An Indirect Fluorescent Antibody Test for the Detection of Cytauxzoon-like Organisms in Experimentally Infected Cats

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ABSTRACT

Antiserum to feline Cytauxzoon-like parasites was used in conjunction with labeled rabbit antisera to feline globulins to detect the presence of Cytauxzoon-like parasites in spleens of experimentally infected cats. Frozen spleen sections from 21 infected cats showed positively fluorescing masses within splenic veins and a diffuse scattering of discretely fluorescing cells in the red and white pulp. The distribution of fluorescence corresponded with the appearance of parasitized reticulo-endothelial cells in histological preparations of spleen tissue. This indirect fluorescent antibody test consistently detected the presence of Cytauxzoon-like parasites in frozen spleen sections from experimentally infected cats.

RÉSUMÉ

Cette expérience visait à rechercher la présence de protozoaires semblables à Cytauxzoon, dans la rate de chats infectés de façon expérimentale. Les auteurs utilisèrent à cette fin un antisérum spécifique pour ce protozoaire et un antisérum spécifique pour les globulines félines, préparé chez le lapin et conjugué ultérieurement.

L'examen microscopique de coupes congelées de la rate des 21 chats expérimentaux révéla la présence de masses fluorescentes dans les veines spléniques et de plusieurs cellules légèrement fluorescentes, éparpillées dans la pulpe blanche et la pulpe rouge. Les points fluorescents correspondaient aux cellules réticulo-endothéliales parasitées que révélaient les coupes histologiques de tissu splénique. Cette épreuve indirecte d'immunofluorescence démontra constamment la présence de protozoaires semblables à Cytauxzoon dans des coupes congelées de la rate des chats expérimentaux.

INTRODUCTION

Wagner (6), in 1976 reported a fatal disease in four domestic cats occurring in heavily wooded areas of southwestern Missouri and northwestern Arkansas. Microscopic observation of parasitic piroplasm or ring forms in peripheral erythrocytes and large numbers of schizonts within reticulo-endothelial (R-E) cells in various organs were considered to be characteristic of *Cytauxzoon* spp. infections. This was the first report of cytauxzoonosis-like infections in cats and the first incidence of cytauxzoonosis-like infection outside of Africa where infection has been reported in the duiker (4), kudu (5), eland (1) and giraffe (2).

This report describes an indirect immunofluorescence test for the diagnosis of a feline cytauxzoonosis-like disease and represents the first serological test available for this disease.

MATERIALS AND METHODS

Transmission of Disease

A *Cytauxzoon*-like infection has been passed serially in over 70 domestic short hair cats in this laboratory by injecting

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fresh blood or triturated spleen from cats showing clinical disease. Cats usually began to develop a febrile response at six to nine days postinoculation (DPI) and usually died between ten and 13 DPI. The method of maintaining the infection was as follows: 1 to 3 ml of blood or triturated spleen were taken from an infected cat at some time after the initial rise in temperature and injected either intravenously (IV) or intraperitoneally (IP) in normal cats. The 21 infected cats used in this study were inoculated IP.

Demonstration of Parasites in Blood Smears

Blood samples from 14 of the infected cats were examined for presence of the *Cytauxzoon*-like organism at certain intervals after the initial rise in body temperature.

Blood smears were made and fixed in methanol for five minutes, stained with 6% Giemsa for 35 minutes, rinsed in distilled water and examined microscopically under the oil immersion lens. The results are given in Table I.

Cell Cultures and Immunizing Inoculum

A spleen from an infected cat was harvested aseptically when the cat was *in extremis*. The spleen was minced with scissors and washed in three changes of phosphate buffered solution (PBS). The cells were then dispersed with 0.5% trypsin and centrifuged at 250 x g for three minutes. The cells were then suspended in 400 ml of growth media per 1 ml packed cells. The growth medium, 199 in Hank's salts, was supplemented with 20% fetal bovine serum, penicillin (200 units/ml), dihydrostreptomycin (200 μg/ml), mycostatin (60 units/ml) and kanamycin (100 μg/ml). Six ml of the cell suspension (1:400) were seeded in each of 50 ml Falcon flasks.

Bone marrow from the long bones of the same infected cat was harvested, washed three times in PBS and centrifuged at 250 x g for 20 minutes. The cells were then suspended in the same medium as were the spleen cells at a dilution of 1:300 and 6 ml amounts of the cell suspension were dispensed into 50 ml Falcon flasks.

