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Research paper

cDNA sequencing and expression of Nramp1 (Slc11a1) in dogs phenotypically resistant or susceptible to visceral leishmaniasis

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ABSTRACT

Nramp1 (Slc11a1) is linked to resistance to *Leishmania* in mice, but its role in canine leishmaniasis is not clear. In this study we sequenced the Nramp1 cDNA from dogs whose macrophages allowed or restricted intracellular growth of *Leishmania chagasi*. Peripheral blood monocyte-derived macrophages were isolated from 29 dogs, cultured and inoculated with *L. chagasi*. This approach resulted in the identification of dogs whose macrophages were resistant or susceptible to *L. chagasi*. Nramp1 cDNA sequences of these dogs were identical. mRNA levels of Nramp1, IFNγ, IL-4 and the subunit p35 of IL-12 were assessed in the spleen of naturally infected symptomatic and asymptomatic dogs in comparison to uninfected controls. Although not statistically significant, asymptomatic dogs had a tendency for higher levels of Nramp1 mRNA ($p = 0.11$). Expression of Nramp1 was then compared between phenotypically resistant and susceptible dogs, without any significant difference between these groups.

1. Introduction

Visceral leishmaniasis (VL) is a vector-borne disease caused by a parasitic protozoa belonging to the family Trypanosomatidae, genus *Leishmania*. It is an important public health problem in Latin America, Southern Europe, Africa, and Asia. *Leishmania* (*Leishmania*) *chagasi* (=*L. infantum*) is the etiological agent of VL. In South America, VL is transmitted through the bite of infected san flies (*Lutzomyia longipalpis*) and domestic dogs are the most important reservoir of the disease. An intense cutaneous parasitism that is often observed in dogs and their presence in the peridomestic environment favor their role as source of infection for *L. longipalpis* (Grimaldi and Tesh, 1993; Ashford et al., 1998; Diniz et al., 2008).

Genetic selection of domestic animals resistant to pathogens has been applied mostly to farm animals, particularly cattle. Identification of genes linked to natural resistance may allow for a better understanding of natural resistance with obvious practical implications. These genes may also function as markers for prediction of genetic resistance against specific diseases (Adams and Templeton, 1998). Genetic selection of dogs naturally resistant against infections is not broadly used as a tool for controlling infectious diseases. However, considering the importance of zoonotic diseases such as VL (Ashford et al., 1998; Diniz et al., 2008), selection of resistant dogs may have a significant impact in controlling this disease in both human and canine populations (Quinnell et al., 2003) since the dog is the most important reservoir of VL. In the mouse,

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Nramp1 (natural resistance associated protein 1) is linked to resistance or susceptibility to intracellular pathogens including *Leishmania* (Vidal et al., 1995), and it is possibly also associated with natural resistance against *Leishmania* in dogs (Altet et al., 2002). Therefore, the study of Nramp1 in dogs may result in the identification of genetic markers for natural resistance against leishmaniasis, which may allow genetic selection of resistant dogs.

The Nramp1 or *Slc11a1* is expressed primarily by macrophages. It was initially identified in the mouse and it was linked to natural resistance against intracellular pathogens such as *Leishmania* (Bradley et al., 1979), *Salmonella* (Lissner et al., 1983), and *Mycobacterium* (Gros et al., 1981). A point mutation identified in the murine Nramp1 render the macrophages susceptible to intracellular multiplication of *Leishmania*, and consequently dissemination of the pathogen within the host (Bradley, 1977; Bradley et al., 1979; Vidal et al., 1995). Nramp1 is a divalent cation transporter (Mn^{2+}, Zn^{2+} and Fe^{2+}) located in the membrane of phagolysosomes in macrophages (Gruenheid et al., 1997). Its mechanism of action is not completely known, but Nramp1 controls the ionic content of the phagolysosome interfering with survival and growth of intracellular pathogens (Canonne-Hergaux et al., 1999; Forbes and Gros, 2001). In addition, Nramp1 has pleiotropic effects on the immune system (Blackwell et al., 2003). The influence of Nramp1 on resistance to different intracellular pathogens has been demonstrated both in vitro (Lissner et al., 1983; Stach et al., 1984; Olivier and Tanner, 1987) and in vivo (Gros et al., 1983; Crocker et al., 1984). In addition, previous studies demonstrated that natural resistance against intracellular pathogens correlates well with intracellular survival of the microorganism in cultured macrophages (Price et al., 1990; Campbell and Adams, 1992; Qureshi et al., 1996). During the course of this study the canine Nramp1 was sequenced by another group and polymorphisms were identified, although the role of these polymorphisms in resistance or susceptibility to visceral leishmaniasis is not completely clear (Altet et al., 2002).

