SECRETED ANTIGENS OF THE AMASTIGOTE AND PROMASTIGOTE FORMS OF *LEISHMANIA INFANTUM* INDUCING A HUMORAL RESPONSE IN HUMANS AND DOGS

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Summary :

To study the antigens secreted by promastigote and amastigote forms of *Leishmania infantum* which are able to induce a humoral response in human patients and dogs, we have carried out immunoprecipitation assays with different supernatants of *in vitro* cultured parasites, metabolically labelled with [^{35}S]methionine, using serum samples from human patients and dogs. In addition, some metabolic labelling experiments were performed daily during the *in vitro* culture parasite's life cycle to follow the time course excretion-secretion of parasitic antigens. The results demonstrated that the two different hosts developed an antibody response against secreted antigens of both stages of *Leishmania infantum*. Nevertheless, the humoral response directed against the excreted-secreted antigens of the promastigote forms was qualitatively and quantitatively different when we compare the human and the dog immune responses. On the other hand, when the excreted-secreted antigens of the amastigote forms are immunoprecipitated with either human or canine immune serum, the humoral response is similar. In addition, the time course study showed that excretion-secretion of antigens was qualitatively and quantitatively modulated during the parasitic *in vitro* life cycle.

KEY WORDS : *Leishmania infantum*, excreted-secreted antigens, humoral immunity, amastigote, promastigote.

ABBREVIATIONS : VL: visceral leishmaniasis; AMES-antigens: amastigote excreted-secreted antigens; PMES-antigens: promastigote excreted-secreted antigens.

Résumé : ANTIGÈNES SÉCRÉTÉS PAR LES FORMES AMASTIGOTES ET PROMASTIGOTES DE *LEISHMANIA INFANTUM* ET INDUISANT UNE RÉPONSE HUMORALE CHEZ L'HOMME ET LE CHIEN

Afin d'étudier les antigènes sécrétés par les formes promastigotes et amastigotes de Leishmania infantum *capable d'induire une réponse humorale nous avons immunoprécipité différents surnageants de culture* in vitro, *marqués à la méthionine[S^{35}], à l'aide de sérums de malades ou de chiens. De plus, certains marquages métaboliques ont été effectués chaque jour et ceci durant un cycle complet de développement* in vitro *de* L. infantum *afin d'avoir un suivi temporel de l'excrétion-sécrétion des antigènes parasitaires. Les résultats obtenus ont montré que les deux différents hôtes développaient une réponse humorale contre les antigènes sécrétés par chacun des deux stades parasitaires de* L. infantum. *Néanmoins, la réponse humorale dirigée contre les antigènes d'excrétion-sécrétion du stade promastigote est qualitativement et quantitativement différente si l'on compare la réponse humorale humaine et canine. Par contre, que l'on immunoprécipite avec un sérum humain ou canin, la réponse humorale dirigée contre les antigènes d'excrétion-sécrétion du stade amastigote est similaire. De plus, l'étude temporelle a montré que l'excrétion-sécrétion des antigènes était qualitativement et quantitativement modulée au cours du cycle parasitaire* in vitro *de* L. infantum.

MOTS CLÉS : Leishmania infantum, antigène d'excrétion-sécrétion, immunité humorale, forme amastigote, forme promastigote.

INTRODUCTION

Leishmania parasites, members of the family of *Trypanosomatidae*, are protozoan parasites which infect macrophages of man and other vertebrate hosts like those of the *Canidae* family. They cause a variety of human diseases ranging from localized self-healing cutaneous lesions to fatal visceral infections. The parasites of the *Leishmania donovani* complex (*L. donovani* and *L. infantum* in the Old World and *L. chagasi* in the New World) are responsible for the visceral leishmaniasis which is characterized, in its active form, by a lack of impaired T-cell-mediated immune responses and a marked hypergammaglobulinaemia (Carvalho *et al.*, 1981; Galvão-Castro *et al.*, 1984). *Leishmania* presents with two morphologically distinct forms: *i*) the flagellated promastigote forms, transmitted to the vertebrate host by the bite of an infected sandfly, are found in the alimentary tract of the vector and, *ii*) in the mammalian host, *Leishmania* replicates exclusively as the aflagellated amastigote form inside the macrophage where they induce strong changes in the cell functions and morphology of macrophages such as the formation of a phagolysosome or the down regulation of class II MHC (for a review see : Alexander & Russell, 1992).

