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IgG1 and IgG2a Profile of Serum Antibodies to *Leishmania major* Amastigote in BALB/c and C57BL/6Mice

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ABSTRACT

It is well accepted that in experimental model of *Leishmania major* infection, BALB/c mice mount a Th2 response and produce IgG1 predominantly whereas C57BL/6mice enhance Th1 response with the biased production of IgG2a antibodies. Therefore, screening for parasite antigens on the basis of reactivity with sera from infected susceptible or resistant mice might be used for the identification of Th1- or Th2-inducing antigens.

In this study, the antigenic profile of *Leishmania major*amastigote that induce IgG1 or IgG2a isotypes in infected BALB/c or C57BL/6 mice were compared.

Western blot analyses revealed that 27, 40, 43, 45 and 114 kDa proteins elicited IgG1 and not IgG2a in both BALB/c and C57BL/6 mice and 55 kDa protein was recognized exclusively by IgG1 of BALB/c mice sera. On the contrary, the bands corresponding to proteins with molecular weights (MW) of 30, 35, 52, 58 and 66 were intensively immunostained with IgG2a from C57BL/6 mice which made them potential candidates for eliciting Th1 response.

In conclusion, the results showed that the generation of the immune responses depended on the mouse strain and some leishmanial antigens had an intrinsic potency to elicit Th2 responses.

Keywords: Amastigote; *Leishmania major*; Western blot

INTRODUCTION

Leishmaniasis is endemic in 98 countries or territories, with more than 350 million people at risk. Published figures indicate an estimated incidence of 2 million new cases per year.[1]The disease is caused by parasites of the genus *Leishmania*, a group of kineto-plastid protozoans.

The parasite has a dimorphic life cycle: Extracellular stage promastigotes multiply and develop within the digestive tract of sand flies and intracellular amastigotes which reside and multiply within the phagolysosomal vacuoles of mammalian phagocyte. Promastigotes are transmitted by a sand fly vector and are inoculated into the host when an infected sand fly takes a blood meal. Once in the host, they are rapidly phagocytosed by macrophages and eventually

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metamorphosed to amastigotes.

The experimental infection of mice with *Leishmania major (L. major)* is one of the best characterized models for the study of the immune response associated with the differentiation of T lymphocytes. The outcome of infection is dependent upon the type of the immune response generated: in resistant strains of mice, *L. major* infection induces a T helper 1(Th1) type of response associated with a high level of IFN-γ which activates macrophages to kill parasites via effector nitric oxide (NO) production. Therefore, lesion heals spontaneously and the animals are protected against further infection; whereas *L. major* infection in susceptible BALB/c mice induces a Th2 response and high levels of IL (interleukin)-4, IL-5, and IL-13, as a result the disease is fatal.[2-4] In addition, IL-10 plays an important role in regulating immune responses to *Leishmania*.[5,6] The CD4+ Th1 and Th2 subsets also participate in B-cell differentiation and immunoglobulin isotype switching. In mice, the level of IgG1 antibodies correlates well with an overall Th2 immune response profile whereas that of IgG2a antibodies indicates an overall Th1 profile.[7,8] Since IgG isotype-dominated response depends on different CD4 T cell subsets, knowledge of serospecificities may provide insight into Th1- or Th2-inducing antigens. Identification of leishmanial antigens is required for the development of vaccines and serodiagnostic tests. The majority of studies evaluated the titers of IgG subclasses against promastigotes. There are few reports that have assessed immune response against amastigoteforms.[9,10] Since these two parasite forms have been shown to express stage-specific molecules and elicit differing host cell response [9-12], the present study has focused on the humoral immune response against amastigote forms of *L. major*.

In this study, the levels of IgG1 and IgG2a antibodies and the antigenic profile of *L.major* amastigote that induce IgG1 or IgG2a isotypes in infected BALB/c or C57BL/6 mice were compared.

MATERIALS AND METHODS

Parasite Culture and Mice Infection

L. major (MHRO/IR/75/ER) promastigotes were obtained by cultivation of lymph node from an infected BALB/c mouse in RPMI medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich) , 2mM L-glutamine (Gibco BRL), 100U/ml penicillin and 100 µg/ml streptomycin. Stationary phase promastigotes were harvested by centrifugation at 3500 rpm for 15 minutes. Female C57BL/6 and BALB/c mice (6 mice in each group), 6-8 week-old, were infected subcutaneously in the base tail with $2x10^6$ 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.

Protein Sample Preparation

Lesion-derived amastigotes were prepared according to Farajniaet al.[13] Briefly, lesions were excised from infected BALB/c mice and homogenized in sterile PBS containing 1 mM PMSF on ice. Amastigotes were released from infected macrophages by passing sequentially through 21, 23 and 25-gauge needles and purified by Percoll gradient centrifugation. Amastigote lysates were prepared through repeated freeze-thawing (4 cycles) and stored at -70^{o}C until use.

