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Antigenicity of the *Leishmania infantum* histones H2B and H4 during canine viscerocutaneous leishmaniasis

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SUMMARY

In this study we show that sera from dogs naturally infected with *Leishmania infantum* contain antibodies that specifically react against the parasite H2B and H4 histones. The *Leishmania* H2B and the amino-terminal region of the histone H4, expressed as fusion proteins, when confronted with sera from canine viscerocutaneous leishmaniasis (VCL) dogs, were recognized by 63% and 47%, respectively. No reactivity was detected when sera from dogs naturally infected with pathogens other than *Leishmania* were used. Using a collection of synthetic peptides covering the complete sequence of both proteins, we have determined that the main linear antigenic determinants are located in the amino-terminal domains of these histones. The humoral response against histones H2B and H4 induced during canine leishmaniasis was found to be specific for *Leishmania* histones, since no cross-reactivity of the VCL sera with mammal histones was observed. Also, a comparative study of the prevalence of antibodies among VCL sera against the four core histones of *L. infantum* was performed. Although a large heterogeneity of the humoral responses against these proteins was found, histones H2A and H3 seem to be more prevalent immunogens than histones H2B and H4 during canine natural leishmaniasis. The origin of the anti-histone humoral response and its possible implications in the pathogenesis of *Leishmania* infection are discussed.

Keywords leishmaniasis histone H2B histone H4 antigenic determinants dog

INTRODUCTION

Leishmaniases are a spectrum of diseases distributed world-wide caused by infection with the protozoan parasites belonging to the genus *Leishmania*. *Leishmania infantum*, a species present in the Mediterranean basin countries, is the aetiologic agent of visceral leishmaniasis (VL) in humans and viscerocutaneous leishmaniasis (VCL) in dogs. Dogs are the main reservoir for *L. infantum*, and several reports suggest a direct correlation between the transmission of the parasite to man and the prevalence of canine VCL [1–4]. In recent years, a rise in the incidence of VL in humans has been observed in several Mediterranean countries, mainly due to the fact that *L. infantum* infection has emerged as an opportunistic infection in AIDS patients (see [5] for review).

The absence in natural infections of any detectable cell-mediated immunity and a hypergammaglobulinaemia are the main immunological features of the VL (see [6] for review). In contrast, there is a marked humoral response in VL patients, including both non-specific immunoglobulins, due to polyclonal B cell activation, and specific anti-*Leishmania* antibodies. The available evidence argues against a protective role of anti-*Leishmania* antibodies in controlling infection and favours the idea that they are involved in the formation of immune complexes [7], which may be detrimental to the host. Deposits of such immune complexes have been observed on different filtration barriers of *Leishmania*-infected animals and they have been suggested to be responsible for several pathologies such as the glomerulonephritis observed during VL [8–10]. On the other hand, glomerulonephritis is a frequent pathology observed in systemic lupus erythematosus (SLE) patients, a pathology that can be induced in animal models by the production of anti-histone and other anti-nucleosomal antibodies [11].

In previous studies, it was found that the *L. infantum* histones H2A and H3 are immunodominant antigens during canine VCL. In fact, it was observed that 78% and 81% of the canine VCL sera have anti-H2A and anti-H3 antibodies, respectively [12,13]. Furthermore, the mapping of the B cell epitopes indicated that

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the antigenic determinants are located in the most divergent regions of these proteins [12,13]. Despite the fact that histones are among the most highly conserved proteins along the evolutionary scale, histones of Trypanosomatids have accumulated substantial sequence differences, mainly at the amino- and carboxyl-terminal regions (reviewed in [14]), to trigger a specific immune response. To better understand the anti-histone immune response induced during *L. infantum* infection, in this study we have extended previous work towards the characterization of the humoral response in VCL dogs against all the four histones forming the nucleosomal core. For that purpose, genes coding for *L. infantum* histone H4 [15] and histone H2B were isolated, characterized, and expressed in *Escherichia coli* as recombinant proteins. The present study shows that histones H4 and H2B are immunogenic during natural canine leishmaniasis and that the B cell epitopes are located in the most divergent regions of the proteins. It was also found that the anti-H2B and anti-H4 antibodies present in sera from VCL dogs do not recognize the counterpart of mammalian origin, an indication that the humoral response is specifically elicited by the parasite histones.

