Masticatory and skeletal muscle myositis in canine leishmaniasis (Leishmania infantum)

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Twenty-four dogs with a parasitologically and serologically established diagnosis of leishmaniasis were studied to investigate the atrophy of the masticatory muscles which commonly occurs in this disease, and to compare the lesions in the masticatory muscles with those in the cranial tibial muscles. The 24 animals were divided into three groups of eight, group A dogs with no muscular atrophy, group B dogs with different degrees of atrophy in the masticatory and skeletal muscles, and group C dogs with similar degrees of atrophy in the masticatory and skeletal muscles. Increased activities of creatine phosphokinase and lactate dehydrogenase were recorded in only some of the dogs in groups B and C, but there were no significant differences between the mean activities in the three groups. Electromyographic changes indicating myopathy and involving both the temporalis and cranial tibial muscles, were observed in two of the dogs in group A, seven of those in group B, and in all the dogs in group C. Muscle histopathology revealed a variable degree of muscle fibre necrosis and atrophy, mononuclear infiltrates and neutrophilic vasculitis in all the dogs except two in group A. Leishmanial amastigotes were found within macrophages and myofibres in 16 of the 22 dogs, in each group. IgG immune complexes were detected in muscle samples, and circulating antibodies against myofibres were detected in serum samples from all the 24 dogs.

CANINE leishmaniasis, a protozoan disease with zoonotic potential, is caused by intracellular parasites of the genus Leishmania (Slappendel and Greene 1990). The disease is endemic in the Mediterranean countries and Portugal but occurs only sporadically in northern Europe and in the United Kingdom, usually when people and their dogs have lived for some time in the Mediterranean area (Molyneux and Ashford 1983, Slappendel and Greene 1990). In the Mediterranean area the causative agent is Leishmania infantum (zymodeme MON-1) which is transmitted by blood- sucking sandflies of the subfamily Phlebotominae (Abranches and others 1991, Jimenez and others 1995). Amastigotes of L infantum multiply inside macrophages of the host’s mononuclear phagocytic system causing an inflammatory reaction and immune-mediated lesions (Chang and others 1985).

A wide variety of clinical signs and lesions involving nearly every organ system, have been described (Slappendel and Greene 1990, Kontos and Koutinas 1993), although there is only one report on muscular pathology (Macri and Guarda 1987). The potential for leishmaniasis to trigger a non-infectious myositis in dogs by modifying the immunogenicity of muscle has been suggested (Blot and Fuhrer 1996). The pathogenesis of the muscular atrophy that commonly affects dogs with the disease has never been fully explained, but simply attributed to the catabolic nature of the disease (Dedet and Belazzoung 1985, Kontos and Koutinas 1993).
TABLE 1: Numbers of dogs in groups A, B and C with abnormally high activities of creatine phosphokinase (CK) and lactate dehydrogenase (LDH) and with electromyographic abnormalities

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>CK (normal range 30-120 IU/litre)</th>
<th>LDH (normal range 60-224 IU/litre)</th>
<th>EMG abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>2, 2</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>7, 6</td>
</tr>
<tr>
<td>C</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>8, 8</td>
</tr>
</tbody>
</table>

Group A No muscular atrophy, Group B Disproportional atrophy between masticatory and skeletal muscles, Group C Generalised muscular atrophy.

This study was designed to investigate the nature of the atrophy of the masticatory muscles in canine leishmaniasis by using histopathological, immunohistochemical and electrodiagnostic techniques. The lesions in the masticatory muscles were compared with those in skeletal muscles to determine whether the myopathy is localised or generalised.

MATERIALS AND METHODS

Twenty-four dogs with leishmaniasis were studied between September 1996 and May 1997. Their ages ranged from one- and a-half to 10 years (median four years) and there were 15 intact males and nine intact females. They were of various breeds. _L. infantum_ amastigotes were found in Giemsa-stained lymph node and/or bone marrow aspiration smears taken from all the dogs. Indirect immunofluorescence tests and enzyme-linked immunosorbent assays for leishmania antibodies were also positive in every case. The dogs had no clinical or laboratory evidence of other concurrent diseases. The severity of the muscular atrophy in the temporalis and skeletal muscles was evaluated independently by two of the authors, and on the basis of the findings the 24 dogs were allotted into three groups of eight: group A consisted of dogs with no atrophy of the temporalis or skeletal muscles; group B consisted of dogs with a much greater degree of atrophy in the temporalis muscles than in the skeletal muscles; and group C consisted of dogs with a similar degree of atrophy in the two groups of muscles (generalised muscular atrophy).