Measurement of Amastigote-Specific Serum IgG and IgG Subclasses

The mice sera were assayed by indirect ELISA for the presence of IgG1 and IgG2a against *L. major* amastigotes. Briefly, ELISA plates (Greiner, Germany) were coated overnight with 2 µg of *L. major* amastigote lysate in PBS, pH 7.2 and then were blocked with PBS containing 1% bovine serum albumin (BSA-PBS). The sera diluted in BSA-PBS (1:100) were added and plates were incubated for 2h at 37^{o}C. For detection of specific total IgG, peroxidase-conjugated goat anti-mouse antibody (Sigma, Germany) was added to each well and the plate was incubated for 1h at 37^{o}C before the addition of tetramethylbenzidine (TMB) substrate (Sigma, Germany). For detection of IgG subclasses, goat anti-mouse IgG1 and IgG2a antibodies (Sigma, Germany) were added and the plates were incubated for 1h at 37^{o}C. Peroxidase-conjugated rabbit anti-goat antibody (Sigma, Germany) was then added to each well and the plates were incubated for 1h at 37^{o}C, before the addition of TMB. Absorbance was read at 450 nm using an ELISA plate reader (Anthos 2020, Austria).

SDS–PAGE and Immunoblotting

Amastigote proteins were separated by

discontinuous SDS-PAGE on 4% stacking over 10% separating gel. Parasite lysate was boiled for 5 minutes in 6X SDS gel-loading buffer consisting of 375mMTris-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.

Proteins were electrophoretically transferred to PVDF membrane (Millipore, Immobilon P) according to the method described by Towbin.[14] After the transfer, the membranes were stained with Panceau 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 of 15 min each in PBS containing 0.1% Triton X-100 (PBS-T), the strips were incubated for 1 hr with gout anti-mouse IgG isotypes (IgG1, IgG2a) (Sigma, Germany). Peroxidase-conjugated rabbit anti-goat antibody (Sigma, Germany) was then 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

Student t-test was applied to compare the levels of each IgG subclass and the total IgG results between the groups. Statistical analyses were considered significant when $p< 0.05$.

RESULTS

Antibody Response

Amastigote-specific serum IgG as well as IgG1 and IgG2a responses were assayed by ELISA, 8 weeks after infection with *L. major*. The IgG level in *L. major* infected BALB/c mice was significantly higher than C57BL/6-infected mice (p=0.02). Likewise, BALB/c mice showed higher IgG1 antibody levels compared to C57BL/6 mice (p=0.001); however, there was no significant difference in IgG2a level between C57BL/6 and BALB/c mice (Fig. 1). The IgG2a/IgG1 ratios were 1.125 and 0.3 in C57BL/6 and BALB/c mice, respectively.

Immunological Detection of Amastigote Proteins

Western Blot analyses were done with IgG1 and IgG2a to test anti-amastigote responses from 8-weeks post-infection sera of C57BL/6 and BALB/c mice (Fig. 2). The results showed that in BALB/c mice, the intensity and number of bands were higher with IgG1 compared to IgG2a. Amastigote proteins recognized by IgG1 but not by IgG2a isotype from BALB/c mice correspond to proteins with molecular weights (MW) 23, 27, 34, 40, 43, 45, 55, 79, 89 and 114 kDa. Bands of 37, 52 and 66 kDa showed equal intensity with IgG2a compared to IgG1.

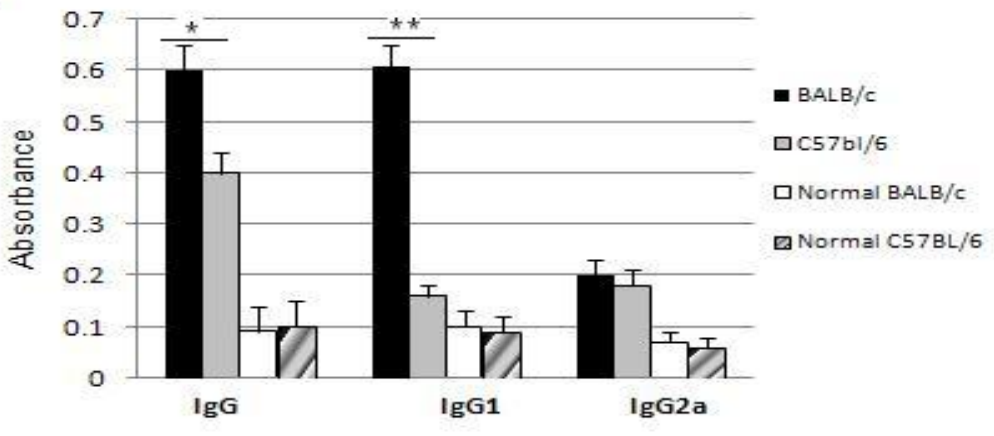

Figure 1. Absorbance values of serum antibodies (IgG, IgG1, IgG2a) from *L. major*-infected BALB/c and C57BL/6 mice against amastigote. Bars represent the mean+ SD of absorbance values of five mice. The absorbance values of total IgG, IgG1, and IgG2a in the serum of *Leishmania*-infected mice were significantly (p<0.05) greater than those of the control mice; however, there was no significant difference in IgG2a level between C57BL/6 and BALB/c mice. * p= 0.02, ** p = 0.001