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 two different regions of Spain: Extremadura (Department of Parasitology, Veterinary School, Extremadura University), and Catalunya (Mataró Veterinary Hospital, Barcelona). Three groups of sera were used. Group I was composed by 46 sera from dogs affected of VCL. All sera were seropositive when tested by indirect immunofluorescence, and the presence of amastigote forms of *L. infantum* was confirmed by Giemsa staining of lymphoid node preparations. Group 2 was composed by 11 sera from *Leishmania*-uninfected dogs, but infected by the following parasitic pathogens: *Mesocestoides* spp. ($n = 1$), *Diphylidium caninum* ($n = 1$), *Uncinaria stenocephala* (n = 1), *Toxocara canis* (n = 1), *Dipetalonema dranunculoides* (n = 1), *Demodex canis* (n = 1), *Babesia canis* (n = 2), and *Ehrlichia canis* (n = 3). Group 3 was composed by sera from four healthy animals.

Cloning and purification of recombinant antigens

The LiH2B cDNA coding for *L. infantum* histone H2B was isolated after screening of a *L. infantum* expression library with the ^{32}P-labelled insert of a *Trypanosoma cruzi* histone EST-clone (kindly provided by Dr W. Degrave, DBBM-Fiocruz, Rio de Janeiro, Brazil). The LiH2B cDNA was cloned into the *Eco*RI site of the pUC18 plasmid and sequenced by the dideoxy chain termination method [16] using the Sequenase Kit (United States Biochemical Corp., Cleveland, OH). Also, the LiH2B cDNA was cloned in the *Eco*RI site of the pMal-c2 expression plasmid (New England Biolabs, Beverly, MA) to over-produce the recombinant protein rLiH2B in *E. coli* cells. Purification of the recombinant protein was performed by affinity chromatography on amylose columns according to the methodology provided by the supplier (New England Biolabs).

For expression of the amino-terminal 38 amino acid region of the H4 of *L. infantum* histone, the corresponding coding region from LiH4-1 cDNA clone [15] was polymerase chain reaction (PCR)-amplified using the following oligonucleotides: sense, 5′-GGAATTCATGGCCAAGGGCAAGCGTTC-3′ (positions 55–74 of the LiH4-1 cDNA); antisense, 5′-CGGGATCC**TTA**GCGCGCCATGCGGCGGACGC-3′ (reverse and complementary to positions 149–168 of the LiH4-1 cDNA). The *Eco*RI and *Bam*HI restriction sites, included to clone the amplified DNA fragment in the pMal-c2 expression vector, are underlined. In the antisense oligonucleotide an ochre stop codon was included (in bold). The recombinant protein, named rLiH4-Nt, was also purified by affinity chromatography on amylose columns. The recombinant proteins rLiH2A [12] and rLiH3 [13] were purified as described elsewhere.

Preparation of nuclear proteins

Preparations of *L. infantum* nuclear fractions were performed according to the methodology described by Ramamoorthy *et al*. [17], with the sole modification that the protease inhibitor TLCK (1·5 mM) was also used along the preparation process. Commercial preparations of calf thymus histones (type II-S) and molecular weight markers were purchased from Sigma Chemical Co. (St Louis, MO).

Protein electrophoresis, immunoblot analysis and FAST-ELISA measurements

SDS–PAGE on 10% polyacrylamide gels was performed according to standard methodology [18], using the Mini-protean system (BioRad, Hercules, CA). For a proper resolution of the histone proteins, calf thymus histones and *L. infantum* nuclear preparations were separated by electrophoresis on linear 10–14% gradient SDS–polyacrylamide gels at 10 mA for 12 h using the Hoefer Scientific Instrument protein system (Pharmacia AB, Stockholm, Sweden).

The immunoblot analysis and the FAST-ELISA assay were performed as previously described [13]. For coating, the protein antigens were used at 2 μg/ml and the synthetic peptides at 100 μg/ml.

Affinity-purification of antibodies

Specific antibodies against *Leishmania* H2B and H4 histones were affinity-purified from a pool of six positive anti-histone sera of VCL dogs. For that purpose approx. 0·5 mg of recombinant protein was covalently bound to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) and packed into a column. Coupling and blocking were carried out according to the manufacturer's instructions. One ml of the mixed sera was 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 antibodies was restored to the original volume of the pooled sera.

