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Characterization of the antigenic determinants of the *Leishmania infantum* histone H3 recognized by antibodies elicited during canine visceral leishmaniasis

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SUMMARY

In the present study we show that sera from dogs naturally infected with the protozoan parasite *Leishmania infantum* contain antibodies that specifically react with the parasite histone H3. Using synthetic peptides covering the complete sequence of the protein we located the linear antigenic determinants within the 40 amino-terminal amino acids of the molecule. In addition to the complete form of the protein (rLiH3), two regions of the *Leishmania* histone H3 were expressed as recombinant proteins: the rLiH3-Nt fragment containing the 39 amino-terminal amino acids and the rLiH3-Ct fragment containing the 90 carboxyl-terminal residues. Competition experiments using the protein fragment rLiH3-Nt as competitor confirmed that the antigenic determinants of histone H3 are confined to the amino-terminal domain. This domain, which is believed to be exposed on the nucleosome surface, is also the most evolutionarily divergent region of the *L. infantum* histone H3. Visceral leishmaniasis (VL) sera do not react with mammalian histones, an indication that the anti-histone response elicited during *Leishmania* infection is triggered by the parasite histone. The results of the prevalence of anti-histone H3 antibodies in canine VL sera together with the sequence-specific characteristics of the amino-terminal region of *L. infantum* histone H3 indicate that the recombinant protein rLiH3-Nt may be of use for diagnosis of canine VL.

Keywords visceral leishmaniasis humoral response antigenic determinants histone H3 dog

INTRODUCTION

Leishmanias are obligate intracellular protozoan parasites of macrophages whose infection causes a spectrum of human and veterinary diseases known as leishmaniasis. In humans, depending on the *Leishmania* species involved, the infection symptoms range from self-healing cutaneous manifestations to highly destructive mucosal lesions and from asymptomatic infections to fatal visceral dissemination [1]. The *L. infantum* parasite is distributed in many areas of the Mediterranean basin causing mainly the visceral form of the disease. Although the incidence of visceral leishmaniasis (VL) in humans is relatively low, the prevalence of VL in canids is very high, ranging from 10% to 37% [2]. Hence, dogs are considered to be the main veterinary reservoir of *L. infantum*, particularly during the long asymptomatic incubation phase preceding the appearance of the clinical symptoms associated with the VL form [3,4]. Studies of populations at risk of *L. infantum* infection in endemic regions demonstrated that the transmission of the parasite to humans has a direct correlation with the incidence of canine VL [5,6]. As reported by Marty *et al.* [5], about 30% of the human population living in the department of Alpes-Maritimes (France), an endemic region of canine VL, showed positive leishmanin reactions. It seems reasonable to believe therefore that the number of asymptomatic *L. infantum* infections are higher than the number of VL clinical cases. In recent years, the VL form of the disease has emerged as an important opportunistic infection in all the major groups of immunodeficient patients, including transplant recipients and AIDS patients. It is estimated that 50% of VL in adults is associated with HIV and that 1–3% of AIDS patients acquire VL in south-west Europe [7]. Hence, it appears that the human form of VL disease is in most cases associated with deficiencies in the host immune system related to cell-mediated immunity [8].

Until now, definitive diagnosis of leishmaniasis has been based on the demonstration of the presence of the parasite in aspirates of spleen, bone marrow, liver or lymph nodes [9]. Because of the frequent absence of clinical signs in the early phases of canine VL and the direct detection of the parasite in aspirates may be troublesome in some cases, the search for rapid and accurate

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indirect methods for the diagnosis of infections became an important field of investigation. The presence of high levels of circulating antibodies against the parasite proteins in sera from dogs infected with *L. infantum* [3,10–12] has indicated that serological techniques could be of great potential use for the diagnosis of canine VL. In fact, several methods of detection such as indirect immunofluorescence and ELISA are currently used [9]; however, these techniques suffer from a relative lack of specificity because of potential cross-reactivities with proteins present in crude parasite extracts used as the source of antigen. In addition to the group formed by *Leishmania*-specific antigens such as gp63 [13], gp46 [14] and Kmp-11 [15], an additional important group of *Leishmania* antigens is constituted by evolutionarily conserved proteins like kinesin [16], actin and tubulin [17], acidic P ribosomal proteins [18–20], histones [21], and particular heat shock proteins (hsp) [22–25]. In spite of the sequence conservation of the latter group of proteins, the mapping of their antigenic determinants revealed that the humoral response, in humans and in canine VL, is triggered by regions of the parasite antigens which are *Leishmania*-specific. It was found, for example, that the anti-hsp70 and anti-hsp83 antibodies elicited during *Leishmania* infection are specifically directed against the parasite proteins [24,25]. Similarly, analysis of antigenic regions of the acidic ribosomal proteins recognized by VL sera indicated that the antibodies are elicited by specific epitopes of the *Leishmania* proteins [18–20]. Regarding the *L. infantum* histone H2A it was found that during VL, specific epitopes of the protein are selected by the host immune system [21]. In a previous work we described also that the *L. infantum* histone H3 may be considered as immunogenic during VL since a cDNA coding for this protein could be isolated after immunoscreening with canine VL sera [26].

