Available online at www.sciencedirect.com
SCIENCE DIRECT®
Experimental Parasitology 109 (2005) 106–114
Experimental Parasitology
www.elsevier.com/locate/yexpr

Leishmania infantum: soluble proteins released by the parasite exert differential effects on host immune response

R. Rosa, O. Roos Rodrigues, C. Marques, G.M. Santos-Gomes*

Unidade de Leishmanioses e Centro de Malária e Outras Doenças Tropicais, Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa, Rua da Junqueira 96, 1349-008 Lisboa, Portugal

Received 10 August 2004; received in revised form 23 November 2004; accepted 24 November 2004

Abstract

The objective of this study was to analyse the modulatory effect of proteins released by cultured *Leishmania infantum* promastigotes on the cellular immune response of infected susceptible (BALB/c) and more resistant (C57BL/6) mice strains after 30 and 45 days of infection. One month after parasite inoculation, *L. infantum* released protein fractions (High, Inter, and Low according to molecular weight) stimulated C57BL/6 mice spleen cells to proliferate and to express cytokines. Following the decrease of parasite load only the Low protein fraction induced a considerable release of IL-4. In BALB/c mice, specific immune response to protein fractions was only observed at the higher parasitic level, with the fraction Inter promoting the production of IL-4 and fractions High and Low inducing high levels of IL-12. These results point out to a role of these proteins fractions in the modulation of host immunity, that depending on the host genetic background and parasite magnitude, seem to be critical in the control of parasite replication levels, thus avoiding premature host death.

Index Descriptors and Abbreviations: cDNA, complementary deoxyribonucleic acid; cpm, counts per minute; Ag, crude *Leishmania* antigen; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; ECM, extracellular matrix; FCS, foetal calf serum; HPRT, hypoxanthine guanine phosphoribosyl transferase; IFN, interferon; IL, interleukin; kDa, kilodaltons; mRNA, messenger RNA; NNN, Novy, McNeal and Nicolle; PCR, polymerase chain reaction; pi, post-infection; pg, picogram; RT, reverse transcription; RNA, ribonucleic acid; SDS–PAGE, sodium dodecyl sulphate—polyacrylamide gel electrophoresis; SI, stimulation index; Th, T helper cell; TGF, transforming growth factor; WS, without stimulation; ZVL, zoonotic visceral leishmaniasis

Keywords: *Leishmania infantum* released proteins; Murine model; Cellular immune response; Real-time semi-quantitative PCR; Cytokine mRNA expression and production; IL-4; IL-12

1. Introduction

Zoonotic visceral leishmaniasis, caused by *Leishmania infantum*, is a veterinary and public health problem in the Mediterranean Basin, South and Central America, and in the Middle East. Canids are the host reservoir for ZVL.

Leishmania have a digenetic life-cycle and are transmitted by the infected sand fly vector to the mammalian host. As the vector ingests a blood meal from the mammalian host, the flagellate promastigotes are deposited in the skin where they interact with a variety of membranes and proteins and finally transform into amastigotes inside the phagolysosomes of the macrophage.

The outcome of leishmanial infections depends primarily on host cell-mediated immune mechanisms and on the parasite virulence. Control of infection has been associated with production of IFN-γ by the host (Carvalho and Badaró, 1985; Pinelli et al., 1994; Sacks et al., 1987) whereas disease progression has been

* Corresponding author. Fax: +351 21 3622458.
E-mail address: santosgomes@ihmt.unl.pt (G.M. Santos-Gomes).

0014-4894/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2004.11.008

correlated with impaired Th1 response (Ghalib et al., 1993; Santos-Gomes et al., 2002). Additionally, other cytokines, such has TGF-β seem to regulate parasite growth, contributing to its multiplication or favouring its reduction, preventing severe injuries in the host organs (Gomes-Pereira et al., 2004; Wilson et al., 1996), and enhancing both parasite and host survival rates.

