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SHORT PAPER

Decreased Lipid Fluidity of the Erythrocyte Membrane in Dogs with Leishmaniasis-associated Anaemia

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Summary

In both man and the dog, anaemia resulting from natural leishmaniasis is often severe and mainly associated with a shortened life span of erythrocytes. Lipid fluidity of erythrocyte membranes from 17 dogs with anaemia caused by visceral leishmaniasis was investigated by means of fluorescence polarization. Results were compared with those from three groups of control animals (10 healthy dogs, seven dogs with visceral leishmaniasis but no anaemia, and 10 dogs with anaemia unrelated to leishmaniasis). Fluorescence polarization values recorded for animals with leishmaniasis-associated anaemia were elevated—indicating reduced erythrocyte membrane fluidity—and significantly higher than in the control groups. Mechanical sequestration by the spleen due to increased cell rigidity, or alterations in receptor/ligand erythrocyte cytoadherence mechanisms, or both, may result from decreased membrane fluidity and hence contribute to the anaemia of *Leishmania*-infected dogs.

Introduction

Visceral leishmaniasis is a life-threatening protozoan disease of the mononuclear phagocyte system of man, endemic in both the Old and New Worlds. In southern European countries it is caused by *Leishmania infantum* and domestic dogs are the main reservoir (Bettini and Gradoni, 1986). These animals may develop a syndrome characterized by chronic development of major viscerocutaneous signs (lymphadenopathy, splenomegaly, exfoliative dermatitis and skin ulcers), which appear with varying degrees of severity after an incubation period of one month to several years.

In both man and the dog, anaemia resulting from natural leishmanial infection is often severe. In human patients suffering from kala-azar, anaemia was found to be associated with shortened life span of erythrocytes (Knight *et al.*, 1967). In a retrospective study of 150 dogs naturally infected by *L. infantum*, Ciaramella *et al.* (1997) documented the presence of anaemia in 60% of animals. In experimental visceral leishmaniasis, anaemia has been reported as an early and constant clinical feature in both canine and hamster hosts (Binhazim *et al.*, 1993). The pathogenesis of this disorder is still poorly understood and probably more than one factor is involved. Immunopathological events resulting from polyclonal B-cell activation, such as production of autoantibodies against erythrocyte membrane antigens, complement activation or immunoglobulin opsonizing factors, have long been suspected to play a role (Woodruff *et al.*, 1972; Kager *et al.*, 1984; Pontes De Carvalho *et al.*, 1986). More recently, decreased life span of erythrocytes from *Leishmania*-infected hamsters has been associated with lipid peroxidation of membranes, due

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Table 1
Haematological features and leishmaniasis serology and parasitology in four groups of dogs

Group (n)	*RBC (× 10^6/μl) (mean ± SEM)*	*Hb (g%) (mean ± SEM)*	*Ht (%) (mean ± SEM)*	*IFAT titre (range)*	*Microscopy/ culture*
L+/A+ (17)	4·0±0·7†	9·1±1·5†	24·9±4·9†	80–2560	Positive‡
L−/A−* (10)	6·4±1·4	12·5±0·7	41·1±5·7	<40	Negative
L+/A−* (7)	6·4±0·3	12·8±0·3	43·8±1·1	40–320	Positive§
L−/A+* (10)	3·6±0·2†	8·3±0·4†	23·6±1·4†	<40	Negative

* Control groups.
† Significantly different from group L−A− ($P<0·01$).
‡ Not done in three animals with IFAT titres ⩾ 160.
§ Not done in one animal with IFAT titre of 80.

to reduced efficacy of the protective antioxidant enzyme system (Biswas *et al.*, 1995). This process may alter the lipid fluidity of erythrocyte membranes (Rice-Evans and Hochstein, 1981), leading to changes in cell shape and plasticity and possibly inducing splenic sequestration and destruction (Weed and Reed, 1966).

