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Apollina Goel · Harpreet Vohra · Grish C. Varshney

Strain-specific recognition of live *Leishmania donovani* promastigotes by homologous antiserum raised against a crude membrane fraction of infected macrophages

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Abstract Surface antigens on *Leishmania* promastigotes and infected macrophages are obvious targets in immunoprophylaxis for leishmanial infection. We have recently demonstrated that the polyclonal antiserum and monoclonal antibodies generated by homologous immunizations with the crude membranes of parasite-infected cells react effectively with the 'neo-antigenic' determinants on the infected cell surface. In the present study, we investigated the utility of such polyclonal antisera for identifying 'minor' surface components of promastigotes. The reactivity of anti-*Leishmania donovani*-(strain RMRI68) infected macrophage membrane (anti-IMm) antiserum was compared with that of anti-promastigote (anti-Pr) antiserum towards the infected macrophage surface and promastigotes of three Indian strains of *L. donovani*, RMRI68, AG83 and DD8. While anti-Pr antiserum showed no reactivity with the infected macrophage surface but reacted strongly with air dried and live promastigotes of all three strains, anti-IMm antiserum reacted with the infected cell surface and, interestingly, specifically recognized live promastigotes of the strain used for infection, i.e., strain RMRI68. The reactivity patterns of the two antisera with the immunodominant components of the *L. donovani* promastigote surface, i.e., purified LPG-KMP11 complex and gp63 molecules, indicated that unlike anti-Pr antiserum, the specificities in anti-IMm antiserum were mainly directed towards molecules other than the LPG-KMP11 complex and gp63. Antiserum generated in a similar fashion against the macrophage membrane of cells infected in vitro with strain AG83 also contained antibodies specific to strain AG83 promastigotes. The present approach may therefore greatly help in identifying specific antigen(s) important in clinical and epidemiological control of leishmaniasis.

A. Goel · G.C. Varshney (✉)
Division of Cell Biology and Immunology,
Institute of Microbial Technology, Sector 39-A,
Chandigarh-160 036, India
e-mail: grish@koel.imtech.ernet.in,
Fax: +91-172-690632/690585

H. Vohra
Department of Experimental Medicine,
Post Graduate Institute of
Medical Education and Research,
Chandigarh-160 012, India

Introduction

Visceral leishmaniasis or kala azar is the most severe form of the major tropical disease leishmaniasis. About 90% of the visceral cases occur only in Bangladesh, India, Nepal, and Sudan and the species mainly responsible for these infections is *Leishmania donovani* (Ashford et al. 1992).

Surface molecules of *Leishmania* promastigotes have been the focus of attention for taxonomic classification, vaccine development, and elucidation of the mechanisms involved during invasion and intracellular survival (Chang and Chaudhuri 1990; Alexander and Russell 1992). The presence of *Leishmania*-specific antigens on the infected macrophage surface has been demonstrated in several studies (Berman and Dwyer 1981; Handman 1990; Tolson et al. 1990; Stierhof et al. 1991). Among these, LPG epitopes have been well studied (Handman 1990; Stierhof et al. 1991).

We recently demonstrated that the polyclonal antiserum or monoclonal antibodies (MAbs) generated by homologous immunization with parasite-infected cell membranes effectively react with neo-antigenic determinants on the infected cell surface (Owais et al. 1995; Choudhury et al. 1997). Based on this, in this paper we have checked if such an immunization strategy is useful in diverting the immune response towards 'minor' antigenic determinant(s) in the absence of major parasite immunogenic components. For this, the reactivity of an antiserum, raised against a crude membrane preparation of in vitro *L. donovani*-infected macrophage membrane (anti-IMm antiserum) was compared with that of anti-promastigote antiserum (anti-Pr antiserum) towards the

infected cell surface as well as promastigote of different strains. Anti-IMm antiserum, unlike anti-Pr antiserum, exhibited specific recognition of live promastigotes of the strain used for infection.

