Lower levels of IgG1 in comparison with IgG2a are associated with protective immunity against *Leishmania tropica* infection in BALB/c mice

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Received 11 January 2015; received in revised form 6 April 2015; accepted 5 May 2015

Available online

**KEYWORDS**  
BALB/c mice; immunity; immunoglobulin G; *Leishmania tropica*

**Background/Purpose:** *Leishmania* (L.) *tropica* is the causative agent of different forms of human leishmaniasis. There is little information about the role of *Leishmania*-specific antibodies in the immune response against *L. tropica* infection. The aim of this study is to evaluate the role of *Leishmania*-specific antibodies and their immunoglobulin G (IgG) isotypes in *L. tropica* infection.

**Methods:** *L. tropica* at two different doses (high dose, \(10^6\) parasites/mouse and low dose, \(10^3\) parasites/mouse) were used for infection of BALB/c mice. BALB/c mice infected with *Leishmania major* were used for comparison. Anti-*Leishmania* antibodies of the IgG1 and IgG2a isotypes were assayed by enzyme-linked immunosorbent assay.

**Results:** Our data showed that (1) a higher parasite dose results in higher levels of antibody. (2) *L. tropica* infection results in a lower IgG1 antibody response, compared with *L. major* infection. (3) The IgG2a/IgG1 antibody response in *L. tropica* infection is higher than that in *L. major* infection.

**Conclusion:** A higher IgG2a/IgG1 ratio is associated with protective immune response in *L. tropica* infection. These data can help to approach the complex profile of immunity against *L. tropica* infection.

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http://dx.doi.org/10.1016/j.jmii.2015.05.007  
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Introduction

Leishmaniasis is a disease resulting from infection by protozoan parasites of the genus *Leishmania* (L.), which can affect man and several species of animals. *Leishmania tropica* is the causative agent of different forms of human leishmaniasis, anthropoponic and visceral leishmaniasis, and a few cases of visceral leishmaniasis in many parts of the world including Iran. *Leishmania major* is a major causative agent of cutaneous leishmaniasis.1

With regard to *Leishmania* parasites, it is generally accepted that protection from related diseases is associated with cellular immune response. In fact, it was reported that removal of B cells or antibodies profoundly alters the course and outcome of infection within genetically susceptible and resistant mouse strains.2 Antibodies are an important component of immune response against pathogens. Antibodies have evolved into classes and subclasses with specific assigned functions such as cytotoxicity, phagocytosis, and release of inflammatory mediators. Both detrimental and beneficial roles for host have been reported for *Leishmania*-specific antibodies. With regard to detrimental roles, it has been reported that B cells and/or antibodies are required for disease susceptibility in BALB/c mice.4 It has also been shown that these antibodies contribute to the progression of *Leishmania* disease through the induction of interleukin-10 (IL-10) production from macrophages.5 The ligation of macrophage Fcγ receptors (FcγR) promoted the production of IL-10 and inhibited the secretion of IL-12. The implication from these studies is that immunoglobulin G (IgG) itself may be an important promoter of the Th2-type immune response.6 The detrimental role of these antibodies was also reported in another study on leishmaniasis in both experimental animals infected with *L. major* and humans with visceral leishmaniasis.7 It has been shown that BALB/c mice lacking the common γ-chain of the Fc receptor can control *L. major* infection and totally resolve cutaneous lesions.7 With regard to the beneficial role of antibodies, immune immunoglobulin G (IgG) production and engagement of dendritic cell FcγR are required for the timely development of Th1/Tc1-dependent immunity and control of experimental cutaneous leishmaniasis in mice.8 The IgG subclasses in BALB/c mice include IgG1, IgG2a, IgG2b, and IgG3.9–12 The IgG subclass expression is influenced by multiple factors, including the prevailing cytokine environment. It is known that interferon-γ (as a Th1 cytokine) and IL-4 (as a Th2 cytokine) induce isotype switching to IgG2a and IgG1, respectively.13, 14 These two IgG isotypes have been used in many studies as surrogate markers of Th1 and Th2 responses against *Leishmania* and other infections.15, 16 Many reports show that during *Leishmania* infections, higher amounts of *Leishmania*-specific antibodies of the IgG1 isotype in comparison with IgG2a are associated with disease progression, and higher amounts of *Leishmania*-specific antibodies of the IgG2a isotype in comparison with IgG1 are associated with protection against the disease.17–23 It is crucial to know whether all antibody responses or only certain isotypes are pathogenic, to tailor these responses to the benefit of the host.24

