Pathological changes in the bone marrow of dogs with leishmaniosis

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Bone marrow aspiration smears from 15 dogs naturally infected with leishmania were evaluated. Three of the dogs showed no clinical signs, six had up to three clinical signs and six had more than three. The most common pathological features of the bone marrow were megakaryocytic dysplasia in 10 of the dogs, erythrophagocytosis in eight, erythroid dysplasia in two and emperipolesis in two. The megakaryocytic and erythroid dysplasia were probably related to an increased number of marrow macrophages producing high levels of tumour necrosis factor α and interferon γ. Six of the dogs with clinical signs showed bone marrow dysplastic features and erythrophagocytosis, suggesting that leishmaniosis could be the unique cause of both conditions.

IN dogs, leishmaniosis, caused by the kinetoplastid protozoan *Leishmania infantum*, an obligatory intracellular parasite of mammalian macrophages, is a severe systemic disease transmitted by the bite of haematophagous phlebotomine sandflies. Domestic dogs are the main reservoir hosts for zoonotic human visceral leishmaniasis in both the Old and New Worlds. The disease in dogs is characterised by the chronic development of viscerocutaneous signs, although these signs develop in less than half of infected dogs. However, whether they show clinical signs or not, infected dogs can infect phlebotomine vectors (Gradoni and others 1987, Molina and others 1994). The appearance and severity of clinical signs depend upon the dog's immunological response and the stage of the disease. The most common signs are lymphadenomegaly, skin lesions, loss of weight, anaemia, ocular lesions and renal failure. Laboratory findings consist of high levels of serum immunoglobulins as a result of polyclonal B cell activation, accompanied by the inversion of the normal albumin:globulin ratio, a moderate normohypochromic anaemia, with low levels of red blood cells, low packed-cell volume and haemoglobin, a moderate neutrophilia and high levels of renal parameters (Ciaramella and others 1997). This paper describes the pathological changes observed in the bone marrow of 15 dogs with leishmaniosis, which were affected clinically to different degrees.

MATERIALS AND METHODS

Nine male and six female dogs of different breeds, aged two to nine years, and weighing 20 to 45 kg were examined. Three of the dogs showed no clinical signs, six showed from one to three clinical signs and the other six showed more than three clinical signs. The diagnosis was confirmed by specific serology with an indirect fluorescent antibody test (IFAT), with a titre of at least 1:80, and by direct observation of the parasite in smears of bone marrow and/or lymph nodes. The dogs had not received antileishmania treatment over the previous six months and had not been given drugs suspected or known to cause myelodysplasia. Complete blood counts, and blood, serum and urine biochemical profiles were obtained from all the dogs, and Coombs' test was applied to the anaemic and thrombocytopenic dogs. The dogs were also tested serologically to exclude diseases such as erhlichiosis, filariosis and babesiosis. Samples of bone marrow were obtained by aspiration biopsy from the third sternebra, using a 16 to 18 G needle, and smears were stained with Diff-Quik. A differential count of 500 cells was made on each of two bone marrow smears from each dog. The myeloid:erythroid (M:E) ratio, the granulocyte maturation ratio and the erythroid maturation ratio were calculated. The percentages of granulocytes, erythroid cells and megakaryocytes with dysplastic features, and the percentages of myeloblasts and rubriblasts were determined. In each series, dysplasia was defined by the occurrence of dysplastic features in over 10 per cent of the cells counted.

Ten normal dogs, six of them female, admitted for neutering, were enrolled as a control group. They were all free of infection, had normal blood counts with no haematological abnormalities and had no history of haematological disease. Samples of bone marrow were aspirated by sternal puncture under general anaesthesia before the surgical procedure began.

RESULTS

The characteristics of the dogs with leishmaniosis and the main findings are summarised in Table 1. The bone marrow smears were satisfactory in terms of their cellularity, thickness and the quality of staining.

Control group

The bone marrow aspirates of the dogs in the control group were normal in terms of their morphology and the maturation of the myeloid and erythroid series and the M:E ratio. Two of the 10 dogs showed occasional erythrophagocytosis, with nuclei being extruded from maturing erythrocytes.