Antigenic macromolecules secreted by microorganisms are important in the establishment of immune and physiologic interactions with the host. *Leishmania* sp. released products have been implicated in different parasite life-cycle stages, either in the vector (Blum and Opperdoes, 1994; Schlein et al., 1991) or in the vertebrate host (El-On et al., 1980; Londner et al., 1983; Mukerji et al., 1986). McGwire et al. (2002, 2003) reported that active metalloprotease gp63, released from a variety of *Leishmania* species, enhanced the parasite's ability in migrating through the organ's ECM. Thus, migration through the ECM and the basement membrane favours parasite dissemination to different organs and tissues (Bandyopadhyay et al., 2002; Ghosh et al., 1999). On the other hand, most of the released proteins are recognised by antibodies of the host (Cibrelus et al., 1999), and the use of *L. infantum* secreted/excreted antigens to treat dogs with leishmaniasis has resulted in a long-lasting clinical improvement (Bourdoiseau et al., 2004). However, the role of *Leishmania* released proteins on the host immune response still needs further clarification.

In this study, isolated spleen leukocytes from two mice strains with different degrees of resistance to *Leishmania* infection were used to assess, in vitro, the influence of *L. infantum* released proteins on the cellular immune responses, by comparing lymphocyte proliferation, and the expression and production of Th1 (IFN-γ), Th2 (IL-4), anti-inflammatory (IL-10), and pro-inflammatory (IL-12) cytokines.

2. Materials and methods

2.1. Parasite, mice, and infection protocol

C57BL/6 and BALB/c female mice, aged 6–8 week, were purchased from Harlan Interfauna Ibérica (Spain) and housed at the IHMT animal facilities, fulfilling the European Union requirements (86/609/CEE), recognised by Portuguese law (DR DL129/92 and Portaria 1005/92).

Leishmania infantum MON-1 (MCAN/PT/95/IMT 205) was maintained by passage in Syrian golden hamsters and amastigotes were isolated from infected spleens. After in vitro transformation, virulent promastigotes collected from the stationary phase of a subculture with less than five passages (Santos-Gomes and Abranches, 1996) was used for mice inoculation, to obtain released proteins from culture supernatants and to prepare crude *Leishmania* antigen.

Infection was performed by intraperitoneal inoculation with 5×10^6 promastigotes per mouse. Two groups of non-infected healthy mice, one of each strain, were maintained as controls. At days 30 and 45 pi, five infected, and three healthy animals of each mouse strain were sacrificed by cervical dislocation and their spleens processed for determination of parasite burden, and isolation of leukocytes. The experiments were repeated three times.

2.2. Isolation of released L. infantum proteins

To isolate *Leishmania* released proteins, supernatants from virulent cultures were collected, after 24 h in a protein-free medium. These supernatants were concentrated by centrifuging using Centricon tubes (Amicom, Millipore, USA). Released proteins were separated according to their molecular weight by SDS–PAGE (Santos-Gomes et al., 2000). Protein bands, localised in three main regions of the gel of different molecular weight, were electroeluted according to their position into three groups: <37 kDa, Low group; 37–75 kDa, Inter group; and >75 kDa, High group. Protein concentration was determined by the Lowry method.

2.3. Estimation of viable parasites

Spleen parasite burden was calculated by limiting dilution assay, as described by Buffet et al. (1995). Briefly, 0.01 g of spleen from each mouse was removed and homogenised individually in Schneider's medium (Gibco-BRL, UK) supplemented with 20% heat-inactivated foetal calf serum (Biochrom, Germany). Fourfold serial dilutions of the infected tissues were distributed in 96-multi well plates (Nunc, Denmark) in which the solid phase of NNN medium plus Schneider's medium 20% FCS were already set. After 15 days at 24 °C, a sample of each well was examined and defined as positive or negative depending on the presence or absence of promastigotes in the well. The number of parasites per gram of tissue was calculated as follows: (reciprocal titre of the highest dilution which contained at least one parasite/weight of homogenised tissue) ×400.

2.4. Lymphocyte proliferation assays

Splenic leukocytes were isolated from spleen homogenates by Ficoll (Gibco-BRL) density sedimentation, washed with Hanks' balanced salt solution (Sigma, UK) and cultured in RPMI 1640 medium supplemented with 10% FCS and 100 U/ml penicillin, 100 mg/ml streptomycin, in U-shaped 96-well tissue culture plates (Nunc). Assays were performed using 2×10^5 cells/well. Cells were stimulated with 40 μg/ml of Ag and 5 μg/ml of High, Inter and Low protein groups. After 72 h of culture in 5% CO_2 humidified atmosphere at 37 °C, the cells