During its life cycle, *Leishmania* organisms secrete various antigens that could be directly implicated in many immunological or biochemical alterations of the

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host cell. Different glycoconjugates or glycoproteins such as lipophosphoglycan (Handman *et al.,* 1984; King *et al.*, 1987) or acid phosphatase (Bates *et al.*, 1990; Ilg *et al.,* 1991) have been shown to be secreted from the promastigote forms of *Leishmania.* Similarly, other studies have demonstrated *i)* the secretion of amastigote antigens into the phagolysosomal compartment via the flagellar pocket of the parasite (for review: Chakraborty & Basu, 1997), *ii)* the presence of *Leishmania*-derived antigens on the surface of the parasitized macrophages (Berman & Dwyer, 1981; Williams *et al.,* 1986). Among all the described amastigote antigens of *Leishmania* species, some of them are well characterized: an acid phosphatase (Bates *et al.,* 1989; Doyle & Dwyer, 1993), a proteophosphoglycan, (Ilg *et al.,* 1995), a sucrase (Blum & Opperdoes, 1994), a glutathione binding 66 kDa polypeptide (Yahiaoui *et al.,* 1993) and a protective LACK antigen (Prina *et al,* 1996).

The recent development of axenic cultures of amastigote forms from different *Leishmania* species (Bates *et al.,* 1992; Lemesre *et al.,* 1997) have provided new opportunities to characterize the antigens excreted by the mammalian stage of the parasite. In this paper, we studied the humoral response of dogs and men against excreted-secreted antigens from *Leishmania infantum* amastigotes and promastigotes forms.

MATERIALS AND METHODS

L. INFANTUM IN VITRO CULTURES

The amastigote and promastigote forms of *L. infantum* MHOM/MA(BE)/67/ITMAP-263 clone 2 were used in the present study.

Axenically grown amastigotes were maintained at 37 +/- 1°C under 5 % CO_2 atmosphere by subpassaging every five days in cell-free medium called MAA/20. MAA/20 consisted of modified medium 199 (Life Technologies, France) balanced with Hanks'salts, supplemented with 0.01 mM bathocuproine disulfonic acid, 3 mM L-cysteine, 5 mM L-glutamine, 0.023 mM hemin, 5 mM D-glucose, 4 mM $NaHCO_3$, 0.5 % soya tryptocasein and 25 mM Hepes. The pH was adjusted at 6.5 and 20 % heat-inactivated fetal calf serum (FCS) was added (Lemesre *et al.*, 1997). The starting inoculum was 5×10^5 amastigotes/ml and 10^7 to 5×10^7 parasites were regularly obtained by day 5.

Promastigote cultures were derived from axenically cultured amastigote stages and were maintained at 25 +/- 1°C by weekly subculturing in medium RPMI 1640 (Life Technologies, France) buffered with 25 mM HEPES and 2 mM $NaHCO_3$, pH 7.2, supplemented with 10 % heat-inactived FCS. The starting inoculum was 5×10^5 promastigotes/ml.

HUMAN AND DOG SERUM SAMPLES

Human sera were obtained from five South-American patients infected with *Leishmania chagasi* (acute leishmaniasis), the causative agent of American visceral leishmaniasis (VL). The VL-infected human sera were: HIM-A1, HIM-A3, HIM-C1, HIM-V1 and HIM-L1. The VL-infected canine sera were obtained from four dogs (acute infection) living in the mediterranean endemic area and infected with *Leishmania infantum.* The canine sera were: CIS-14, CIS-15, CIS-16 and CIS-18. Sera from healthy subjects (human or dog) were used as negative controls.

METABOLIC RADIOLABELLING

Two different procedures of metabolic radiolabelling were performed. In a first experiment, the *Leishmania* parasites were labelled during a complete *in vitro* life cycle. When the *in vitro* cultures were initiated, 100 µCi/ml of [^{35}S]-methionine were added to MAA/20 medium and the labelled parasites were collected after five days for the amastigote forms and seven days for the promastigote forms. These labelled antigens were called "one cycle antigens".

In a second experiment, promastigotes and amastigotes were cultured in RPMI 1640 with 10 % FCS and in MAA/20 with 20 % FCS respectively, and supplemented with [^{35}S]-methionine (100 µCi/ml) for 24 hours. The same procedure was also utilized for the promastigote forms, except that it was carried out during seven days. On day 0, five culture flasks were initiated with the amastigotes and seven with the promastigotes (one flask for each day of *in vitro* culture) and, for each parasitic form, [^{35}S]-methionine was only added to one culture flask. On day 1, the labelled *in vitro* culture supernatants and parasites were collected. The culture supernatants were separated from the parasite pellet by centrifugation at 2,500 g for five minutes. The supernatants were then centrifugated at 15,000 g for 20 min and filtered through a diam. 0.2 µm membrane (Acrodisc, Gelman Science). The parasitic pellets and the supernatants were stored at – 80°C until used. The same day (day 1), [^{35}S]methionine was added to a second flask of *in vitro* culture of promastigotes and amastigotes; after 24 hours labelling (day 2), the supernatants and the parasites were collected as described above. This procedure was repeated every day during the complete *in vitro* culture life cycle (five days for amastigote forms and seven days for promastigotes forms). Thus, culture supernatants and parasites were each collected one day after labelling.

