

Western Blot Analysis of Sera from *Leishmania major*-Infected BALB/c and C57BL/6 Mice

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In *Leishmania (L.) major*-infected BALB/c mice Th2-type cells results in disease progression, whereas C57BL/6-infected mice mount a Th1-type response, which leads to control of the infection. Th2 response correlates with IgG1 whereas, Th1 response supports switching to IgG2a subclasses. Since IgG isotype-dominated response depends on different CD4 + T cell subsets, we studied the antigenic profile of *L. major* promastigotes that induce IgG1, IgG2a and IgG2b isotypes in BALB/c and C57BL/6 mice to provide insight into Th1 and Th2 inducing antigens. Humoral immune responses were studied in sera from *L. major*-infected BALB/c and C57BL/6 mice by ELISA and immunoblot analysis. These techniques were used to detect IgG, IgG1, IgG2a, and IgG2b antibodies against crude promastigote antigens. BALB/c mice showed higher IgG, IgG1 and IgG2a antibody levels compared to C57BL/6 mice ($p < 0.01$). The IgG2a/IgG1 ratio was higher in C57BL/6 mice. There was no significant difference in IgG2b level between susceptible (BALB/c) and resistant (C57BL/6) mice. Western blot analysis revealed that 27, 32, 117, 125 and 153 kDa bands reacted only with IgG1 from both mice strains. The 41, 45, 47, 52 and 55 kDa bands reacted only with IgG2a from C57BL/6. A very similar pattern of immunostaining was observed for IgG2a and IgG2b in each mouse strain. The results from both mouse strains indicate that some antigens react exclusively with IgG1, the antibody, which may be involved in generation of a predominant Th2 response. The antigens that reacted strongly with IgG2a in C57BL/6 are potential candidates for eliciting protective Th1 response.

Keywords: *Leishmania major*, Promastigote, Western blot analysis.

INTRODUCTION

Leishmania, a protozoan parasite, is responsible for a variety of diseases known as leishmaniasis ranging from self-healing cutaneous lesions to a fatal visceral disease. The mechanisms by which the parasite induces such a broad range of diseases are mostly unknown and involve both the pathogen and the host factors. Experimentally *Leishmania (L.) major*-infected mice are widely used to study the immune responses elicited against the disease. Infection with *L. major* in resistant C57BL/6 mice is controlled at the site of primary infection and the lesion is finally healed. Susceptible BALB/c mice, on the other hand, fail to control parasite growth and its fatal visceral spread. In these mice models, resistance and susceptibility against *Leishmania* infection are determined by functionally distinct T-

cell subsets. A predominant T helper 1 (Th1) response is identified by interferon-Gamma (IFN γ) and tumor necrosis factor alpha (TNF α) release. This response leads to the activation of infected macrophages to kill parasites and subsequently will control the infection. In contrast, a Th2 response, mediated by interleukin (IL)-4, IL-5, and IL-13, increases susceptibility to infection and permits disease progression [1-3]. In addition, IL-10 is an important mediator shared by multiple T-cell subsets,

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including T cells with regulatory functions (Treg ce, which play a major role in *L. major* reactivation [4, 5]. Furthermore, Th1 and Th2 subsets participate in B cell differentiation and immunoglobulin isotypes switching. In mice, IFN γ , which is the key cytokine of Th1 response, promotes the production of IgG2a, while IL-4, a Th2-related cytokine, induces the production of IgG1 by B cells. It is widely accepted that a high ratio of specific IgG2a/IgG1 is associated with a dominated Th1-type response. Conversely, a comparatively low IgG2a/IgG1 ratio results from preferential activation of a non-protective Th2 response [6-8]. The protein profile of *L. major* promastigote, which induce IgG1 and/or IgG2a production in *Leishmania*-infected susceptible and resistant mice, has not been studied yet.

Several factors influence the development of effector Th1 and Th2 cells. The parasite antigens are among these important factors. Screening of the parasite antigens based on reactivity with infected susceptible or resistant mice sera might be used to identify Th1- or Th2-inducing antigens. In the present study screening for parasite antigens on the basis of reactivity with IgG subclasses (IgG1, IgG2a and IgG2b) from both infected susceptible and resistant mice was used to identify Th1 or Th2-inducing antigens.

