The Diagnostic Significance of a Positive Direct Antiglobulin Test in Anemic Cats

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ABSTRACT

The preparation of a feline Coombs serum (rabbit antifeline gamma globulin) is described. The direct antiglobulin test using this serum was performed on 20 anemic and 20 healthy control cats. Red cell membrane antibodies were detected in cats with feline leukemia virus infection and in others with inflammatory and neoplastic diseases. A low titre of cold agglutinating antibody was present in a high proportion of the control cats. Positive direct antiglobulin tests were noted in cats without overt hemolytic disease.

It was concluded that the direct antiglobulin test in anemic cats has certain diagnostic limitations. A positive reaction should be interpreted cautiously especially when there is no clinical or laboratory evidence to support a diagnosis of autoimmune hemolytic anemia.

Key words: Cat, anemia, Coombs test, direct antiglobulin test, autoimmune hemolytic anemia, feline leukemia virus.

RÉSUMÉ

Cet article décrit la préparation de gamma-globulines antifélines, chez le lapin, et leur utilisation dans le test de Coombs effectué avec les hématies de 20 chats anémiques et de 20 témoins. Ce test permit de détecter des anticorps contre la membrane des hématies des chats atteints de leucémie ou d'une maladie hémolytique subclinique et de celles d'autres chats qui souffraient de conditions inflammatoires ou néoplasiques. Il révéla aussi la présence d'un faible taux d'anticorps qui agglutinaient à froid, chez une forte proportion des témoins.

Les auteurs conclurent que le test de Coombs comporte certaines restrictions pour le diagnostic des anémies félines. L'interprétation d'une réaction positive commande la prudence, surtout en l'absence d'une évidence clinique ou de laboratoire qui supporterait un diagnostic d'anémie hémolytique autoimmune.

Mots clés: chat, anémie, test de Coombs, épreuve directe à l'antiglobuline, anémie hémolytique autoimmune, virus de la leucémie féline.

INTRODUCTION

Anemia is common in the cat and can occur as a result of blood loss, hemolysis, renal disease, chronic inflammatory or neoplastic disease, and feline leukemia virus (Felv) infection (1). A diagnosis of autoimmune hemolytic anemia in the dog is usually confirmed by a positive direct antiglobulin test (DAT).

In man, although autoimmune hemolytic disease (AHD) is frequently associated with chronic lymphocytic leukemia, lymphosarcoma and reticulum cell sarcoma, transient increases in cold agglutinating antibodies can occur with a variety of infectious diseases, in particular mycoplasma pneumonia, when hemolysis is often minimal (2,3). In the dog, in addition to lymphoid and myeloid neoplasms, numerous inflammatory diseases can be accompanied by anemia and a positive DAT. Again hemolysis may be minimal (4). Coombs positive hemolytic anemia has been reported in the cat in association with Felv infection and lymphosarcoma (5,6).

The purpose of this paper is to describe the preparation of a feline Coombs reagent and to evaluate DAT results in anemic cats.

MATERIALS AND METHODS

PREPARATION OF A FELINE COOMBS REAGENT (RABBIT ANTIFELINE GAMMA GLOBULIN)

Preparation of feline gamma globulin for immunization of rabbits

A healthy donor cat was bled (30 mL) and the serum heated at 56°C for 30 minutes before being absorbed with packed rabbit erythrocytes washed three times with normal saline (pH 7.2-7.4). This procedure was repeated five times over 48 hours. The serum was then tested against a 2-8% suspension of healthy feline erythrocytes which had been washed three times in normal saline. If agglutination occurred at this point the procedure was repeated using different donor serum.

Precipitation of the absorbed serum with saturated ammonium chloride was followed by dialysis against 30 mL of borate saline buffer (BSB) at 4°C. The dialysed precipitin was centrifuged at 3500 rpm for 30 minutes at 4°C before being transferred to vials for storage at -20°C (7).

The gamma (γ) globulins were separated by Sephadex gel chromatography using Sephadex G-200 (8). The fractions were collected at hourly intervals for 12 hours. The peak fractions were pooled and the eluate collected from five filtrations was used to immunize the rabbits.

The purity of the immunizing fraction was assessed later by double diffusion immunoelectrophoresis on agar

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slides (1% Noble agar with 5 mM EDTA added, DIFCO Laboratories, Detroit, Michigan) using the rabbit antifeline gamma globulin as the antiserum. The slides were stained with amido black and excess stain was removed with 5-7% aqueous acetic acid (7).

