood type. The data indicate that type AB erythrocytes have immunologic features of both type A and type B. However, type AB erythrocytes seem to have decreased expression (or decreased number of sites) of both the A and B antigens compared with type A or type B erythrocytes. All TLC, TLC immunostains, lectin immunoblots, and Western blots in these experiments were performed in semiquantitative manner. In each experiment, equal amounts of erythrocyte membrane proteins, or glycolipids extracted from equal amounts of erythrocytes from type A, B, and AB cats, were run in each lane so that a semiquantitative comparison could be made. Results presented in Figs 1 and 2 demonstrate that the type AB erythrocyte has less NeuGc-GD3 than the type A erythrocyte. Results presented in Figs 1 and 3 show that the type AB erythrocyte has less NeuAc-GD3 than the type B erythrocyte. Figure 5 illustrates that the

![Image of TLC and Western blot analyses of feline erythrocyte membrane proteins](image-url)
type AB erythrocyte has fewer glycoprotein-binding sites for \textit{T vulgaris} lectin than the type B erythrocyte. Figures 4 and 6 illustrate that the type AB erythrocyte has fewer binding sites for feline alloimmune antibodies than the corresponding type A or type B erythrocytes. MoAb 32-27 TLC immunostaining (Fig. 2) shows that the type AB erythrocyte lacks two ganglioside species that are present on the type A erythrocyte. We have proposed that these two gangliosides are [NeuGe], disialylparagloboside and NeuAc-NeuGc-disialylparagloboside.

The mode of inheritance of the AB type is unknown. It is hypothesized that type A and type B are due to the action of two different alleles at the same gene locus, with type A being completely dominant to B.6 No O (null) phenotype has been documented. We have proposed a model for the inheritance of the A and B antigens based on the fact that the enzyme CMP-N-acetylneuraminic acid hydroxylase converts NeuAc to NeuGc.20 In this model, type A cats have CMP-N-acetylneuraminic acid hydroxylase and type B cats lack the enzyme. This model is compatible with the mode of inheritance and the apparent lack of a null allele. Limited breeding experiments indicate that type AB offspring are produced only when one of the parents is type AB.1 Type AB cats may have a mutated form of CMP-N-acetylneuraminic acid hydroxylase, which alters substrate specificity, substrate binding, or enzyme kinetics. Alternatively, type AB cats may have a gene that alters expression of the enzyme at the transcriptional or translational level. Another possibility is a mutation in a sialyltransferase enzyme that alters the ultimate expression of NeuGc on the surface of the AB erythrocyte.

NeuGc is a blood group substance in some breeds of East Asian dogs and is inherited as an autosomal dominant trait over NeuAc.31 The neuraminic acid species are the terminal sugar residues on canine erythrocyte hematosides. In a study of 1,591 dogs representing 56 breeds, NeuGc was limited to several Oriental breeds, despite the dominant mode of inheritance.32 NeuGc was not found in erythrocyte glycolipids of any European breeds examined. Canine erythrocytes expressing NeuGc-hematoside also had traces of NeuAc-hematoside, but erythrocytes expressing NeuAc-hematoside had no detectable NeuGc-hematoside. Sera from dogs with NeuAc-hematoside agglutinated erythrocytes from dogs with NeuGc-hematoside. The agglutination was inhibited by NeuGc-hematoside. Reciprocal sera was not found. Canine erythrocytes strongly expressing both NeuGc and NeuAc (analogous to the feline type AB) have not been documented.

Erythrocyte ganglioside polymorphisms have also been described in inbred strains of mice and in horses and cattle.34 It is unknown if these polymorphisms may also be related to blood group systems in these species. Forms of neuraminic acids are species- and tissue-specific and are developmentally regulated.35

Human erythrocyte membranes contain only NeuAc, which is responsible for the specificity of some Mn blood group antigens.37 Antibodies against NeuGc (Hanganutziu-Deicher antibodies) have been recognized in patients with serum sickness, and found in human sera from patients with infectious and immune mediated diseases and cancer.38 The occurrence of these antibodies in cancer patients has been used to advantage to produce human MoAbs with both NeuGc and NeuAc binding specificities.39 Altered ganglioside metabolism and expression on neoplastic cells is an area of much current research. NeuGc containing gangliosides have been isolated from human colon cancer and a novel pathway for the expression of NeuGc containing ganglioside antigens in cultured human melanoma and astrocytoma cells has been described.41 The role of neuraminic acids as antigens in a wide variety of biologic systems and the role of gangliosides in cancer have been reviewed.

Comparison of blood group A, B, and particularly the unique type AB cats will provide an interesting and useful model to study the biosynthesis and tissue-specific expression of NeuAc and NeuGc containing gangliosides and sialylglycoproteins.

REFERENCES

N-glycolylneuraminic acid and N-acetylneuraminic acid define feline blood group A and B antigens

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