The dose of *Leishmania* parasites used for inoculation is an important factor determining the course and outcome of disease in murine models of leishmaniasis.25 A low dose of 100 parasites results in protective immunity against the parasite,17 whereas a high dose of 105–107 parasites results in progressive disease in BALB/c mice.25 Therefore, the infective dose of parasite may acts as a variable in the induction of antibody response against *Leishmania* parasites. *L. tropica* and *L. major* differ from each other with regard to pathogenicity in experimental models. In BALB/c mice, *L. major* produces rapidly progressing, nonhealing, and destructive lesions, whereas lesions of *L. tropica* are nonulcerative and eventually controlled.26, 27 Although the immune response against *L. major* infection has been studied in-depth,28 there is limited information about the immunology of *L. tropica* infection. Thus, experimental models of *L. tropica* infection can help in increasing the knowledge about this infection.

The aim of this study is to evaluate the role of *Leishmania*-specific antibodies and their IgG isotypes in *L. tropica* infection. For this purpose, we determined the kinetics of production of *Leishmania*-specific antibodies during the course of *L. tropica* infection in BALB/c mice. Two different doses of parasites [high dose (108 parasites/mouse) and low dose (103 parasites/mouse)] were used for inoculation. *L. major* infection in BALB/c mice was used as a well-defined model to validate our assay variables and the *L. major* data were compared with those of *L. tropica*.

Methods

Parasite and antigen

The *L. tropica* strain MHOM/AF/88/KK27 isolated from a patient with cutaneous leishmaniasis in Afghanistan was kindly donated by Dr D. Sacks (Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, MD, USA). The *L. major* strain MRHO/IR/75/ER isolate from Iran was a gift from Dr M. Mohabati (School of Public Health, Tehran University of Medical Sciences, Tehran, Iran). Procedures followed for parasite culture and their species confirmation were the same as reported elsewhere.29, 30

Parasites were cultured in Novy–MacNeal–Nicolle (NNN) media and were injected into BALB/c mice and retrieved from their lymph node for preservation of their virulence. The *in vitro* cultivation of parasite, after isolation from mice, was kept to less than three passages in NNN media to maintain their virulence. Soluble leishmanial antigens (SLAs) were prepared as follows: *L. tropica* was cultivated in liquid media consisting of Roswell Park Memorial Institute-1640 (RPMI-1640), 10% fetal bovine serum, 1% L-glutamine, 100 IU/mL penicillin, and 1 μg/mL streptomycin. Parasites were harvested at the stationary phase of growth curve, and washed three times with phosphate-buffered saline (PBS). Protease inhibitor (cComplete, Mini, EDTA free, Roche Diagnostics, Mannheim, Germany) was added to the parasite suspension. The parasites (concentration, 2 × 109/mL) were subjected to 10 rounds of freeze and thaw cycles, centrifuged at 16,000g (at 4°C for 20 minutes), and the supernatant was aliquoted in small volumes and stored at −70°C until use.
**Study animals and blood collection**

Female BALB/c mice (4–6 weeks old) were purchased from the animal production facility of Pasteur Institute of Iran (Tehran, Iran). Blood samples were obtained by the retro-orbital bleeding procedure. The study protocol was approved by the Ethics Committee of the Pasteur Institute of Iran.

**Infection**

The parasites (10-μL volume) were intradermally injected into the right ear of mice. The course of lesion development was monitored by measuring ear thickness using a dial-gauge caliper (Mitutoyo, Kawasaki, Kanagawa, Japan) at 2-week intervals.