After five days, the spleen cell cultures from the infected cat were retarded in growth (30% confluence, compared with 75% confluence in control spleen cultures) and the bone marrow cells, although attached to the vessel surface, did not show any proliferation. The infected spleen and bone marrow cells were removed from the flasks with rubber policemen and were mixed with a 1:4 dilution of African green monkey kidney (Vero) cell[1] monolayers. These cultures reached confluence after six days and were removed from the flasks with rubber policemen and passed at 1:4 dilution with growth media. The "infected" Vero cells were maintained through the third passage.

Production of Antiserum

Cat 4538 was successfully immunized against the feline *Cytauxzoon* by inoculating it with parasites of cell culture origin. It was inoculated IV with 3 ml of cell suspension from the primary "infected" Vero cell cultures described. After two weeks it was subcutaneously inoculated with 3 ml of third passage "infected" Vero cell suspension. Consequently, it was challenged ten times with 1 to 3 ml of infective blood from cats showing clinical signs of cytauxzoonosis. Blood smears from all of these cats had piroplasms (Piroplasms were not always present. However, schizonts were invariably found in some major organ or organs). It was also challenged by IP inoculation with 5 ml of ground spleen from an infected cat. In contrast to results obtained with the immunized cat, all other cats inoculated with fresh infective blood or spleen developed clinical signs of the cytauxzoonsis-like disease and died.

The serum harvested from weekly bleedings of the immunized cat was collected at weekly intervals, pooled and stored at –70°C.

Fluorescein Isothiocyanate (F.I.T.C.) Labeled Anti-feline Globulin[2]

Commercially conjugated fluorescein rabbit anti-feline immunoglobulin was used as

[1]American Type Culture Collection, Rockville, Maryland.

[2]Sylvana, Millburn, New Jersey.

the labeled secondary antiserum in the indirect fluorescent antibody (IFA) test. The conjugate was absorbed once with 75 mg of normal cat liver powder for each milliliter of conjugate. The liver powder was prepared by acetone dehydration. The conjugate was diluted 1:4 with a counterstaining solution before use.

COUNTERSTAIN

Normal bovine serum conjugated with Bacto-FA rhodamine[3] (No. 2340-56-8) was used to suppress the nonspecific tissue fluorescence. The stock solution of rhodamine was diluted 1:15 in PBS before use.

INFECTED SPLEENS

Infected cats were either euthanized by exsanguination at the terminal stage of disease or succumbed to infection. Carcasses were stored at refrigeration temperature until necropsy. The approximate delays between the time of death and the postmortem examinations are given in Table I. Samples of spleen were placed in PBS until the completion of the necropsy. Tissues were then quick-frozen in liquid nitrogen and stored at –70°C until sectioning. Duplicate samples of all tissues were fixed in 10% phosphate buffered formalin for histological examination.

CONTROLS

In tests for specificity of the reaction, spleens from the following ten cats were processed and tested along with spleens from experimentally infected cats: three normal cats, two cats experimentally inoculated with feline panleukopenia virus (FPLV), one cat that died with colitis of undetermined origin, three cats that died of respiratory disease and one cat that died with an acute suppurative peritonitis. Duplicate slides of all samples were stained with (a) the immune serum, (b) normal feline serum and (c) saline for use as (a) principals and (b, c) controls for nonspecific staining and autofluorescence. In addition, several known positive spleen sections were also stained with cat FPL immune serum.

PREPARATION AND STAINING OF SAMPLES

Frozen spleen sections were cut at 8 microns, placed on glass slides and allowed to air dry. Samples were then fixed for five minutes in cold acetone and again air dried.

The primary reagents (immune serum, normal serum, saline or FPLV antisera) were applied to the slides, which were then placed in a moisture chamber at 37°C. After incubation for 50 minutes, the slides were dipped in distilled water and rinsed with agitation in three changes of PBS for a total of 15 minutes. The secondary reagent (F.I.T.C. labeled rabbit antifeline globulin) was then applied to the sample, which was incubated in a moisture chamber at 37°C for 50 minutes. The slides were again dipped in distilled water and rinsed for 15 minutes in three changes of PBS. After a final rinse in distilled water, slides were completely dried and mounted with coverslips (90% glycerol in 10% PBS was used as the mounting medium).

HISTOLOGY

Samples of spleen were fixed in 10% phosphate buffered formalin, embedded in paraffin sectioned at 3 to 6 microns thickness and stained with hematoxylin and eosin (H & E).

