Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against Leishmania donovani infections

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Abstract

The A2 genes of Leishmania donovani encode amastigote-specific A2 proteins, which are considered to be virulence factors required for the survival of this protozoan parasite in the mammalian host. The A2 genes are present within a multigene family and corresponding A2 proteins are composed predominantly of multiple copies of a 10 amino acid repeat sequences. A2-specific antibodies have been detected in the sera of patients suffering from visceral leishmaniasis (VL) and it has been shown that generation of A2 deficient L. donovani resulted in an avirulent phenotype. In this report, we show that immunization of mice with recombinant A2 protein conferred significant protection against challenge infection with L. donovani. The protection correlated with in vitro splenocyte proliferation, production of IFN-γ in response to A2 protein and the presence of A2-specific antibodies in the sera of immunized mice. These data demonstrate that A2 represents a potential antigen for protection against infection with L. donovani and VL. © 2001 Published by Elsevier Science Ltd.

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1. Introduction

Leishmaniasis is a spectrum of diseases caused by infection with different species of the protozoan parasite Leishmania [1–4]. These diseases range from self-limiting cutaneous leishmaniasis (CL) to visceral leishmaniasis (VL), also known as Kala-azar, which is a fatal infection if not treated successfully. Leishmaniasis effects over 12 million people in 88 countries, over 350 million are at risk, and over 2 million new cases emerge every year [1–3]. Different species of sandfly transmit Leishmania and reservoirs include canine, wild rodents, and human. Within the insect host, Leishmania is present as flagellated promastigote form and upon infecting the mammalian host it differentiates into the smaller aflagellated round amastigote stage and multiplies in the phagolysosome vacuole of macrophages. Leishmaniasis is difficult to treat and there is increasing resistance developing against the currently available drugs [5]. New disease foci are identified every year in different parts of the world and this may be due to the emerging resistance of sandflies towards insecticides [6] and resistance of the parasite to the existing chemotherapy. In developing and underdeveloped parts of the world, acquired immunosuppressive syndromes (including AIDS) add to the higher risk of leishmaniasis [7]. Based on these and other observations, there is clearly an urgent need for vaccine development against this disease and in particular against fatal Kala-azar.

Several vaccine clinical trials against CL have been undertaken [8–10] and more recently a trial has been carried out against VL in the Sudan [11]. Most experimental vaccines against leishmaniasis have been either live strains [12–14], defined subunit vaccines [15–18] or crude fractions of the parasite [19]. Recently, DNA vaccines have resulted in protection in experimental CL and have appeared to preferentially induce a Th1 immune response [20]. There are considerably more protective antigens described for L. major infections (example [21–23]) as compared to Leishmania donovani in this respect [24–27]. All of the experimental vaccine candidates for L. donovani are thought to produce T-cell mediated responses and high antigen-specific antibody titres [24,25,27]. Since few experimental vaccination studies have been carried out against VL, we have therefore examined the possibility of using the A2 antigen of L. donovani as a vaccine candidate against VL.

A2 genes are expressed specifically in L. donovani amastigotes [28]. The A2 genes are present within a multigene family and the corresponding A2 proteins are composed of predominantly multiple copies of a 10 amino acid repeat sequence. The A2 proteins are abundant in amastigotes and range in molecular weight from 42 to 100 kDa
depending on the number of repeats within each protein species [29,30]. A2-specific antibodies have been identified in the sera of patients suffering from VL [31]. It has been shown that generation of A2 deficient L. donovani resulted in avirulent amastigotes, which could not survive in a mouse model but were able to multiply in axenic culture as promastigotes [32]. All these above findings tempted us to investigate the potential of using the A2 antigen as a vaccine candidate against L. donovani infection.

2. Materials and methods

2.1. Leishmania strain and mice

L. donovani Sudanese 1S2D promastigotes and amastigotes were cultured as described previously [32]. Female BALB/c (Lshs, H-2d) and C57B/6 mice (4–6 weeks old) were obtained from Charles River, Canada.