**Study design**

Mice were randomly divided into four experimental groups. Each group consisted of 20 mice. High (10^6 parasites/mouse) and low (10^3 parasites/mouse) doses were used for infecting mice with both *L. major* and *L. tropica*. The experimental groups were as follows: (1) low-dose *L. major*, (2) high-dose *L. major*, (3) low-dose *L. tropica*, and (4) high-dose *L. tropica*. Blood sample was obtained from four mice in each group at three intervals after the induction of infection (at Weeks 1, 4, and 16). Anti-**Leishmania** antibodies of the IgG1 and IgG2a isotypes were assayed by enzyme-linked immunosorbent assay (ELISA). The whole experiment was carried out twice (each experiment consisted of 80 mice) and data from one representative experiment are presented.

**Enzyme-linked immunosorbent assay**

The following factors were optimized in our ELISA setting: concentration of antigen (range, 0.2 μg/well, 0.5 μg/well, and 1 μg/well), positive and negative serum dilutions (range, 1/100, 1/500, and 1/1000), and dilution of horse-radish peroxidase (HRP)-conjugated antiserum IgG1 and IgG2a isotypes (1/1000 and 1/2000, respectively). The 96-well ELISA plates (Greiner, MICROLON, Kremsmünster, Austria) were coated with 0.2 μg/well of SLA and incubated at 4°C overnight. The plates were then washed three times with PBST (250 μL/well; PBS + 0.05% v/v Tween 20) and soaked for 1 minute in an ELISA plate washing system (Anthos Fluido; Asys-hitech GmbH, Eugendorf, Austria). Plates were blocked using 100 μL/well of 1% w/v bovine serum albumin (BSA in PBS) at 37°C for 2 hours. After the washing step (as above), the sera at the 1/400 dilution (i.e., 1 unit of each serum in 399 units of 1% w/v of BSA) were added (100 μL/well) to plates. Plates were incubated at 37°C for 2 hours. The plates were washed as mentioned earlier. Then, 100 μL of HRP-conjugated antiserum IgG1 (BD Pharmingen, Heidelberg, Germany) or IgG2a (BD Pharmingen, Heidelberg, Germany) antibodies at the 1/2000 dilution were added to the previously assigned wells, and the plates were incubated at 37°C for 2 hours. The plates were then washed according to the method mentioned earlier and 3,3',5,5'-tetramethylbenzidine (100 μL/well) as HRP substrate was added to each plate. Plates were incubated at room temperature for 15 minutes in a dark place. The enzyme−substrate reaction was terminated by adding a stop solution (100 μL/well; 2N H2SO4) and absorbance of wells was read using a microplate reader (Anthos 2020; Eugendorf, Austria) at optical densities of 620 nm and 450 nm. The final values were calculated by subtracting the 620-nm values from the 450-nm values. Each sample was used in triplicate. Positive and negative control sera were included in each run.

**Statistical analysis**

Lesion diameter, optical density (OD) of ELISA results, and isotype ratios were compared between experimental groups by one-tailed two-sample equal variance t test. A p value of 0.05 or less was taken to be significant.

**Results**

**Course of *L. tropica* infection in BALB/c mice**

The course of infection was determined by measuring the ear thickness. The results are shown in Figure 1. The course of infection for *L. major* and *L. tropica* was as expected: *L. major* infection, irrespective of whether a high or low dose was injected, resulted in progressive lesions, whereas lesions of *L. tropica* infection, irrespective of the dose, displayed a healing pattern. Therefore, high and low doses of both *L. major* and *L. tropica* did not change the outcome of disease in BALB/c mice. High doses of the parasites resulted in early onset of lesions, whereas the low doses resulted in late onset of lesions. The results were similar to those reported previously.27

![Figure 1. Lesion development after *Leishmania tropica* and *Leishmania major* infection. High (10^6) and low (10^3) doses of parasites were injected into the ear of BALB/c mice. Ear thickness was determined at 2-week intervals. Each point is mean ± standard deviation of values of 7–16 mice/group. The differences were statistically significant between high doses of *L. tropica* and *L. major* in all time points shown. Statistically significant difference between low doses of *L. tropica* and *L. major* were obtained from Week 14 onward.](dx.doi.org/10.1016/j.jmii.2015.05.007)
Validation of ELISA setting by testing different dilutions of sera

Three serum dilutions (1/400, 1/4000, and 1/40,000) were used for all sera samples to validate the results. Testing of these three serum dilutions enables the quantification of antibody levels in sera containing high levels of antibody. The 1/400 dilution of these titer sera containing high levels of antibody resulted in high OD, which was not within the assay limit of our ELISA setting, but further dilutions of these sera yielded OD within the assay range. Data obtained from the 1/400 dilution of sera were used for comparison of all experimental groups, unless otherwise stated. Representative data obtained from different dilutions of sera are presented in Figure 2. The results showed that our ELISA settings are reliable.