Likewise, in C57BL/6 mice, more antigens from amastigote were recognized by IgG1 compared to IgG2a; however the intensity of bands were almost similar. The bands of 27, 36, 40, 43, 45, 49, 70, 79, 89 and 114 kDa were immunostained exclusively with IgG1. The IgG2a immune responses with proteins of 35, 52, 58 and 66 kDa were more intense than or equal to IgG1 responses. Interestingly, the protein with MW of approximately 30 kDa was only recognized by IgG2a.

Comparison of immunoblot profiles between C57BL/6 and BALB/c mice indicated that most of immunoreactive proteins of amastigote with sera from both strains of mice were in the range of 30-70 kDa. Moreover, IgG2a from C57bl/c mice reacted with more proteins compared to that of BALB/c mice. Proteins with MWs of 25, 32, 40, 43, 45 and 114 kDa only reacted with IgG1 isotype from both BALB/c and C57b/c mice. The immunostaining of 55kDa protein was solely observed with BALB/c mice sera and exclusively with IgG1 isotype. On the contrary, the 36, 49 and 70 kDa proteins exclusively reacted with IgG1 from C57BL/6, but not from BALB/c mice.

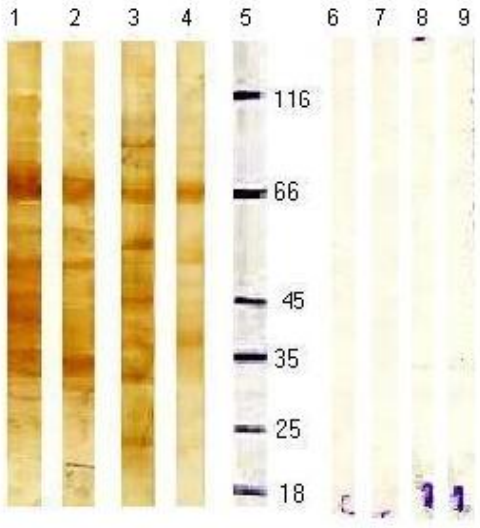

Figure 2. Immune response of C57BL/6 and BALB/c mice to amastigote antigens. Amastigote antigens immunoblotted with sera from *L. major* infected (lanes 1,2) and uninfected (lanes 6, 7) C57BL/6 mice and *L. major* infected (Lanes 3,4) and uninfected (8, 9) BALB/c mice. Lanes 1, 3, 6, 8: anti IgG1; Lanes 2, 4, 7, 9: anti IgG2a, Lane 5: MW marker. Representative data from 3 repeated experiments.

DISCUSSION

The development of a vaccine against leishmaniasis involves the identification of molecules that modulate the immune response against the parasite. Although over 94% of *Leishmania* genes are expressed in both life stages i.e. promastigotes and amastigotes, there are some genes that are differentially expressed in each stage.[9,10] The majority of studies have investigated immune response to promastigote antigens and fewer reports have assessed the antigenic profile of amastigote forms. This might be owing to the fact that the promastigotes are easily obtained from the liquid cultures; however there is no culture system for *L. major* amastigotes, the form found in the infected mammalian host. It would be of interest to identify amastigote proteins that elicit protective immune responses against the infection.

C57BL/6 and BALB/c mice as resistant and susceptible models for *L. major* infection, respectively, have been widely used for study of immune responses against leishmaniasis. Cellular immunity is thought to be essential for controlling *Leishmania*; however *Leishmania*-specific humoral immune responses are also present during the infection.[15,16]

BALB/c mice infected with *L. major* mount a Th2 response and produce IgG1 predominantly whereas C57BL/6mice enhance Th1 response with the biased production of IgG2a antibodies.[2,4] Indeed in mice, IFN-γ which is the hallmark cytokine of Th1 response, promotes the production of IgG2a while IL-4, a Th2-related cytokine, induces the production of IgG1 from the B cells.[7,8] Therefore, screening for parasite antigens on the basis of reactivity with sera from infected susceptible or resistant mice might be used for the identification of Th1- or Th2-inducing antigens. Indeed many studies have reported proteins from *L. major* which preferentially induce IgG1 or IgG2a in mice, however to the best of our knowledge this is the first report that investigated the antigen binding pattern of IgG1 and IgG2a of sera from *L. major*-infected BALB/c and C57BL/6 mice.