MATERIALS AND METHODS

Parasite culture. *L. major* Promastigotes (MH-OM/IR /75/ER) were obtained by cultivation in RPMI medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Sigma-Aldrich, USA), 2 mM L-glutamine (Gibco BRL), 100 U/ml penicillin and 100 μ g/ml streptomycin at 25°C. Parasites in stationary phase were harvested and washed three times by centrifugation at 1100 g in phosphate-buffered saline (PBS, pH 7.2) for 15 min before cell lysis. Promastigote lysates were prepared through repeated freeze-thawing and were stored at -70°C until used.

Mice infection. Female, 6-8 week-old C57BL/6 and BALB/c mice were infected subcutaneously in

the base of tail with 2×10^6 stationary phase promastigotes of *L. major* and their sera were obtained 8 weeks after the infection. Uninfected mice were used as controls. Experimental protocols were approved by the Institutional Research and Ethics Committee.

ELISA for *L. major*-specific total IgG and IgG subclasses. The mice sera were tested for the presence of *Leishmania*-specific IgG, IgG1, IgG2a and IgG2b by indirect ELISA. Briefly, wells of microtiter plates (Greiner, Germany) were coated overnight with 10^6 promastigotes suspended in PBS (pH 7.2) and then were blocked with PBS containing 1% bovine serum albumin (PBS-BSA). Diluted sera in PBS-BSA (1:100) were added to wells, and plates were incubated for 2 h at 37°C. To detect specific total IgG, peroxidase-conjugated goat anti-mouse antibody (Sigma, Germany) was added to each well and the plate was incubated for 1 h at 37°C before the addition of tetramethylbenzidine (TMB) substrate (Sigma, Germany). To detect IgG subclasses, goat anti-mouse IgG1, IgG2a, and IgG2b antibodies (Sigma, USA) were added and the plates were incubated for 1 h at 37°C. Later, peroxidase-conjugated rabbit anti-goat antibody (Sigma, Germany) was added to each well and the plates were incubated for 1 h at 37°C, before addition of TMB. Absorbance was read at 450 nm using an ELISA plate reader (Anthos 2020, Austria). Sera from five mice per group were pooled and samples were run in triplicates.

Western blot analysis of antibody-reactive leishmanial antigens. Discontinuous SDS-PAGE was performed on 4% stacking gels over 10% separating gels. Parasites lysates were boiled at 100°C for 5 min in 6X SDS gel-loading buffer consisting of 375 mM Tris-HCl (pH 6.8), 12% SDS, 60% glycerol, 30% 2-mercaptoethanol, and 0.6% bromophenol blue. Gels were run until the bromophenol tracking blue reached the bottom of the gel. The electrophoretic transfer of antigens from gel to PVDF membrane (BioRad, Hercules, CA, USA) was performed according to the method described by Towbin [9]. After the transfer, the membranes

were stained with Ponceau S and cut into strips. Nonspecific binding sites were blocked by 3% BSA in PBS for 1 h. The strips were subsequently incubated for 2 h with 1:100 diluted murine sera. After three washes, 15 min each, with PBS containing 0.1% Triton X-100 (PBS-T), the strips were incubated for 1 h with goat anti-mouse IgG isotypes (IgG1, IgG2a, IgG2b) (Sigma, Germany). Later peroxidase-conjugated rabbit anti-goat antibody (Sigma, Germany) was added and the strips were developed with diaminobenzidine (DAB) (Sigma, Germany). Following scanning, Biorad Quantity one software (Bio-Rad Laboratories, Hercules, CA) was used to analyze the immunoblot bands. During the analysis, the authenticity of each band was validated by visual inspection and edited where necessary. All experiments were repeated three times.

Statistical analysis. Mann-Whitney U-test was applied to compare the levels of total IgG and each IgG subclass results between the groups. Statistical analyses were considered significant when $P < 0.05$.

RESULTS

Serum IgG and IgG subclasses response.