IMMUNOPRECIPITATION ASSAYS

The radiolabelled cells were extensively washed by centrifugation at 2,500 g in culture medium without

serum and then incubated in lysis buffer (2 % Triton X-100, 0.6 M KCl, 5 mM EDTA (ethylenediamine-tetra-acetic acid), 3 mM phenylmethylsulfonyl fluoride, 1 % aprotinin, 2.5 % iodoacetamide in Tris-buffered saline [TBS: 50 mM Tris, 100 mM NaCl, pH 7.8]). The lysate was kept on ice for one hour and then centrifuged at 15,000 g for 10 min at 4°C. The Triton X-100 insoluble fraction was discarded and the supernatant (radiolabelled *L. infantum* lysate) was used immediatly or kept at – 80°C. The radiolabelled supernatants were concentrated by lyophilization.

The radiolabelled *L. infantum* lysate (10^6 cpm) or 100 µl of concentrated *L. infantum* supernatant (2 ml equivalent of labelled supernatant) were each mixed with 2 µl of human or dog immune serum and incubated overnight at 4°C. Then, the antigen-antibody complexes were precipitated by adding 75 µl protein A-Sepharose CL4B beads (Pharmacia; Uppsala, Sweden) diluted 1: 1 in TBS, pH 7.2, with constant stirring at 37°C for one hour. After incubation, the complexes were washed: twice with TEN buffer 1 (20 mM Tris, 5 mM EDTA, 100 mM NaCl, pH 7.5) supplemented with 1 % NP-40 and 2.5 % bovine serum albumin, four times with TEN buffer 2 (20 mM Tris, 5 mM EDTA, 2 M NaCl, pH 7.5) containing 1 % NP-40 and twice with TEN buffer 1 containing 1 % NP-40. The excess buffer was removed, and the immunoprecipitated antigens were prepared for SDS-PAGE and run on 12.5 % polyacrylamide gels under reducing conditions (Laemmli, 1970). The gels were dried, treated by immersion in Amplify (Amersham, France), and exposed to X-OMAT XAR-5 films (Eastman Kodak Co., Rochester, N.Y.). The molecular mass standards (^{14}C-methylated protein marker) were obtained from Amersham.

RESULTS

ONE CYCLE *L. INFANTUM* PROMASTIGOTE ANTIGENS RECOGNIZED BY HUMAN AND CANINE IMMUNE SERA

Five human sera (Fig. 1A) and four canine sera (Fig. 1B) have been respectively used to immunoprecipitate one cycle promastigote antigens. When the promastigote antigens were immunoprecipitated by the different VL-infected human sera (Fig. 1A, lanes 2-6), numerous common bands were found. The major antigens corresponded to molecular weights of 155, 120-118 doublet, 97, 78, 72, 67, 63, 50, 46, 44, 38, 34 and 26 kDa. Nevertheless, HIM-C1 (Fig. 1A, lane 4) and HIM-V1 (Fig. 1A, lane 5) sera strongly immunoprecipitated a 63 kDa antigen. When the same antigens were immunoprecipitated by VL-infected canine sera (Fig. 1B, lanes 2-5), the major common bands were 72, 70, 50, 48, 46, 34-32 doublet and 22 kDa. Nevertheless, on a general point of view, there were numerous qualitative and/or quantitative differences between *L. infantum* promastigote antigens