Four healthy and leishmania-negative dogs, kept as blood donors, were used as controls. Control samples of serum and muscle, needed for the immunohistochemical procedures, were obtained from these dogs, and serum biochemistry and electrodiagnostic tests were also applied to them.

The serum activities of the muscle enzymes creatinine phosphokinase (CK) and lactate dehydrogenase (LDH) were measured spectrophotometrically (Hitachi V-2000). Electromyographic studies (Neuropack Four) were made with the dogs under general anaesthesia. A needle electromyogram (EMG), including at least 10 recordings, was obtained from the right temporalis and cranial tibial muscles of all 24 dogs. In three randomly selected animals of each group, tibial nerve conduction velocity was also measured.

Muscle biopsy samples were obtained, without the use of clamps, from the temporalis and cranial tibial muscles, and fixed in 10 per cent neutral buffered formalin. Longitudinal and transverse sections, cut at 5 μm, were stained with haematoxylin and eosin. The same staining technique was also applied to fresh frozen sections of biopsy specimens collected at the same time and cut at 10 μm. For the detection of deposits of immune complexes on the myofibres, sections of these frozen muscle samples were fixed for three minutes in acetone at 4°C and left to dry at room temperature overnight. They were then rinsed for 15 minutes with phosphate-buffered saline (PBS) 0-01M at pH 7-2 and incubated for 45 minutes at room temperature with 0·5 ml staphylococcal protein A conjugated to horseradish peroxidase (SPA-HRP; Fluka) diluted in 0·01M PBS at a concentration of 25 μg/ml. Subsequently, the sections were rinsed with 0·01M PBS for 15 minutes and then incubated with Karnovsky’s dianisobenzidine medium for 30 minutes. Finally, they were rinsed with distilled water for 10 minutes, dehydrated with alcohol, cleared in xylol, and mounted with Permount. For
the detection of circulating antibodies directed against myofibres, frozen muscle sections from the control dogs were incubated overnight with the serum from each of the 24 affected dogs, diluted 1:50 in a solution containing 1 per cent bovine serum albumin (BSA) and 1 per cent sodium azide in 0·01M PBS, pH 7·2. The sections were then washed with 0·01M PBS and incubated with SPA-HRPO as described by Engel and others (1977), Pflugfelder and others (1981), and Shelton and others (1985).

The Kruskal-Wallis one-way analysis of variance was used to compare the groups for increased activities of CK and LDH in the serum, the presence of abnormal EMGs, and the presence of deposits of immune complexes on myofibres, and circulating antibodies against myofibres. With the exception of the muscular enzymes, all the comparisons were made separately for the temporalis and cranial tibial muscles (Lehmann 1975). When differences were identified with the Kruskal-Wallis (chi-squared) test, a multivariate analysis was done to determine the exact differences between the three groups. The differences in all these variables (except the muscular enzymes) between the two muscles but within the same group of dogs were tested by using the Wilcoxon rank test (Lehmann 1975). The statistical software used was the SAS System release 6.12 for Windows 1996 (SAS Institute).

RESULTS

A wide variety of clinical signs indicating multiple organ dysfunction was observed among the 24 dogs. Their description is beyond the scope of this study. The only manifestation of muscle involvement observed in all the dogs of groups B and C was muscular atrophy (Fig 1).

Above normal activities of CK and LDH were recorded only in some of the dogs in groups B and C (Table 1, Fig 2). In particular, there was higher than normal CK activity in four group B and two group C dogs, and a high activity of LDH in one dog in each of the two groups, both dogs also having a high serum CK activity. However, there were no significant differences between the mean activities of the two enzymes in groups A, B and C (Table 1, Fig 2).

FIG 3: Histopathological changes in the cranial tibial muscle of a dog with leishmaniasis (group B). Myofibre degeneration, regeneration (small arrows) and necrosis (large arrow) together with interstitial and perivascular (arrowhead) mononuclear infiltrates. Longitudinal section, haematoxylin and eosin x 300

FIG 4: Vascular changes in the cranial tibial muscle of a dog with leishmaniasis and polymyositis (group B). Neutrophilic vasculitis. A hyaline thrombus obliterates the lumen of the upper blood vessel. Haematoxylin and eosin x 120