The presence and levels of anti-*Leishmania* antibodies (IgG, IgG1, IgG2a, IgG2b) in sera were analyzed by ELISA 8 weeks after infection with *L.*

major (Fig. 1). The absorbance values of total IgG, IgG1, IgG2a, and IgG2b in the sera of *Leishmania*-infected mice were significantly greater than those of the control mice ($P < 0.05$). Moreover, BALB/c mice showed higher IgG, IgG1 and IgG2a antibody levels compared to C57BL/6 mice ($P < 0.01$). In BALB/c mice the antibody response was predominantly of IgG1 subclass; while IgG1 and IgG2a were almost equally produced in C57BL/6 mice. The IgG2a/IgG1 ratio was 1.1 and 0.4 in C57BL/6 and BALB/c mice, respectively. There was no significant difference in IgG2b level between susceptible and resistant mice.

Immunoblotting analysis of antibody-reactive *Leishmania* antigens. *L. major* promastigote antigens were immunoblotted with sera from BALB/c and C57BL/6 mice 8 weeks post-infection (Fig. 2). Western blot analysis in BALB/c mice revealed that the same antigens were recognized by IgG and IgG1. However, the antigen binding patterns of IgG1 and IgG2a were different, i.e., more bands were recognized by IgG1 compared to IgG2a and the intensity of bands was also higher for IgG1. The 27, 32, 117, 125, 139, 146, 149, 153, 161, and 167 kDa bands exclusively reacted with IgG1 but not with IgG2a. None of the bands were exclusively recognized by IgG2a.

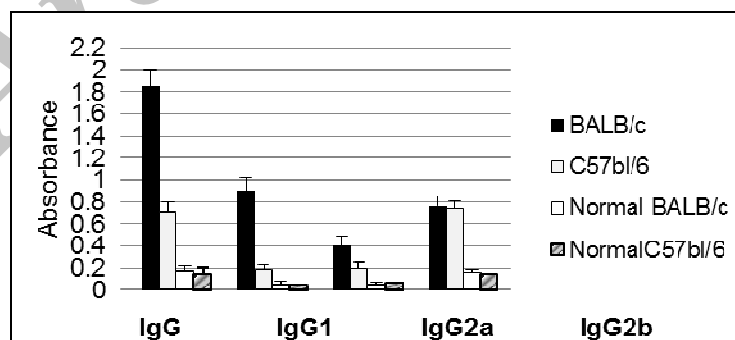


Fig. 1. Absorbance values of IgG, IgG1, IgG2a, and IgG2b from *L. major*-infected and uninfected BALB/c and C57BL/6 mice. Sera from 5 mice per group were pooled. Bars represent the mean+SD of absorbance values of triplicates. The representative data from 3 repeated experiments.

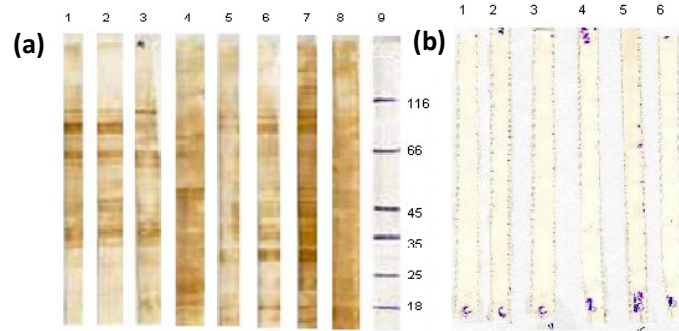


Fig. 2. (a) IgG subclass profile in *L. major*-infected C57BL/6 and BALB/c mice. Promastigote antigens immunoblotted with sera from C57BL/6 (lanes 1, 2, 3, and 4) and BALB/c mice (lanes 5, 6, 7, and 8) 8 weeks after infection; lanes 1 and 5, anti IgG2b; lanes 2 and 6, anti IgG2a; lanes 3 and 7, anti IgG1; lanes 4 and 8, anti IgG; lane 9, MW marker; (b) IgG subclass profile in uninfected C57BL/6 (Lanes 1, 2, and 3) and BALB/c (lanes 4, 5, and 6) mice; lanes 1 and 4, anti IgG2b; lanes 2 and 5, anti IgG2a; lanes 3 and 6, anti IgG1. Sera from five mice per group were pooled. The representative data was obtained from 3 repeated experiments.