2.2. A2 immunization and challenge infection

A2 was purified from E. coli BL-21 containing pET16b/A2 plasmid. Endotoxin free recombinant A2 protein was used for vaccination and other studies. Mice were injected i.p. with A2 protein combined with 100 μg heat killed Propionibactrum acnes (Elkins, Sinn, Cherry Hill, NJ) as the adjuvant for the first injection and subsequent boosts were with A2 protein in PBS in the absence of adjuvants. For the vaccination studies, the antibody response experiments, and for passive immunization studies, each mouse received 10 μg of recombinant A2 protein for the first injection and 5 μg each for the two boosts with 3-week intervals between each injection. Control mice received only 100 μg heat killed P. acnes as the adjuvant for the first injection and subsequent boosts were with PBS. Mice were bled 3 weeks following the final injections and serum from the mice in each group (n = 4) were pooled. For the vaccination experiment, mice were immunized as above and then challenged 3 weeks after the final boost and euthanized for liver biopsies 4 weeks following challenge. For challenge infection, 2 × 10⁷ stationary phase cultured promastigotes of L. donovani (1S2D) were injected in the tail vein in 100 μl PBS per mice. For passive immunization, 3 weeks after the final boost 8×10⁶ splenocytes were collected and transferred to naive mice by tail iv. One week after the transfer mice were challenged with 2×10⁹ L. donovani promastigotes and 4 weeks after the challenge infection mice were killed and parasite burden were measured by liver touch biopsy. For the cell proliferation and cytokine production assays, mice were immunized with 10 μg recombinant A2 protein and 100 μg heat killed P. acnes in the first injection and 5 μg of A2 protein in PBS for 1 boost injection at 2-week intervals. Control mice received only 100 μg heat killed P. acnes for the first injection and the subsequent boost was with PBS. Two weeks after the boost, mice were euthanized and spleens were isolated. Spleens from mice in the same group (four per group) were pooled together.

2.3. Vaccination analysis

Four weeks following challenge infection, mice were euthanized and liver touch biopsies were microscopically examined after fixing and staining the slides with Giemsa [33]. Leishman donovan unit (LDU) were calculated as LDU = (number of amastigotes/number of liver nuclei) × weight of liver in milligrams [34]. Protection studies were performed in 4 mice per group and the experiment was repeated three times with similar results.

2.4. ELISA

The method for end point titration was described elsewhere [35]. In brief, 5 ng of recombinant A2 protein was coated per well in a 96 well plate at 4°C overnight in 50 μl binding buffer (0.1 M NaPO₄, pH 9.0). The wells were washed three times with PBS-T (PBS, 0.1% Tween 20) and blocked with 200 μl of 3% bovine serum albumin (BSA) in PBS-T for 2 h at 37°C. Wells were then washed three times with PBS-T and incubated with 100 μl of diluted serum (serially diluted at 2-fold starting at 1:20 in PBS-T, 1% BSA) for 2 h at 37°C. Wells were then washed three times with PBS-T and incubated with 1:2000 diluted HRPO conjugated anti-mouse goat antibody in PBS-T, 1% BSA for 1 h at 37°C. After washing three times with PBS-T, the color was developed with TMB-ELISA (Life Technology) as manufacturers recommendation. The cut-off was determined as: 3 × (mean blank OD ± S.D. of blanks).

For cytokine capture ELISA of IL-4 and IFN-γ, 5 × 10⁵ per single spleen cell suspensions in RPMI-1640 were stimulated with 50 ng/ml recombinant A2 antigen and culture supernatant were collected after 96 h. The concentration of IFN-γ and IL-4 in the resulting supernatant was determined as described previously [36] using biotinylated capture antibody followed by streptavidin conjugated to HRPO (Pharmingen).