Infected dogs showing no clinical signs

In two of these three dogs, the anti-leishmania IFAT titre was 1:80, and in the other it was 1:320. In all three dogs, the peripheral blood count showed mild neutrophilia, and in one eosinophilia and normocytic normochromic hyporegenerative anaemia were also observed. The dogs' total protein concentration and albumin:globulin ratio were normal, as were the results of Coombs' test. The bone marrow smears showed neutrophilic hyperplasia in two of the three dogs, and myeloid hyperplasia in the third (neutrophilic and eosinophilic hyperplasia). One dog showed erythrophagocytosis (Fig 1).

Infected dogs showing one to three clinical signs

The anti-leishmania IFAT titres of the six dogs showing one to three clinical signs of leishmaniosis ranged from 1:320 to 1:2560. Two of the dogs showed moderate thrombocytopenia. One of the dogs had a mild proteinuria (300 mg/l). In two of the dogs the concentration of total protein was significantly higher and the albumin:globulin ratio lower than normal values. Coombs' test was negative in all cases. Bone marrow changes consisted of megakaryocytic dysplasia in all six dogs,

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TABLE 1: Demographic, serological and clinical characteristics of 15 dogs with leishmaniosis and the pathological changes in their bone marrow

Dog	Age (years)	Clinical condition	IFAT titre	Haemogram	Biochemical/ urinary profile	Bone marrow pathology
Male beagle	2	No clinical signs	1:80	Neutrophilia	Normal	Neutrophilic hyperplasia, erythrophagocytosis
Female crossbreed	3	No clinical signs	1:80	Neutrophilia	Normal	Neutrophilic hyperplasia
Female crossbreed	5	No clinical signs	1:320	Anaemia, neutrophilia, eosinophilia	Normal	Myeloid hyperplasia
Female dobermann	3	≤3 clinical signs	1:320	Thrombocytopenia	Normal	Megakaryocytic dysplasia, eosinophilic hyperplasia, erythrophagocytosis
Male crossbreed	6	≤3 clinical signs	1:2560	Thrombocytopenia	Mild proteinuria A:G ratio: 0·28	Megakaryocytic dysplasia, erythrophagocytosis
Male bassetthound	6	≤3 clinical signs	1:640	Normal	Normal	Megakaryocytic dysplasia, emperipolesis
Female bassetthound	6	≤3 clinical signs	1:1280	Normal	Normal	Megakaryocytic dysplasia, erythrophagocytosis
Male breton	2	≤3 clinical signs	1:320	Normal	Normal	Megakaryocytic dysplasia, erythrophagocytosis
Male beagle	5	≤3 clinical signs	1:640	Normal	A:G ratio: 0·31	Megakaryocytic dysplasia
Male crossbreed	2	>3 clinical signs	1:640	Anaemia, thrombocytopenia	A:G ratio: 0·25	Megakaryocytic dysplasia, erythroid hypoplasia, erythrophagocytosis
Female English setter	3	>3 clinical signs	1:2560	Anaemia, thrombocytopenia	A:G ratio: 0·63	Megakaryocytic dysplasia, erythroid dysplasia, erythrophagocytosis
Male dobermann	6	>3 clinical signs	1:1280	Anaemia, thrombocytopenia	Hyperazotaemia, hypercreatininaemia, proteinuria A:G ratio: 0·20	Erythroid hypoplasia, erythrophagocytosis, emperipolesis
Male beagle	2	>3 clinical signs	1:2560	Anaemia, thrombocytopenia	A:G ratio: 0·65	Erythroid dysplasia
Male dobermann	3	>3 clinical signs	1:320	Thrombocytopenia	Normal	Megakaryocytic dysplasia
Female labrador	9	>3 clinical signs	1:640	Normal	A:G ratio: 0·42	Megakaryocytic dysplasia

A:G Albumin:globulin, IFAT Indirect fluorescent antibody test

erythrophagocytosis in four, megakaryocytic emperipolesis in one (Figs 2, 3) and eosinophilic hyperplasia in one.