FIG 5: Longitudinal section of the cranial tibial muscle of a dog with leishmaniasis and polymyositis (group C). (a) Segmental necrosis of a myofibre invaded by parasitised macrophages (arrowheads) and a regenerative process taking place in the left adjacent myofibre (arrows). Haematoxylin and eosin. x 480. (b) Higher resolution of (a) showing large numbers of Leishmania amastigotes (arrows) in the cytoplasm of macrophages. Haematoxylin and eosin. x 720

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The electromyograph (EMG) abnormalities, which had a multifocal distribution, included increased insertion activity and abnormal spontaneous activity in the form of positive sharp waves, fibrillation potentials and bizarre high frequency discharges. These EMG findings were observed in two of the dogs in group A, seven of those in group B and in all the dogs of group C (Table 1). In one group B animal, abnormal EMGs were recorded only in the temporals, while the cranial tibial muscle was electrically silent. The incidence of abnormal EMG recordings was significantly lower in the dogs of group A than in those of groups B and C for both the temporals and cranial tibial muscles (P<0.05). The velocity of tibial nerve conduction was within the normal range (64.5 to 69.3 m/s) (Walker and others 1979) in all the nine dogs examined (range 67 to 69.2 m/s, mean 68.1 m/s), six of them with and three of them without cranial tibial muscle atrophy.

A wide variety of microscopical lesions was observed in the muscle biopsies from all the dogs except two of the group A dogs. Myofibre necrosis, degeneration, regeneration and/or atrophy in various combinations, together with fibrosis and infiltrations of mononuclear cells with an interstitial and/or perivascular distribution were the most prominent changes (Fig 3). The predominant infiltrating cells were macrophages and lymphocytes together with a small number of plasma cells. These lesions, in almost all the samples examined, had a multifocal distribution. Neutrophilic vasculitis, accompanied by destruction of the vascular wall, with enlarged endothelial cells and mild to severe thrombosis, was found in both the temporals and cranial tibial muscles of one group A dog, two group B dogs, and two group C dogs (Fig 4). Leishmanial amastigotes were found within macrophages and myofibres in 16 of the 24 dogs (six group A, seven group B and three group C dogs) (Fig 5).

IgG immune complexes were detected in muscle samples, and circulating antibodies against myofibres were detected in serum samples, with a variable intensity of reaction, in all the 24 dogs (Fig 6). Positive staining was observed both within the myofibres and the sarcolemma. Similar muscle biopsies and serum samples taken from the control dogs and treated in an identical manner, showed no staining (Fig 6). Conversely, all the specimens from the 24 affected dogs, and those from the control dogs had a positive non-specific staining of perimysial connective tissue and vascular wall when both immuno histochemical techniques were used.

Finally, the four control dogs had normal serum CK and LDH activities, and tibial nerve conduction velocities, and had no abnormal EMG findings or histological lesions.

**DISCUSSION**

The diagnosis of canine polymyositis is based on the fulfilment of four criteria extrapolated from human medicine (Bohan and Peter 1975a, b). They are compatible clinical signs, high serum activities of muscle enzymes, EMG abnormalities and histological evidence of muscle necrosis and inflammation. The diagnosis would be definite if all four criteria were met, probable if only three were met, and possible if only two of the criteria were met (Kornegay and others 1980). According to these criteria, five of these 24 dogs (three in group B and two in group C) had a definite diagnosis of polymyositis, 11 dogs (five in group B and six in group C) had a probable diagnosis and two group A dogs had a possible diagnosis of polymyositis.

The only clinical sign indicating muscular damage that was observed in the 16 dogs in groups B and C was muscular atrophy. Other more typical signs, such as muscle swelling and pain, disturbances of locomotion and stance, and exercise intolerance were not detected in any of them. These observations imply that the polymyositis in canine leishmaniasis is a low-grade, subclinical and chronic myopathy, which may lead to muscular atrophy. In the experience of the authors, this progressive muscular atrophy is more prominent in the masticatory muscles, whereas in the skeletal muscles it is subtle and could easily be attributed to the general catabolic nature of the disease. The atrophic myositis of the masticatory muscles associated with leishmaniasis is almost never accompanied by dysfunctional states of the jaw, in contrast to the idiopathic form of the disease (trismus), and trigeminal neuropathy (jaw drop) which are the two major differential diagnoses (Smith 1989, Gilmour and others 1992). The same holds true for the skeletal part of the disease, in which neither locomotive signs nor megaesophagus have been observed, whereas they are common in polymyositis of other aetiology (Kornegay and others 1980, Blot 1996).