In this study we describe the mapping of the antigenic determinants of the *L. infantum* histone H3 protein. We observed that the protein is an immunodominant antigen during canine VL and that the antigenic determinants are exclusively located in the amino-terminal domain. The relevance of these findings is shown by the fact that the amino-terminal domain is the less evolutionarily conserved region of the protein [26]. Since we detected that sera from VL dogs do not recognize the histone H3 of mammalian origin, we believe that the anti-H3 antibodies circulating in sera from dogs are specifically directed against the parasite histone. Given the specificity of the humoral response we expressed the amino-terminal region of histone H3 as a recombinant protein to test its usefulness for serodiagnosis of VL.

MATERIALS AND METHODS

Parasites and sera

Promastigotes of *L. infantum* (LEM 75; zymodeme 1) were grown at 26°C in RPMI 1640 medium (GIBCO, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; Flow Labs, Irvine, UK).

Canine sera were collected in the Extremadura region of Spain. Animals were clinically and analytically evaluated at the Department of Parasitology (Veterinary School, Extremadura University, Spain). Sera from 26 dogs suffering from VL were assayed. All sera were positive when tested by indirect immunofluorescence. The presence of amastigote forms of the parasites in these animals was confirmed by direct observation in popliteal and prescapular lymphoid nodes. Control sera were obtained from 10 healthy animals. Also, five serum specimens were obtained from dogs suffering from various diseases other than leishmaniasis: OP1, dog naturally infected by *Mesocestoides* spp., *Diphylidium caninum*, *Uncinaria stenocephala*, and *Toxocara canis*; OP2, dog infected by *Dipetalonema dranunculoides*; OP3, dog infected by *Demodex canis*; OP4, dog infected by *Dipe. dranunculoides*; and OP5, dog parasited by *Babesia canis*.

Cloning and purification of recombinant antigens

The cDNA coding for *L. infantum* histone H3 described previously [26] was cloned into the *Eco*RI site of the pMal-cRI expression plasmid to produce the plasmid pMal-H3. Purification of the recombinant protein, namely rLiH3, expressed in the pMal-H3 vector was performed by affinity chromatography on amylose columns according to the methodology provided by the supplier (New England Biolabs, Inc., Cambridge, MA). For the cloning of the amino-terminal coding region of the histone H3 the pMal-H3 clone was digested with *Eco*RI and *Sma*I enzymes. Then the 149 bp long DNA fragment was cloned in a modified pMal-c2 expression plasmid (New England Biolabs). The modifications made on plasmid pMal-c2 were as follows. The vector was digested with *Bam*HI and the restriction site refilled to a blunt-end using the klenow fragment of DNA Polymerase I [27]. The vector was afterwards digested with *Eco*RI. This new clone coded for a recombinant protein, named rLiH3-Nt, encompassing the 39 amino-terminal amino acids of the *L. infantum* histone H3. The cloning procedure is illustrated in Fig. 4a. Purification of the rLiH3-Nt protein was also performed by affinity chromatography on amylose columns. The purification of the protein was done at 4°C using 1 mM PMSF and 10 μg/ml of aprotinin, leupeptin and pepstatin to maintain protein stability.

For the expression of the 90 carboxyl-terminal amino acids of H3 the original pMaL-LiH3 clone expressing the complete protein was digested with *Sma*I and *Ecl*136II restriction enzymes. The resulting product was religated using appropriate conditions [27]. The recombinant protein was named rLiH3-Ct. In aqueous solution, the recombinant protein rLiH3-Ct aggregates and therefore it cannot be purified by amylose-affinity column. rLiH3-Ct was purified from other bacterial proteins by five steps of extractions with a buffer containing 20 mM Tris–HCl pH 7·4, 200 mM NaCl, 1 mM EDTA and 1% Triton X-100. The recombinant protein remained insoluble during treatment. For SDS–PAGE analysis the insoluble pellet was resuspended in 2 × Laemmli's buffer [28]. The MBP protein, used as control, was obtained by affinity purification after induction of *Escherichia coli* cultures harbouring the pMal-c2 plasmid.

Protein samples, electrophoresis and immunoblot analysis

Preparations of the *L. infantum* nuclear fractions were performed according to the methodology described [29] modified by the addition of 1·5 mM of the TLCK protease inhibitor. Commercial preparations of calf thymus histones (type II-S) were purchased from Sigma Chemical Co. (St Louis, MO). SDS–PAGE on 10% polyacrylamide gels was performed using standard conditions [28] in a Mini-protean system (BioRad, Hercules, CA). For best resolution the calf thymus histones and *L. infantum* nuclear fractions were electrophoresed on linear 10–14% gradient SDS-polyacrylamide gels at 10 mA for 12 h using the Hoefer Scientific Instrument protein system.