Infected dogs showing more than three clinical signs

The anti-leishmania IFAT titres of the six dogs showing more than three clinical signs of leishmaniosis ranged from 1:320 to 1:2560. Peripheral blood counts revealed hyporegenerative normocytic hypochromic anaemia in four of the dogs and thrombocytopenia in four; one anaemic and thrombocytopenic dog also had hyperazotaemia (20·6 mmol/l), hypercreatininaemia (185 µmol/l) and proteinuria (1 g/l). In five of the six dogs, serum protein electrophoresis revealed a high concentration of total proteins, with hypoalbuminaemia and hyperglobulinaemia, and a low albumin:globulin ratio. Coombs' test was negative in all cases. Bone marrow changes consisted of megakaryocytic dysplasia in four of the dogs (Figs 4, 5), erythroid hypoplasia in two, erythroid dysplasia in two (Figs 6, 7) and erythrophagocytosis in three of the dogs (Fig 8). Macrophages phagocytising erythroblasts and leishmania amastigotes were also detected (Fig 9). One dog had emperipolesis.

DISCUSSION

Myelodysplastic syndromes are a heterogeneous group of acquired changes in haematopoietic stem cells that occur in

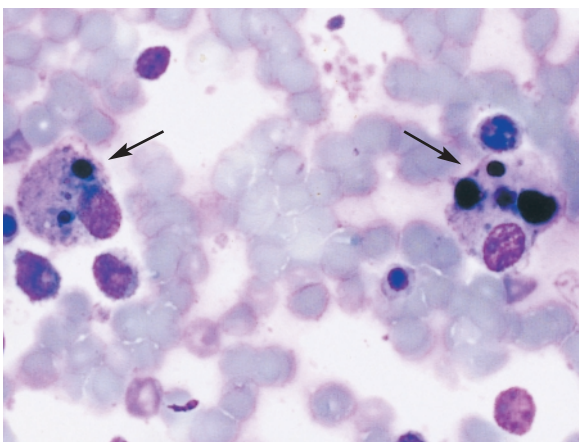

FIG 1: Erythrophagocytosis of a dog with no clinical signs of leishmaniosis, showing two large mononuclear cells with phagocytised erythroblasts (arrows). Diff Quik. x 1000

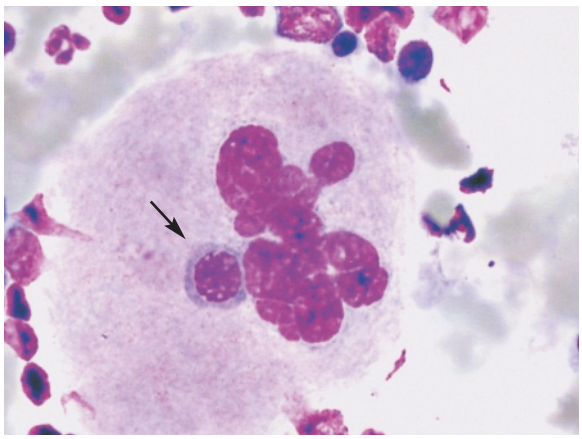

FIG 2: Megakaryocytic emperipolesis in a dog with one to three clinical signs of leishmaniosis, showing a megakaryocyte with hyperlobulated nucleus and a basophil erythroblast (arrow). Diff Quik. x 1000

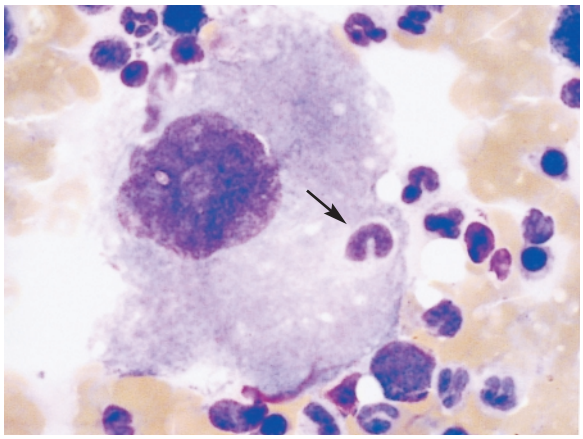

FIG 3: Megakaryocytic emperipolesis in a dog with one to three clinical signs of leishmaniosis, showing a megakaryocyte with a neutrophilic band (arrow). Diff Quik. x 1000