Lipid bilayer fluidity of normal and pathological cellular membranes can be measured by means of fluorescence probes which, once incorporated into the membrane, determine a fluorescence polarization which is inversely related to fluidity (Schachter *et al.*, 1982; Sorensen, 1988). This study evaluated erythrocyte membrane fluidity in dogs with anaemia caused by visceral leishmaniasis. Three groups of dogs were used for comparison (healthy animals, dogs with visceral leishmaniasis but no anaemia, and dogs with anaemia unrelated to leishmaniasis).

Materials and Methods

Leishmania *Serology and Parasitology*

Serum samples were examined by an immunofluorescence antibody test (IFAT) with *L. infantum* promastigotes as antigen and a serum dilution of 1 in 40 as threshold titre (Pozio *et al.*, 1981). Lymph node or bone marrow aspirate samples were smeared on slides and stained with Giemsa's stain for microscopical examination, and also cultured in blood-agar based media.

Dogs

Forty-four domestic dogs of various breeds and ages from the Naples area of Italy were included in the study (Table 1). Group L+/A+ consisted of 17 dogs, shown by serological and parasitological methods to have visceral leishmaniasis, and shown by red blood cell (RBC) count, haemoglobin (Hb) value, and haematocrit (Ht) value to be anaemic. Control group L−/A− consisted of 10 clinically healthy animals with no leishmaniasis or anaemia as judged by the methods listed above. Control group L+/A− consisted of seven dogs with leishmaniasis but no anaemia. Control group L−/A+ consisted of 10 dogs with no leishmaniasis but with anaemia due to a nutritional deficit or to intestinal worms, or both. Concomitant systemic infectious diseases, such as ehrlichiosis, babesiosis, hepatozoonosis and toxoplasmosis, were excluded in all dogs by means of routine microscopical and serological methods.

Fluorescence Polarization Study

Erythrocytes were obtained from heparinized blood samples by centrifugation for 10 min at 500 ***g*** at room temperature. The cells were washed three times by centrifugation in phosphate-buffered saline (pH 7.4) and resuspended in the same buffer at 0·2% concentration. Each sample was examined in triplicate with a fluorescent probe rapidly incorporated into membranes of living cells (Illinger *et al.*, 1995). An aliquot of 4 μM 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH; Sigma), prepared from a 4 mM stock solution in dimethylformamide, was added to the erythrocyte suspensions at 1:1 ratio (v/v) and incubated for 65 min at 37°C. Fluorescence was measured at 37°C with an LS-3 Luminescence Spectrometer (Perkin-Elmer Corp., Norwalk, Connecticut, USA) equipped with polarizers in both

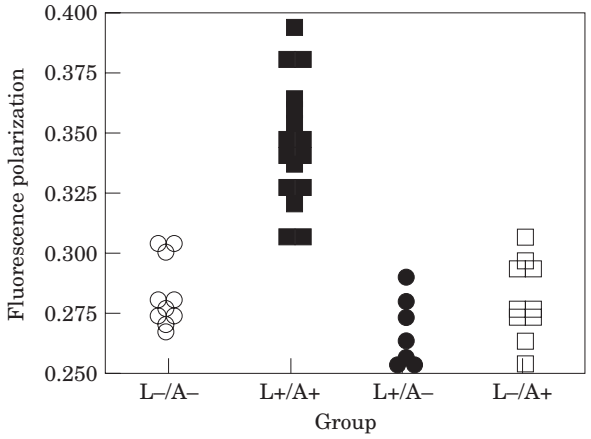

Fig. 1. Distribution of fluorescence polarization of erythrocyte membranes in four groups of dogs. Filled symbols represent *Leishmania*-infected animals, square symbols refer to dogs with anaemia. L = *Leishmania*-infected; A = anaemic.

excitation and emission beams. Excitation and emission wavelengths were set at 360 and 455 nm, respectively. The polarization value, P, of the fluorescence signal was measured as follows: $P = I_w - I_{vh} \cdot G / I_w + G \cdot I_{vh}$, where I_w and I_{vh} are the parallel (vertical) and perpendicular (horizontal) components of the emitted light when excited with vertically polarized light; G is a correction factor for the optical system and is equal to the ratio I_{hv}/I_{hh}, where I_{hv} and I_{hh} are the vertical and horizontal components of emission from horizontally polarized exciting light (Shinitzky and Barenholz, 1978).