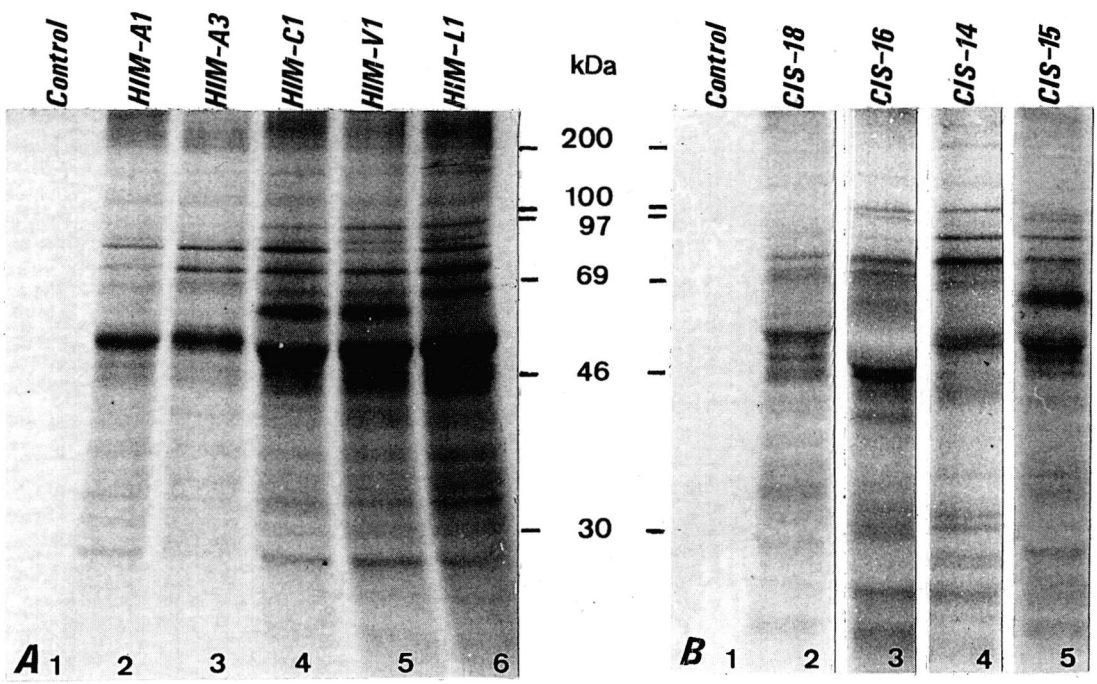

Fig. 1. – Immunoprecipitation of one cycle antigens of *L. infantum* promastigotes with VL-infected sera. (**A**) VL-infected human sera: lane 1, healthy human serum; lane 2, HIM-A1; lane 3, HIM-A3; lane 4, HIM-C1; lane 5, HIM-V1 and lane 6, HIM-L1. (**B**) VL-infected canine sera: lane 1, healthy dog serum; lane 2, CIS-18; lane 3, CIS-16; lane 4, CIS-14 and lane 5, CIS-15.

immunoprecipited by each canine serum. For example, serum CIS-15 (Fig. 1B, lane 5) stongly reacted with a 63 kDa antigen.

ONE CYCLE *L. INFANTUM* AMASTIGOTE ANTIGENS RECOGNIZED BY HUMAN AND CANINE IMMUNE SERA

Similarly, the human sera (Fig. 2A) and canine sera (Fig. 2B) have been used to immunoprecipitate one cycle amastigote antigens. Eleven major common bands, corresponding to proteins of 155, 96, 78, 72, 66, 50, 46, 38, 34, 32 and 26 kDa were immunoprecipitated with both human and canine sera. As demonstrated on the one cycle *L. infantum* promastigote antigens, HIM-V1 (Fig. 2A, lane 5) exhibited the strongest humoral responses against the one cycle *L. infantum* amastigote antigens. Based on the results obtained with the immunoprecipitations of one cycle *L. infantum* antigens, HIM-V1 human serum and CIS-15 canine serum were chosen to immunoprecipitate excreted-secreted antigens of *L. infantum* promastigote and amastigote forms.

PROMASTIGOTE EXCRETED-SECRETED ANTIGENS RECOGNIZED BY HUMAN AND CANINE IMMUNE SERA

The labelled excreted-secreted antigens collected every day of the *in vitro* life cycle (from day 1 to day 7) were immunoprecipitated with human (HIM-V1) and canine (CIS-15) serum (Figs. 3A and 3B). All along the *in vitro* *L. infantum* life cycle, the major promastigote excreted-secreted antigens (PMES-antigens) inducing humoral response in human were a 63-65 kDa doublet. On day 1, the major immunoprecipitated antigen had a molecular weight of 63 kDa and three other minor antigens could be detected (78, 70 and 65 kDa). The excretion of the 65 kDa antigen increased to reach a maximum at day 4 of culture, whereas, the excretion of the 78 and 70 kDa antigens was quantitatively stable until day 4. On day 4, the PMES-antigens that could be immunoprecipitated were 95, 78, 70, 63-65 doublet, 50 and 46 kDa. On day 5 to 7, the patterns of immunoprecipitated antigens looked like those evidenced on day 1 to 3, excepted that there was no qualitative difference between the antigens of 63 and 65 kDa.