Frozen spleen sections from ten infected cats and three controls were fixed in methanol for five minutes and stained for 45 minutes with a 6% Giemsa solution.

RESULTS

Results of the IFA test along with supportive data from hemotological and histological examinations are described below and summarized in Table I.

[3]Difco Laboratories, Detroit, Michigan.

TABLE I. Results of the Indirect Fluorescent Antibody Test and Other Diagnostic Criteria for Detection of Cytauxzoonosis in Cats

Cat#	Blood Smears[a] Examination	Clinical[b] Diagnosis	Histological[c] Examination	Indirect F.A. Test	Postmortem[d] Delay (hrs.)
1.	+	Cytauxzoonosis	+	+	0
2.	+	"	NE	+	0
3.	−	"	+	+	0
4.	+	"	NE	+	0
5.	−	"	+	+	12
6	−	"	+	+	48
7.	−	"	+	+	0
8.	+	"	+	+	30
9.	NE[e]	"	+	+	12
10.	NE	"	+	+	12
11.	+	"	+	+	0
12.	NE	"	+	+	52
13.	+	"	+	+	24
14.	−	"	+	+	12
15.	+	"	+	+	10
16.	+	"	+	+	10
17.	+	"	NE	+	50
18.	NE	"	NE	+	12
19.	NE	"	NE	+	12
20.	NE	"	NE	+	12
21.	NE	"	NE	+	0
22.	−	Normal	−	−	0
23.	−	"	−	−	0
24.	NE	"	NE	−	4
25.	NE	Respiratory disease	NE	−	12
26.	−	" "	−	−	12
27.	−	" "	NE	−	12
28.	−	Colitis	−	−	24
29.	−	Panleukopenia[f]	−	−	0
30.	−	"	−	−	0
31.	NE	Peritonitis	−	−	12

[a]Blood smears from infected cats checked after the initial temperature rise (Note: less than half of the cats dying of this disease showed a parasitemia)
[b]Clinical diagnosis based on clinical signs and gross pathology
[c]Histological examination is graded + or − depending on the presence or absence of infected R−E cells in spleen sections
[d]Approximate time elapse between death and necropsy
[e]NE = not examined
Experimentally infected

Fluorescence Microscopy

Small, discretely fluorescing structures were observed diffusely scattered throughout the red and white pulp of spleens from all cats infected with the *Cytauxzoon*-like parasites. These structures were interpreted to be fluorescing parasites within the cytoplasm of infected R-E cells. The amount of antigen within the cell cytoplasm varied from cell to cell as evidenced by the variation in size and shape of the fluorescing area. Many cells had a dark spot situated close to the cell border corresponding to the size and location of the nuclei (Fig. 2B). Infected cells were distributed randomly throughout the red pulp and an occasional fluorescing cell was observed within the white pulp.

A striking feature often observed in these spleens was the large accumulations of fluorescing R-E cells within the splenic veins and venous sinuses (Figs. 1, 2A and 2B). Aggregates of fluorescing cells which often occluded the lumen of infected vessels had the same morphology as the cells seen within the red and white pulp but were more likely to show complete cytoplasmic fluorescence. Fluorescing cells were observed both closely associated with vessel walls and free within the lumen. Trabecular veins appeared to be most severely affected.

Only two infected spleens tested with normal serum, saline or heterologous antiserum (FPL antiserum) showed weak fluorescence. All spleen samples from uninoculated control cats were negative.

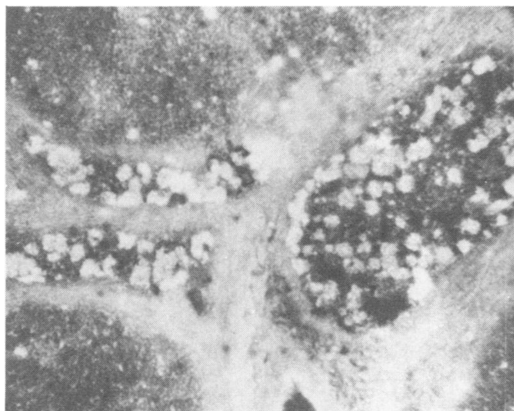
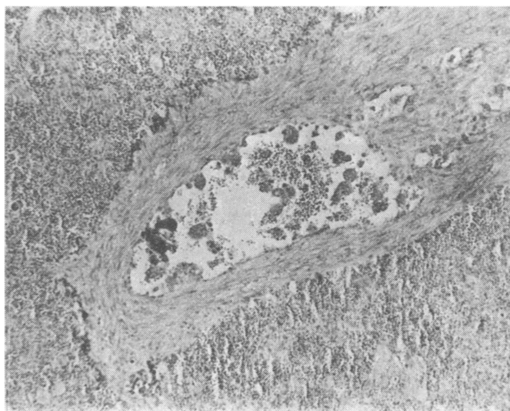