Data Analysis

Results were expressed as mean value ± SEM. The unpaired Student *t*-test was used for statistical analysis at the significance level of $P \leqslant 0{\cdot}01$.

Results

Fluorescence polarization results are shown in Fig. 1. For the three groups employed as controls, distributions of fluorescence polarization values were very similar: $0{\cdot}28 \pm 0{\cdot}01$ (range 0·27–0·30) in group L−/A−, $0{\cdot}27 \pm 0{\cdot}02$ (range 0·25–0·29) in group L+/A−, and $0{\cdot}29 \pm 0{\cdot}02$ (range 0·26–0·31) in group L−/A+. Values recorded for the animals with leishmaniasis-associated anaemia (group L+/A+) were, however, much higher ($0{\cdot}34 \pm 0{\cdot}03$, range 0·31–0·39) and significantly different from those of the control groups ($P<0{\cdot}01$).

Discussion

The increase in fluorescence polarization above control values (+18%), which corresponds to a decrease in membrane fluidity of the same magnitude, appeared to be very elevated by comparison with measurements on pathological canine erythrocyte membranes recorded in the literature (Mehta *et al.*, 1991).

The maintenance of membrane fluidity within narrow physiological limits is probably a prerequisite for proper functioning and long-term survival of a cell. Lipids play a key role in determining the membrane fluidity, and changes in lipid composition have been reported to alter important cellular functions (Stubbs and Smith, 1984). Among the techniques used to study the dynamic properties of the membrane lipid matrix, fluorescence polarization with fluorescent probes such as TMA-DPH is a rapid and powerful routine method for assessing the overall structural order in membrane lipids (Van Blitterswijk *et al.*, 1987; Illinger *et al.*, 1995). So far, only one study has been reported on fluidity changes in canine eythrocyte membranes, in animals with a non-infectious disease (Mehta *et al.*, 1991). No studies of cell membrane fluidity are available in respect of clinical cases of human or canine leishmaniasis.

The findings in the four groups of dogs strongly indicated that a significant change in erythrocyte membrane fluidity was directly related to both leishmaniasis and anaemia. These results accord with conclusions from the experimental study of Biswas *et al.* (1995) who, in *Leishmania*-infected hamsters, showed a clear association between presence of anaemia, decrease in erythocyte membrane fluidity and lipid peroxidation, this last event probably being caused by the demonstrated decrease in activity of protective enzymes such as superoxide dismutase, catalase and glutathione reductase. This biochemical injury, however, remains to be confirmed in *Leishmania*-infected dogs. In addition to impairment of the enzyme protective system, the role of oxygen-free radicals should be taken into account, as these products increased in the peripheral blood of dogs with leishmaniasis (De Luna *et al.*, 1996). Interestingly, none of the animals of group L−/A+, with anaemia due to an iron-deficient diet or to infection with intestinal worms, showed significant changes in membrane fluidity, thus suggesting a specific role of *Leishmania* infection in this pathological event.

How anaemia results, at least in part, from changes in erythrocyte membrane fluidity remains unknown. On the one hand, decreased membrane

fluidity greatly increases cell rigidity, which may lead to mechanical sequestration in the spleen (Rosenmund *et al.*, 1975). On the other, receptor-ligand cytoadherence mechanisms may play a role, since alterations in lipid fluidity have been reported to affect receptor and protein exposure (Shinitzky and Rivnay, 1977). In patients affected by acute myeloid leukemia, increased membrane fluidity of blast cells was directly related to bone marrow egress and inversely associated with adhesion to fibronectin and fibroblastic extracellular matrix (Berger *et al.*, 1994). Bone-marrow suppressive factors have long been postulated in leishmaniasis-associated anaemia (Woodruff *et al.*, 1972), but whether alterations in receptor/ligand erythrocyte cytoadherence mechanisms play a part in canine leishmaniasis remains to be elucidated.

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