The immunoprecipitations carried out with canine serum on the same labelled antigens, confirmed the timing of antigen excretion-secretion described above, i.e, an increase from day 1 to day 3, a maximum on day 4 and decrease from day 5 to 7 of culture. Nevertheless, CIS-15 canine serum was able to immunoprecipitate more excreted-secreted antigens than HIM-V1 human serum. The major antigens (detected on day 4) displayed a molecular mass of 120, 78, 74, 72, 65, 63, 50, 44, 40, 38 and 32 kDa. These results showed that the day 4 of *in vitro* culture (logarithmic growth phase) corresponded, quantitatively, to the most important

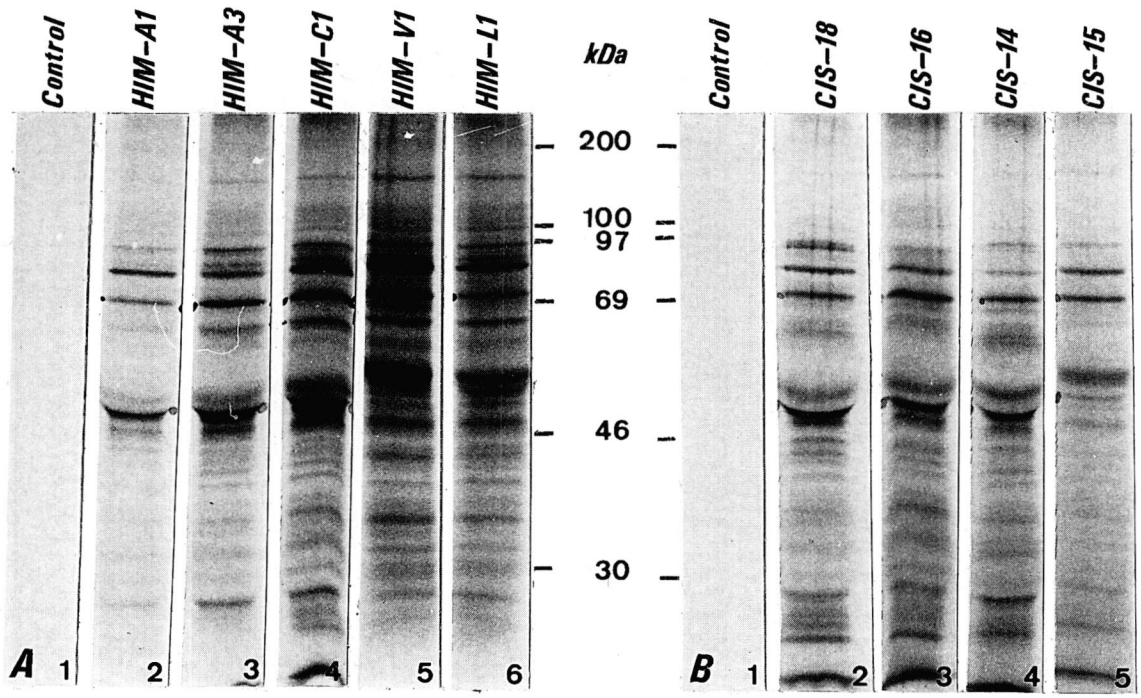

Fig. 2. – Immunoprecipitation of one cycle antigens of *L. infantum* amastigotes with VL-infected sera. (**A**) VL-infected human sera : lane 1, healthy human serum; lane 2, HIM-A1; lane 3, HIM-A3; lane 4, HIM-C1; lane 5, HIM-V1 and lane 6, HIM-L1. (**B**) VL-infected canine sera : lane 1, healthy dog serum; lane 2, CIS-18; lane 3, CIS-16; lane 4, CIS-14 and lane 5, CIS-15.