Materials and methods

Parasites and animals

L. donovani strains RMRI68 (Sodhi et al. 1992; Choudhury et al. 1997), AG83 (MHOM/IN/83/AG83), and DD8 (MHOM/IN/80/DD8) were isolates from Indian patients with kala azar. Parasites were maintained in vitro in NNN medium with a few mass cultivations in RPMI 1640 (Gibco, Grand Island, N.Y.) supplemented with 10% (v/v) fetal calf serum (FCS, Sera Lab) (RPMI complete medium). Promastigotes were grown at 26 °C and the actively motile forms, in the stationary phase of growth, were used for the experiments.

Inbred BALB/c mice (6–10 weeks old) were used in the study. These were procured from Jackson Laboratory, Bar Harbor, USA and were reared in the Central Animal Facility of the Institute.

Monoclonal antibodies, glycoprotein 63 and lipophosphoglycan

CA7AE and L98 MAbs, which recognize the repeating disaccharide portions of leishmanial LPG and KMP11, respectively (Tolson et al. 1989; Jardim et al. 1991), were kindly provided by Dr. T.W. Pearson (University of Victoria, British Columbia, Canada). *L. donovani* LPG-KMP11 complex was kindly given by Dr. S.J. Turco (University of Kentucky, Lexington, Ky.) and gp63 was a gift from Dr. K.P. Chang (UHS Chicago Medical School, North Chicago, Ill.).

Preparation of crude membrane fraction of peritoneal macrophages in vitro infected with *L. donovani*

The method followed was essentially that described in our earlier paper (Choudhury et al. 1997). The infection load obtained, using 2 h infection followed by a 4-h post-infection period, with strain RMRI68 and strain AG83, was 3–4 parasites/cell with a 60% level of infection and 1–2 parasites/cell with a 90% level of infection, respectively.

Generation of polyclonal antisera

Polyclonal antisera were generated in inbred BALB/c mice by immunizing each mouse intraperitoneally with either a crude membrane preparation from $1–2 \times 10^7$ macrophages (homologous) infected with promastigotes (Choudhury et al. 1997) or $1–2 \times 10^7$ heat-killed (56 °C, 30 min) promastigotes without adjuvant (Tolson et al. 1989). Boosters were given every 3rd week. After five to six boosters, the antisera were collected, pooled and heat inactivated at 56 °C for 30 min. Pre-immune sera and/or normal macrophage (cultured in vitro for 6 h) membrane antisera served as controls.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed with promastigotes, LPG-KMP11 complex, and gp63. For promastigotes, intact cells (2×10^5 cells/50 µl per well) were coupled to poly-L-lysine-coated flat-bottomed polyvinylchloride microplates followed by glutaraldehyde fixation (Tolson et al. 1989). The purified gp63 (1 µg/well) or LPG-KMP11 complex (10 ng/well) were coated by drying in a 96-well plate. The assay was performed taking appropriate dilutions of polyclonal antisera or ascites fluid of MAbs, followed by incubations with horseradish-peroxidase-conjugated goat anti-mouse IgG (1:2000 dilution, Bio-Rad) as the secondary antibody and 2,2′-azino-bis(3-ethylbenz-thiazoline sulfonic acid) as a substrate.

Direct agglutination test

This was performed with stained *L. donovani* promastigotes as described earlier (Harith et al. 1988).

Immunofluorescence assay (IFA)

A previously published protocol (Williams et al. 1986) was followed. Cells were incubated at 4 °C (live) or at 37 °C (air dried) for 1 h at different dilutions of test or control sera followed by incubation with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (1:300 dilution, Dakopatts, Denmark). For macrophages, an additional step of blocking the Fc receptors with normal rabbit immunoglobulins (100 µg/ml) for 1 h was included prior to test antibody incubations (Choudhury et al. 1997). Samples were analyzed for FITC fluorescence with a fluorescence microscope (Leitz, Germany).