In contrast to BALB/c mice, the number of antigens that recognized by IgG and IgG1 from C57BL/6 mice sera was different. The antibody binding patterns of IgG, IgG1 and IgG2a were different as well. Most of the antigens immunostained with IgG1 and IgG2a from C57BL/6 mice were within the range of 35-100 kDa. The antigens, which exclusively recognized by IgG1 but not by IgG2a isotype, corresponded to the bands with MWs of 18, 23, 27, 29, 32, 44, 57, 109, 117, 125, and 153 kDa. On the contrary, the bands of 41, 45, 47, 52, and 55 kDa reacted with IgG2a, but not with IgG1. Furthermore, in C57BL/6 mice, IgG2a response against 45, 83, and 88 kDa antigenic bands was dominant compared to IgG1 response.

Comparison of immunoblot profiles of C57BL/6 and BALB/c mice indicated that the antigen binding patterns of BALB/c and C57BL/6 mice were different. IgG from BALB/c mice sera recognized more antigens compared to the sera from C57BL/6. Antigens with MWs of 16, 50, 66, 97, 146, 149, 161, and 167 kDa were exclusively IgG inducer only in BALB/c mice. Moreover, the bands with molecular weights of about 27, 32, 117, 125, and 153 kDa reacted only with IgG1 from both mice strains. A very similar pattern of immunostaining was observed for IgG2a and IgG2b in both mouse strains.

DISCUSSION

In experimental models of leishmaniasis, *Leishmania* antigens elicit both humoral and cell mediated responses during the infection [10, 11]. *L. major* infection in C57BL/6 resistant mice results in protective Th1 response, characterized by IFN γ release and a biased IgG2a antibody production; while infection in susceptible BALB/c mice is associated with inappropriate Th2 response and predominant IL-4 production that supports switching to IgG1 subclasses [1-3].

Several studies attempted to correlate the humoral immune response to *Leishmania* infections with different clinical manifestations. IgG subclasses of *Leishmania*-specific antibodies in dogs infected with *L. chagasi* and *L. braziliensis* and patients with *L. braziliensis* infection, have been investigated by ELISA [12-14]. In these studies whole parasite proteins have been used as antigens and the profile of proteins has not been investigated. More recently the proteins of *L. chagasi* which reacted with sera from dogs with different clinical presentations, i.e., acutely infected, chronically infected, and uninfected controls have been identified. The results showed that some proteins sets were specifically recognized by sera from a specific group of infected dogs [15]. The patterns of anti-*Leishmania* se-

rospecificities in patients infected with *L. donovani* has also been studied, however, the subclasses of IgG have not been considered [16]. Moreover, many studies have reported proteins from *L. major*, which preferentially induce IgG1 or IgG2a in mice [17-19]. To the best of our knowledge, this is the first report that investigates the antigen binding pattern of IgG1, IgG2a, and IgG2b in sera from *L. major*-infected BALB/c and C57BL/6 mice.

In the present study the ELISA results consistently showed higher titers of anti-*Leishmania* antibodies in BALB/c mice sera compared to C57BL/6 mice. Our results is in agreement with the finding that IgG contributes to disease progression in leishmaniasis, similar to a study, which has shown that IgG-deficient BALB/c mice were more resistant to infection with *L. major* than normal BALB/c mice [9]. Moreover, BALB/c mice are more susceptible to *L. shawi* infection and show greater parasite density and humoral immune response compared to C57BL/6 [20].