The goal of this study was to compare the cDNA sequence of the canine Nramp1 between dogs that are phenotypically resistant or susceptible to visceral leishmaniasis, and assess expression of Nramp1 in naturally infected susceptible or resistant dogs.

2. Material and methods

2.1. Dogs and experimental design

Blood samples were obtained from 29 dogs for macrophage phenotyping and Nramp1 cDNA cloning and sequencing (described below). These dogs were all serologically negative for visceral leishmaniasis by both indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA). They were adult, healthy, from both genders, and mostly large breed dogs as detailed in Supplementary Table 1. This experimental protocol has been approved by the institutional Committee for Ethical Use of Experimental Animals (CETEA-UFMG, protocol 12/02).

Expression of Nramp1 and selected cytokines was measured in the spleen of symptomatic or asymptomatic dogs. Fifteen dogs from the Center for Zoonosis Control (Ribeirão das Neves, Brazil) were divided into three groups: (i) serologically positive for *Leishmania* by indirect fluorescence and ELISA without any clinical sign or lesion suggestive of visceral leishmaniasis ($n = 5$); (ii) serologically positive and symptomatic dogs, with clinical signs and lesions suggestive of visceral leishmaniasis ($n = 5$), such as emaciation, lymphadenopathy, splenomegaly, skin lesions, and onicogriphosis; (iii) control group ($n = 5$) with serologically negative dogs without clinical signs or lesions of visceral leishmaniasis. All these dogs were euthanatized as part of the official program for zoonosis and VL control. Samples of the spleen were collected immediately after euthanasia, snap frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

Other 10 dogs from the Center for Zoonosis Control (Ribeirão das Neves, Brazil) were also sampled for the analysis of Nramp1 and cytokine expression in the spleen. Five of these dogs were considered phenotypically resistant based on the criteria that they remained asymptomatic for at least 6 months after testing serologically positive for *Leishmania* (resistant group, $n = 5$). The other five dogs were also serologically positive for *Leishmania* for at least 6 months and developed several clinical signs and lesions of visceral leishmaniasis (susceptible group, $n = 5$). These dogs were also euthanatized as part of the official VL control program. Samples of the spleen were collected immediately after euthanasia, snap frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

2.2. Isolation of peripheral blood monocyte-derived macrophages and experimental inoculation

Canine macrophages were isolated and cultured from 29 adult healthy dogs of both genders as previously described (Bueno et al., 2005). This protocol yields more than 80% of cells with phenotypic features of macrophages (Bueno et al., 2005). Ten days is the time required for differentiation of monocytes into macrophages (Wardley et al., 1980). Therefore, after 10 days in culture, the flasks were placed onto ice for 30 min followed by agitation for harvesting the cells, which were seeded onto a chamber slide (Lab-Tek, NalgeNunc). Macrophages were inoculated with *Leishmania* (*Leishmania*) *chagasi* promastigotes (international code MCAN/BR/2002/BH400) with a multiplicity of infection (MOI) of 10 in duplicates. This strain was isolated from the spleen of a naturally infected dog from Belo Horizonte, Minas Gerais, Brazil, and cultured in α-MEM medium, pH 7.4, supplemented with 10% fetal bovine serum and penicillin (50 IU/ml) at 24 °C. Twenty-four or 72 h after infection, the cells were stained with a modified Giemsa staining system (Diff-Quick Laborclin, Pinhais, Brazil), and the infection rate established by counting 200 randomly selected macrophages from each chamber (two chambers per dog). The number of intracellular amastigotes was semi-quantitatively score from 1 to 6 according to the following criteria: 1 = 1–4 amastigotes/macrophage (a/m); 2 = 5–8 a/m; 3 = 9–12 a/m; 4 = 13–16 a/m; 5 = 17–20 a/m; 6 = >20 a/m.