For immunoblot analysis the electrophoresed proteins were transferred to nitrocellulose membranes (Amersham, Aylesbury, UK). The transfers were blocked with 5% non-fat dried milk

powder in PBS and 0·5% Tween 20. The filters were sequentially probed with primary and secondary antisera in blocking solution. A peroxidase immunoconjugate (Nordic Immunology, Tilburg, The Netherlands) was used as second antibody and the specific binding was revealed with the Western blotting detection ECL system (Amersham).

Falcon assay screening test (FAST)-ELISA measurements

The Falcon assay screening test (FAST)-ELISA (Becton Dickinson, Lincoln Park, NJ) was used instead of the classic ELISA. The sensitization of the lids was performed overnight at room temperature using 100 μl of the antigen diluted in PBS. The antigen concentration was 2 μg/ml for all recombinant proteins and 100 μg/ml for synthetic peptides. After sensitization, the lids were washed three times by immersion in 200 μl of PBS–0·5% Tween-20. Afterwards, the antigen-coated lids were incubated for 1 h with the blocking solution (5% non-fat dried milk powder in PBS–0·5% Tween-20). The sera to be assayed were diluted 1:200 in blocking solution. The lids were immersed in the microtitre plates containing the diluted sera and incubated for 2 h at room temperature with shaking. After exposure to antibody, lids were washed as described above. As secondary antibody, horseradish peroxidase (HRP)-labelled antibodies (dilution 1:2000) were used. After incubation for 1 h at room temperature and washing, the lids were developed by orthophenylenediamine as substrate. Absorbance was read at 450 nm. In the competition experiments, sera at 1:200 dilution were first incubated for 12 h at room temperature with increasing amounts of the recombinant proteins or of peptides and assayed in FAST-ELISA plates coated with 100 μl of the protein rLiH3 at a concentration of 2 μg/ml. The inhibition molar ratios were calculated as the ratio between the molar concentration of the inhibitor (preincubated with the sera) and the molar concentration of rLiH3 (used to coat the FAST-ELISA lids). Competition experiments were performed using five different canine VL sera.

Affinity purification of antibodies

Specific antibodies against the *Leishmania* H3 histone were affinity-purified from a pool of 10 positive anti-H3 VL sera on a column using the rLiH3 as antigen. For that purpose 0·5 mg of the recombinant protein were covalently bound to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) and packed into a column. Coupling and blocking were carried out according to the manufacturer's instructions. Mixed sera (2 ml) were passed through the antigen column. After washing, the specific antibodies were eluted from the column with 0·1 glycine pH 2·8. Finally, the antibody preparation was equilibrated to pH 7·5 with 1 M Tris–HCl. The solution of the antibody was restored to the original volume of the pooled sera.

Synthesis of peptides

A library of overlapping peptides covering the whole *L. infantum* histone H3 sequence was synthesized by the simultaneous multiple solid-phase synthetic method using a polyamine resin and FMOC chemistry [30]. Purity was checked by amino acid analysis and high performance liquid chromatography (HPLC).

RESULTS

Reactivity of sera from VL dogs against the recombinant L. infantum *histone H3*

In order to test the antigenicity of *L. infantum* histone H3, a histone H3 coding cDNA [26] was cloned in the pMal-cRI expression vector and used to overexpress the protein in *E. coli* cultures. After affinity chromatography, a 56-kD recombinant fusion protein (rLiH3) was isolated (Fig. 1a). The reactivity of a collection of canine sera against the protein rLiH3 was assayed by FAST-ELISA. The results showed that while the mean reactivity value of sera from healthy animals (n = 10) was 0·08 (s.d. = 0·02), the mean reactivity of VL sera (n = 26) was 0·51 (s.d. = 0·185). As shown in Fig. 1b, 81% (21/26) of VL sera reacted against the rLiH3 protein with reactivity values over the mean value of control sera + 3 s.d. The high s.d. obtained for the VL sera illustrates the heterogeneity of the humoral response against the rLiH3 protein in the infected canine population assayed.