Flow cytometry

Live promastigotes after primary and secondary antibody incubations were subjected to flow cytometric analysis. Samples were fixed with 0.5% paraformaldehyde (Polyscience Inc., USA) and analyzed for FITC fluorescence using log amplifiers on LYSYS II software of FACScan (Becton Dickinson, Mountain View, Calif.). Ten thousand cells (events) were acquired after 'live' gating on the log FSC/SSC parameter and the fluorescence was measured on gated cells only. The analysis of mean fluorescence intensity (MFI) was done on histograms where abscissa and ordinate denote log FITC fluorescence and relative cell counts, respectively.

Results

In our earlier studies we reported that the anti-IMm antiserum reacted with the infected cell surface (Choudhury et al. 1997). In this study, IFA revealed that this antiserum also recognized the intracellular parasites. Anti-Pr antiserum was reactive with the intracellular parasites but not with infected cell membrane (Fig. 1). Further, both antisera reacted well with the air-dried promastigotes of *L. donovani* strains RMRI68, AG83, and DD8 (Fig. 2). Live promastigotes, however, exhibited different patterns with the two antisera (1:20 dilution). Flow cytometry showed that the anti-Pr antiserum recognized the live promastigotes of all the three strains. The anti-IMm antiserum specifically recognized live promastigotes of strain RMRI68 (used for infection) without any detectable fluorescence with strains AG83 and DD8 (Fig. 3). IFA confirmed these results (Fig. 4) This strain-specific reactivity pattern was not due to some unique characteristics of strain RMRI68, as antisera generated against AG83-promastigote-infected macrophage membranes reacted specifically, at 1:50 dilution, with live promastigotes of strain AG83 (Fig. 5). However, at a 1:20 dilution, the antiserum exhibited a slight cross-reactivity with live promastigotes of strain DD8 (data not shown).

Promastigotes of all three strains reacted well with CA7AE (anti-LPG) as revealed by the direct agglutina-

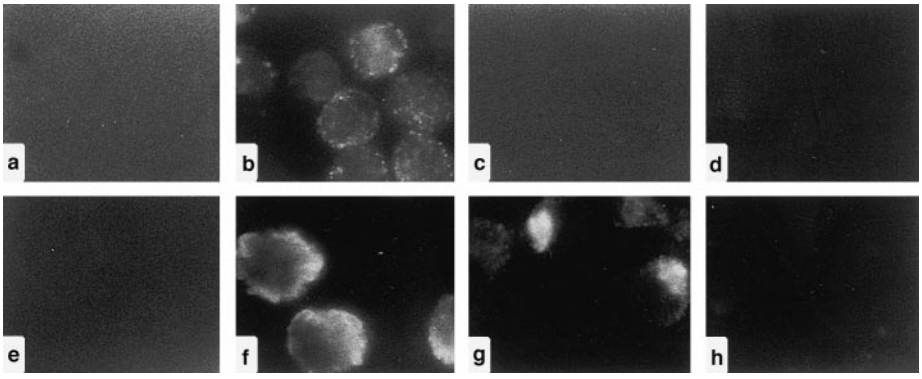

Fig. 1 Fluorescence micrographs of *Leishmania donovani*-(strain RMRI68) infected macrophages. Live (**a–d**) and air-dried (**e–h**) infected macrophages were treated with a 1:20 dilution of pre-immune serum (**a, e**), anti-infected macrophage membrane (anti-IMm) antiserum (**b, f**), anti-promastigote (anti-Pr) antiserum (**c, g**) and anti-NMm (normal macrophage membrane) antiserum (**d, h**), followed by incubation with FITC-conjugated secondary antibody