Lower IgG response to *L. major* after protective vaccination with formalin-killed promastigotes has been shown to be associated with reduced lesion size [21]. Higher antibody levels in BALB/c mice can be attributed to the high concentrations of Th2 type cytokines, i.e., antibody promoting cytokines such as IL-4, IL-5 and IL-10 in *L. major*-infected BALB/c mice. The titers of specific IgG1 and IgG2a subclasses also differ in the resistant and susceptible mice. The IgG2a/IgG1 ratio in C57BL/6 mice was higher than BALB/c mice. These results are in line with studies, which correlate lower IgG1 and/or higher IgG2a antibody levels to Th1 response and a disease-controlling phenotype [17, 18, 19, 22]. For instance, immunization of BALB/c mice with a cocktail of gp63 and Hsp70 protein generated a protective response that correlated with high IgG2a/IgG1 ratio [23]. It has been shown that IgG1, but not IgG2a is pathogenic in *L. mexicana* infection. The mice lacking IgG1 could control the infection better than the wild types [24].

The factors, which determine the differentiation of naive CD4+ T cells into either Th1 or Th2 cells, are not completely known. However, it is widely accepted that antigen per se is an important determinant. In the present study the possible contribution of parasite antigens to elicit either Th1 or Th2 immune response was indirectly evaluated through the analysis of the IgG subclasses induced during the infection. Western blot analysis of serum showed that overall proteins were recognized by BALB/c mice sera compared to C57BL/6 mice sera. C57BL/6 mice showed limited antibody reactivity to antigens with MWs below 35 kDa and above 100 kDa, therefore generation of the immune responses depends on the mice strains. In addition, our results revealed that the number of bands or IgG1 was higher than IgG2a for both BALB/c and C57BL/6 mice sera. Furthermore, the intensity of immunoreactive bands with IgG1 in BALB/c mice was higher than IgG2a; while in C57BL/6 mice the intensity of bands immunostained with IgG2a and IgG1 was almost the same. These results are in consistent with the ELISA results which showed higher concentration of IgG1 compared to IgG2a in BALB/c mice and almost the same concentration of IgG1 and IgG2a in C57BL/6 mice.

The antigens with relative MWs of 139, 146, 149, 161, and 167 kDa could exclusively elicit IgG1 response only in BALB/c mice and the bands of 27, 32, 117, 125, and 153 kDa reacted exclusively with IgG1 in both mice strains.

In an attempt to identify new immunogenic proteins Mohammadi *et al.* (2006) investigated the reactivity of procyclic and metacyclic promastigote antigens with sera from both resistant and susceptible *L. major*-infected mice and reported two bands 140 and 152 kDa as metacyclic specific proteins. They did not compare the blotting profile of two mice strains. The 139 and 153 kDa proteins of our study are very close to 140 and 152 kDa antigens; however, 140 and 152 kDa proteins in contrast to our results were recognized by both IgG1 and IgG2a from BALB/c and C57BL/6 mice [25]. As

IgG1 is a Th2 related and pathogenic isotype, our findings suggest that the antigens, which were recognized by IgG1 from both mice strains, may be involved in the generation and maintenance of a predominant Th2 response. Moreover, the bands, which were recognized exclusively by IgG1, might be exacerbatory (counter protective) antigen. Interestingly, the antigens with MWs of 32 and 125 kDa were also present in amastigote form of the parasite and exclusively reacted with IgG1 from both resistant and susceptible mice strains [26]. It has been shown that some leishmanial antigens have an intrinsic potency to elicit Th2 responses.

For instance, vaccination of mice with *Leishmania* meta 1 protein and the plasmid bearing the meta 1 gene induced an immune response of the Th2 type; and the natural response of BALB/c mice against *Leishmania* histones and LACK protein after infection with *L. major* seems to be of the Th2 type [14, 19, 27]. On the other hand, the bands of 41, 45, 47, 52, and 55 kDa, which exclusively immunostained with IgG2a from C57BL/c, are potential candidates for eliciting Th1 response. The band of 52 kDa was also present in amastigote form and reacted stronger with IgG2a than IgG1 from sera of both mice strains [26]. The similar pattern of IgG2a and IgG2b is in accordance with studies, which state that IgG2b isotype is also associated with a Th1 response.

In conclusion, the results indicate that the antigen binding patterns of sera from *L. major*-infected BALB/c and C57BL/6 mice were different. Nevertheless, there are antigens which react exclusively with IgG1 in both mice strains and those which react strongly with IgG2a. Further investigation is required to verify the roles of these antigens as protective or counter protective types in *Leishmania* infections.

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