Immunization program and preparation of the Coombs reagent

Two rabbits were immunized intravenously with the pooled peak fraction of feline γ globulin over a six week period, giving 0.1 mL the first week, 0.25 mL the second week and increasing the dose by 0.25 mL each successive week thereafter. Week 7 onwards, the rabbits were bled once weekly for two weeks and then bimonthly. A 0.5 mL booster injection was given every two weeks. Anaphylactic reactions were not observed using this protocol.

The rabbit serum was heated at 56° C for 30 minutes and absorbed five times over 48 hours with packed erythrocytes obtained from several healthy donor cats. This was performed at 4° C and 25° C to remove nonspecific agglutinins. The resulting serum, which was the Western College of Veterinary Medicine (WCVM) Coombs reagent in this study, was then frozen in 0.5 mL aliquots.

A commercially prepared rabbit antifeline IgG (Cappel Laboratories, 237 Lacey Street, P.O. Box 37, West Chester, Pennsylvania 19380) was similarly heated, absorbed and frozen. The specificity of both reagents against whole feline serum was assessed by immunoelectrophoresis (7).

THE DIRECT ANTIGLOBULIN TEST

The DAT was performed, using the two sera described above, on erythrocytes from 20 anemic and 20 healthy control cats.

The patients' erythrocytes were washed three times in phosphate buffered saline (PBS) until hemolysis or agglutination was no longer visible. A 1-2% solution of the packed erythrocytes in normal saline (pH 7.0-7.4) was added to centrifuge tubes containing an equal volume (0.1 mL) of increasing dilutions of the Coombs serum (1:2 up to 1:1024). Each series of dilutions was prepared in triplicate with three normal saline controls. The first series was incubated at 4° C, the second at 25° C and the third at 37° C. After 30 minutes, all the tubes were observed for signs of macroscopic and microscopic agglutination.

Agglutination in each of the first five tubes (1:2 to 1:32 dilutions inclusive) was considered a "strongly" positive reaction; less than this was a "weakly" positive reaction. The degree of agglutination in each tube was graded on a scale of 1 to 4.

FELINE LEUKEMIA VIRUS ASSAY

The Felv assays were performed using a commercially available test kit (Leukassay* F, Pitman Moore Inc., Washington Crossing, New Jersey).

BONE MARROW ASPIRATES

Bone marrow aspirates were obtained from the trochanteric fossa (9). The slides were stained with Wright's-Giemsa.

POPULATIONS STUDIED

Anemic Cats

Twenty anemic cats, presented to the Small Animal Clinic, WCVM, University of Saskatchewan with a PCV equal to or less than 0.24 L/L were included in this study. The data base for each cat, included a complete blood count (CBC), blood urea nitrogen (BUN), serum alanine amino transferase (ALT) and urine analysis. A DAT, bone marrow aspirate, Felv assay and serum protein electrophoresis were also performed.

Control Cats

Twenty healthy, Felv negative cats of varying ages, presented to WCVM for elective surgical procedures, made up the control group. Blood was taken for a DAT, CBC and BUN.

RESULTS

AGAR GEL IMMUNOELECTROPHORESIS

Agar gel immunoelectrophoresis confirmed that the commercial serum was a pure preparation of rabbit antifeline IgG (Fig. 1). In comparison, however, the WCVM serum had activity against feline IgG, IgM and other serum proteins in the β and α-2 zones (Fig. 2). These fractions were not detected in the globulin preparation used for immunizing the rabbits perhaps due to insufficient concentration. Since heating serum at 56° C for 30 minutes does not inactivate the C_3 component of complement (10), it is possible the WCVM serum also contained antibody against C_3.

The WCVM serum consistently gave positive reactions at one higher dilution than the commercial reagent. This may have been a direct result of its broader spectrum of activity or may have reflected a difference in antibody concentration between the two sera.

ANEMIC CATS

In total, 80% (16/20) of the anemic cats were positive on the DAT. Sixty percent (12/20) were Felv positive, and of these twelve cats, three were strongly positive, eight were weakly positive and one was negative on the DAT. One of the eight weak reactions was positive only at 4° C with both sera. The anemia was considered to be a direct myelodysplastic effect of Felv infection in five of the twelve cats which had a concurrent cytopenia and/or megaloblastic erythroid precursors in a bone marrow aspirate. In seven, the anemia was associated with other diseases (Table I). The remaining 40% (8/20) of cats were Felv negative

