Molecular & Biochemical Parasitology 159 (2008) 121–129

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Immuno stimulating glycophosphosphingolipid antigen from *Leishmania donovani* is recognized by visceral leishmaniasis patient sera

Siddhartha Kumar Bhaumik, Manoj Kumar Singh, Subir Karmakar, Tripti De*

Division of Infectious Disease and Immunology, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata 700032, India

ARTICLE INFO

Article history:
Received 31 August 2007
Received in revised form 28 February 2008
Accepted 1 March 2008
Available online 18 March 2008

Keywords:
Leishmania donovani
Glycophosphoshingo lipid antigen
Immune modulation
Infected macrophage
Patient sera

ABSTRACT

Surface antigens on *Leishmania promastigotes* and infected macrophages are obvious targets in immunoprophylaxis for leishmanial infection. The glycophosphosphingolipid (GSPL) antigen isolated from *Leishmania donovani* surface membrane was recognized by sera from patients with visceral leishmaniasis. GSPL was also expressed on the membrane of parasite-infected macrophages. The effect of GSPL on the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) was studied using the macrophage cell line J774.1. In addition, induction of IFNγ, IL4, IL10, IL12 secretion in presence of GSPL was investigated in PBMC from normal individuals. ROS and RNI in addition to IFNγ and IL12 were induced by GSPL. Though there was a moderate induction of IL10, there was very little induction of the Th2 cytokine IL4. GSPL also induced blood cells to proliferate. The data suggests that this functionally important antigen of *L. donovani* may be used as a candidate vaccine.

1. Introduction

Leishmania are obligate intercellular parasites of the macrophages. They cause a number of important human disease ranging from self-healing cutaneous lesions to diffuse cutaneous and mucosal manifestations or disseminated and often fatal visceral leishmaniasis. Some 350 million people live in endemic areas and are at risk of developing the clinical disease [1]. Infection is initiated by the transfer of insect vector-borne promastigotes. These are taken up by phagocytic cells and transform into the obligate intracellular amastigotes. Uncontrolled replication of the amastigotes cause disease. To survive within the hostile environment of the macrophages, these parasites have developed strategies to prevent activation of an effective immune response. In order to do this, leishmania have evolved a number of sophisticated mechanisms to subvert the normal macrophage functions like antigen presentation [2,3], production of microbial agents like reactive nitrogen [4–6] and oxygen intermediates [5–9] and cytokine production [10,11].

It is generally accepted that the nature of the T cell response is one of the crucial factors controlling experimental and human leishmaniasis [12,13]. Hence, identification of parasite antigens that elicitate a protective immune response is crucial for the development of an immunization protocol. We had earlier purified a glycophosphosphingolipid (GSPL) antigen from *Leishmania donovani* surface membrane [14]. Here, we tried to investigate the biological role of GSPL. Our findings indicated that GSPL was expressed on the surface of the infected macrophage, was recognized by patient sera and could elicitate a protective immune response.

2. Materials and method

2.1. Parasites

L. donovani strain AG83 (MHOM/IN/83/AG83) was maintained in Medium 199 containing 10% heat inactivated fetal calf serum (FCS) as described before [15].

2.2. Purification of glycophosphosphingolipid antigen

GSPL was purified from AG 83 promastigote membrane as described earlier [14]. In short, late log phase promastigote membrane (1 g wet weight) was extracted with 19 vol of chloroform:methanol:ethyl acetate:pyridine:4.5N ammonia:water

Abbreviations: LD, *Leishmania donovani*; GSPL, glycophosphosphingolipid; Macrophage, Mϕ; p.i., post infection; RCA-1, *Ricinus communis* agglutinin-1; Solvent A, chloroform:methanol:ethyl acetate:pyridine:4.5N ammonia:water (15:15:5:0.5:0.5:0.5, v/v); Solvent B, 0.01 M phosphate buffer, pH 6.4, containing 0.05N ammonium hydroxide and 0.1% sodium salt of taurodeoxycholic acid; Solvent C, chloroform:methanol:0.25N ammonia in 0.25% KCl (65:45:9); Solvent D, pyridine:ethyl acetate:acetic acid:0.25% KCl (36:36:7:21, v/v); Solvent E, 1-butanol:pyridine:0.25% KCl (3:2:1, v/v); L-NMMA, N^G-monomethyl-L-arginine; PB, polymixin B; complete DMEM medium, DMEM medium with 2 mM L-glutamine and $NaHCO_3$, supplemented with 10% heat inactivated fetal calf serum, 50 nm 2-β-mercaptoethanol, 25 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin.