Synthesis of peptides

A library of overlapping peptides was synthesized by the simultaneous multiple solid-phase synthetic method using a polyamine resin and FMOC chemistry [19]. The peptides had a purity of 96% as detected by mass spectroscopy, amino acid analysis and high performance liquid chromatography (HPLC).

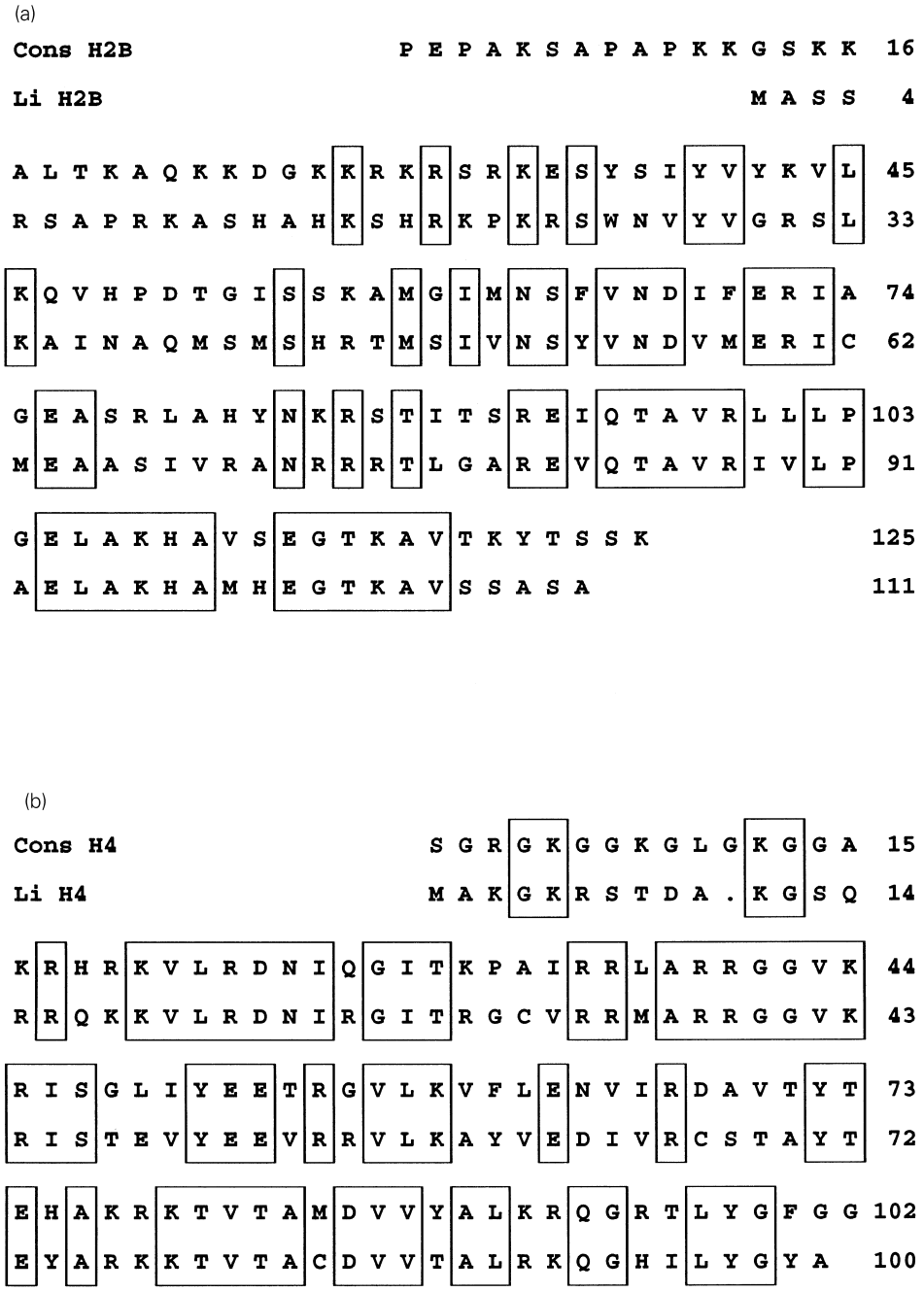

Fig. 1. Amino acid sequence alignment of *Leishmania infantum* histones H2B (a) and H4 (b) with consensus sequences for histones H2B and H4 (Cons [20]);. Identical residues are boxed. A gap (indicated by a dot) has been introduced in the *Leishmania* histone H4 sequence (LiH4) to maximize the alignment.