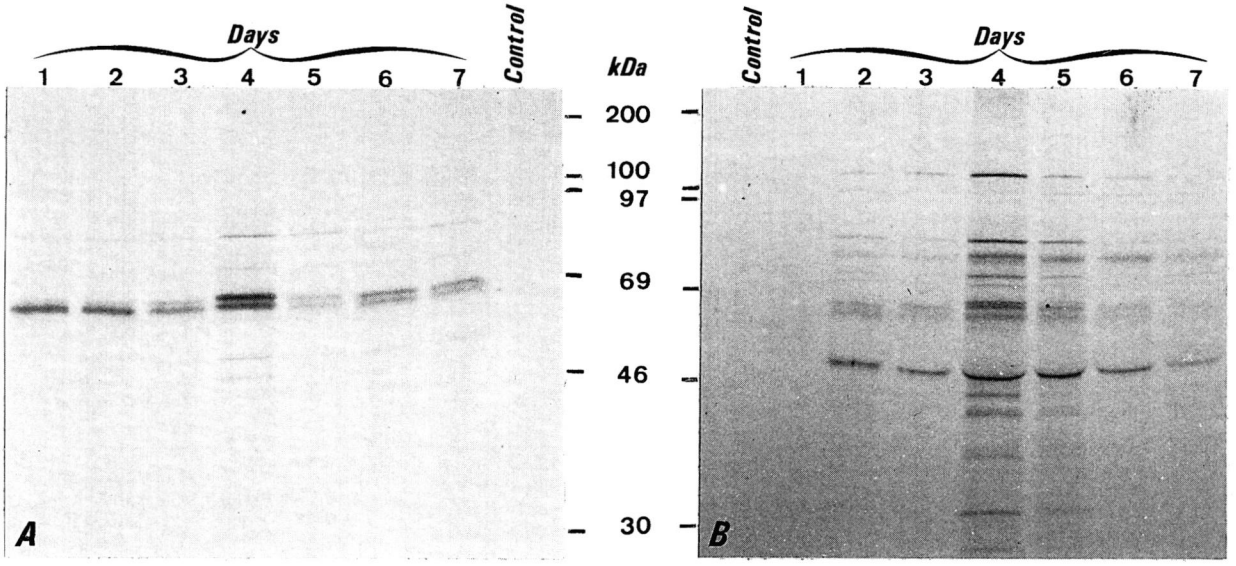

Fig. 3. – Analysis of the humoral response of man (**A**) and dog (**B**) against promastigote excreted-secreted antigens. Immunoprecipitations were performed with HIM-V1 and CIS-15 sera on radiolabelled culture supernatants collected every day of the *in vitro* life cycle. Lanes 1 to 7 correspond to each day of the *in vitro* culture life cycle.

period for *L. infantum* promastigote antigen excretion-secretion.

The human humoral response against PMES-antigens was predominantly directed against a 63-65 kDa excreted-secreted antigen doublet, whereas the canine humoral response was more complex and directed against a greater number of excreted-secreted antigens of *L. infantum* promastigotes.

AMASTIGOTE EXCRETED-SECRETED ANTIGENS RECOGNIZED BY HUMAN AND CANINE IMMUNE SERA

The same immunoprecipitation protocol was applied on radiolabelled amastigote excreted-secreted antigens (AMES-antigens) collected from day 1 to day 5 of *in vitro* culture (Figs. 4A and 4B). In contrast to PMES-antigens, a similar pattern was observed with human (Fig. 4A) and canine serum (fig. 4B). The major AMES-antigens inducing a humoral response had a molecular mass of 90, 67, 65 and 63 kDa. However, other minor antigens (98, 81, 72, 46 and 44 kDa) were also detected. The maximum of excretion-secretion antigens was observed on day 2 to 3 (logarithmic growth phase) of axenic *in vitro* cultures of *L. infantum* amastigotes. The chronological appearance of the antigens showed that on day 1 (latent growth phase), the 67 kDa antigen was the major secreted antigen. From the beginning of the logarithmic growth phase (day 2), to the end of the logarithmic growth phase (day 4), a strong secretion-excretion of the 67 kDa antigen was observed and an increase in the excretion of the 65 and 63 kDa antigens was evidenced. During the same time lapse (from day 2 to 4), there was a qualitative decrease of all the others immunoprecipitated antigens. During the stationary growth phase (day 5), the secretion-excretion of the 67 kDa antigen was residual and all the others excreted-secreted antigens had almost disappeared.

DISCUSSION

The excreted-secreted antigens of *Leishmania* are implicated to have a role all along the life cycle of the parasite. In the sandfly vector, at least three enzymes are known to be secreted by promastigotes: chitinase, N-acetylglucasaminidase and sucrase. Chitinase and N-acetylglucasaminidase intervene in the lysis of the chitin framework of the peritrophic membrane which permits the forward migration of the promastigotes (Schlein *et al.*, 1991), the secreted sucrase plays an important role in the nutrition and development of promastigotes in the insect gut (Blum & Opperdoes, 1994). Early after the infection of the mammalian host, lipophosphoglycan released by the promastigotes, promotes intracellular survival by protecting *Leishmania* promastigote against early intralysosomal degradation (Mauël, 1996) and on a general point of view, the excreted-secreted antigens of the promastigote stages have also been implicated in facilitating macrophage infection by the parasite (Mukerji *et al.*, 1986), and in decreasing monocyte and lymphocyte differentiation (El-On *et al.*, 1980; Londner *et al.*, 1983). More recently, Peters *et al.* (1997), have shown that the secretion of proteophosphoglycan by

