Short communication

Nucleoside hydrolase from *Leishmania (L.*) donovani* is an antigen diagnostic for visceral leishmaniasis☆

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Received 13 November 2001; accepted in revised form 10 January 2002

Keywords: Recombinant antigen; Kala-azar; Zoonotic visceral leishmaniasis; Diagnosis

In this study, we have isolated a gene encoding the nucleoside hydrolase (NH) of *Leishmania (L.*) donovani* which is a component of the GP36 antigen. We show that recombinant *Ld*NH is useful for the diagnosis of clinical kala-azar in dogs since it reacts preferentially with the IgG1 subtype of immunoglobulin that is enhanced in kala-azar susceptible dogs and in advanced disease.

In the New World kala-azar (visceral leishmaniasis, VL) is a canid zoonosis and one of the most important emerging diseases [1]. In human and canine kala-azar antibodies to *Leishmania* appear in the circulation soon after infection during an early subclinical period [2]. The control of zoonotic VL (ZVL) in northeastern Brazil has centered on the detection of infected dogs by serological assays using immunofluorescence (IF) and elimination of seropositive dogs [3]. Seropositivity detected by IF usually correlates well with parasitological evidence of infection [3–5]. However due to its low sensitivity this method underestimates the true prevalence of canine infection. Enzyme linked immunosorbent assay (ELISA) [3,6,7], dot-ELISA [8], Western blot [9], rapid immunochromatographic tests [10] and DNA-based techniques [3,11] represent improved and more sensitive methods of detection.

The fucose–mannose ligand (FML) is an antigenic glycoprotein complex from the promastigote form of *L. (L.*) donovani* that is used for the diagnosis, prognosis and blood bank control of human kala-azar [12–14] and the diagnosis and prognosis of canine VL [15]. In addition, vaccination with FML conferred protection in mice [16,17], hamsters [18] and dogs [19]. The major immunoprotective component of FML is a 36-kDa glycoprotein fraction designated GP36 [20] that is recognized specifically by sera from human kala-azar patients [21]. Thus, the GP36 fraction of FML is a useful antigen for diagnostic assays [21] and as a potential candidate for a second-generation vaccine [20].

To obtain recombinant protein for further studies on the antigenicity and vaccine potential of GP36, we cloned the gene that encodes the immunogenic component of the GP36 fraction of FML. Briefly, the GP36 fraction was isolated from FML [20] and the sequence of a tryptic peptide, EVGTKPAAFMLQILDFYTK, was obtained. Hybridization screening of a *L. (L.*) donovani* genomic library with two degenerate oligonucleotides yielded a double-positive cosmid clone. The cosmid harbored a hybridizing, single-copy gene whose ORF translated into a 314-amino acid protein that contained the entire sequenced peptide fragment (data not shown). The sequence showed 96.2% similarity to the *Leishmania major* NH [22] and other kinetoplastid NHs.

The *Ld*NH was expressed as a maltose-binding protein fusion protein in *Escherichia coli*. The *Ld*NH fusion protein was purified by affinity chromatography,

Abbreviations: FML, fucose–mannose ligand; GP 36, 36-kDa glycoprotein; IF, immunofluorescence; Ld, *Leishmania donovani*; NH, nucleoside hydrolase; rNH, recombinant NH; VL, visceral leishmaniasis; ZVL, zoonotic VL.

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followed by Factor Xa cleavage. Further purification of the \( rLdNH \) by ion exchange chromatography yielded a high purity product that was recognized in Western blot analysis by polyclonal anti-GP36 serum and sera from symptomatic dogs (data not shown). Thus, we conclude that \( LdNH \) presents epitopes that are recognized in cases of kala-azar.

To determine the usefulness of the cloned antigen in a diagnostic assay, the ability of \( LdNH \) and FML to be recognized by sera from canine kala-azar cases was compared by a microtiter plate ELISA assay. Titrations of the antigens (2–0.03125 \( \mu \)g per well) against pools of normal, asymptomatic and symptomatic kala-azar dog sera were performed. All FML and \( LdNH \) antigen concentrations were able to distinguish the three groups of sera (data not shown). Fig. 1 shows the response of pools of sera against 2 \( \mu \)g of each antigen. While FML antigen reacted strongly with sera from all infected animals (either symptomatic or asymptomatic), \( rLdNH \) was recognized mainly by sera from kala-azar symptomatic dogs. A minor response against \( rLdNH \) was developed by sera from asymptomatic dogs while normal healthy animals remain below the cut-off for FML. The mean average of symptomatic sera developed against 2 \( \mu \)g of \( rLdNH \) (Abs 492 nm = 0.927) was lower than that developed by FML antigen at the same concentration (Abs 492 nm = 1.189). A possible explanation for this result is that each antigen may react with different antibody fractions.

To analyze the immunoglobulin response, the individual total IgG, IgG1 and IgG2 reactivities of each symptomatic dog sera sample against the \( LdNH \) antigen were determined in the ELISA assay. The absorbency values corresponding to IgG1 and IgG2 antibodies of symptomatic dogs are represented in Fig. 2. The levels of IgG1 antibodies were higher than IgG2 in the majority of the samples (18/25) (mean average of absorbencies for IgG = 0.951 ± 0.458 and for IgG2 = 0.611 ± 0.368) indicating that \( LdNH \) is an antigenic marker mainly recognized by IgG1, the immunoglobulin subtype related to the advancement of disease [23]. Fig. 3 shows that the IgG1 absorbency values increase with the number of dog symptoms. The correlation coefficient analysis (Pearson bivariate) gave a significant result (\( P = 0.035 \)), while no correlation was detected between levels of IgG2 and total IgG, whether the \( LdNH \) was used at 0.5 or 1 \( \mu \)g per well. Also, the IgG1 rate was correlated with IgG2 (\( P = 0.001 \)) but not with total IgG (\( P = 0.926 \)). In contrast, IgG2 was correlated to total IgG (\( P = 0.003 \)).

