Recombinant single-chain canine interleukin 12 induces interferon gamma mRNA expression in peripheral blood mononuclear cells of dogs with visceral leishmaniasis

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Abstract

Canine visceral leishmaniasis poses important concerns for public health and veterinary medicine in many areas of the world. Resistance to it seems to be associated with cellular specific immune responses of the so-called Th1 type. Interleukin-12 (IL-12) is one of the most potent inducers of Th1 type of immune responses to co-administered antigens. Herein, the cloning of canine IL-12, as a single-chain fusion protein (sccaIL-12), and its expression in biologically active form in COS-7 cells is reported. Supernatants from these cells stimulated the expression of comparable amounts of interferon gamma mRNA in peripheral blood mononuclear cells from dogs with natural visceral leishmaniasis. In addition, after stimulation with sccaIL-12, there was no difference between interferon gamma mRNA expressions in peripheral blood mononuclear cells of dogs with visceral leishmaniasis and from normal healthy control animals.

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1. Introduction

Leishmania infantum/chagasi frequently causes a generalized disease in the Mediterranean basin and in South America, the visceral leishmaniasis (VL), which can be fatal for both people and dogs (Deane and Deane, 1955; Corredor et al., 1989; Abranches et al., 1991; Molina et al., 1994). Infected dogs, which are often found in endemic areas in frequencies exceeding 30%, are considered to be the major reservoirs of the parasite (Ashford et al., 1995; Berrahal et al., 1996). Canine VL is also an important veterinary problem, since treatment of affected dogs with the currently available drugs is expensive, toxic, and promotes only transient clinical cure, as relapses after

Abbreviations: VL, visceral leishmaniasis; sccaIL-12, single-chain canine IL-12; PVDF, polyvinylidene difluoride; HPRT, hypoxanthine phosphoribosyltransferase

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treatment withdrawal are the rule (Baneth and Shaw, 2002).

Protective immune responses against VL in dogs seems to be cellular, of the so-called Th1 type (Pinelli et al., 1995), and the predominant cytokine expressed after specific stimulation is the protection-associated interferon gamma (IFN-\(\gamma\)) cytokine (Pinelli et al., 1995; Whitton, 1998). IL-12 has been shown to induce Th1 responses against many intracellular organisms (Whitton, 1998). In this paper, the cloning and expression of biologically active recombinant single-chain canine IL-12 (sccaIL-12) is described. In addition, the ability of sccaIL-12 to stimulate IFN-\(\gamma\) mRNA expression by peripheral blood mononuclear cells (PBMC) from dogs with visceral leishmaniasis was compared to its ability to stimulate the same expression from the PBMC of uninfected healthy control dogs.

2. Material and methods

2.1. Cloning of sccaIL-12 cDNA

Primers were designed based upon previously described cDNA sequences for canine IL-12 (Okano et al., 1997) and used to clone full length cDNAs coding for IL-12 p35 and p40. Details of the cloning and expression of p35 and p40 in Escherichia coli will be reported elsewhere (Oliveira et al., in preparation). Briefly, cDNA was synthesized from total RNA obtained from canine PBMC stimulated with 10 \(\mu\)g/ml of bacterial lipopolysaccharide (Sigma Chemical Co., St. Louis, MO), through reverse transcriptase reaction (RT). cDNA coding for the full extent of p35 and p40 chains were obtained from total cDNA. For this, the primers Ca-p35f 5'-ATGTGCCCGCCGCGCAG, Ca-p35r 5'-TTAGGAAAGATTCAGAAGATCTCAG, and Ca-p40f 5'-ATGCATCCTCAGGACACAGATGC were used. The cDNA encoding each subunit of IL-12 was separately cloned into the pCR2.1 plasmid (TA cloning kit, Invitrogen). The insert was then subcloned into the pcDNA3.1 plasmid (Invitrogen), generating a construct named pcDNA3.1–sccaIL-12, which was also sequenced for confirmation of successful cloning.