Mapping of the antigenic determinants of the L. infantum *histone H3*

In order to determine the region(s) involved in the antibody recognition of the *L. infantum* histone H3 a collection of peptides overlapping by 10 amino acids were synthesized (Fig. 2). The collection of peptides was screened with 12 anti-rLiH3-positive sera. Only peptides P1, P2 and P3 were significantly recognized by VL sera (Fig. 2), while there was no reactivity against peptides P4–P12. None of the peptides was recognized by sera from healthy dogs as an indication that the recognition of peptides P1–P3 was specific for VL sera. We observed, moreover, that VL sera negative for the rLiH3 protein were also negative when assayed against the synthetic peptides (data not shown). Thus, it can be concluded that the linear antigenic determinants of *L. infantum* histone H3 recognized by VL sera are located within the 40 amino-terminal amino acids of the protein. Interestingly, sequence comparison between the *L. infantum* histone H3 sequence and the consensus

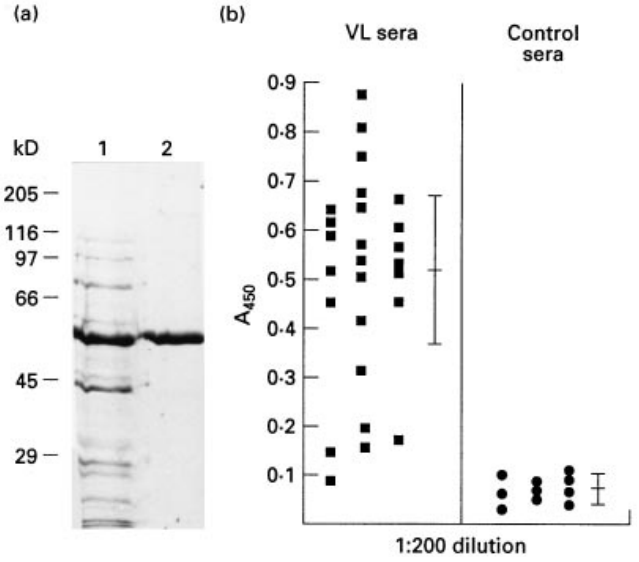

Fig. 1. Reactivity of the visceral leishmaniasis (VL) canine sera against the *L. infantum* histone H3 protein. (a) SDS–PAGE analysis of the rLiH3 expression products. Lane 1, lysate of the *Escherichia coli* cells harbouring the recombinant fusion protein MBP-rLiH3; lane 2, MBP-rLiH3 after purification through the amylose column. (b) Reactivity analysis of VL (■) and normal (●) canine sera against the affinity-purified rLiH3 protein. Each point represents the mean of triplicate values for the same sera.

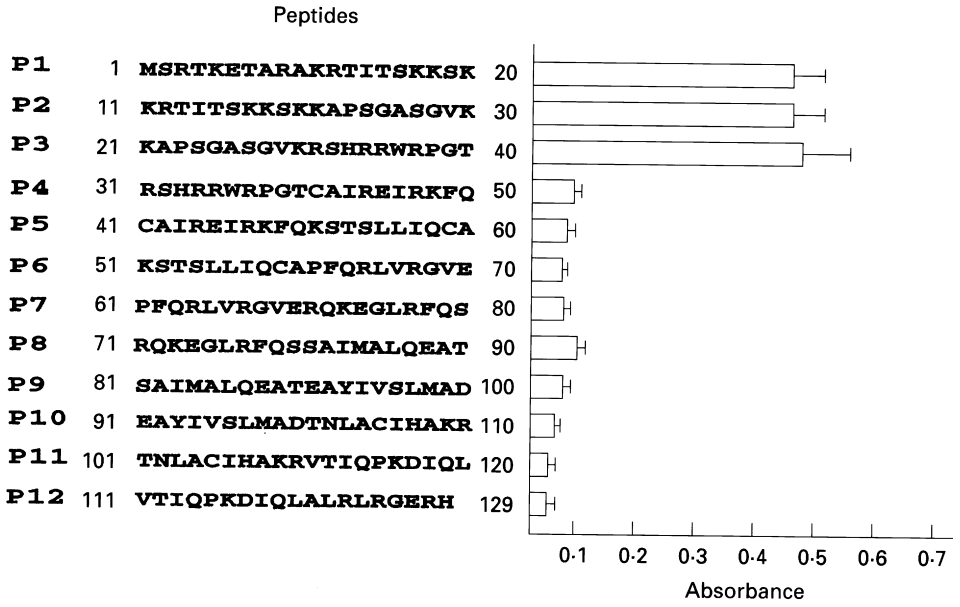

Fig. 2. Reactivity of visceral leishmaniasis (VL) canine sera against the synthetic peptides, 20-mer long overlapping by 10 residues, covering the entire *L. infantum* protein. The OD_{450} values are the mean of reactivities obtained with 12 VL sera against each peptide. The sera were assayed by Falcon assay screening test (FAST)-ELISA at 1:200 dilution.

sequence for histones H3 revealed a great number of sequence differences in this region, with many amino acid substitutions and a seven amino acid gap [26]. This highly divergent region of the protein is assumed to be exposed on the nucleosome surface. As stated above, the peptides covering the more evolutionarily conserved region of the histone H3 (P4–P12), that seems to be functionally implicated in the interaction with the other nucleosomal histones, were not recognized by the canine VL sera.