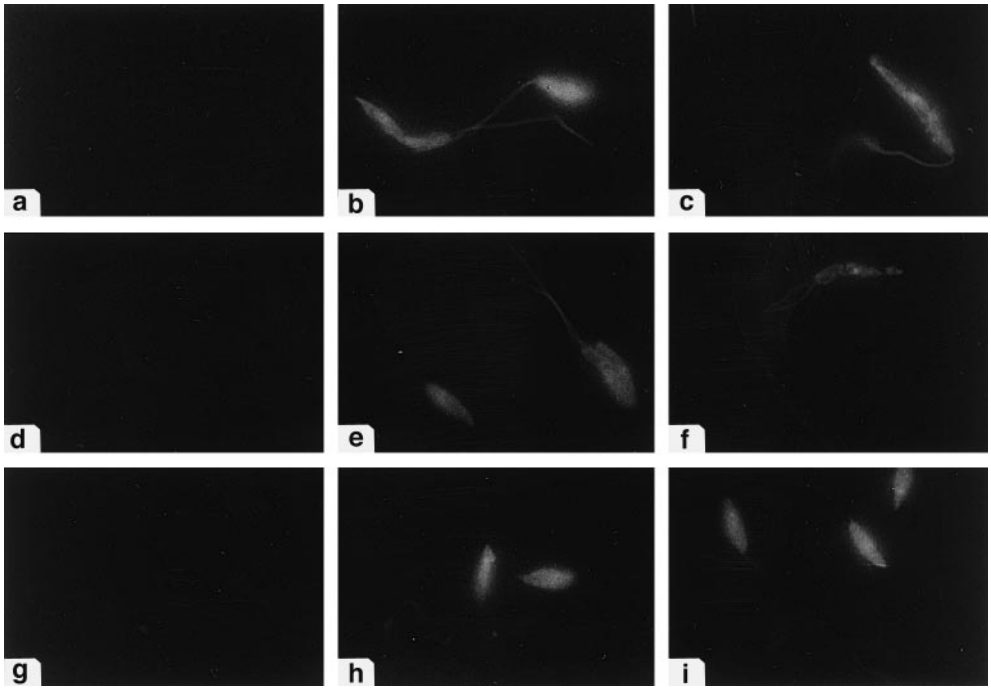

Fig. 2 Fluorescence micrographs of air-dried promastigotes treated with test antisera raised using strain RMRI68. Promastigotes of strains RMRI68 (**a–c**), AG83 (**d–f**), and DD8 (**g–i**) were treated with a 1:20 dilution of pre-immune serum (**a, d, g**), anti-Pr antiserum (**b, e, h**), and anti-IMm antiserum (**c, f, i**), followed by incubation with FITC-conjugated secondary antibody

tion test IFA and ELISA. L98 (anti-KMP11) did not agglutinate promastigotes of any strain but recognized the parasite of all three strains in ELISA and IFA. Macrophages infected with the three strains were recognized, at a low level, by CA7AE as checked by flow cytometry (results not shown). Anti-Pr antiserum also reacted very well with the LPG-KMP11 complex and with the gp63 molecule, whereas the anti-IMm antiserum exhibited either low reactivity with LPG-KMP11 complex or negligible reactivity with the gp63 molecule (Fig. 6). This indicated that the specificities in anti-IMm antiserum, unlike those of anti-Pr antiserum, were mainly directed towards molecule(s) other than the LPG-KMP11 complex and gp63.

Discussion

It is well known that polyclonal antisera and MAbs raised against promastigotes of one *Leishmania* species often cross-react with similar surface determinants of other species (Ramasamy et al. 1983; Gardiner et al. 1984). In our recent studies, we have demonstrated that the polyclonal antiserum and monoclonal antibodies, generated by homologous immunizations with the crude membrane preparation of parasite-infected cells, react effectively with the parasite-derived antigens on the infected cell surface (Owais et al. 1995; Choudhury et al. 1997). The aim of the present study was to check if such polyclonal antiserum, generated using in vitro *L. donovani*-infected macrophage membranes, reacts with the 'minor' component(s) on the promastigote surface. An early period of the in vitro infection (2 h followed by 4 h) of macrophages was chosen since it is more appropriate for the expression of promastigote-like antigen(s) on the infected cell surface (Tolson et al. 1990; Stierof et al. 1991).