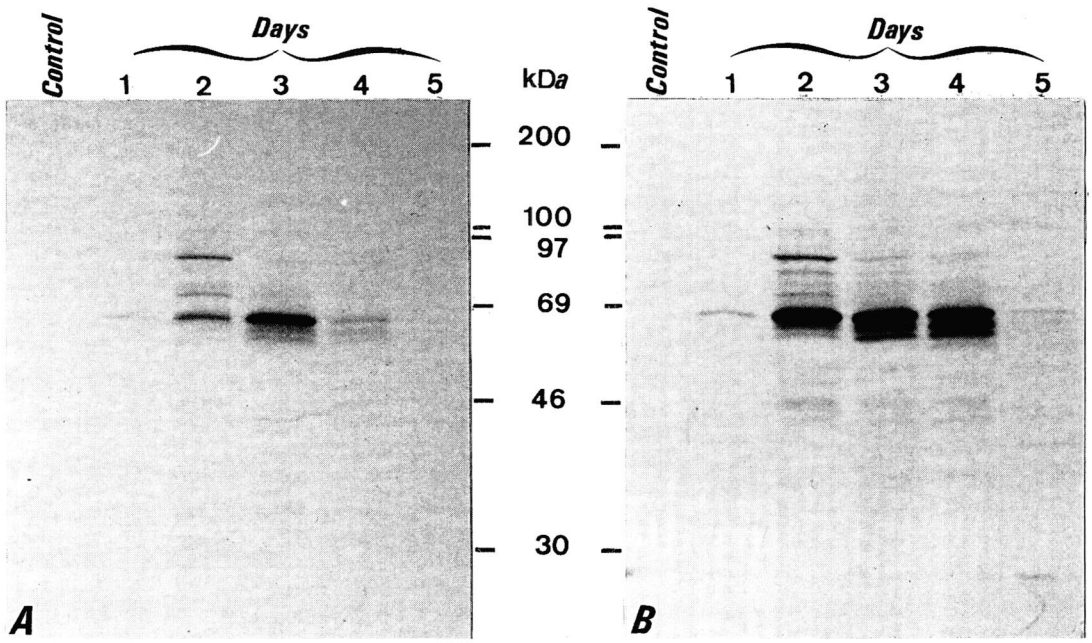

Fig. 4. – Analysis of the humoral response of man (**A**) and dog (**B**) against amastigote excreted-secreted antigens. Immunoprecipitations were performed with HIM-V1 and CIS-15 sera on radiolabelled culture supernatants collected every day of the *in vitro* life cycle. Lanes 1 to 5 correspond to each day of the *in vitro* culture life cycle.

L. mexicana amastigotes caused vacuolization of peritoneal macrophages *in vitro*.

In an attempt to define molecules for immunodiagnostic or vaccination purposes, several authors have studied the humoral response to *Leishmania* promastigote antigens by using immunoblotting techniques with sera from patients affected with leishmaniasis (Rolland-Burger *et al.*, 1991; Mary *et al.*, 1992), and patients cured of visceral leishmaniasis or with an asymptomatic infection (White *et al.*, 1992). However, as far as we know, no studies have been carried out on the *L. infantum* excreted-secreted antigens, which provide evidence about the humoral immune responses in patients and dogs having visceral leishmaniasis. Our studies illustrate that, regardless the host serum utilized, the excreted-secreted antigens of the promastigote forms or of the intramacrophagic amastigotes are able to induce an humoral response.

The major PMES-antigens able to induce a strong humoral response in man have an apparent molecular weight of 63 and 65 kDa. We have observed that in the promastigote stage of *L. infantum*, the 65 kDa excreted-secreted antigen is first, though slightly, secreted during the latent phase, while the secretion strongly increases on day 4 of the *in vitro* culture (log phase). During the metacyclogenesis phase (days 6 to 7) the excretion of this excreted-secreted antigen remains predominant. In contrast to the 65 kDa antigen, the 63 kDa antigen is more abundantly expressed at the beginning than at the end of the *in vitro* culture. Thus, the immunoprecipitation assays seem to demonstrate two different roles, for the 63 kDa and 65 kDa excreted-secreted antigens, during the promastigote life cycle. Differences in the expression of these two excreted-secreted antigens of *L. infantum* promastigotes might have a meaning in terms of parasite virulence and/or infectivity. The 63 kDa antigen could be implicated in the establishment of the parasitaemia, whereas the 65 kDa antigen could be involved in the differentiation of promastigote forms to metacyclic forms. The latter hypotheses are in agreement with the studies of Kweider *et al.* (1989) who have demonstrated that the development of metacyclic promastigotes of *L. braziliensis* is associated with the increasing expression of gp65.