In the present study, the humoral immune responses of BALB/c and C57BL/6 mice infected with *L. major* towards amastigote proteins were compared and the antigenic profile of *L. major* amastigote that induces IgG1 or IgG2a isotypes was determined by western blot analysis.

The ELISA results in our study showed higher serum anti-amastigote IgG level in BALB/c mice compared to that of C57bl/c mice. Since the infection was limited in C57BL/6 mice and was progressive in BALB/c mice, there was a positive correlation between the titers of anti-*Leishmania* amastigote antibodies and the disease progression. This was in agreement with studies that showed vaccinated and protected mice against *Leishmania* developed lower antibody compared to unprotected controls[17-19]. Higher antibody levels in BALB/c mice can be attributed to the fact that the concentrations of Th2 type cytokines which are antibody promoting cytokines such as IL-4, IL-5 and IL-10 are high in L. major-infected BALB/c mice. There was also a marked difference in the level of IgG1 between the resistant and the susceptible mice. In BALB/c mice, the antibody response was predominantly of IgG1 isotype; however, both C57BL/6 and BALB/c mice produced similar levels of IgG2a.Since IL-4 is a main cytokine in *L. major*-infected BALB/c mice and this cytokine was found to induce switching to the IgG1 and to inhibit IgG2a production, it was surprising that the titers of both isotypes increased in the presence of a strong Th2 response associated with BALB/c mice. Furthermore, the IgG2a/IgG1 ratio was higher in C57BL/6 mice compared to BALB/c mice. These findings extend other studies that have indicated higher IgG2a and/or lower IgG1 antibody levels toward promastigote antigens are indicative of a Th1 response and a disease-controlling phenotype.[17,20,21] For instance, immunization of BALB/c mice with Leish-111f polyprotein induced high IgG2a/IgG1 ratio that correlated with a dominant Th1 response and a better control of the disease.[22]

Several factors influence the development of effector Th1 and Th2 cells. One of the important determinants is the antigen. Screening of antigens which are preferentially recognized by Th1 or Th2 response is impractical while the B cell repertoire is conventionally and rapidly analyzed by the western blot methods which permit antibody based detection of the proteins, separated by SDS-PAGE. In the present study, the screening for amastigote antigens on the basis of reactivity with IgG isotypes from infected susceptible and resistant mice were carried out. The results of IgG1 and IgG2a blotting profiles revealed that there are more immunoreactive proteins with BALB/c mouse serum compared to that of C57bl/c mouse. This is consistent with the finding that sera from BALB/c but not C57BL/6 mice recognize several antigens excreted by axenic amastigotes of *L. amazonensis.*[23] In addition, our results showed that in both mice strains, the antigen binding pattern of IgG1 and IgG2a were different. The number and intensity of bands were higher with IgG1 compared to IgG2a with BALB/c mice sera. Likewise, IgG1 from C57BL/6 sera recognized more proteins compared to IgG2a, however the intensity of bands immunostained with IgG1 and IgG2a was similar. These results are in line with ELISA results which showed higher concentration of IgG1 than IgG2a in BALB/c mice and almost equal concentration of IgG1 and IgG2a in C57BL/6 mice. Western blot analysis revealed that most of immunoreactive proteins of amastigote reacted with IgG1 from both mice strains. It has been indicated that IgG1, but not IgG2a, is pathogenic *in vivo.*[24]Moreover, amastigote antigens have been suggested to induce a suppressive cellular immune response in mice, favoring the spread of the infection.[11,25]

The results presented in this work revealed that 27, 40, 43, 45 and 114 kDa proteins have elicited IgG1 and not IgG2a in both BALB/c and C57BL/6 mice. Considering that IgG1 is a Th2 related and pathogenic isotype, our findings suggest that the above-mentioned antigens may be involved in the generation and maintenance of a predominant Th2 response. Moreover, 55 kDa protein which was recognized exclusively by IgG1 of BALB/c mice sera might be exacerbatory (counter protective) antigen. 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 gene induces an immune response of the Th2-type and the natural response of BALB/c mice against *Leishmania*histones and LACK protein after challenging with *L. major* seems to be of the Th2 type.[20,26,27] On the contrary, the bands corresponding to proteins with MWs of 30, 35, 52, 58 and 66 were intensively immunostained with IgG2a from C57BL/6 mice which made them potential candidates for eliciting Th1 response.

In conclusion, variations in the specificity pattern of the immune responses seen with sera from BALB/c and C57bl/c mice strongly suggest that different amastigotes proteins can act as antigens in these strains. Nevertheless, there are proteins which react exclusively with IgG1 in both strains of mice and proteins which

react strongly with IgG2a. Further investigation is required to verify the roles of these proteins as protective or counter protective antigens in *Leishmania* infection.

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