2.2. Expression of sccaIL-12 in mammalian cells

For transfection of COS-7 cells, the pcDNA3.1–sccaIL-12 plasmid construct, as well as the pcDNA3.1 plasmid without any insert (negative control), were purified using Qiagen-tips (Qiagen, Valencia, CA), following the manufacturer’s instructions. Transfections with 2 \(\mu\)g of plasmid, either pcDNA3.1–sccaIL-12 or pcDNA3.1, per 100 mm Petri dish, cellular metabolical labeling with \(\mathrm{S}^{35}\) methionine and immunoprecipitation with an anti-human IL-12 goat antibodies (Sigma Chemical Co., Saint Louis, MO) were carried out, essentially, as described by McGonigle et al. (2002). In one experiment, 2 days after transfection, the cell supernatants were removed and stored at \(-20\) \(^\circ\)C until used. Western blot analysis were performed with the anti-human IL-12 antibody, referred to above, as described elsewhere (Oliveira et al., in preparation).

2.3. Animals

Four healthy dogs from a non-endemic area for canine visceral leishmaniasis (Salvador, Bahia, Brazil) and six dogs with clinical manifestations of visceral leishmaniasis (Table 1), from the endemic area of Monte Gordo, Bahia, Brazil, were used. Leishmania was detected in all sick dogs and in none of the healthy control dogs after culturing splenic aspirates in NNN modified medium (Lightner et al., 1983). All animals were kept in a Leishmania-insect vector-free kennel. All experiments were performed in accordance with the Oswaldo Cruz Foundation guidelines for experimentation on animals.

2.4. Detection of canine IFN-\(\gamma\) mRNA

PBMC were purified from heparinized venous blood by centrifugation on a Fycoll-Hypaque (Sigma) solution. PBMC were then cultured in 2 ml of RPMI supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Inc., Utah, USA), 2 mM L-glutamine (Life Technologies) and 50 \(\mu\)g/ml
gentamycin in six-well plates (2 \times 10^7 cells/well) for 24 h, containing 12.5% of supernatants from COS-7 cells transfected with either pcDNA3.1–sccaIL-12 or with the pcDNA3.1 plasmid (without insert). Total RNA was extracted using Trizol (Life Technologies) following the manufacturer’s protocol. For RT-PCR, initially, cDNAs were synthesized using the GeneAmp RNA PCR kit (Perkin Elmer, New Jersey, NJ), following the manufacturer’s instructions, from 1 \mu g of total RNA. PCR designed for amplification of a segment of cDNA coding for IFN-\(\gamma\) and another for the housekeeping hypoxanthine phosphoribosyltransferase (HPRT) was carried out in a single test tube. The primers used to amplify a fragment of IFN-\(\gamma\) and HPRT cDNA were 5’-GGTGTTGCTCTTTTTGC, 5’-ACTCCTTTTCCGCTTCC (Devos et al., 1992), 5’-TATGGACAGGACTGAACGTCTTGC and 5’-GACACAAACATGATTCAAATCCCTGA (Dutra et al., 1997). The PCR conditions were: 35 cycles of 95 °C for 1 min, 60 °C for 2 min, 72 °C for 2 min, and then 72 °C for 10 min. These 35 cycles generated signals inside the linear region of the amplification curve. The RT-PCR products were resolved in a 1% agarose gel in 1 \times TAE buffer with 0.5 \mu g/ml ethidium bromide (Sambrook et al., 1989). The gel image was captured in an Eagle Eye II still video system (Stratagene, La Jolla, CA) and analyzed using the EagleSight, Version 3.22, software (Stratagene). Kinetic studies showed that the IFN-\(\gamma\) mRNA expression reached a maximum plateau at 18 h, which shows little decrease by 48 h (data not shown). Samples of gel-purified cDNA were sequenced to confirm the specificity of RT-PCR.

2.5. Expression of RT-PCR results

The computer software attributed a number of pixels to each band in the agarose gel, corresponding to amplified IFN-\(\gamma\) or HPRT cDNA, according to their intensities. Results were expressed as the ratio of IFN-\(\gamma\)/HPRT pixels originated from each sample. Non-paired \(t\)-test was used for statistical analysis, as the data showed normal distribution.

