American tegumentary leishmaniasis (ATL), which is caused by *Leishmania Viannia braziliensis*, shows distinct epidemiological patterns that might occur in primary forest environments that are colonized by sandflies and by wild mammals as well as in regions that have long been deforested, where vectors and reservoirs have adapted to the modified environments in rural and urban areas, and where there is domiciliar and peridomiciliar transmission (Marzochi, 1992; Marzochi & Marzochi, 1994). In endemic areas from Rio de Janeiro, horses and dogs are frequently found infected with *L. (V.) braziliensis* (Alencar, 1959; Falqueto *et al.*, 1984; Vexenat *et al.*, 1986; Aguilar *et al.*, 1989; Yoshida *et al.*, 1990; Passos *et al.*, 1993). Restriction fragment length polymorphisms of kDNA analysis has shown the same genotypic pattern of the parasite circulating in humans and dogs (Pacheco *et al.*, 1986).

The clinical features of canine tegumentary leishmaniasis (CTL) are similar to the ones of the human disease (Pirmez *et al.*, 1988b). Single or multiple skin lesions occur in the form of ulcerations with irregular raised borders. Canine tegumentary leishmaniasis usually shows a high amount of parasites. The lesions tend to heal spontaneously but they recur months later in the same location, mostly during the colder periods (Marzochi, 1992), which accounts for the persistence of *Leishmania* in the locus.

*Leishmania* is a mononuclear phagocyte system (MPS) cellular parasite that can induce humoral and cellular immune responses. In the assays involving natural and experimental canine tegumentary leishmaniasis...
the observed serial antibody titers are relatively low—around 1:80 (Coutinho et al., 1985; Maywald et al., 1996)—and the delayed hypersensitivity skin test shows negative (Marzochi & Barbosa Santos, 1988). Treatment of dogs infected by *L. (V.) braziliensis* could be an alternative control measure. However the usual drug N-methylglucamine antimonate is inaccesible when it is administered by systemic route (Pirmez et al., 1988a). Oliveira Neto et al. (1997) reported to have been successful in a longitudinal study of the treatment of ATL patients by means of intraleional inoculation with N-methylglucamine antimonate (Glucantime®). The purpose of the present study was to evaluate the effectiveness of intraleional therapy with Glucantime® in the treatment of canine tegumentary leishmaniasis in a field assay which took place in endemic areas of the state of Rio de Janeiro, Brazil.

**MATERIALS AND METHODS**

**DOGS**

There have been selected 25 adult mongrel dogs—four females (16.0 %) and 21 males (84.0 %)—that had been naturally infected. Nine (37.5 %) of the animals showed simple cutaneous lesions, four (16.0 %) had multiple ulcers, six (24.0 %) of them had mucosal lesions just in the muzzle and six (24.0 %) of them had mucosal lesions associated to cutaneous lesions. The skin lesions were located mainly on the inside and outside of their ears (54.2 %), and in 4.2 % of the cases they were located on the back and/or scrotal bag (12.5 %) (Table I). The animals came from endemic ATL areas of the municipalities of Rio Bonito (2/8.33 %), Paracambi (10/41.67 %) and Angra dos Reis (12/50 %) in the state of Rio de Janeiro.

Canine tegumentary leishmaniasis diagnosis was based on the features of the skin lesions, on the observation of *Leishmania* in the material that was taken from the lesions or on its isolation and on positive serology and intradermoreactions.

**PARASITE DIAGNOSIS**

An in print of the suspicious lesions of all the animals was performed for biopsies, and was followed by Giemsa staining or by vacuum aspirative punction with culture media-filled tubes for the isolation of *Leishmania*, as described by Marzochi et al. (1993). The

<table>
<thead>
<tr>
<th>Type/location of lesions</th>
<th>Amount of lesions</th>
<th>Amount of dogs</th>
<th>Amount of dogs/doses and results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple Ulcer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ears</td>
<td>6</td>
<td>6</td>
<td>6/1°C</td>
</tr>
<tr>
<td>scrotum</td>
<td>3</td>
<td>3</td>
<td>2/2°C; 1/1D</td>
</tr>
<tr>
<td>Sub-total</td>
<td>9</td>
<td>9</td>
<td>6/1°C; 2/2°C; 1/1D</td>
</tr>
<tr>
<td>Multiple Ulcers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>back</td>
<td>4</td>
<td>1</td>
<td>1/3F</td>
</tr>
<tr>
<td>ear</td>
<td>4</td>
<td>2</td>
<td>2/1C</td>
</tr>
<tr>
<td>ears + scrotum</td>
<td>3</td>
<td>1</td>
<td>1/3C</td>
</tr>
<tr>
<td>Sub-total</td>
<td>11</td>
<td>4</td>
<td>1/3F; 2/1C; 1/3C</td>
</tr>
<tr>
<td>Simple Mucosal Ulcer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>muzzle</td>
<td>6</td>
<td>6</td>
<td>4/3F; 1/3F; 1/1D</td>
</tr>
<tr>
<td>Associated Mucosal Ulcer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>muzzle + ears</td>
<td>7</td>
<td>5</td>
<td>3/3F; 1/3F; 1/1D</td>
</tr>
<tr>
<td>muzzle + scrotum</td>
<td>1</td>
<td>1</td>
<td>1/3F</td>
</tr>
<tr>
<td>Sub-total</td>
<td>14</td>
<td>12</td>
<td>4/3F; 1/3F; 1/1D</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>25</td>
<td>9/1°C; 2/2°C; 9/3C</td>
</tr>
</tbody>
</table>