RESULTS

Expression and antigenicity of the L. infantum *histones H2B and H4*

In order to obtain the *Leishmania* histones H2B and H4, as recombinant proteins, the corresponding cDNAs were cloned in bacterial expression vectors. The cDNA coding for the *L. infantum* H2B was isolated by screening of a *L. infantum* expression library using as probe an EST-clone derived from the *T. cruzi* H2B gene. The sequence of this clone, namely LiH2B, was determined and submitted to the EMBL/GenBank/DDBJ databases with the accession number Y13396. The cDNA LiH2B insert is 894 nucleotides long possessing an open reading frame from positions 64 to 396 where the putative *L. infantum* histone H2B is encoded. Figure 1a shows the deduced amino acid sequence of the parasite histone H2B and its comparison with the consensus sequence of the histones H2B reported by Wells [20]; both sequences shared a 49% identity. The identity values reached a value of 85% with the *L. enriettii* histone H2B [21] and 72% with the *T. cruzi* histone H2B [22]. For expression of the *L. infantum* histone H4 the LiH4-1

Table 1. FAST-ELISA reactivity of viscerocutaneous leishmaniasis (VCL) sera with histone H2B peptides

Peptides										
B1 1–20	B2 11–30	B3 21–40	B4 31–50	B5 41–60	B6 51–70	B7 61–80	B8 71–90	B9 81–100	B10 91–112	rLiH2B
0·500	–	–	0·610	0·120	–	–	–	–	0·120	0·630
0·200	0·480	0·120	–	–	–	–	0·600	0·650	–	0·887
–	0·230	–	0·200	–	0·107	0·120	–	–	0·120	0·720
–	–	–	0·230	–	–	–	0·100	–	–	0·800
0·400	0·370	0·130	0·140	–	–	0·110	–	–	–	0·400
–	–	–	0·600	–	–	–	–	0·147	–	0·460
–	0·380	–	–	–	–	–	–	–	0·130	0·565

Canine VCL sera were diluted 1:100 and allowed to react with FAST-ELISA plates coated with peptides (100 μg/ml) or protein (2 μg/ml). Absorbance values below background values, detected in absence of antigen (0·05), were considered as negative (–).

cDNA, previously described [15], was used. Figure 1b shows the deduced amino acid sequence of the *L. infantum* histone H4 and its comparison with the consensus sequence of the histones H4 reported by Wells [20]. It is remarkable that there is a large divergence of the amino-terminal region of the *Leishmania* histone H4 with regard to that of the consensus sequence.

For expression of *L. infantum* histones H2B and H4 in *E. coli* cells, the corresponding cDNAs were subcloned in frame into the *Eco*RI site of the plasmid vector pMal-cR2. The pMal-LiH2B recombinant plasmid over-expressed a fusion protein (named rLiH2B) with a molecular weight of 54 kD corresponding to that expected for the fusion protein formed by the maltose binding protein (MBP; 42 kD) and the H2B (12 kD) moieties. The antigenicity of the *L. infantum* histone H2B during canine leishmaniasis was assayed by studying the reactivity of a collection of canine sera against the recombinant protein rLiH2B. Of the VCL sera 63% (29/46) recognized the protein rLiH2B with reactivity values higher than the mean absorbance value + 3 s.d. (cut-off value = 0·12) of sera from dogs having infections others than leishmaniasis (see Materials and Methods for description of the sera).