Anti-H3 antibodies present in VL sera are specific for the parasite histone H3

Although as indicated above the anti-H3 histone antibodies, present in sera from *L. infantum*-infected dogs, seem to be directed exclusively against the most divergent region of the protein, it could not be excluded that antibodies present in VL sera would recognize also histones of mammalian origin. It is known that, in fact, histones are among the most evolutionarily conserved proteins. For that purpose, nuclear extracts of *L. infantum* were resolved on linear 10–14% gradient SDS–PAGE gels and made to react with a pool of six canine VL sera by Western blotting. The coomassie blue staining of the gel (Fig. 3a) showed that the nuclear extracts were enriched in parasite histones and that the pattern was similar to that recently reported for *L. mexicana* histones [31]. Commercial calf thymus histones were used as a representative source of mammalian histones (Fig. 3, lanes 1). Figure 3 shows that while VL sera strongly reacted with several protein bands in the lane containing the *L. infantum* nuclear extracts, sera did not show any reactivity with calf thymus histones as an indication that the anti-histone antibodies of the VL sera are specifically directed against the parasite histones. The result was in agreement with the finding that antibodies present in VL sera are directed against the divergent amino-terminal of the *L. infantum* histone H3, and that the sera do not have antibodies against the conserved region. In order to identify the position of the *L. infantum* histone H3 on the gel, anti-H3 histone antibodies purified from the pool of sera were probed with a blot similar to that from Fig. 3b. Figure 3c shows that a single reacting band could be detected corresponding to one of the most reactive bands recognized by VL sera (Fig. 3b). The observation that the purified antibodies did not cross-react with the calf histone H3 (Fig. 3c) reinforces the conclusion that anti-H3 antibodies elicited during *Leishmania* infection are highly specific against the parasite histone.

Cloning, expression and purification of the amino-terminal and carboxyl-terminal regions of the L. infantum *histone H3*

In order to determine the diagnostic value of the amino-terminal

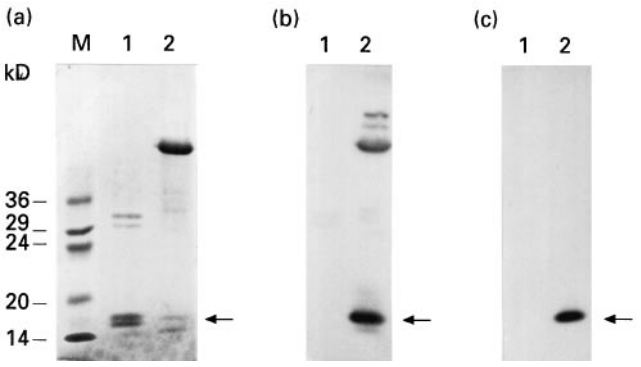

Fig. 3. Analysis of the specificity of the anti-H3 antibodies present in canine visceral leishmaniasis (VL) sera. Calf thymus histones (6 μg; lane 1) and 20 μg of *L. infantum* nuclear protein extracts (lane 2) were electrophoresed on linear 10–14% gradient SDS-polyacrylamide gels. (a) Coomassie blue staining of the gel. Molecular weight markers are shown in kD (lane M). Similar gels were blotted and probed with a pool of six VL sera (b) or with the anti-H3 affinity-purified antibodies from the same pool of VL sera (c). The position of the *L. infantum* histone H3 protein band is indicated by an arrow.

region of the histone H3, containing the linear antigenic determinants as detected by epitope mapping, and to test for the presence of conformational antigenic determinants involving other regions of the protein, both the amino-terminal and carboxyl-terminal regions were expressed separately using a series of plasmid constructs (Fig. 4). The DNA segment coding for the 39 amino acids long amino-terminal region of H3 was obtained by *Eco*RI+*Sma*I double digestion of the pMal-LiB6 clone [26] and subsequent cloning of the *Eco*RI+*Sma*I fragment into the expression vector pMal-c2 (see Materials and Methods for additional cloning details). The region coding for the globular C-terminal domain was obtained by *Sma*I+*Ecl*136II double digestion of the pMal-LiB6 clone. The new vector obtained after subsequent religation of the plasmid contains the DNA expressing the 90 carboxyl-terminal amino acids of the *L. infantum* histone H3. The recombinant proteins expressed by these new constructs were produced in *E. coli* cultures, as illustrated in Fig. 4b. The recombinant protein rLiH3-Nt could be purified by affinity chromatography (Fig. 4b), although protease inhibitors and low temperatures during purification were needed for a good yield (see Materials and Methods). The rLiH3-Ct region of the protein, on the other hand, showed a clear tendency to form aggregates of an insoluble nature. Partial purification was achieved by repeated extraction with Triton X-100 of the soluble protein from the insoluble pellet that contains the protein rLiH3-Ct (Fig. 4b, lane 6).