Symbols: Cure: C; Failure: F; Death: D.

Table I. - Classification of the dogs as to type, location and amount of lesions, amount of necessary intraleional Glucantime® doses, and result.
medium that was used contained a solid phase of enriched blood agar (NNN) with a liquid phase of a heart and brain infusion (BHI), supplemented with 20% fetal calf serum and streptomycin 250 μg/ml and 5-fluorocytosine 500 μg/ml. The culture was kept at 26 °C and its content was examined every 5 days.

**ENZYME ELECTROPHORESIS**

The samples of *Leishmania* sp. that were isolated in a culture medium were characterized by means of electrophoretical analysis by isoenzyme, as described by Momen *et al.* (1985). The parasites were collected in a stationary phase of growth and centrifuged at 700 g for ten minutes at 4 °C in a 0.15M-EDTA 0.1 M (SE) salt solution. Biochemical analysis was undertaken on an agarose gel which contained nine enzymes: 6-phosphogluconate dehydrogenase (6PGDH E.C.1.1.43), glucose phosphate isomerase (GPI E.C.5.3.1.9), isocitrate dehydrogenase (IDH E.C.1.1.42), glucose-6-phosphate-dehydrogenase (G6PDH E.C.1.1.1.49), malate dehydrogenase (MDH E.C.1.1.37), nucleosidase (NH E.C.3.2.2.1), phosphoglucomutase (PGM E.C.1.4.1.9), peptidase 3 (PEP3 E.C.3.4.11) and proline aminopeptidase (PEP-D E.C.3.4.13.9). The samples of *L. (V) braziliensis* (MHOM/BR/75/M2903) and *L. (L) chagasi* (MHOM/BR/74/PP75) were used as reference.

**SEROLOGIC AND CELLULAR EVALUATION**

The sera that have been collected by means of venous puncture were analysed before the treatment and six months after it with the indirect fluorescence antibody test (IFAT), according to Coutinho *et al.* (1985). The antigen that was used in the reaction had been prepared with a promastigote suspension of a *Leishmania* sample that was phenotypically similar to *L. major* (MHOM/BR/76/JOF), which was characterized by Momen *et al.* (1985). The sera were prepared in serial twofold dilutions of 1:40 in a phosphate buffer saline (PBS pH 7.2). The titers equal to or greater than 1:40 were considered positive.

Cellular immunity was detected before treatment and after blood samples *in vivo* by means of a skin test with the antigen called IMUNOLEISH, which was prepared with a sample of *L. (V) braziliensis* (Marzochi & Barbosa Santos, 1988). Each animal received intradermally on the inside of the back limb 0.1 ml of the antigen suspension, which contained 200 μg of the total protein. The reading was done 48 hours later and an induction of 5.0 mm or more was considered positive.

**INTRALESIONAL THERAPY**

The N-methylglucamine antimonate (Glucantime®, Rhône-Poulenc Rorer, France) was administered to each skin lesion. A dose of 85 mg SbV₅/lesion or 1.0 ml of a 5.0 ml solution which contained 425 mg SbV₅ was slowly injected into the cardinal points around each lesion with a 13 x 4.0 gauge needle.

The dogs were divided into three groups according to the number of series (up to 3) that had been necessary for the healing of the lesions: nine animals had the need for one dose only (Group 1), two of them received two doses (Group 2) and 11 others received three doses at intervals of twenty days between each series. During the experiment two animals died due to adverse infections.

The criteria that was adopted to define cure was the complete epithelization of the lesions which was checked six months after the treatment.

**STATISTICAL ANALYSIS**

The McNemar test for comparison of IFAT observed titers was undertaken before and after the treatment.