The non-reactivity of the anti-Pr (strain RMRI68) antiserum with the infected macrophage surface was in accordance with an earlier report (Berman and Dwyer

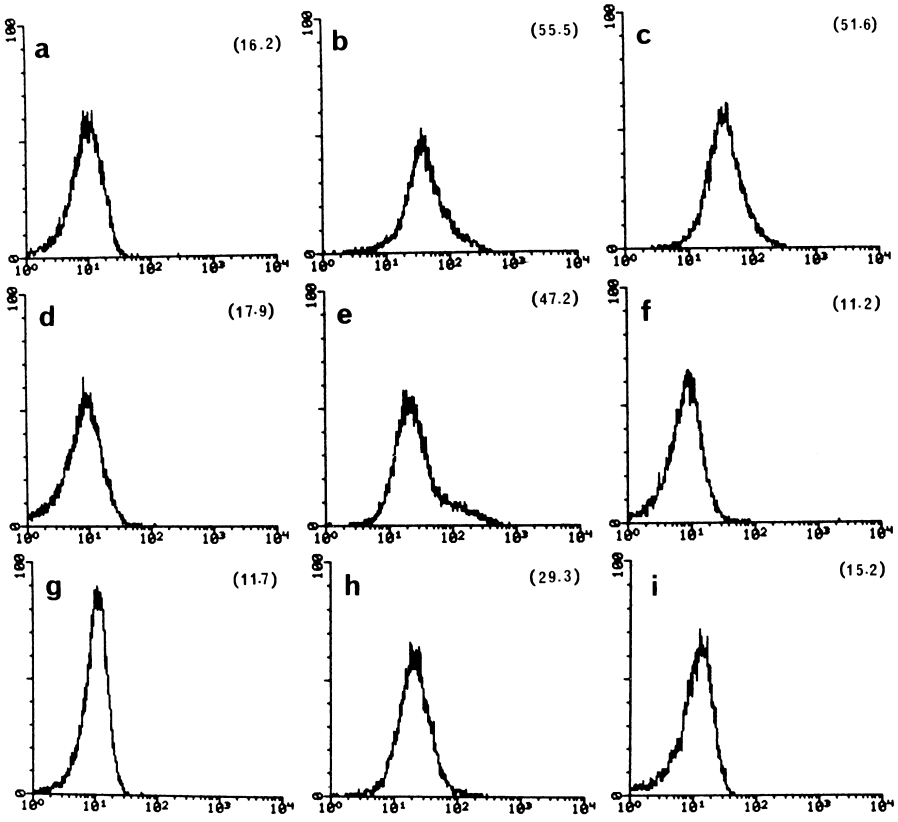

Fig. 3 Flow cytometric analysis of live promastigotes treated with test antisera raised using strain RMRI68. Promastigotes of strains RMRI68 (**a–c**), AG83 (**d–f**), and DD8 (**g–i**) were treated with a 1:20 dilution of pre-immune serum (**a, d, g**), anti-Pr antiserum (**b, e, h**), and anti-IMm antiserum (**c, f, i**), followed by binding of FITC-conjugated secondary antibody. *Values shown in parentheses* are the mean fluorescence intensity (MFI) scores