Higher parasite dose results in higher levels of antibody

Two doses of parasites (high and low) were used for infection of mice. The antibody titer of IgG1 and IgG2a against the high-parasite dose is significantly higher than the antibody titer of the same isotype against the low-parasite dose of both species at 4 weeks and 16 weeks after the infection. Figure 3 shows the antibody response against two doses (high and low) of *L. major* and Figure 4 shows the data for two doses (high and low) of *L. tropica*. These results show that antibody responses against both *Leishmania* species are dependent on the infective dose and our assay system is sufficiently optimized for detecting the differences between the low and high doses.

*L. tropica* infection results in lower IgG1 antibody response in comparison with *L. major* infection

Mice were infected with high doses of *L. tropica* and *L. major*. The antibody responses of IgG1 and IgG2a against *L. tropica* infection were lower, compared with those against *L. major* infection (Figure 5). The difference in IgG1 response was statistically significant at 16 weeks after the infection. However, the difference in IgG2a response did not reach the significance level ($p = 0.07$). When low doses of *L. tropica* and *L. major* were used to infect the mice, the IgG1 and IgG2a responses were quite similar to IgG1 and IgG2a responses against the high dose of these two *Leishmania* species (Figure 6).

Figure 2. IgG1 and IgG2a levels of the three different dilutions of sera of mice infected with high doses of *Leishmania tropica* and *Leishmania major*. OD = optical density.

Figure 3. Higher infective dose of *Leishmania major* resulted in higher levels of anti-*Leishmania* antibody. *Leishmania*-specific antibody titers are shown at indicated time points after infection of BALB/c mice with low (10³ parasites/mouse) or high dose (10⁶ parasites/mouse) of *L. major*. Antibody titers are presented as optical density (OD). Each value is mean ± standard deviation of values of four mice/group. The asterisk shows statistically significant difference between the *L. major* and *Leishmania tropica* groups (*$p \leq 0.001$). The open circle shows that the OD of each serum in this group was higher than the assay limit at the 1/400 dilution. The 1/4000 dilution showed OD in the assay range of the test and at this dilution the OD of the *L. major* high-dose group was also higher than that of the *L. major* low-dose group statistically.
IgG isotypes in Leishmania tropica infection

Figures 4 and 5. Levels of IgG2a and IgG1 in response to Leishmania tropica and Leishmania major at high infective dose. Leishmania-specific antibody titers at indicated time points after infection of BALB/c mice with a high dose (10^6 parasites/mouse) or low dose (10^5 parasites/mouse) of L. tropica and L. major. Antibody titers are presented as optical density (OD). Each value is mean ± standard deviation of values of four mice/group. The asterisk shows statistically significant difference between the Leishmania major and L. tropica groups (*p < 0.001).

Levels of IgG2a/IgG1 antibodies in response to L. tropica and L. major infections

The levels of IgG2a and IgG1 antibodies produced against the high doses of L. tropica and L. major are shown in Figure 5. The data show that more IgG1 in comparison with IgG2a is produced in response to L. major infection, but more IgG2a in comparison with IgG1 is produced against L. tropica infection. The amount of IgG2a/IgG1 antibodies produced in response to L. tropica is significantly higher than that in response to L. major at 16 weeks after the infection. Similar results were obtained when the low doses of L. major and L. tropica were used for infection. The levels of IgG isotypes produced in response to low-dose infections are presented in Figure 6. As is the case with high-dose infection, in response to low-dose infection, the IgG2a/IgG1 ratio is higher in L. tropica infection in comparison with L. major infection, and the difference was statistically significant at 16 weeks after the infection. This ratio (IgG2a/IgG1) does not differ significantly between mice infected with high or low dose of L. major (Figure 7). Similarly, for L. tropica, this ratio (IgG2a/IgG1) was not statistically different between high and low doses of L. tropica infection (Figure 7).