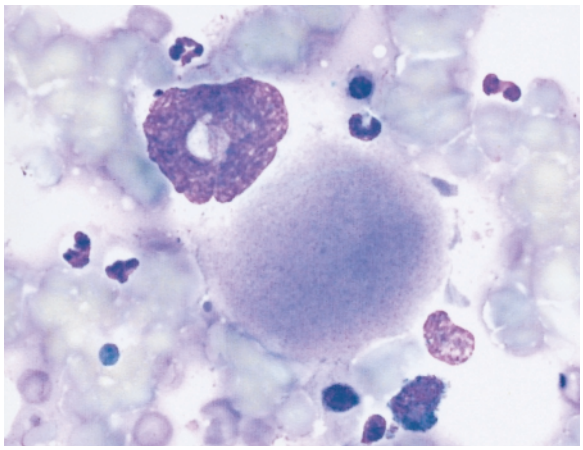

FIG 5: Megakaryocytic dysplasia in a dog with more than three clinical signs or leishmaniosis, showing a megakaryocyte with a disorganised and expelled nucleus. Diff Quik. x 1000

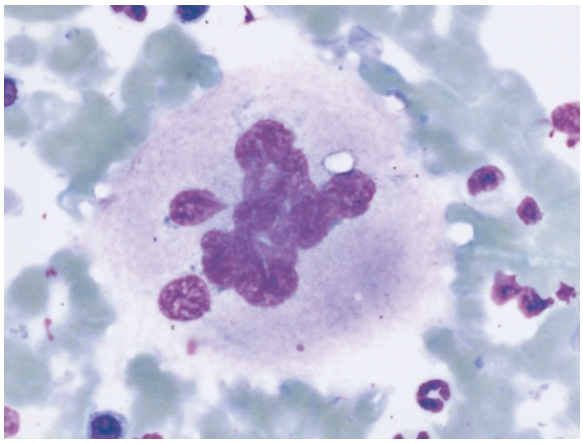

FIG 4: Megakaryocytic dysplasia in a dog with more than three clinical signs of leishmaniosis, showing a megakaryocyte with a hyperlobulated and fragmented nucleus. Diff Quik. x 1000

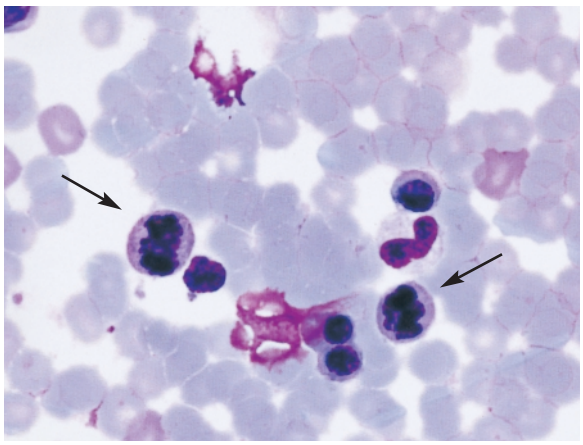

FIG 6: Erythroid dysplasia in a dog with more than three clinical signs of leishmaniosis, showing two erythroblasts with atypical mitoses (arrows). Diff Quik. x 1000

dogs and cats as well as in people. The disorders may be primary or secondary, and are characterised by anaemia and/or leucopenia and thrombocytopenia, together with single or multilineage dysplastic changes in the bone marrow. Secondary causes of myelodysplasia have not been studied extensively; they include myelofibrosis, immune-mediated thrombocytopenia, immune-mediated haemolytic anaemia, polycythaemia vera, pyometra, thrombopathia and some antineoplastic drugs (Weiss and Aird 2001). High serum levels of tumour necrosis factor alpha (TNFα) and interferon gamma (INFγ), which play a pivotal role in myelodysplastic syndromes, together with other proapoptotic cytokines have been reported in dogs and cats (Walton and others 1996). In human visceral leishmaniosis bone marrow changes including haemophagocytic syndrome and trilineage myelodysplasia have been reported (Gagnaire and others 2000, De Fusco and others 2002, Yarali and others 2002, Kopterides and others 2003).