Since repeated attempts to express the cDNA LiH4-1, coding for *L. infantum* histone H4 [15] into plasmid pMal-c2, failed to yield *E. coli* colonies expressing the recombinant histone H4, other expression vectors were used. We tried to clone the cDNA LiH4-1 into plasmid pMS, a modified pUR vector [23], and into plasmid pQE (Qiagen, Chatsworth, CA). In any of these vectors expression of the cDNA LiH4-1 could be achieved, although the restriction analysis of the recombinant clones indicated that the cDNA had been inserted in the right orientation. Therefore, it is plausible that the expression failed because the *L. infantum* histone H4 results highly toxic for the *E. coli* cells. Hence, we tried to clone and express the amino-terminal region of this protein since, as described below, the epitope mapping using synthetic peptides indicated that the amino-terminal domain of *L. infantum* histone H4 was the most immunodominant region of the molecule. In order to clone and express the amino-terminal region of the histone H4 a DNA fragment coding for the 38 amino-terminal residues of the protein was PCR-amplified from the LiH4-1 cDNA (nucleotides 55–168) and cloned into the pMal-c2 vector to yield plasmid pMal-LiH4-Nt. A protein band of 45-kD was over-expressed by the *E. coli* cells transformed by plasmid pMal-LiH4-Nt. The fusion protein, named rLiH4-Nt, was purified by affinity chromatography and used to analyse its immune recognition by sera from dogs with leishmaniasis. Twenty-two out of 46 VCL sera (47%) showed reactivity values above the cut-off value (0·10) of sera from dogs with infections other than leishmaniasis, indicating that during natural canine *Leishmania* infection anti-histone H4 antibodies are elicited.

Mapping of the linear antigenic determinants of the L. infantum *histones H2B and H4*

In order to define the location of the main antigenic determinants recognized by the sera from the VCL dogs a collection of peptides (20mer, overlapping by 10 amino acids), derived from the amino acid sequences of the histones H2B and H4 (Fig. 1), were synthesized and assayed individually by FAST-ELISA against the VCL sera. Table 1 shows the reactivity of seven VCL sera against peptides B1-B10 (histone H2B). All peptides were recognized, at least by a serum, although peptides B1 (amino acids 1–20), B2 (amino acids 11–30) and B4 (amino acids 31–50) were recognized more frequently and showed the highest reactivity values. Thus, the results shown in Table 1 point to the existence, at least, of two main antigenic determinants in the *L. infantum* histone H2B, both located in the first amino-terminal 50 residues. One antigenic determinant would be located in the amino-terminal shared by peptides B1 and B2, while the other seems to be located in the amino acid sequence of peptide B4. None of the peptides was recognized by either the control sera or the sera from dogs with infections other than leishmaniasis (data not shown). Also, the sera from the VCL dogs that did not recognize the recombinant protein rLiH2B did not react with any of the peptides B1–B10, indicating that these peptides are specifically recognized by the anti-H2B antibodies present in the VCL sera.

The epitope mapping of the *Leishmania* histone H4 was also performed by analysing the reactivity of the VCL sera against peptides C1–C9 (Table 2). The results indicated the existence of a prominent antigenic determinant which is located in the first 30 amino acids of the protein, a region covered by peptides C1 and C2. Remarkably, this antigenic determinant is located in the most divergent region of the *L. infantum* histone H4 (Fig. 1b). Although showing a lower frequency of recognition, another antigenic determinant was located in the region covered by peptides C7 and C8 (from amino acid position 61–90, Table 2).

Table 2. FAST-ELISA reactivity of viscerocutaneous leishmaniasis (VCL) sera with histone H4 peptides

Peptides									
C1 1–20	C2 11–30	C3 21–40	C4 31–50	C5 41–60	C6 51–70	C7 61–80	C8 71–90	C9 81–100	rLiH4-Nt
0·400	0·670	–	–	–	–	–	0·220	–	0·620
0·234	0·440	–	–	–	–	–	–	–	0·480
0·310	0·930	–	–	–	–	0·610	0·120	–	0·510
0·340	–	–	–	–	–	–	–	–	0·490
0·415	0·100	–	–	–	–	0·160	0·235	–	0·800
0·230	0·220	–	–	–	–	0·111	–	–	0·328
0·460	0·912	–	–	–	–	–	–	–	0·750

Canine VCL sera were diluted 1:100 and allowed to react with FAST-ELISA plates coated with peptides (100 μg/ml) or protein (2 μg/ml). Absorbance values below background values, detected in absence of antigen (0·05), were considered as negative (–).