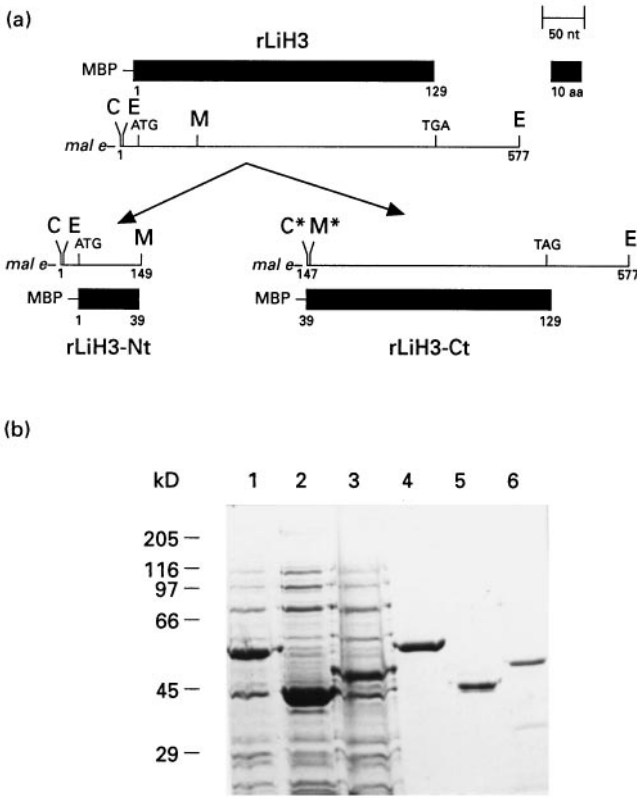

Fig. 4. (a) Diagrammatic representation of the cloning procedure leading to the expression of the amino- and carboxyl-regions of the *Leishmania infantum* histone H3. The restriction map of the LiH3-coding cDNA is shown. Restriction sites are: C, *Ecl*136II; E, *Eco*RI; M, *Sma*I. ATG and TAG sites indicate the locations of initiation and termination codons, respectively. The new recombinant pMal-c2 clones, expressing proteins rLiH3-Nt and rLiH3-Ct, were constructed as indicated. C* and M* represent the corresponding restriction sites, which were lost during construction of the pMal-LiH3-Ct clone. Recombinant proteins rLiH3, rLiH3-Nt and rLiH3-Ct are indicated by black boxes. (b) Coomassie blue staining of the recombinant proteins separated on 10% SDS–PAGE gels. Lane 1, lysate of *Escherichia coli* cells harbouring the protein MBP-rLiH3; lane 2, lysate of *E. coli* cells harbouring the protein MBP-rLiH3-Nt; lane 3, lysate of *E. coli* cells harbouring the protein MBP-rLiH3-Ct; lane 4, affinity purified MBP-rLiH3; lane 5, affinity-purified MBP-rLiH3-Nt; lane 6, Triton X-100-purified MBP-rLiH3-Ct. Molecular weight markers in kD are indicated on the left.

Anti-H3 antibodies present in VL sera are directed against the histone amino-terminal domain

The reactivity of the VL sera against the amino- and carboxyl-terminal regions of the *L. infantum* histone H3, expressed as recombinant proteins, was analysed by Western blotting (Fig. 5a). The recombinant MBP (lane 1), resulting from expression of the wild-type plasmid pMal-c2, the rLiH3 (lane 2), the rLiH3-Nt (lane 3) and rLiH3-Ct (lane 4) proteins were eletrophoresed on polyacrylamide gels and blotted. It was observed that the pool of 10 canine VL sera reacted with the amino-terminal region (lane 3), but that it did not show any reactivity with the carboxyl-terminal region (lane 4). Thus, in agreement with the epitope mapping described above (see Fig. 2), the linear antigenic determinants of the *L. infantum* histone H3 must be located exclusively in the amino-terminal region (amino acids 1–40).

To exclude the existence of conformational epitopes involving other regions of the protein, competition experiments using the recombinant proteins and synthetic peptides were performed (Fig. 5b). VL sera were preincubated with known amounts of either rLiH3, rLiH3-Nt or an equimolar mixture of peptides P1, P2 and P3. Then, the reactivities of those sera against the rLiH3 protein were assayed by FAST-ELISA. Figure 5b shows that preincubation of sera with rLiH3 or rLiH3-Nt promoted an effective inhibition of reactivity against rLiH3. A 100% inhibition was obtained when the protein rLiH3-Nt was used at a competitor:coating antigen molar ratio of 1:1. These results indicate also that the antigenic determinants of the *L. infantum* histone H3 are encompassed in the amino-terminal region of the protein. However, the mixture of the three peptides, even at a 100-fold excess, inhibited only partially (about 20%) the reactivity of VL sera. We think that the low inhibition values may be interpreted as if the peptides do not fully conform to the antigenic determinants present in the native protein.