Minor dysplastic changes have been described in bone marrow aspirates from 54 haematologically normal elderly human patients; they involved mainly megakaryocytes, and to a smaller extent, erythroblasts and granulocytes, but no emperipolesis was observed (Girodon and others 2001).

In dogs with leishmaniosis, the main blood changes are anaemia and/or thrombocytopenia, whereas in human patients pancytopenia is often observed.

In this study, 10 of the dogs showed megakaryocytic dysplasia, characterised by dwarf megakaryocytes, and large megakaryocytes, with nuclear abnormalities including hypolobulation, hyperlobulation and disorganised nuclei; in six of them these changes were associated with mild thrombocytopenia. All the dogs with megakaryocytic dysplasia had clinical signs of leishmaniosis and had anti-leishmania IFAT titres of greater than 1:160; this clinical condition is believed to be associated with a Th-2 immune response, with increased production of interleukin 4 and interleukin 10. In contrast, dogs showing no clinical signs usually have a Th-1 immune response characterised by high levels of TNFα, INFγ and interleukin 2 (Pinelli and others 1994, Cabral and others 1998). The present data therefore contrast with these reports that give the Th-1 cytokines, for example, TNFα and INFγ, a pivotal role in the development of myelodysplasia. In this study, the bone marrow aspirates from the 12 dogs that showed clinical signs all had a large number of macrophages, which may have produced high levels of the cytokines responsible for dysplasia. However, the pathway of the immune response in dogs with leishmaniosis needs further investigation. It is possible that the thrombocytopenia in dogs with megakaryocytic dysplasia may be caused by a low rate of production of thrombocytes and by an increased disruption of abnormal platelets by the spleen.

Emperipolesis was observed in two of the dogs, which both showed several signs of leishmaniosis. Emperipolesis differs from phagocytosis in that the intracytoplasmic cells survive for a short time within the megakaryocytes. The significance of this finding is uncertain. Increased emperipolesis has been reported in human beings with various conditions, including active blood loss, carcinomas, myeloproliferative disorders and reactive thrombocytosis (Cashell and Buss 1992). It is possible that in the dogs with leishman-

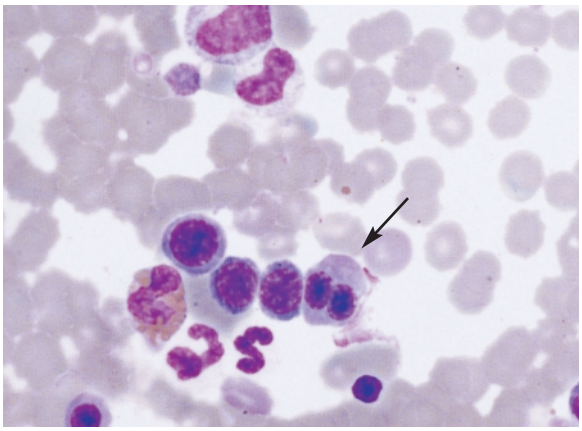

FIG 7: Erythroid dysplasia in a dog with more than three clinical signs of leishmaniosis, showing an erythroblast with an atypical mitosis (arrow). Diff Quik. x 1000

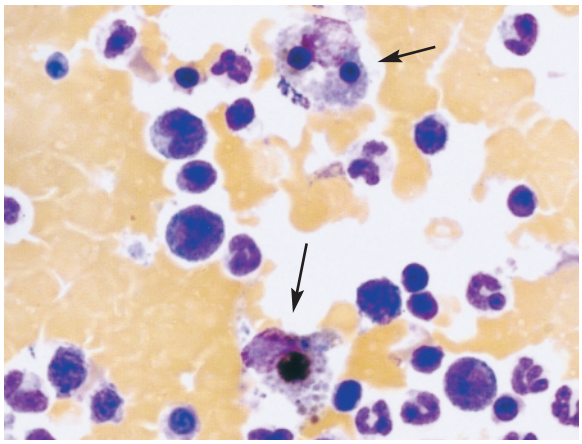

FIG 8: Erythrophagocytosis in a dog with more than three clinical signs of leishmaniosis, showing two large mononuclear cells with phagocytised erythroblasts (arrows). Diff Quik. x 1000

iosis emperipolesis may be another sign of bone marrow abnormality.