Specificity of the anti-histone antibodies

Since histones are among the most highly conserved proteins in nature, an interesting question to analyse is the specificity of recognition of the anti-histone antibodies elicited during canine leishmaniasis. In previous works, we demonstrated that the anti-H2A and anti-H3 antibodies present in canine VCL sera recognized specifically the *Leishmania* histones [12,13]. In order to investigate the specificity of the anti-H2B and the anti-H4 antibodies present in the VL dogs a pool of six VCL sera, selected by their reactivities against both histones, was incubated with a Western blot containing calf thymus histones and *L. infantum* nuclear extracts (Fig. 2). The results indicate that the VCL sera reacted with the *Leishmania* nuclear extracts but that they did not cross-react with histones of mammalian origin. The purification by affinity chromatography of the anti-H2B and anti-H4 antibody fractions from VCL sera (panels αH2B and αH4, respectively) allowed the identification of the position in the gel of the *L. infantum* histones H2B and H4. Each one of the affinity-purified antibody fractions reacted specifically with the putative bands corresponding to the *Leishmania* histones H2B and H4, but they did not react with the mammal counterparts. It may be concluded therefore that the anti-histone antibodies, present in the sera of VCL dogs, are specifically directed against the *Leishmania* histones.

VCL sera recognize the four core histones of L. infantum

In this study it has been shown that antibodies against *L. infantum* histones H2B and H4 are present in the sera of dogs with leishmaniasis, completing previous data in which the antigenicity of *L. infantum* histones H2A and H3 during canine VCL was demonstrated [12,13]. Therefore, these findings indicate that all of the nucleosomal core histones from the parasite become antigenic during canine leishmaniasis. This conclusion is further illustrated by the data shown in Fig. 3. The four *L. infantum* histones (H2A, H2B, H3, and H4), expressed in *E. coli* as MBP fusion proteins, were separated by SDS–PAGE (Fig. 3a), blotted onto a nitrocellulose membrane and incubated with a pool of VCL sera. As shown in Fig. 3b, the four recombinant proteins were recognized by a mixture of VCL sera, demonstrating that antibodies for the different *L. infantum* histones are elicited during canine leishmaniasis.

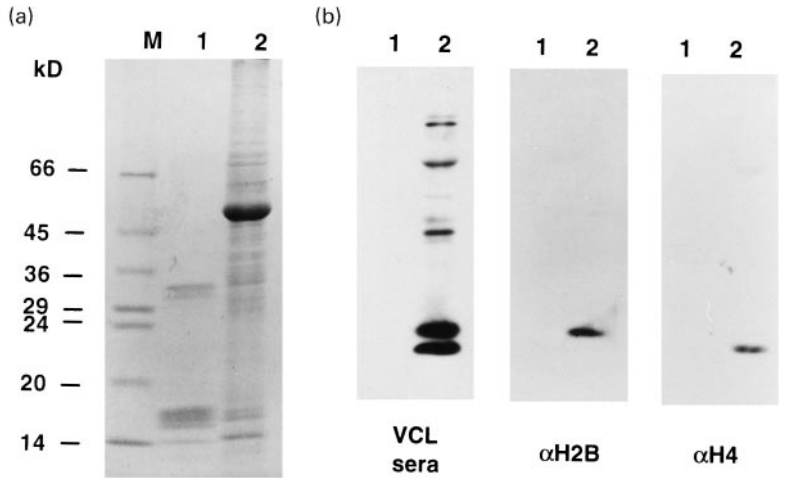

Fig. 2. Specificity of anti-histone antibodies present in viscerocutaneous leishmaniasis (VCL) sera. Calf thymus histone (6 μg; lane 1) and 20 μg of *Leishmania infantum* nuclear protein extracts (lane 2) were electrophoresed on linear 10–14% gradient SDS–PAGE gels. (a) Coomassie blue staining of the gel. Molecular weight markers are shown in kD (lane M). (b) Equivalent gels were blotted and probed either with a pool of six VCL sera (panel VCL sera), with the affinity-purified anti-H2B antibody fraction of VCL sera (panel αH2B), or with the affinity-purified anti-H4 antibody fraction of VCL sera (panel αH4).