Immunoprecipitations of *L. infantum* promastigotes excreted-secreted antigens with canine infection serum has shown a humoral response directed against a greater number of antigens than that with human serum. In addition to the 63 kDa and 65 kDa excreted-secreted antigens majoritarely recognized by human serum, the canine serum allowed the detection of antigens in the 70-120 kDa range and in the low molecular mass range. Our data demonstrate that the promastigotes predominantly release excreted-secreted antigens on day 4, during the log phase of the *in vitro* culture. This stronger humoral response of dogs against leishmanial excreted-secreted antigens is in accordance

with the studies of different authors (Abranches *et al.*, 1991; Rolland *et al.*, 1994; Correia *et al.*, 1996). In this case, the authors studied the humoral response of infected dogs against total antigenic extracts of the promastigote forms and, by western blotting, they have shown that the strongest humoral response was directed against the 30, 45 kDa, the 58-84 kDa and the 94 kDa regions. If we compare the human and the canine humoral responses, the hypothesis that the canine response could be directed against antigens of lyzed promastigotes must be discarded. As a matter of fact, if the immunoprecipitation samples had contained mainly lysis antigens, the immunoprecipitation patterns of promastigote excreted-secreted antigens with human or canine sera should have been similar to that of the immunoprecipitation patterns observed when using the total promastigote antigens. Indeed, we can observe that the human antibody response is completely different when using total or excreted-secreted antigens.

The success of having axenically grown amastigotes with biochemical and molecular characteristics similar to those of macrophage-derived amastigotes has allowed new developments in the studies of excreted-secreted antigens from the amastigote stage. In contrast to the data obtained with the immunoprecipitations on the excreted-secreted antigens of the promastigote form of *L. infantum,* the pattern of immunoprecipitated amastigote excreted-secreted antigens and their secretion timing were quite comparable when human or canine sera were used. Working with axenically grown amastigotes implicates that at least three different types of excreted-secreted antigens may be observed. The excreted-secreted antigens present in/on the parasitophorous vacuole, in/on the macrophage, and outside the host-cell. Even if the results obtained do not allow for discrimination between these different excreted-secreted antigens, it can be assumed that the excreted-secreted antigens immunoprecipitated during the first two days of *in vitro* culture could be directly involved in the parasite establishment inside the parasitophorous vacuole. Lang *et al.* (1994) have suggested that the parasitophorous vacuole acquires its biochemical characteristics within 48 hours after infection. In accordance with these results, it is demonstrated here that the immunoprecipitation profile of the excreted-secreted antigens was qualitatively complex on day 2 and remained more simple on days 3 and 4. In addition, Lang *et al.* (1994) have shown that a GTP-binding protein rab (marker of the prelysosomal compartment) was highly expressed in/on the parasitophorous vacuole. In a similar manner, when polyclonal antibodies directed against excreted-secreted antigens, were used to screen a cDNA library of *L infantum* amastigote stages, among the different cDNA clones obtained, one of them expressed an excreted-secreted GTP-binding protein rab (unpublished results).

With regard to the excretion timing of the amastigote excreted-secreted antigens during the *in vitro* life cycle, a strong humoral response against a 67, 65 and 63 kDa triplet was observed from day 2 to day 4 of culture. A polyclonal antibody directed against the major surface glycoprotein gp63 is able to recognize these excreted-secreted antigens (data not shown). Different authors [Medina-Acosta *et al.* (1989, 1993), Frommel *et al.* (1990)] have demonstrated that the gp63 can be found in the amastigote stage. The amastigote gp63, unlike the promastigote form of the protein, lacks a phosphatidyl inositol membrane anchor and is an hydrophilic protein that can be found in the flagellar pocket of the parasite. Moreover, Frommel *et al.* (1990) demonstrated that at least two higher-Mr gp63 bands can be detected in an amastigote lysate. Medina-Acosta *et al.* (1993) have shown that the gp63 genes expressed in the amastigote encode an extended (41 amino acids) carboxyl terminus. In addition, Frommel *et al.* (1990) have also supposed that these differences in the molecular weigth possibly reflected differences in the post-translational processing of the amastigote gp63. In this experiment, it can be observed that, although the 67 kDa antigen was expressed from day 2 to day 4, the 65 and 63 kDa excreted-secreted antigens were only expressed on days 3 and 4 and could not be immunoprecipitated on day 2. That seems to indicate that these different gp63 proteins and/or post-translational modifications probably possess a biological significance for the development of the parasite inside the host-cell.

In conclusion, we demonstrated the release of extracellular antigens from *L. infantum* amastigote and promastigote forms and the existence of a host humoral response directed against these excreted-secreted antigens. Moreover, the time course studies have shown that the secretion of leishmanial antigens exhibits both qualitative and quantitative variations according to the day of the *in vitro* cycle. Currently, studies are underway to demonstrate the efficiency of excreted-excreted antigens to induce an immunoprotection against *L. infantum.* Moreover, the biochemical characterization of excreted-secreted antigens will allow us to speculate about their possible functions in the survival of amastigotes within the macrophage.

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