* Corresponding author. Tel.: +91 33 2473 3491/0492; fax: +91 33 24730284.
E-mail address: triptide@iicb.res.in (T. De).

0166-6851/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2008.03.001

(15:15:5:0.5:0.5:0.5, v/v; Solvent A). Anionic glycolipids were eluted from a DEAE-Sephadex A-25 column with a gradient of KCl in 0.01 M phosphate buffer, pH 6.4, containing 0.05N ammonium hydroxide and 0.1% sodium salt of taurodeoxycholic acid (Solvent B). The anionic glycolipids were loaded onto a silicic acid column and the glycophosphosphingolipid (GSPL) was eluted with C:M (4:6, v/v) and further purified on a RCA-1-Sepharose 4B affinity column. GSPL was eluted with 0.1 M galactose in Solvent B. Purity of GSPL was checked by HP-TLC developed in three different solvent systems, chloroform:methanol:0.25N ammonia in 0.25% KCl (65:45:9, v/v; Solvent C), pyridine:ethyl acetate:acetic acid:0.25% KCl (36:36:7:21, v/v; Solvent D,), and 1-butanol:pyridine:0.25% KCl (3:2:1, v/v; Solvent E). Plates were sprayed with either the diphenyl amine reagent for glycolipids [16] or Dittmer and Lester reagent for phospholipids [17].

2.3. Preparation of antisera

Antibodies against GSPL was raised in rabbits by four subcutaneous injections of GSPL (100 μg) micelles given at 15 days interval. Micelles were prepared by ultrasonication of 100 μg GSPL in 500 μl PBS for 45 min [18]. The antisera was collected, and heat inactivated at 56 °C for 30 min. Pre-immune sera served as controls.

2.4. In vitro infection of macrophages and confocal microscopy

The macrophage cell line J774.1 was suspended in DMEM medium with 2 mM L-glutamine and $NaHCO_3$, supplemented with 10% heat inactivated fetal calf serum, 50 nm 2-β-mercaptoethanol, 25 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin (complete DMEM medium). Cells at a concentration of 5×10^5 per ml were allowed to adhere on glass cover slips in 24-well tissue culture trays (Falcon, Becton Dickenson, USA), for 2 h at 37 °C and were infected with promastigotes at a ratio of 1:5. Parasites were allowed to infect for 6 h at 37 °C in 5% CO_2, free organisms were removed by vigorous washing, and the cells were incubated at 37 °C in 5% CO_2, overnight. Cells were washed in serum-free medium supplemented with 1.8% de-fatted BSA, and fixed by the addition of 2% paraformaldehyde (8 min on ice). Cells were incubated with normal rabbit immunglobulins (10 μg/10^6 cells) for 1 h to block the Fc receptors, followed by incubation with GSPL antibody for 2 h and then with fluorescein isothyocyanate (FITC) conjugated goat anti rabbit IgG. Cells were analyzed by confocal laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