Two of the anaemic dogs with more than three clinical signs had erythroid dysplasia consisting of abnormal erythrocyte maturation and morphology. Nuclear fragmentation, multinucleated cells, nuclear and cytoplasmic asynchrony and atypical mitosis were also observed. In dogs with leishmaniosis, anaemia is usually attributed to an inflammatory condition and in some cases to an immune-mediated mechanism. Erythroid dysplasia could be a further pathogenic mechanism of anaemia in dogs with leishmaniosis.

Erythrophagocytosis was observed in eight of the dogs, one of which showed no clinical signs. Bone marrow smears showed numerous macrophages phagocytising one or more erythroblasts. Bone marrow macrophages normally remove erythrocytes to some extent, but the degree to which this phagocytic activity occurs, and the cell types that are phagocytised differentiate this normal process from inappropriate haemophagia (Rothstein 1993). In normal bone marrow from dogs and cats, erythrocytophagia is rare and generally involves only mature erythrocytes or extruded nuclei; conversely, prominent haemophagia in bone marrow that involves haematopoietic precursors is considered abnormal (Tyler and others 1994). Haemophagic histiocytosis is a reactive proliferation of polyclonal histiocytes secondary to infection, for example, with canine parvovirus, *Salmonella* species, or feline calicivirus, neoplasia or metabolic disorders (Walton and others 1996). The condition is a rare complication that can lead to a misdiagnosis of visceral leishmaniosis when this parasitic disease is uncommon in a country (De Fusco and others 2002).

In the present study, the erythrophagocytosis could represent an abnormal response of macrophages to the production of T cell cytokines necessary to enhance the phagocytosis of amastigotes, as shown by the simultaneous presence of erythroblasts and *Leishmania* species amastigotes phagocytised by macrophages. Erythrophagocytosis was also observed in one of the dogs showing no clinical signs, in which macrophage activity was probably increased.

Six of the dogs showing clinical signs had dysplastic features and erythrophagocytosis in their bone marrow, suggesting that leishmaniosis could be the sole cause of both conditions.

The results of this study suggest that changes in the bone marrow, particularly dysplastic changes, could be related to the progression of leishmaniosis. Further studies are necessary to understand whether an examination of bone marrow samples from dogs with leishmaniosis could help to identify animals with no clinical signs that might develop overt clinical disease.

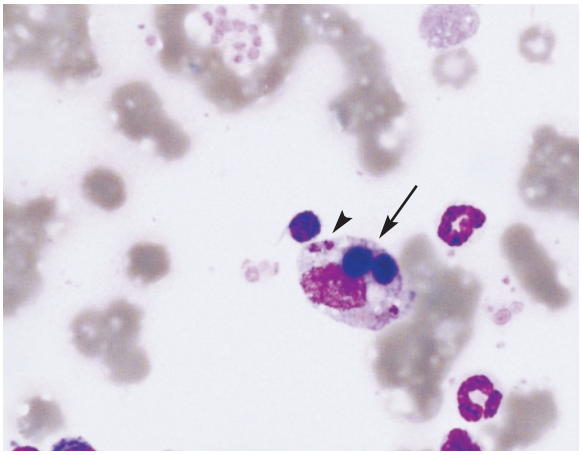

FIG 9: Erythrophagocytosis in a dog with more than three clinical signs of leishmaniosis, showing a large mononuclear cell phagocytising two erythroblasts (arrow) and three *Leishmania* species amastigotes (arrowhead). Diff Quik. x 1000

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