In the present study we have isolated the gene for \( LdNH \), which we demonstrate is an antigenic component of the GP36 fraction of FML [20,21]. Sera from symptomatic dogs show a predominant IgG1 antibody response against NH in the majority of animals. This result and the association of an IgG1 response with symptomatic disease [23,24] supports further development of FML-derived vaccine and diagnostic reagents. In addition to its potential for diagnosis and vaccination, the absence of NH activity in mammals [25] makes it a potential target for selective drug design.

The purified \( rLdNH \) obtained from \( E. coli \) maintained its antigenicity, as evidenced by its reaction with sera from symptomatic dogs. Further, an ELISA assay using low concentrations of antigen showed that while FML reacts with sera of both symptomatic and asymptomatic dogs, the \( LdNH \) recognizes primarily cases of clinical symptomatic confirmed disease. This result suggests that the \( LdNH \) exposes epitopes reacting specifically with the antibody fraction synthesized during the intensification of the disease. This antibody fraction was characterized as the IgG1 subtype and, as found in other studies (see below), the level of anti-\( LdNH \) IgG1 antibodies in infected animals is correlated with the increase in the number of symptoms due to advancement of the disease.

The control of VL in Northeastern Brazil in the past 30 years has centered on the detection of infected dogs by serological methods and the elimination of seropositive dogs. The optimization of the control campaign
relies on the improvement of the sensitivity of the diagnostic tool used [6–8,15,26]. Some seropositive dogs are infected and asymptomatic or oligosymptomatic [2,4]; some infected dogs spontaneously recover [27]. Furthermore, evidence correlating dog symptoms and infectiousness to sandflies is controversial. Although a correlation between increasing symptoms and infectiousness has recently been reported [28], both asymptomatic and symptomatic naturally-infected dogs proved to be infective to sandflies [4]. Cabral et al. [29] and Pinelli et al. [30] described stronger antibody synthesis only in symptomatic dogs, both naturally and experimentally infected. Solano-Gallego et al. [24] found higher titers in symptomatic than in asymptomatic dogs. As observed for humans [31], the differential increase in several IgG immunoglobulin subclasses might be marker of overt kala-azar. Dogs infected with *L. infantum* produced both IgG1 and IgG2 antibodies with IgG1 being associated with disease (symptomatic dogs, non- or low-responsive to chemotherapy) and IgG2 being associated to asymptomatic infections [23]. Solano-Gallego et al. [24] analyzed the *L. infantum*-specific IgG, IgG1 and IgG2 antibody response in healthy and ill dogs of an endemic area of Spain. Although a great variation of antibody levels was recorded, the authors observed that in a group of naturally infected asymptomatic dogs, only IgG1 remained stable for a 5 years period. Furthermore, IgG1 levels were especially decreased in the group of symptomatic dogs that were responsive to chemotherapy treatment and remain stable in the unresponsive group suggesting a correlation between IgG1 increase, symptoms and disease.

Since infectiousness to sandflies may be related to dog symptoms [28] and the development of clinical disease is related to the increase of IgG1 subtype of anti-*L. donovani* antibodies [23,24], the identification of an *L.
endemic areas contributes to the improvement of tools used in epidemiological control of kala-azar. Serological testing of dogs in endemic areas using the FML-ELISA assay and the LD/NH ELISA will help in elucidating the real incidence of canine disease.

Future studies on the LD/NH potential to discriminate dog infectiousness might also be helpful, improving decisions for control programs. The molecular cloning of LD/NH has provided insights into the immune response to ZVL and constitutes a promising advance for the development of a battery of quality molecular diagnostic tests to be used in the control of this emerging disease.

Acknowledgements

C.B. Palatnik de Sousa. received financial support from: National Foundation of Health (Brazilian Ministry of Health), PCDEN, PNUD-FNS, FINEP, CNPQ, CAPES, MCT/PRONEX (Brazilian Ministry of Science and Technology), RHA-E-CNPQ, ‘José Bonifácio’ University Foundation of the Federal University of Rio de Janeiro, FAPERJ, and CEPG-UFRJ. D.M. Santana received fellowships from CAPES, RSG-UNDP/World Bank/WHO—Special Program for Research and Training in Tropical Diseases (TDR ID no. 98–1147), and ‘José Bonifácio’ University Foundation of the Federal University of Rio de Janeiro. D.A. Campbell received support from Margaret H. Moffat. We thank Buddy Ullman for the cosmid library; Sabrina Dyall, Larry Feldman, Otávio Nóbrega, Kumaran Ramamurthy, Guy Roberts, Arun Venkatesan, Mike Yu and Gusti Zeiner for suggestions; and Dr L.Y. Hashimoto Okada from Clínica Veterinária de Cães e Gatos, Araçatuba, São Paulo, Brazil for the donation of dog sample sera.

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