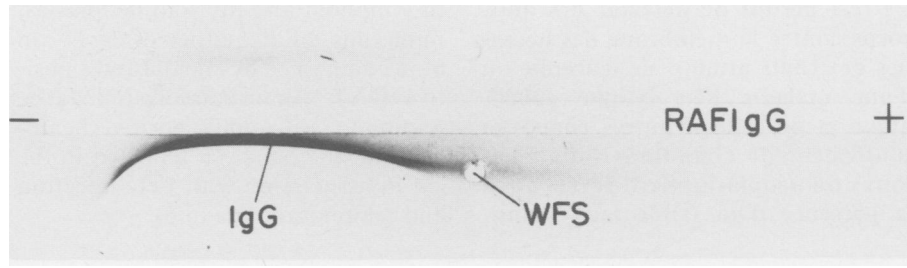

Fig. 1. Immunoelectrophoresis of a purified preparation of rabbit antifeline IgG (RAF IgG) placed in trough against whole feline serum (WFS) in the lower well. The precipitin line (IgG) confirms that the commercial serum has activity against IgG only. Cathode (−) and anode (+) are marked.

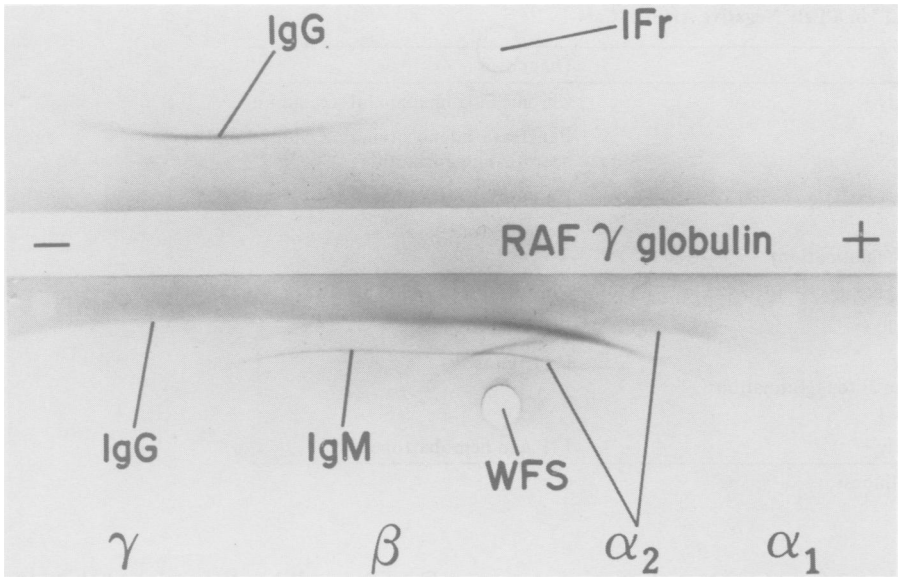

Fig. 2. Immunoelectrophoresis. The immunizing fraction (IFr) is in the upper well; whole feline serum (WFS) is in the lower well, with WCVM rabbit antifeline gamma globulin (WCVM) in the trough. Note that although the immunizing fraction appears to contain only IgG, the WCVM serum has activity against IgG, IgM and other proteins in the β and α-2 zones (all shown with lines). Cathode (-) and anode (+) are marked.

and anemic for a variety of reasons. Two were strongly positive, three were weakly positive and one was negative on the DAT. Two cats, one of which had hemobartonellosis, had autoagglutination so severe the DAT could not be performed. These cats were assumed to have red cell antibody (Table II).

CONTROL CATS

Forty percent (8/20) of the control cats were weakly positive on the DAT using a 1:2 dilution of the WCVM serum at 4° C. One other cat (5%) was weakly positive with both sera. The remaining 55% (11/20) were negative on the DAT.

INCIDENTAL FINDINGS

Six anemic cats showing one or more cytopenias on a CBC were Felv positive and five out of six were DAT positive. Six cats with megaloblastic erythroid precursors in bone marrow aspirates were Felv positive and DAT positive. Three cats with myeloproliferative disease were Felv positive and DAT positive. (Table I).

Two of the three cats with hemobartonellosis were Felv negative. This included one with feline infectious peritonitis (FIP). Autoagglutination occurred in six cats including the three with hemobartonellosis.

DISCUSSION

Coombs positive hemolytic anemia

TABLE I. Result of DAT[a] in 12 Felv Positive Anemic Cats

Cat No.	DAT	Megaloblastosis of erythroid bone marrow precursors	Cytopenia	Diagnosis
1	Weakly +			Hemobartonellosis Cardiomyopathy Glomerulonephritis
3	Strongly +	+	Neutropenia	Myeloproliferative disease
4	Strongly +	+		Myeloproliferative disease
7	Strongly +	+	Neutropenia Thrombocytopenia	
8	Weakly +[b]	+	Neutropenia Thrombocytopenia	
10	Weakly +			Glomerulonephritis aplastic anemia
11	Weakly +			Polyarthritis
12	Weakly +			
14	Weakly +	+	Thrombocytopenia	Myeloproliferative disease
16	Weakly +	+		
17	-		Neutropenia	
20	Weakly +		Thrombocytopenia	Chronic interstitial nephritis Glomerulonephritis splenic amyloidosis