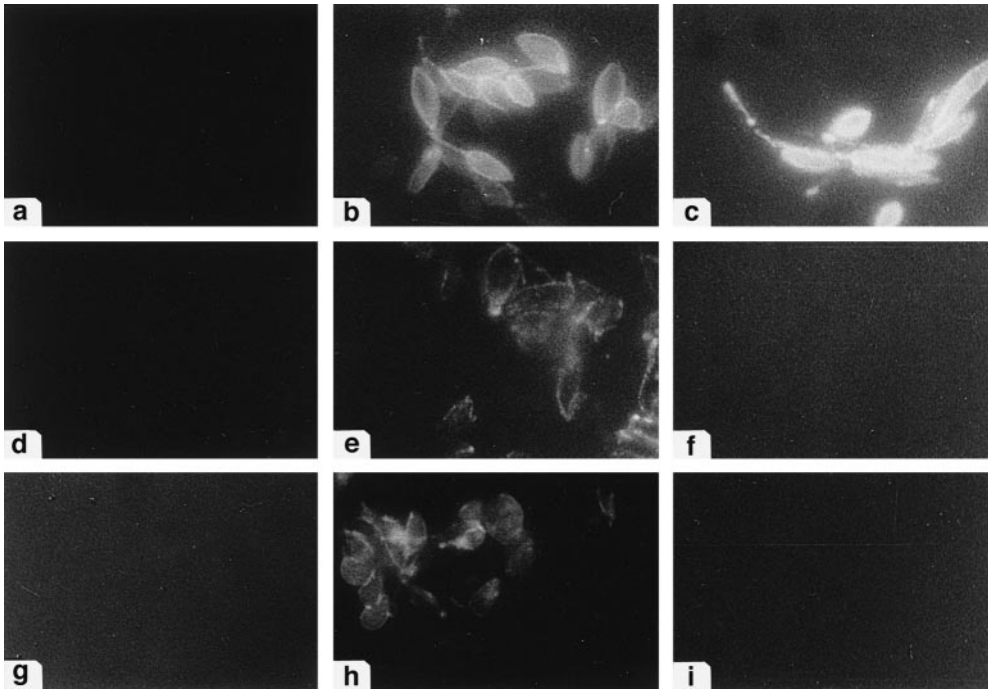

Fig. 4 Fluorescence micrographs of live promastigotes treated with test antisera raised using strain RMRI68. Promastigotes of strain RMRI68 (**a–c**), AG83 (**d–f**), and DD8 (**g–i**) were treated with a 1:20 dilution of pre-immune serum (**a, d, g**), anti-Pr antiserum (**b, e, h**), and anti-IMm antiserum (**c, f, i**), followed by incubation with FITC-conjugated secondary antibody

1981). The observation that the anti-Pr antiserum reacted with the promastigotes (air dried or live) of all three strains, RMRI68, AG83, and DD8, implied that the immune response remained mainly directed towards the cross-reactive immunodominant determinant(s) (Gardiner et al. 1984; Colomer-Gould et al. 1985). This was further corroborated by an earlier report, where anti-Pr antiserum failed to detect 'minor' stage-specific determinant(s) of promastigotes (Kweider et al. 1987). On the other hand, anti-IMm antisera included reac-