Only six of the 18 dogs with a definite or possible diagnosis of polymyositis, had high activities of CK and/or LDH in serum. This finding is not surprising because increased muscle enzyme activity is thought to be an unreliable indicator of canine polymyositis (Blot 1996). De Vere and Bradley (1975) reported that 64 per cent of human patients with polymyositis had high activities of muscle enzymes. High serum CK activity was recorded in nine of 13 dogs with masticatory myositis (Gilmour and others 1992) and in three of
nine dogs with idiopathic polymyositis (Kornegay and others 1980). Abnormalities of the EMG similar to those observed in these dogs, also occur in various myopathies, including muscular dystrophy and other congenital myopathies, dermatomyositis, steroid myopathy, polymyositis of a different aetiology and neurogenic myopathies (Braud 1997). The last possibility was rejected because of the normal nerve conduction velocities detected in the nine randomly selected dogs representing all three groups (Walker and others 1979, Smith 1989). Similarly, the other myopathies could be excluded when the clinical signs, history and laboratory evaluations were considered.

Histological evidence of muscle necrosis and inflammation was detected in all 18 dogs with polymyositis, and in four group A animals that did not show any of the other diagnostic criteria (Bohan and Peter 1975a, b). It is postulated that these four dogs had an early stage polymyositis that would eventually have progressed to the atrophic form.

Canine inflammatory myopathies are usually classified as infectious (toxoplasmosis, neosporosis, trypansomiasis, hepatitis, leprotriosis etc) and immune-mediated (idiopathic polymyositis, masticatory and extraocular myositis, dermatomyositis, systemic lupus erythematosus, drug-induced polymyositis etc) (Kornegay and others 1980, Blot and Fuhrer 1996). Direct muscular injury from the offending organisms and/or their toxins has been proposed as the main cause of the pathology in infectious myositis (Wouda and others 1995, Wouda and others 1997). In immune-mediated myositis, the presence of circulating IgG antibodies against myofibres and the deposition or the formation of immune complexes in both skeletal and masticatory muscles has been either suggested or confirmed as the cause (Griffiths and others 1973, Orvis and Cardinet 1981, Haupt and others 1985, Shelton and others 1985, 1987). It is evident that in dogs with polymyositis induced by leishmaniasis both causes may be involved because the presence of L infansuitum amastigotes in the muscle biopsies from 16 of the 24 dogs is consistent with direct muscular damage, and the detection of IgG antibodies against myofibres in the sera, and the deposition of immune complexes in the muscles of all the 24 dogs indicates an immune-mediated pathology. In general, antimuscle antibodies are directed towards myofilibrillar proteins which are normal constituents of the immune system (Smith 1989). It is postulated that the cross-reactivity of antibodies directed against leishmania antigens with sarcollermal antigens, and/or the deposition of immune complexes, may be mechanisms for the initial sarcollermal injury, and result in the leakage of intracellular proteins from myofibres. Lyambert and Daniilova (1975) reported that there was cross-antigenicity between mammalian muscles and certain bacterial species (Streptococcus pyogenes and Staphylococcus species). The only two dogs with normal muscle histopathology and EMG belonged to group A, the group with no muscle atrophy. However, even in these dogs muscle antibodies were detected in both the serum and muscles. It is possible that these antibodies appear before any microscopic or electrophysiological evidence of myositis is visible or detectable.

The chronic ischaemic changes, possibly induced by neotrophilic vasculitis, that occurred in two group B dogs and two group C dogs, may have contributed to the development of muscular atrophy. The detection of mild to severe thrombosis, as a consequence of vasculitis, could underlie this change. In canine leishmaniasis vasculitis is usually the result of an immune-mediated process consistent with a type III hypersensitivity reaction (Affolter 1997).

In these dogs, leishmaniasis induced a progressive polymyositis affecting both the masticatory and the skeletal muscles. In most cases, the electromyographic and histopathological changes detected in both groups of muscles were consistent with those observed in other myositis. However, the exact pathogenesis remains uncertain and further studies are needed.

References
Short Communications

Antimicrobial resistance in enteric porcine 
Escherichia coli strains in Spain

E. Mateu, M. Martín

ANTIMICROBIAL resistance is one of the main concerns for health professionals dealing with bacterial diseases. The current trend in the EU and worldwide is to reduce antibiotic use in animal production in favour of good husbandry practices and vaccination, as means to raise healthier animals. This trend towards antibiotic-free animal products is increasingly appreciated by the consumers and public demand is noticeably rising. However, at present, it appears impossible to raise animals under high productivity conditions without the use of antimicrobials and the key issue is a rational use of these substances.