Discussion

Our findings show that the antibody response against L. tropica infection is lower than that against L. major infection in BALB/c mice. The different doses of parasites (from 10^3 to 10^6/mouse) do not change the relatively lower levels of antibody produced in response to L. tropica infection, compared with L. major.

The presence of higher levels of anti-Leishmania antibodies against L. major infection in our study is concordant with induction of Th2 response and ensuing high levels of anti-Leishmania antibodies in response to L. major infection in BALB/c mice. Lower levels of anti-Leishmania antibodies produced against L. tropica infection suggest that the Th2 response is lower against L. tropica infection in comparison with L. major infection in BALB/c mice.

Our results show that higher levels of Leishmania-specific IgG1 antibodies are associated with increasing thicknesses of lesions in BALB/c mice (i.e., IgG1 is associated with progressive leishmaniasis). These findings are similar to other reports. The levels of IgG1 anti-Leishmania antibodies produced in response to L. tropica infection are lower than those produced in response to L. major. This is an important difference with regard to the immune response against these two Leishmania species. This indicates that the IgG1 isotype of anti-Leishmania antibodies is associated with disease exacerbation and lower levels of this isotype is associated with protection against the disease in BALB/c mice. These findings are consistent with other reports regarding L. major infection. These data suggest that the IgG1 isotype of anti-Leishmania antibodies has a detrimental role, and that lower levels of...
IgG1 are associated with protective immunity against \textit{L. tropica} infection. The ratio of IgG2a to IgG1 shows the combined effect of IgG2a and IgG1. Our results show that there is a sharp difference in this ratio between \textit{L. tropica} and \textit{L. major} infections. The IgG2a level is higher than that of IgG1 in response to \textit{L. tropica} (i.e., IgG2a/IgG1 ratio > 1). However, the IgG1 level is higher than that of IgG2a in response to \textit{L. major} (i.e., IgG2a/IgG1 ratio < 1). These data suggest that the protective immune response against \textit{L. tropica} needs not only a low level of IgG1, but also a high level of IgG2a, compared with IgG1. It is possible that the IgG2a response has a beneficial effect and that the IgG1 response counteracts its effects. The beneficial effect of IgG2a response may occur only when the IgG1 response diminishes.

Our findings clearly show that higher levels of IgG2a, compared with IgG1, are associated with beneficial effects in \textit{L. tropica}-infected BALB/c mice. This is the first report on \textit{L. tropica} infection. These findings are consistent with those of other reports that a higher ratio of the IgG2a/IgG1 isotype is associated with a protective immunity against \textit{L. major} infection in BALB/c mice, irrespective of whether the healing resulted from low doses of parasites\textsuperscript{17,19} or the healing resulted from vaccination.\textsuperscript{18,20–23} Therefore, our findings on \textit{L. tropica} infection in BALB/c mice in addition to other reports suggest that high levels of IgG2a and low levels of IgG1 isotype of anti-Leishmania antibodies in this in-bred mice strain are associated with protective immunity against different \textit{Leishmania} species, at least for \textit{L. tropica}, \textit{L. major},\textsuperscript{19} and \textit{Leishmania donovani}.\textsuperscript{17}

In summary, we conclude that higher IgG2a/IgG1 ratio is associated with protective immune response in \textit{L. tropica} infection. These data can help to approach the complex profile of immunity against \textit{L. tropica} infection.

**Conflicts of interest**

All contributing authors declare no conflicts of interest.

**Acknowledgments**

This research was supported by the Pasteur Institute of Iran (Grant No. 595).

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IgG isotypes in *Leishmania tropica* infection


Please cite this article in press as: Rostamian M, et al., Lower levels of IgG1 in comparison with IgG2a are associated with protective immunity against *Leishmania tropica* infection in BALB/c mice, Journal of Microbiology, Immunology and Infection (2015), http://dx.doi.org/10.1016/j.jmii.2015.05.007