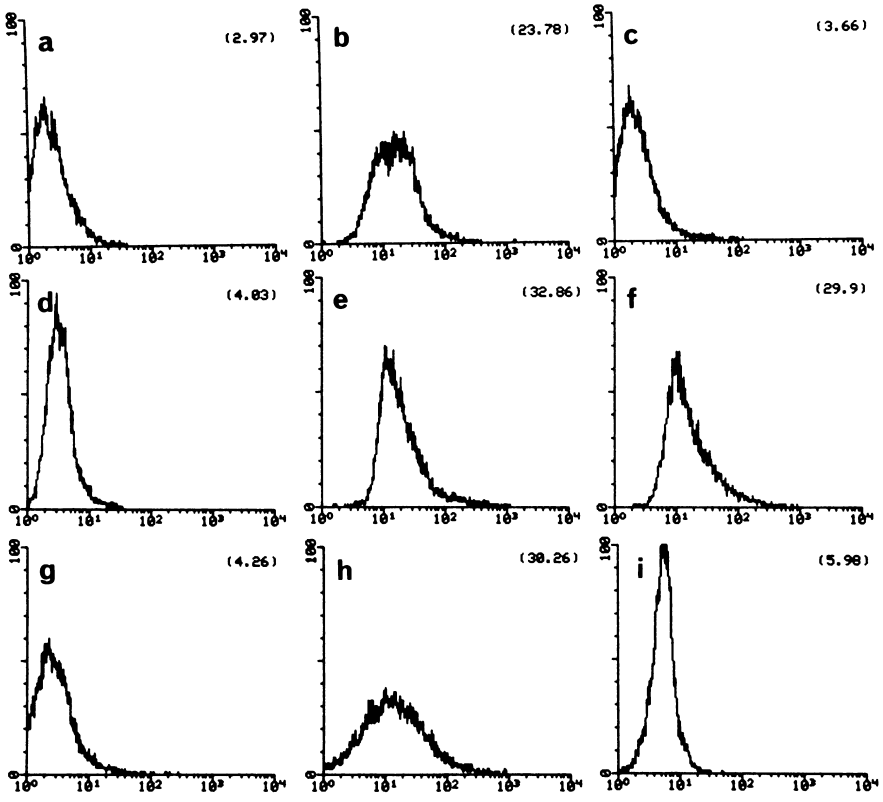

Fig. 5 Flow cytometric analysis of live promastigotes treated with test antisera raised using strain AG83. Promastigotes of strains RMRI68 (**a–c**), AG83 (**d–f**), and DD8 (**g–i**) were treated with a 1:50 dilution of pre-immune serum (**a, d, g**), anti-Pr antiserum (**b, e, h**), and anti-IMm antiserum (**c, f, i**), followed by binding of FITC-conjugated secondary antibody. *Values shown in parentheses* are the mean fluorescence intensity (MFI) scores

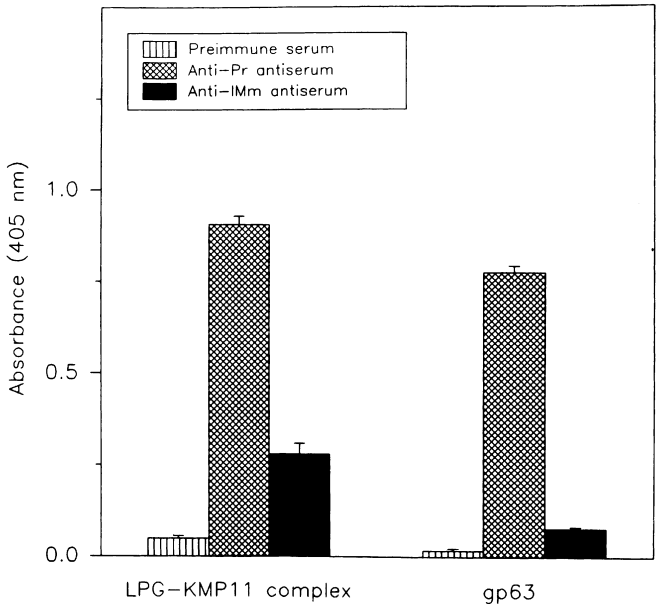

Fig. 6 Reactivity of polyclonal antisera raised using strain RMRI68 with *L. donovani* LPG-KMP11 complex and gp63 in indirect ELISA. Wells were coated with the antigen and the binding of the polyclonal antisera and pre-immune sera (1:50 dilution) was analyzed as described in Materials and methods

tivity specific to the live promastigotes of the strain used for in vitro infection of macrophages.

gp63 and LPG are extensively studied surface molecules of *Leishmania* promastigotes (Chang and Choudhury 1990), LPG has been identified on the *L. donovani*-infected macrophage surface as early as 5–10 min post-infection (Tolson et al. 1990). However, it might not represent the major parasite component on the surface of macrophages infected with *L. donovani* strain RMRI68 at this early time point (6 h) of infection, as shown by the following observations: (1) the non-reactivity of anti-Pr antiserum with the infected macrophage surface, (2) the low reactivity of anti-IMm antiserum with the LPG-KMP11 complex, in contrast to anti-Pr antiserum and (3) the low level binding of CA7AE on the infected macrophage surface. It remains to be seen whether strain-specific antibodies might be due to fine differences in strain LPG structure. However, the similar reactivity patterns of CA7AE and L98 (anti-KMP11) with the three *L. donovani* strains ruled out the participation of the repeating phosphorylated disaccharide unit of LPG and its associated molecule KMP11 in strain-specific recognition of promastigotes. We are in the process of identifying and characterizing the antigenic determinant(s) that are responsible for conferring strain specificity on *L. donovani* promastigotes.

To our knowledge, we have described for the first time a simple approach to generate an immune response against *L. donovani*-promastigote-associated 'minor' antigen(s) responsible for strain specificity. In addition, the approach may provide a simple tool to (1) investigate the possible existence of antigenic diversity among neo-antigenic determinant(s) expressed on the surface of *Leishmania*-infected cells and (2) study minor antigenic determinants relevant for clinical and epidemiological

control of leishmaniasis and other intracellular infections such as tuberculosis.

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