3. Results and discussion

Comparison of the cloned p35 and p40 sequences with those previously described (Okano et al., 1997) showed full identity, except for a single nucleotide difference at position 95 (T > C) for p40 cDNA, resulting in a conservative change from a valine to an alanine residue.

An anti-human IL-12 goat antibodies had been previously shown to react with canine IL-12 p35 and p40 expressed in E. coli (Oliveira et al., unpublished data). The precipitate, obtained with the use of this antibody in a lysate (Fig. 1) or culture supernatant (not shown) of pcDNA3.1–sccaIL-12-transfected COS-7 cells, produced a single band of approximately 73 kDa in SDS-PAGE. The finding of this single band indicates the lack of any isolated p40 expression and, therefore, the absence of an undesired formation of p40 homodimers (Mattner et al., 1993; Gillessen et al., 1995; Ling et al., 1995).

Stimulation of PBMC from four healthy adult dogs with the supernatant from pcDNA3.1–sccaIL-12-
transfected COS-7 cell cultures caused significantly higher ($P < 0.01$) expressions of IFN-$\gamma$ mRNA (mean of pixels in gel bands $\pm$ S.E.M.: 0.505 $\pm$ 0.097) than stimulation with the supernatants from control empty plasmid pcDNA3.1-transfected COS-7 cells (0.135 $\pm$ 0.009, Fig. 2). Comparable results ($P > 0.8$) were obtained with PBMC from six dogs with polysymptomatic visceral leishmaniasis, in which the mean of pixels in gel bands corresponding to IFN-$\gamma$ mRNA $\pm$ S.E.M. in the presence of supernatants from pcDNA3.1–sccaIL-12-transfected COS-7 cells (0.596 $\pm$ 0.119) were significantly higher ($P < 0.01$) than the mean of pixels in gel bands corresponding to IFN-$\gamma$ mRNA $\pm$ S.E.M. in the presence of control supernatants (0.179 $\pm$ 0.036).

sccaIL-12 is therefore able to induce expression of IFN-$\gamma$ mRNA in PBMC from dogs with visceral leishmaniasis, even when these cells fail to proliferate when stimulated with crude Leishmania antigen (Barrouin et al., unpublished results). This indicates that, if proper amounts of IL-12 are administered to a dog with VL, they would induce the production of IFN-$\gamma$, encouraging the testing of purified IL-12, perhaps in combination with conventional chemotherapy, as a therapeutic agent for canine VL. The induced IFN-$\gamma$ could play a protective role. The proposed experiment would also help to determine if the suppression in cell mediated immune responses in dogs with VL (Moreno et al., 1999; Rhalem et al., 1999) can be reversed, thereby preventing recrudescence of disease and further transmission.

The data reported herein contrast with data from two studies, carried out on Sudanese (Ghalib et al., 1995) and Brazilian (Bacellar et al., 1996) human patients. In these reports, PBMC from patients with acute visceral leishmaniasis produce gamma interferon when stimulated in vitro with a combination of Leishmania antigens and IL-12, but not when stimulated with IL-12 alone. However, a figure in Ghalib and collaborators’ report showed that IL-12 induced interferon gamma production by PBMC in
the absence of Leishmania antigens (mean amount of IFN-γ ± S.D.: approximately 2 ± 0.2 ng/ml) as compared with cultures with medium alone (undetectable IFN-γ). A possible explanation for the apparent discrepancy between the results on human patients mentioned above, and those reported herein, could be ascribed to the possibility that the infection of dogs by L. chagasi/infantum would lead to a higher amount of Leishmania antigens carried over in vivo by canine PBMC than by human PBMC, reducing the need to an in vitro addition of Leishmania antigens to canine PBMC cultures.

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References


Oliveira, G.G.S., Santos, L.R., Chang, Y.-F., MacDonough, S.P., Pontes-de-Carvalho, L.C. Cloning and expression of both subunits of canine Interleukin 12 in E. coil (Manuscript in preparation).