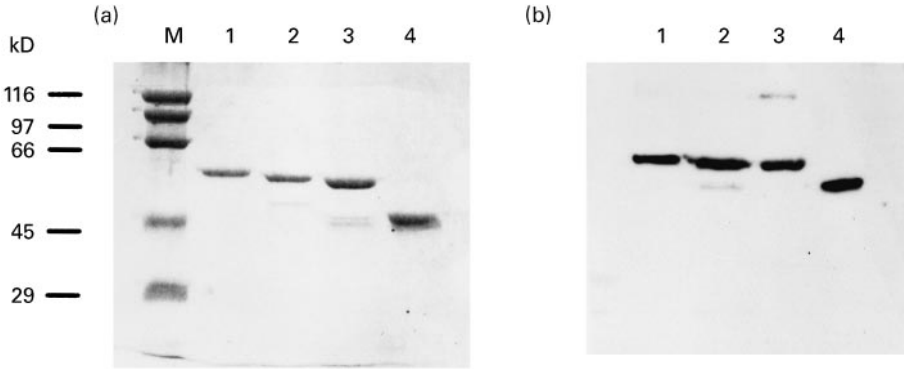

Fig. 3. Reactivity of viscerocutaneous leishmaniasis (VCL) sera against all four core histones of *Leishmania infantum*. One miscrogram of the following recombinant proteins was loaded on 10% SDS–PAGE gels: rLiH2A (lane 1), rLiH2B (lane 2), rLiH3 (lane 3), and rLiH4-Nt (lane 4). Lane M contains the molecular weight markers. (a) Coomassie blue staining of the gel. (b) An equivalent gel was blotted to a nitrocellulose filter and probed with a pool of six VCL sera (1:100 dilution).

In order to analyse whether during canine leishmaniasis there is specificity in eliciting anti-histone antibodies against any particular type of *Leishmania* histones, the reactivity of VCL sera against each one of the four core histones was determined individually.

Table 3. Reactivity of viscerocutaneous leishmaniasis (VCL) sera against the four core histones

		Histone class			
Group	Sera	rLiH2A	rLiH2B	rLiH3	rLiH4-Nt
I	1	0·450	0·128	0·200	0·250
	2	0·610	0·290	0·450	0·550
	3	0·470	0·270	0·176	0·260
	4	0·550	0·360	0·590	0·250
	5	0·390	0·130	0·320	0·150
	6	0·630	0·210	0·800	0·220
II	7	0·530	–	0·630	0·180
	8	0·470	0·235	0·137	–
	9	0·430	0·330	–	0·450
	10	0·690	0·150	0·400	–
	11	0·120	0·195	–	0·180
	12	0·690	0·120	0·470	–
	13	–	0·315	0·190	0·300
III	14	0·350	0·190	–	–
	15	–	0·130	0·300	–
	16	0·310	0·200	–	–
	17	0·145	–	–	0·300
	18	0·210	–	0·100	–
	19	0·150	–	0·130	–
	20	0·200	–	0·800	–
IV	21	–	–	0·240	–
	22	–	–	0·200	–
V	23	–	–	–	–
	24	–	–	–	–
	25	–	–	–	–

Canine VCL sera were diluted 1:500 and allowed to react with the four fusion recombinant histones (2 μg/ml) in FAST-ELISA plates. Absorbance values below background values, detected in absence of antigen (0·05), were considered as negative (–).

Table 3 summarizes the reactivity values showed by 25 VCL sera against each one of the recombinant histones H2A, H2B, H3 and H4. It was found that 24% (6/25) of the sera reacted with the four recombinant proteins, 28% (7/25) of the sera reacted with three histones types, 28% (7/28) of the sera recognized two of them, and that 8% (2/25) of the sera reacted with only one of the histones; 12% (3/25) of the VCL sera did not react with any one of the histones. A statistical analysis indicated that there was no correlation between the presence of a particular antibody against a given histone with an antibody against another histone. Thus, by linear regression analysis of the data shown in Table 3 it was determined that the r values were always $< 0{\cdot}6$ for the four core histones when the reactivity to any one of the histones was compared with the reactivity to any other histone type. In addition, although from the high percentage of VCL sera (36%) that reacted with either all or none of the four histones it seemed that anti-histone antibodies were elicited coordinately, this fact was not found to be of statistical significance. Thus, the individual response against the histones must probably occur as independent events.

On the other hand, when the percentages of recognition of the individual histones by VCL sera were analysed it was observed that histone H2A was the most frequently recognized (72%), followed by histone H3 (68%), histone H2B (60%), and histone H4 (44%). The mean reactivity values shown by the VCL sera at a given dilution were similar against histones H2A (0·386; s.d.$=0{\cdot}278$), H3 (0·357; s.d.$=0{\cdot}22$) and H4 (0·302; s.d.$=0{\cdot}11$), whereas the mean reactivity value of the VCL sera against histone H2B was the lowest (0·147; s.d.$=0{\cdot}119$).