Isotype-specific antibodies were purchased from Sigma and antigen mediated ELISA were performed according to suppliers instructions. In brief, 100 ng of recombinant A2 protein in 100 μl were coated over night at 4°C in 0.1 M phosphate buffer pH 9.0 and blocked with 200 μl of 3% BSA in PBST for 1 h at room temperature and washed three times with PBST. Mouse sera (100 μl) diluted to 1:100 in PBST was added to the wells and incubated at room temperature for 2 h then washed three times with PBST. Goat anti-mouse isotype antibodies were incubated at 1:1,000 dilution for 1 h washed again and rabbit anti-goat-HRPO at 1:5,000 dilution was incubated for 0.5 h and the color was developed with TMB-ELISA. All samples were run in triplicates.
2.5. Cell proliferation assay

Single cell suspensions of isolated splenocytes (4 × 10^6 cells/ml) were stimulated with 0.5 μg/ml of recombinant A2 in 200 μl in a 96 well plate at 37 °C, 5% CO_2 for 72 h and pulsed for additional 18 h with 1 μCi of [3H] thymidine per well. The plate was harvested and the amount of incorporated [3H] thymidine was measured in a β-counter. Results are represented as the difference in counts obtained between the A2-stimulated and non-stimulated controls.

2.6. Western blot analysis of A2

The SDS-PAGE (12%) was run with 1 μg of recombinant A2 protein in each lane. The resolved proteins were then transferred to a nitrocellulose filter in the presence of 20% v/v methanol, 25 mM Tris, pH 8.2, 190 mM glycine at 30 V for 12 h. Filters were washed then incubated directly in anti-A2 C9 hybridoma supernatant [30] with 5% milk in PBS-T for 2 h at 22 °C then washed and incubated in the presence of horse radish peroxidase labeled anti-mouse IgG in PBS-T at room temperature for 1 h. The membrane was then incubated in Amersham ECL detection solution for 1 min and then exposed to X-ray film followed by autoradiography.

2.7. Infection of macrophages with amastigotes

Bone marrow derived macrophages (BMMs) were obtained from femurs of 6–8-week-old female BALB/c mice as previously described [19]. Quiescent BMM (10^6 cells/ml) were infected with cultured amastigotes at a ratio of 1:1 amastigote per macrophage for 24 h in polystyrene tubes. The infected BMMs were washed extensively for four times with 50 volume PBS at 900 rpm for 10 min. Internalization of parasites was measured by microscopic count of Giemsa-stained cytocentrifuged slides. The sera were de-complimented by incubating at 65 °C for 2 h in a water bath.

2.8. Statistical analysis

Significance of difference was examined by Student’s t-test using "GraphPad PRISM" (version 3.02) software with 99% confidence intervals and a value of P < 0.05 was considered statistically significant. The P-values and S.E. values reported were determined from the replicate measurements (minimum 3) within each experiment. Each experiment was repeated two or more times with similar outcomes.

3. Results

3.1. Immunization with A2 protein protects mice from L. donovani infection

We initially determined whether immunization with the recombinant A2 protein was protective against infection from L. donovani in BALB/c mice. As described in the introduction, the A2 protein is a L. donovani amastigote-specific gene product which is highly expressed in infected macrophages. Mice were immunized with recombinant A2 protein as described in Section 2 and 3 weeks after the final injection; BALB/c mice were challenged with L. donovani promastigotes. The degree of protection against infection was evaluated by amastigote levels in the liver touch biopsies represented as LDU. As shown in Fig. 1, A2 protein immunization had reduced the LDU by 89% over the control mice or recombinant GST protein-immunized mice (P < 0.0001). These data demonstrate that vaccination with the recombinant A2 antigen provided a significant level of protection against infection.

3.2. High-specific antibody titer generated in mice immunized with A2

The above observations demonstrated that the recombinant A2 protein immunization provided a significant level of protection against infection. It was therefore necessary to characterize the immune response generated against the A2 antigen. To determine the titer of anti-A2 antibodies in each immunized group of mice, an ELISA end point titration was performed. As shown in Fig. 2A, the antibody response against A2 was much higher in the mice immunized with A2 antigen with a reciprocal end point titre reaching 2560 as compared to mice immunized with adjuvant only.

To confirm that the antibody response was generated against A2, the sera (1:500 dilution) were also tested by Western blot analysis against recombinant A2 protein. As shown in the Fig. 2B, the sera from the mice immunized with recombinant A2 protein demonstrated a specific anti-A2 antibody response. These Western blot data confirmed the ELISA results in demonstrating that A2 vaccination did generate a strong anti-A2 antibody response.