Analysis of the diagnostic value of the recombinant protein rLiH3-Nt

The reactivity of 26 canine VL sera against the rLiH3-Nt was evaluated by FAST-ELISA (Fig. 6). As expected from the results shown in Fig. 1, 81% (21/26) of sera reacted with the rLiH3-Nt protein with absorbance values over the mean of the control sera + 3 s.d. The reactivity values of each one of the VL sera against either the rLiH3 or the rLiH3-Nt were similar, as an indication that the rLiH3-Nt maintains the sensitivity of the complete protein. In order to test the specificity of rLiH3-Nt for VL sera, reactivity of five sera from dogs with infectious diseases other than leishmaniasis (see Materials and Methods for sera description) was determined by FAST-ELISA. The reactivity values obtained with those sera (mean value = 0·17; s.d. = 0·04) were slightly higher than the reactivity shown by control sera (mean value = 0·04; s.d. = 0·02), but were signifi-

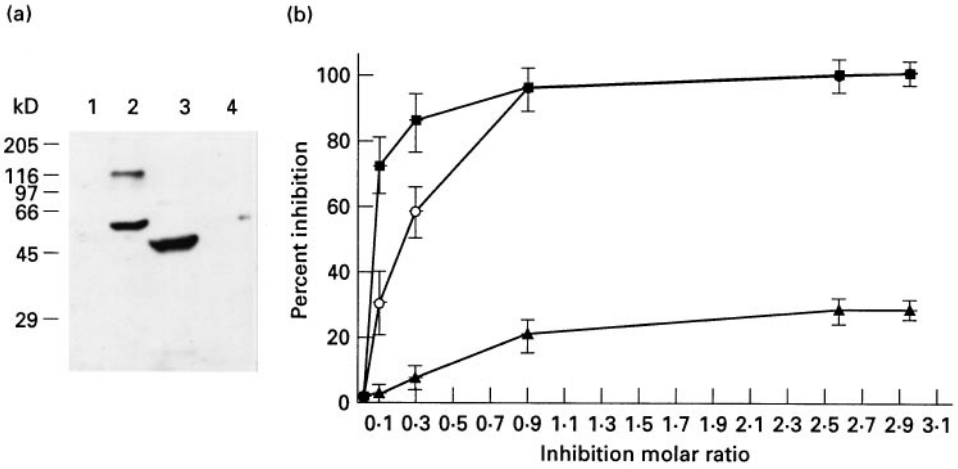

Fig. 5. (a) Western blot analysis of the antigenicity of the different subregions of the *Leishmania infantum* histone H3. Approximately 2 μg of each purified recombinant protein were electrophoresed on 10% polyacrylamide gels, blotted and probed with a pool of 10 visceral leishmaniasis (VL) sera (1:100 dilution). Lane 1, MBP; lane 2, MBP-rLiH3; lane 3, MBP-rLiH3-Nt; lane 4, MBP-rLiH3-Ct. Molecular weight markers in kD are indicated on the left. (b) Competition of antibody binding to recombinant rLiH3 protein. The Falcon assay screening test (FAST)-ELISA lids were coated with 2 pmol of rLiH3. Serum samples (1:200 dilution) were preincubated with increasing amounts of either the protein rLiH3 (■), the rLiH3-Nt protein (○) or an equimolar mixture of the synthetic peptides P1, P2 and P3 (▲). The represented values (mean ± s.e.m.) were calculated from the results of the competition experiments performed with five different VL sera. The inhibition molar ratios (moles of inhibitor/moles of coating antigen) were calculated considering the amount of antigen (2 pmol) used to coat the FAST-ELISA lids.

cantly lower than the reactivities of VL sera (mean value = 0·49; s.d. = 0·12).

DISCUSSION

The data presented in this study demonstrate that a high percentage of sera from dogs infected with the protozoan parasite *L. infantum* possesses antibodies reacting with the histone H3. Our results indicate that 81% of canine VL sera recognize the parasite H3 histone when expressed as a recombinant protein. Moreover, when the specificity of the anti-histone antibodies present in VL sera was assayed against histones of mammalian origin it was found that they only react with *Leishmania* histones. Histones seem to be important immunogens during canine leishmaniasis, since we have recently reported also the high prevalence of antibodies reacting with *Leishmania* histone H2A in sera from VL dogs [21]. Our data indicate that, also, the humoral response against histones elicited during *Leishmania* infection is highly specific, since cross-reactivity with mammalian histones was not observed. The specificity of the humoral response may correlate with the fact that *Leishmania* histones have relevant differences in their primary structure relative to histones from eukaryotic organisms others than kinetoplastids. Thus, the comparison between *L. infantum* histone H3 and the consensus sequence of the eukaryotic histones H3 [32] yielded a 63% sequence identity, with most of the sequence changes being grouped in the amino-terminal region [26]. We found that the antigenic determinants of the *L. infantum* histone H3 are located in this highly divergent amino-terminal domain. Peptides covering the first 40 amino acids were recognized by canine VL sera, whereas sera failed to react with peptides covering the rest of the protein. In a similar way, when the amino- and carboxyl-terminal fragments of *L. infantum* histone H3 gene were expressed as recombinant proteins, we observed that only the fragment including the 39 amino-terminal amino acids (rLiH3-Nt) was recognized by VL sera. In contrast, none of the VL sera reacted with a recombinant protein encompassing the 90 carboxyl-terminal amino acids (rLiH3-Ct). Thus, we can conclude that the amino-terminal region of the histone H3 contains all the antigenic