DISCUSSION

In the present work we have shown that antibodies reacting with *L. infantum* histones H2B and H4 are elicited during canine leishmaniasis. Prevalence studies indicated that about 63% of the VCL sera have anti-histone H2B antibodies and that anti-histone H4 antibodies are present in about 47% of those sera. Western blot analysis indicated, furthermore, that the anti-H4 and anti-H2B antibodies were elicited specifically against the parasite histones and that there was not cross-reactivity with mammal histones. Thus, together with previous reports in which the antigenic

properties of the *L. infantum* histones H2A and H3 during canine leishmaniasis were described [12,13], this study provides an overview of the immunogenic character of the parasite histones. Now, it can be stated that during canine VCL all the four core histones of *L. infantum* are targets of the humoral immune response and that the response is elicited by the parasite proteins. Interestingly, preliminary data from our laboratory suggest that a similar humoral response occurs also during visceral leishmaniasis in human patients.

Our data show that according to the frequency of recognition, histones H2A, H2B and H3 showed similar percentages of recognition (72%, 60% and 68%, respectively) and that the amino-terminal of the H4 histone was recognized by 44% of the sera. The VCL sera showed similar reactivity values against histones H2A, H3 and H4, whereas histone H2B was recognized with lower reactivity values. Thus, from both prevalence and reactivity data, it can be concluded that histones H2A and H3 are more immunogenic than histones H2B and H4 during canine *Leishmania* infection. However, the high degree of variability in the recognition of individual histones shown by each of the VCL sera was remarkable, suggesting that the different anti-histone antibodies are not elicited in a sequential way during canine leishmaniasis.

Although the presence of anti-histone antibodies in the sera of VCL dogs has been well established in this and previous works [12,13], it remains to be understood why antibodies are specifically induced against these proteins during *Leishmania* infection. Classically, production of anti-histone antibodies has been considered as a hallmark of autoimmune processes such as SLE. Although the anti-histone humoral response elicited during either SLE or leishmaniasis is quite different in terms of specificity, it is likely that the production of anti-histone antibodies in both processes can be triggered by the same mechanism. In recent years it became clear that the nucleosome is an important autoantigen responsible for the anti-histone humoral response elicited in SLE patients [11,24–26]. Although more experimental approaches must be performed to ascertain the origin of the anti-histone humoral response observed during canine leishmaniasis, the present results favour the idea that also during VL the *Leishmania* nucleosomes act as the immunogenic stimulus. This hypothesis is also based on the fact that the anti-histone antibodies present in the sera from VCL dogs are directed not against the internal regions of histones, involved in the stabilization of the nucleosomal core, but against epitopes presumed to be exposed at the surface. Thus, in this and previous works [12,13] we showed that the most immunodominant epitopes are located at the amino-terminal ends of the histones H2B, H3 and H4, and at both ends of histone H2A. Furthermore, it has been observed that the antigenic determinants of *L. infantum* histones are located in the most divergent regions of the molecules. As indicated by Galanti *et al*. [14], the sequence divergence of the Trypanosomatid histones appears to reflect the particular functional roles of the different protein domains. Thus, while the globular regions, responsible for the major histone–histone and DNA–histone interactions, of the trypanosome core histones are relatively conserved, the amino-terminal domains of these chromosomal proteins, which seem not to be involved in these interactions, are highly divergent. Although knowledge of the structure of Trypanosomatid chromatin is scanty, proteolysis studies using immobilized proteases have indicated that the amino terminal regions of histones H3 and H4 are probably located on the surface of the nucleosomal particle [27].

Another interesting question to answer is the possible involvement of anti-histone antibodies and *Leishmania* histones in some of the pathological processes associated with leishmaniasis. Due to their abundance and their physical properties it would not be unreasonable to think that the *Leishmania* histones forming immune complexes with the anti-histone antibodies could trigger pathological alterations such as glomerulonephritis. In fact, during *Leishmania* experimental infection in hamsters, a direct correlation has been observed between the appearance of kidney lesions and the deposition of immune complexes in that tissue [8]. Although it remains to be demonstrated, it is likely that parasite histones may be an important component of the immune complexes present in visceral leishmaniasis patients [7]. Studies on murine SLE models have demonstrated the presence of histones in the glomerular immune deposits, showing that histones are involved in the pathogenesis of lupus nephritis [28]. Recently, the presence of antibodies against *Plasmodium falciparum* histones in malaria patients has also been described, and a possible role of these antibodies in the pathology of malaria has been suggested [29].

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