The main concern regarding antibiotic usage in animal husbandry is the emergence of resistant bacterial strains that potentially could infect human beings or may transfer their resistance genes to human pathogens. Much attention has been paid to the emergence of multiresistant strains such as Salmonella typhimurium DT104 or vancomycin-resistant enterococci, and many researchers have claimed that the use of antimicrobial compounds in animal farming is one of the factors responsible for such resistances (van den Bogard and others 1997, Thefall and others 1998, Welton and others 1998, Kruse and others 1999). A knowledge of the resistance profiles of a given bacterium can help to select appropriate treatments for the infections caused by it, which reduces failure rates and saves time and money. For all these reasons, monitoring of antimicrobial resistances in bacteria of animal origin is of the utmost importance. This study describes the antimicrobial susceptibility profiles of porcine Escherichia coli strains isolated from diarrhoea outbreaks in Spain.

Eighty-six E coli strains isolated in the Veterinary Faculty of Barcelona were analysed to determine their antimicrobial susceptibility profiles. All strains had been isolated from small bowel samples or faeces samples submitted to the laboratory between autumn 1997 and June 1999 from unweaned or nursery pigs suffering from diarrhoea or oedema disease. These cases came from most Spanish regions and were submitted for determination of an antimicrobial profile of the causal strain. Preliminary identification was achieved on the basis of the morphological and basic biochemical behaviour of the strain, and further confirmation was carried out by means of the API20E identification system (BioMérieux; Barcelona). Whenever possible, strains were typed using polyclonal antisera against fimbrial and capsular antigens of E coli (polyvalent antisera A, B and C; Central Veterinary Laboratory) to confirm their potential pathogenicity.

Once properly classified, antimicrobial testing was performed in Mueller-Hinton agar (Difco Laboratories) by the Kirby-Bauer microdilution technique according to the United States National Committee for Clinical Laboratory Standards guidelines (NCCLS 1984), with the exception of aminoglycosides which was preincubated for one hour at 4°C as recommended by the manufacturer. The antimicrobial compounds tested were amoxicillin (BioMérieux), cefsulodin (Oxoid), enrofloxacin (Bayer Químico-Farmacéutica), colistin (BioMérieux), neomycin (BioMérieux), apramycin (Elanco-Lilly) and a combination of sulphonamide and trimethoprims (BioMérieux). Measurement of growth inhibition areas allowed the classification of each strain as susceptible, intermediate or resistant, according to data provided by the manufacturer of the microdiluants. Comparison of the proportions of susceptible strains by year was performed by means of a chi-square test using Epi-Info v 6.01 (Dean and others 1994).

Strains found to be resistant to colistin in the disk diffusion test were tested to determine the minimum inhibitory concentration (MIC) of the compound by means of the broth microdilution test performed in U-bottomed, 96-well plates. Overnight cultures of the testing strains in brain-heart infusion broth were diluted in sterile saline to a concentration equivalent to 0.5 on the McFarland scale (approximately 10^5 colony-forming units/ml). These suspensions were further diluted in Isosensitest broth (Oxoid Laboratories) to provide an inoculum containing approximately 10^3 cfu/well in a 100 μl volume. Colistin (Sigma-Aldrich) was diluted in test media using the method described by Ericsson and Sherris (1971) and 100 μl/well was dispensed to provide a range of dilutions from 64 to 1 mg/litre in wells containing uninoculated culture media, wells containing bacterial cultures without antibiotics and wells containing just antibiotics. The plates were incubated for 18 hours at 37°C. The minimum concentration of antibiotic at which no growth (no button or turbidity) was observed was considered to be the MIC. For the purpose of this study, strains showing a MIC higher than 8 mg/litre were considered to be resistant. To assure an adequate concentration of the inocula, bacterial suspensions used in the assay were diluted in sterile saline to a decimal series until 10^-6 and 100 μl of each dilution were further seeded onto agar plates. The number of E coli colonies was counted after 20 hours of incubation at 37°C. All experiments were performed three times and the techniques were validated using E coli KCC 2922 as a control.

In the disk diffusion tests, the antimicrobial compounds for which a lower proportion of resistant strains were found were colistin with 83 per cent susceptible strains and ceftriaxone with 78-8 per cent susceptible and 16-5 per cent intermediate strains. In the case of colistin, resistant strains were not detected until late autumn 1998 and, in the first quarter of 1999, eight out of 30 strains were resistant to this compound.
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