**RESULTS**

All ulcerated lesions that were examined were positive for *Leishmania*. The stained in-prints showed 21 (84.0%) dogs to be positive and it was possible to isolate the parasites by means of the vacuum-closed culture-medium filled tubes (Marzochi *et al.* 1993) in 19 (86.6%) of the cases. Eight samples of *Leishmania* were analysed through enzyme electrophoresis with the use of nine enzyme loci. All samples presented isoenzymatic profiles that were compatible with *L. (V) braziliensis* (Fig. 1).

In the three observed groups the titers of serial antibodies that were detected before the treatment varied.
between 1:40 and 1:80 except in two cases in which the encountered titers were 1:160 and 1:320. Six months after intraleisonal therapy the detected serology in 14 dogs (56.0 %) remained positive with no changes in the titers (≤ 1:180). Four (16.0 %) animals showed a decrease in two serologic titers and five (20.0 %) showed negative serology (Fig. 2). A statistical difference in serologic titers, that was detected in both periods (p ≤ 0.005, α = 0.05), was observed. Delayed hypersensitivity reactions were positive among all dogs and their diameters were of 8.0 mm (s.d. = 1.5 mm).

It was possible to treat every lesion of the 19 (86.6 %) of the 22 dogs that remained under study. The epithelization of the ulcers was complete and only one residual erithema remained. The dogs were weekly observed for six months. Within this period it was possible to notice that the animals that had skin lesions on their ears had the need for just one dose of Glucantime® (Group 1). On the other hand, Groups 2 and 3, which had the need for two or three doses of the drug, consisted of dogs that had cutaneous lesions on the muzzle and on the scrotum. The lesions that healed in the shortest period – in less than 30 days – were located on the ears. The more persistent ones were located on the muzzle and on the scrotal bag – they healed in about 60 days.

**DISCUSSION**

The aim of this study was to evaluate the effectiveness of canine tegumentary leishmaniasis treatment with intraleisonal administration of Glucantime®. The dogs under study had been naturally infected by *L.(V.) braziliensis* according to the undertaken isoenzymatic characterization. To the moment, this species remains solely responsible for the ATL that is found in Rio de Janeiro (Grimaldi & Tesh, 1993).

Systemic treatment with antimonials has given inconsistent results on canine leishmaniasis (Marzochi et al., 1985; Pirmez et al., 1988a). The intraleisonal treatment of human cutaneous leishmaniasis was acknowledged by WHO (1990) which recommended its use. The obtained results have shown that it was possible to treat canine tegumentary leishmaniasis with Glucantime® by intraleisonal N-methylglucamine antimonate. The rate of clinical cure six months after the treatment was of 86.6 %. Pirmez et al. (1988a), in a similar experiment in which they administered the same drug intramuscularly, obtained 80.9 % of cure after three months with 42.8 % of resurgence at the period of five months.

Serological reactions are auxiliary instruments for the diagnosis and the observation of the treatment of infectious diseases. Positive serial titers after antimonial tegumentary leishmaniasis therapy might anticipate the upsurge of secondary lesions in the future. However, in most of human cases, there has been observed a decrease in the IFAT detected serological levels that reach negative results and that show no metastatic oral and nasal mucocae lesions (Mendonça et al., 1988).

As to the results obtained in the IFAT of the dogs' serial samples after treatment, it has been observed that, although most of them (14/62.7 %) kept the previous titers – and this includes the ones that have not been cured – 4 (16.0 %) of them showed a decrease of two serological titers and in 5 (21 %) of them serology was negative. With regard to the 4 (20 %) dogs whose treatment has failed, there is a possibility that renewed doses of Glucantime® might contribute to the disappearance of the lesions and to the decrease of the antibody titers.

*In vitro* activity of the amastigotes has proved to be a good gauge of *in vivo* activity for several leishmancide compounds (Croft, 1986). The evaluation of Glucantime® in canine peritoneal macrophages that have been infected by *L.(V.) braziliensis*, *L.(L.)amazonensis* and *L.(L.)brazagliast* was analysed by Madeira et al., 1997 (submitted). The assay has shown pentavalent antimonoy to be more efficient than Berenil® (aromatic diamidines) in macrophage infections by *L.(V.) braziliensis* with LD$_{50}$ equal to 0.5 g/ml.

The unresponsiveness to antimonial drugs in human visceral and mucocutaneous leishmaniasis is a serious clinical problem (Marsden et al., 1984) and its prevalence appears to be increasing (Marsden, 1985). Intraleisonal tegumentary leishmaniasis therapy has been administered to human beings with success (Gadelha et al., 1985; Oliveira Neto et al., 1997; Tallab et al., 1996). In the present study, the adequate amounts of intraleisonally administrated Glucantime® were apparently able to reach the desired tissue and intracellular...
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