[a]DAT — Direct antiglobulin test
[b]Weak positive at 4° C with both sera

TABLE II. Results of DAT[a] in 8 Felv Negative Anemic Cats

Cat No.	DAT	Diagnosis
2	Weakly +	Chronic inflammation (abscessation)
5	Strongly +	Pancreatic adenocarcinoma with necrotizing pancreatitis
6	—	Hemorrhage (trauma)
9	ND[b] (autoagglutination)	Hemobartonellosis
13	Strongly +	Chronic interstitial nephritis
15	Weakly +	Warfarin toxicity
18	ND (severe autoagglutination at 4° C)	Undiagnosed
19	Weakly +	FIP and hemobartonellosis

[a]DAT — Direct antiglobulin test
[b]ND — Not done

has been reported in the cat in association with Felv infection and lymphosarcoma (5,6). In the dog, numerous infectious, inflammatory, and neoplastic diseases can be accompanied by anemia and a positive DAT, although hemolysis may be minimal (4). Our study revealed that a similar situation exists in the cat. Positive reactions were obtained in cats with Felv infection, hemobartonellosis, feline infectious peritonitis, chronic interstitial nephritis, pancreatic adenocarcinoma, and chronic abscessation. Only in those cats with hemobartonellosis was severe hemolysis evident although varying degrees of autoagglutination were noted in some Felv positive and negative anemic cats.

Myeloproliferative disorders in the cat are often preceded by a marrow suppressive phase (hematopoietic dysplasia) (6,11,12). This so-called preleukemic state is characterized by a progressive nonregenerative anemia, and frequently, a peripheral macrocytosis associated with megaloblastosis of bone marrow precursors. Maturation arrest may be evident in other cell lines resulting in cytopenias (12,13,14). From our results it appears likely that these cats will be Felv positive and DAT positive.

The exact mechanism of anemia as a nonneoplastic manifestation of Felv infection has not been established. C-type virus particles have been identified in leukocytes, platelets, erythrocytes and marrow cells of cats with refractory anemias (15). Since replication of virus can occur at the erythrocyte plasma membrane and mouse erythrocytes can acquire viral antigens following murine leukemia virus (MLV) infection (16,17), it is possible that immune-mediated destruction of red blood cells might partly account for the anemia observed in Felv infected cats and result in a positive DAT.

In man, healthy individuals can have a low serum concentration of a cold reactive IgM with activity against the erythrocyte I antigen. This activity can increase in certain pathological states such as mycoplasma pneumonia and lymphoproliferative disorders although there is often no evidence of hemolysis. Red cells from these patients commonly react with anti-C_3 Coombs serum (2,3). IgM cannot be detected since it readily dissociates from the red cell. Forty percent of the control cats in this study exhibited a low titre of agglutinating antibody on their red cells at 4° C. It is interesting to note that these antibodies were only detected with the WCVM serum. This may have been the result of activity against complement, in particular the C_3 component. Another explanation would be that the WCVM serum possessed a higher concentration of antibody protein. Alternatively, it is possible that we failed to remove all nonspecific agglutinins present in the serum. However, this is unlikely, since it was absorbed five times at 4° C and 25° C with red cells from several healthy donor cats.

The argument for using a Coombs serum with a broad spectrum of activity is equivocal. Such a reagent may give a higher percentage of positive DATS, making the relationship to immune-mediated hemolysis uncertain (4). An antiserum with activity against transferrin or nonspecific absorption of serum proteins by damaged erythrocytes may result in false positive reactions (18).

Cats with hemobartonellosis commonly show marked agglutination and hemolysis *in vitro*. Often the agglutinating antibody cannot be removed by washing the red cells in physiological saline. This is diagnostic of immune-mediated hemolysis and precludes the necessity of performing a DAT (18).

In conclusion, the DAT demonstrates antibodies directed against antigens on the red cell membrane. A positive reaction in the cat should not be regarded as evidence of hemolysis and can occur with a number of infectious, inflammatory, and neoplastic diseases including those initiated by or associated with Felv infection. Deposition of IgG or C_3 on the red cell membrane may result in decreased cell lifespan. A high proportion of healthy cats possess a low titre of cold agglutinating antibody which may only be detected if the Coombs serum has a broad spectrum of activity.

The DAT in the cat, therefore, has obvious diagnostic limitations. Conservative interpretation of a positive reaction is advisable especially when there is no clinical evidence of hemolysis.

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