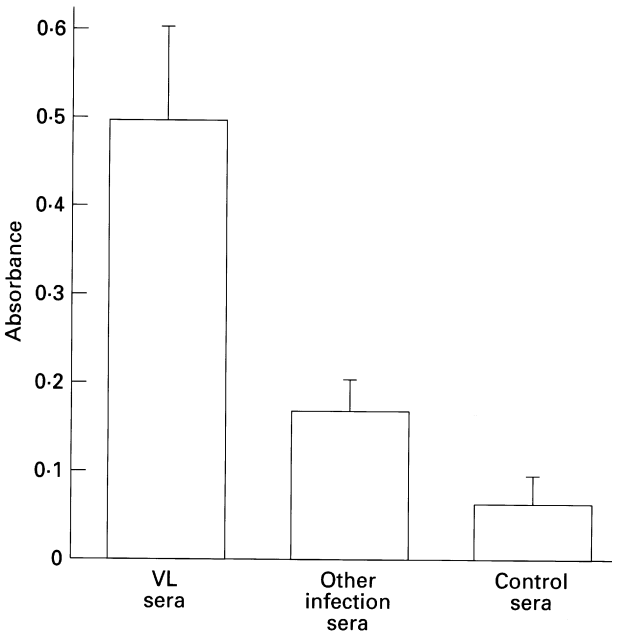

Fig. 6. Falcon assay screening test (FAST)-ELISA evaluation of the diagnostic value of the recombinant protein rLiH3-Nt. Mean absorbance values (+ s.e.m.) of sera from dogs infected with *Leishmania* (VL, n = 26), dogs infected with helminths and protozoa other than *Leishmania* (n = 5) and from control dogs (n = 10). Sera were assayed at 1:200 dilution.

determinants recognized by canine VL sera. This conclusion was further supported by the fact that rLiH3-Nt fully inhibits the binding of canine VL anti-H3 antibodies to the complete *Leishmania* histone H3. The specificity of epitope selection must be a general phenomenon in leishmaniasis, since using similar approaches we determined that the antigenic determinants of the *Leishmania* H2A are located in the most divergent regions of this protein [21]. The absence of antibodies in canine VL sera cross-reacting with mammalian histones suggests, moreover, that the humoral response generated during *Leishmania* infection does not break the host self-tolerance control.

Another feature of the anti-histone humoral response generated during *Leishmania* infection is that the immunogenic histone regions seem to be restricted to those exposed on the nucleosome surface. The structure of the *Leishmania* H3 histone resembles that predicted for the remaining H3 histones, with a hydrophilic amino-terminal regulatory region exposed outside the nucleosome and a globular carboxyl-terminal region that interacts with H4 histone for nucleosome formation [33]. Similarly, the antigenic determinants of the *L. infantum* histone H2A recognized by VL sera correspond to the regions thought to be exposed on the nucleosome surface, i.e. the amino- and carboxyl-terminal domains [21]. Since during the course of some autoimmune diseases like systemic lupus erythematosus (SLE) the elicited anti-histone antibodies are directed mainly against the histone 'tails' exposed on the nucleosomes [34], it has been suggested that chromatin cores rather than free histones would be responsible for the triggering of the humoral response [35]. Taking into account the equivalent locations of the antigenic determinants of the histones which are recognized by both SLE and VL sera, it is likely that a similar mechanism of epitope selection must operate during both pathological processes.

The high prevalence of anti-H3 antibodies in sera of VL dogs indicates that the H3 protein has potential usefulness as a tool for serodiagnosis of leishmaniasis. The use of the complete H3 recombinant protein for serodiagnosis of VL could have serious drawbacks because of its potential cross-reactivity with sera from animals having other diseases where anti-histone antibodies are induced, as, for example, canine SLE [36,37]. Since anti-H3 antibodies in SLE dogs are essentially directed against the globular region of H3, the cross-reactivity problem can be circumvented if the amino-terminal region of the histone H3 is used instead of the complete protein. As we have demonstrated, the recombinant protein rLiH3-Nt (containing the 39 amino-terminal amino acids of *L. infantum* histone H3) will confer specificity to the serological tests, while it mantains the sensitivity of the entire histone H3.

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