2.5. Macrophage membrane preparation, TLC and immunostaining

To obtain the infected macrophage membrane, J774.1 cells were infected as mentioned in Section 2.4. Twelve hours p.i., leishmania infected macrophages were washed extensively with PBS, and suspended in Tris–HCl pH 8.0/0.25 M sucrose and 1 mM PMSF, homogenized, and centrifuged in isotonic sucrose gradient. The plasma membrane fraction was prepared according to Aronson and Touster [19,20]. Total lipid was extracted from the membrane with Solvent A and 20 μg of the extracted lipid was subjected to HP-TLC in Solvent C. Plates were dried and soaked in 0.02% polyisobutyl methacrylate for 1 min and air dried. Plates were then incubated in the blocking buffer (1% BSA/1% polyvinyl pyrolidine/0.02% sodium azide) at 37 °C for 30 min. Plates were rinsed thoroughly with the washing buffer (PBS/1% Tween 20) and incubated with GSPL antiboby (1:400) at 4 °C overnight. After washing with the washing buffer, plates were incubated with HRP-conjugated secondary antibody at 37 °C for 1.5 h. Plates were developed with the peroxidase substrate of 2,2′-azino-bis (3-ethy l benzthiazoline-6-sulfonic acid). With the onset of color development, the reaction was stopped by rinsing with double-distilled water several times.

2.6. Blood sample collection and in vitro culture

Peripheral blood (PB) was obtained by sterile venipuncture, and three different samples were separated. (A) 5 ml of PB was collected without heparin and the serum was separated by centrifugation for use in immunoblots; (B) 10 ml of PB was collected in tubes with heparin and centrifuged at 1600 × *g* for 10 min, and the *buffy coat* (BC) was isolated and seeded directly in culture medium; and (C) 5 ml of PB was collected in EDTA and the mononuclear cells (PBMC) were isolated by density centrifugation through Ficoll (Pharmacia Biotech Inc., Piscataway, NJ).

Five hundred μL of BC and 250 μl of PBMC were seeded in 10 and 5 ml, respectively, of Schneider's Insect Culture Medium (Sigma, St. Louis, MO) supplemented with 20% heat-inactivated foetal calf serum and gentamicin solution, 25 μg/ml (Sigma, St. Louis, MO). Cultures were maintained at 22 °C, examined by inverted microscopy twice a week, and subcultured every 2 weeks for 6 months before being considered negative.

2.7. TLC and immunoblotting with patient sera

Purified GSPL (20 μg) was subjected to HP-TLC in Solvent C. Plates were dried and were subjected to immunoblotting as described in Section 2.5 using diluted patient serum samples (1:100).

2.8. Measurement of respiratory burst activity

Respiratory burst activity of GSPL stimulated macrophages were measured essentially as described previously [15]. In short, the macrophage cell line J774.1 was cultured in 24-well plates (10^5 cells/ml, 1 ml/well) and incubated at 37 °C in 5% CO_2 for 12 h to adhere and non-adherent cells were removed. Cells were incubated in the presence or absence of GSPL at the indicated concentrations, for 12 h at 37 °C in 5% CO_2. Cells were washed and incubated in presence or absence of 10 ng of LPS for 12 h. Oxygen consumption by the stimulated macrophages was measured by using a oxygraph equipped with a Clark-type electrode according to the method of Weening et al. [21]. Respiratory burst activity of the macrophages was expressed as nmol of O_2 consumed per 10^5 cells.

2.9. Measurement of NO

The macrophage cell line J774.1 was cultured in 24-well plates as described above. GSPL at different concentrations in presence or absence of 0.2 mM L-NMMA (iNOS inhibitor) were added to the wells. The supernatants were collected after 18 h and analyzed for the presence of nitrite (NO_2^-) by Griess Reagent as described before [22]. Measurement of nitrite was an indication of NO produced by these cells.

2.10. PBMC culture and cytokine ELISA

Peripheral blood from 9 normal healthy individuals was collected and PBMC isolated from whole blood by density centrifugation through Ficoll (Pharmacia Biotech Inc., Piscataway, NJ). For determination of cytokine production, PBMC were cultured in triplicate wells at 1–2 × 10^6 cells/ml in medium containing 10% FCS with or without GSPL (at the indicated concentrations). Supernatants were analyzed for secreted cytokine by standard ELISA after 24 (for IL12) or 72 (for IL4, IL10, IFNγ) h of culture according to the manufacturer's instructions (BD-Biosciences). Cytokine produced