Fig. 1. Fluorescing reticuloendothelial (R-E) cells lining blood vessel of spleen of cat infected with the Cytauxzoon parasite. These cells are filled with the schizont stage of the parasite. Note that some swollen R-E cells are still attached to the endothelial wall, while others are free in the lumen.



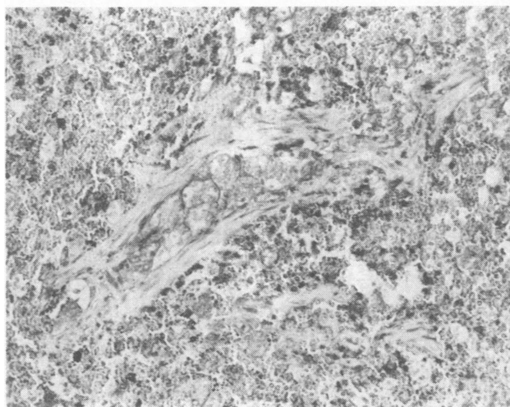


Fig. 2A and B. Splenic sections comparable to Fig. 1 (H & E stain). Both sections show reticuluoendothelial (R-E) cells swollen with the schizont stage of the parasite. Fig. 2A shows R-E cell masses still attached to the endothelium. Other schizont masses are free in the lumen of the blood vessel. Figure 2B shows a blood vessel completely occluded with R-E cells filled with schizonts. The eccentrically located nuclei are clearly discernible.

Histological Findings

Variable numbers of parasitized cells were seen dispersed throughout the red and white pulp. Large accumulations of parasitized R-E cells occupied the lumens of many of the splenic veins and sinuses (Figs. 2A and 2B). Many of these vessels (especially trabecular veins) were partly or completely occluded by these accumulations of swollen cells. Parasitized R-E cells usually had a clearly discernible cytoplasmic membrane and a swollen, lightly basophilic cytoplasm that tended to be amorphous. Occasionally, numerous small round basophilic structures were seen within the cytoplasm of these swollen R-E cells. These basophilic structures were interpreted to be merozoites of the cytauxzoon. Nuclei often had indistinct membranes but could be identified by their prominent nucleoli. Nuclei were eccentrically located and occasionally vesicular in appearance (Fig. 2B).

DISCUSSION

Diagnosis of *Cytauxzoon*-like infections in cats (6) has been dependent upon the demonstration of the erythrocytic stages of the parasite in blood smears and/or schizonts in histological tissue sections. Our own experience and the experience of others (personal communication from J. E. Wagner) has been that infected cats developed very low parasitemias (usually less than 4% infected red blood cells) and we were not able to demonstrate definite intra-erythrocytic parasites in many cases. Infection was detected in stained blood smears of only nine of 14 (64.3%) infected cats examined in this study. Both histological examination and the IFA test detected infection in 14 of 14 and 21 of 21 infected cats respectively (Table I). Diagnosis by IFA staining even after the cats had been dead for up to 52 hours indicated that the parasitic antigens could be detected in the tissue of cats dead for more than two days.

In two infected spleen samples a slightly positive reaction was observed when normal

serum and saline were applied as the primary reagents. This reaction was presumed to be due to the presence of specific antibodies that had been produced *in vivo*. Serum taken from these cats in the late stages of the disease showed specific fluorescent antibody activity. We can assume that, if a cat produces antibodies to the parasite and they react *in vivo*, tissues from such a cat will be positive by the indirect method without the use of immune serum.

The usefulness of the IFA method for testing serum samples from infected cats against known positive antigen is presently being studied. Preliminary results indicate that not all cats develop detectable titers before death. Those cats that show an antibody response tend to have low titers that develop very late in the course of the disease and are insufficient to bring about recovery.

The use of an IFA method for testing serum samples from the *Cytauxzoon* infected or recovered ungulates in Africa remains to be evaluated.

At the present time this is the only available serological test by means of which antibodies against this *Cytauxzoon*-like parasite may be detected. It may be useful in determining the specificity of this infection where another feline protozoan disease such as *Babesia felis* is also a possibility.

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