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Fig. 1. Infection levels following A2 protein vaccination as determined by LDU. BALB/c mice were immunized with recombinant A2 or recombinant GST protein three times at 3-week intervals as described in Section 2. Three weeks following the final injection, the mice were challenged i.v. with 2 × 10^8 L. donovani promastigotes. Four weeks after the challenge infection, mice were killed and LDU was calculated from liver biopsies. The mean LDU ± S.E. is shown (n = 4 mice per group). This result is the representative of three independent experiments.
3.3. Antigen-specific splenocyte proliferation in the mice immunized with recombinant A2 antigen

We next examined the lymphocyte proliferation response to A2 antigen in a mixed splenocyte reaction as described in Section 2. Lymphocytes from a mixed splenocyte preparation were stimulated with recombinant A2 protein in vitro and thymidine incorporation measured. As shown in Fig. 3, thymidine uptake was much higher in splenocytes collected from mice vaccinated with the recombinant A2 antigen. Immunization with the adjuvant alone or PBS resulted in minimal splenocyte proliferation in response to stimulation with A2 protein. Thymidine incorporation was also negligible over background in the former groups when stimulated with an irrelevant recombinant GST antigen (data not shown).

3.4. Induction of IFN-γ production in response to A2 protein stimulation in splenocytes of immunized mice

It has been established that protection against *L. donovani* infection requires an IFN-γ-activated immune response generated against the parasite [37,38] and production of IFN-γ rather than IL-4 determines the degree of resistance of *L. donovani* infection [39]. We therefore determined whether immunization with the recombinant A2 protein resulted in increased IFN-γ or IL-4 production in response to A2 challenge. As demonstrated in Fig. 4A, splenocytes from mice vaccinated with A2 secreted significantly higher level of IFN-γ (*P < 0.0001*) when stimulated with A2 than splenocytes collected from control mice. Moreover, the release of IL-4 was not significantly higher in the recombinant A2 antigen-immunized mice than control mice following stimulation with A2.

It has been previously shown that IFN-γ production, a marker of Th1 cellular response, directly correlates with a higher IgG2a antibody subclass against the antigen [40], whereas IL-4, a Th2 marker, is associated with generation of IgG1 [41]. We therefore investigated the A2 antigen-specific IgG subclass antibody levels in immunized mice as described in Section 2. As shown in Fig. 4B, all of the A2 antigen-specific IgG subclass titres were significantly higher in mice immunized with recombinant A2 protein than in the control group. These data argue that A2 immunization resulted in stimulating both Th1 and Th2 response against the A2 protein.

Taken together, the A2 antigen immunization data show that the A2 is protective against *L. donovani* infection and was able to stimulate both an antibody response as well as induce IFN-γ production in response to recombinant A2 protein. These data strongly argue that the A2 antigen has the prerequisite characteristics for delivering a protective immune response against *L. donovani* infection.
Fig. 4. Panel A, IFN-γ and IL-4 release assay in splenocytes from A2 protein-immunized mice. Mice were immunized with A2. Splenocytes were stimulated with recombinant A2 for 96 h and concentrations of IFN-γ and IL-4 in the culture supernatants was determined. The data is represented as the mean ± S.E. Each sample was examined in triplicate and these results are representative of two experiments. Note that the IFN-γ and IL-4 are represented on different scales. Panel B, IgG isotype assay. The A2-specific IgG isotype titre was determined by ELISA. The relative subclass titre is represented as OD values and the data is representative of two experiments. Control mice received only adjuvant as described in Section 2.

3.5. Adaptive transfer of splenocytes from A2-vaccinated mice protects against L. donovani infection

Protection against L. donovani infection is thought to be predominantly T-cell mediated as demonstrated by adaptive transfer of immune spleen cells to naive mice [42]. Thus, adaptive transfer of spleen cells from A2-immunized mice was carried out in both BALB/c and C57BL/6 mice. As shown in the Fig. 5, mice demonstrated a significant level of protection when passively immunized with spleen cells from A2-vaccinated mice in comparison to the control group of mice which received spleen cells from adjuvant-immunized mice. The LDU was reduced by 50% (P = 0.0215) and 55% (P = 0.0044) for BALB/c and C57BL/6 mice, respectively. These results confirm that irrespective of the strain of mice, A2 antigen passive immunization imparts significant protection against challenge infection.

3.6. Anti-A2 antibodies and complements block amastigote internalization by macrophages in vitro

BMMs from BALA/c mice represent an appropriate cell type to measure infection by Leishmania in vitro. We

Fig. 5. Infection levels in mice challenged with L. donovani following adoptive transfer of splenocytes from A2-vaccinated mice. BALB/c and C57Bl/6 mice were immunized with A2 protein and 3 weeks following the final boost, spleen cells were collected and transferred to naive mice. One week after the transfer, mice were challenged with L. donovani promastigotes and 4 weeks after the challenge infection, mice were killed and LDU was calculated from liver biopsies. The mean LDU ± S.E is shown (n = 4 mice per group). This result is the representative of two independent experiments.
in human and experimental mice is associated with an increased titre of polyclonal and parasite-specific antibodies [43–46], absence of delayed type of hypersensitivity (DTH), and increased IFN-γ production by PBMCs [37]. Various experimental and clinical studies have shown that parasite-specific cellular responses are required to protect against Kala-azar [47]. With respect to CL, it has been documented that susceptible to infection with promastigotes or amastigotes of L. donovani in infection in the presence of anti-A2 sera (Fig. 6) may indicate a potential role of anti-A2 sera (Fig. 6) in the in vitro macrophage infection with amastigotes in presence of anti-A2 sera (Fig. 6) in the in vitro macrophage infection with amastigotes in presence of anti-A2 sera (Fig. 6) as we observed in this report. Moreover, both Th1 and Th2 responses coexisted in cured VL patients [53,54] and a consistent correlation between IFN-γ production and disease protection in human and experimental VL is observed [38]. These previous findings are consistent with our observations that protection with A2 antigen is due to IFN-γ, TNF-α and IL-2, which results in a recovery from infection [48–51].

Unlike for CL infections, vaccine induced protection against VL in experimental murine model does not correlate with the differential production of Th1 and Th2 cytokines [52] as we observed in this report. Moreover, both Th1 and Th2 responses coexisted in cured VL patients [53,54] and a consistent correlation between IFN-γ production and disease protection in human and experimental VL is observed [38]. These previous findings are consistent with our observations that protection with A2 antigen is due to IFN-γ, TNF-α and IL-2, which results in a recovery from infection [48–51].

As described in Section 1, the A2 amastigote-specific protein of L. donovani is a virulence factor [32]. Electron microscopy of L. donovani amastigotes stained with gold beads conjugated with an anti-A2 monoclonal antibody shows A2 protein throughout the amastigote and also is present on or along the amastigote surface as concentrated patches [J Clos, personal communication]. Results from the in vitro macrophage infection with amastigotes in presence of anti-A2 sera (Fig. 6) may indicate a potential role of A2 in the binding and internalization of amastigotes in macrophages. The internalization of promastigotes into macrophages has been shown to be mediated by mannose receptor [55], fibronectin receptor [56], complement receptor CR1 and CR3 [55,57]. However, internalization of amastigotes into macrophages remains controversial. Some
reports showed no contribution of eponans to the infection of amastigotes [58], whereas other studies have demonstrated role of both Fc receptor and CR3 for macrophage infection [59,60]. However, the in vitro internalization experiment reported within this study may indicate a possible role of A2 in internalization process and thus argue that A2 immunization could protect against inter-macrophage infection and disease progression.

In summary, there are a number of factors which support the argument that A2 proteins could represent an effective vaccine against VL. These proteins contain multiple repeat subunits and are expressed at much higher levels in amastigote stage than in promastigotes [30,32]; A2 deficient L. donovani resulted in a virulent phenotype [32]; A2-specific antibodies are present in Kala-azar patients’ sera [31]. Taken together, the A2 immunization (both direct and passive) shows protection against visceral infection which is associated with IFN-γ production, coexpression of both Th1 and Th2, strong humoral response and reduced internalization of amastigotes into macrophages. These data argue that A2 is a strong vaccine candidate against VL or Kala-azar.

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References


