Assessment of Immunity Induced in Mice by Glycoproteins Derived from Different Strains and Species of Leishmania


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A comparative study was undertaken on the immunogenic properties of 63kDa glycoproteins obtained from five different strains/species of Leishmania and assessed in C57BL/10 mice. The humoral immune response was assessed by ELISA against the five different antigens of the immunized animals. The cellular immune response was derived from Leishmania. The response was found to be species-specific in all of determined by means of the cytokine profiles secreted by the spleen cells of immunized animals. The presence of γ-IFN and IL-2, and the absence of IL-4 in the supernatants of cells stimulated by L. amazonensis antigen established that the cellular response is of Th1 type. The five glycoproteins tested were equally effective in protecting C57BL/10 mice against challenge by L. amazonensis. About 50% of the immunized animals were protected for six months.

Key words: Leishmania - glycoproteins - monoclonal antibodies

Human leishmaniases are diseases caused by several species belonging to the protozoan genus Leishmania. The clinical spectrum induced by different species of the genus ranges from a single cutaneous lesion that may undergo spontaneous cure to mucocutaneous lesions that can cause grossly disfiguring lesions or diffuse cutaneous lesions that are virtually impossible to treat, and various visceral forms that can be lethal.

American cutaneous leishmaniasis (ACL) is endemic in Central and South America (Garrido 1983). Within these areas, disease prevalence is often high and (WHO 1980) increasing. The available chemotherapeutic agents are toxic and are sometimes ineffective even after long treatment periods (Marsden 1985). Transmission control by using insecticides against the insect hosts (Diptera: Phlebotomidae) of the parasite is virtually impossible. Most of the sand fly species associated with ACL have sylvatic habits.

In recent years, there has been increasing interest in the potential of a vaccine as a mean of controlling and containing ACL. Modabber (1989) has pointed out that vaccination is the simplest and most convenient mean of protecting man against infection by Leishmania. Because of the forest-dwelling habits of both the insect and the non-human mammalian host of Leishmania species, vaccination is probably the cheapest long-term mean of control.

Attempts to obtain effective vaccines, and to define their immunogenic elements, have been carried out in several parts of the world. In Brazil, the first attempt to protect a human population against ACL was carried out in the State of São Paulo (Pessoa & Pestana 1940). Although the results obtained were promising, the potential of vaccination was not further explored until Mayrink et al. (1979) prepared a vaccine composed of killed promastigotes of five strains of Leishmania, each one having been isolated in different endemic areas of ACL in Brazil.

Since the results of the first field trial (Mayrink et al. 1979) were published, modifications have been made in the composition of the vaccine and method of administration. Studies have also been undertaken on storage and stability, and the duration of protection in immunized individuals (Mayrink et al. 1979, 1985, Antunes et al. 1986, Nascimento et al. 1990, Costa et al. 1992). Using the Montenegro antigen developed by Melo et al. (1977), the results of these field trials have shown that about 50% of vaccinated individuals are protected against ACL. None of the vaccinated patients developed adverse side effects.

Earlier studies (Costa et al. 1990, Nascimento et al. 1990, Tavares et al. 1990, Guimarães et al. 1992) have shown that the 63kDa glycoprotein...
immunoprotection in mice. However, Steinkraus and Langer (1992) found that the GP63 of different species of Leishmania have different genetic code sequences. Against this background, it became necessary to examine and compare the immunostimulator effect of GP63 derived from each of the five strains incorporated in the vaccine.

This report deals with humoral and cellular immune responses observed in C57BL/10 mice immunized with GP63 isolated from each of the component strains of the vaccine. Levels of protection were assessed by challenging immunized mice with L. amazonensis and comparing cross-reactivity of each strain with anti-GP63 monoclonal antibodies.

MATERIALS AND METHODS

Parasites - The strains and species used in the present study were the five incorporated into the vaccine of Mayrink et al. (1979): L. (L.) amazonensis (IFLA/BR/67/PH8); L. (L.) mexicana (MHOM/BR/60/BH06); L. (Viannia) guyanensis (MHOM/BR/70/M1176); L. sp. (MHOM/BR/71/BH49 - major-like); L. sp. (MHOM/BR/73/BH121 - major-like). Promastigotes were grown in LIT culture medium at 23°C with transfers every 14 days. L. amazonensis was used for the challenge infection. Promastigote cultures in NNN/LIT medium were initiated from biopsies taken from the border of a leishmanial lesion on the snout of experimentally infected golden hamsters. The challenge infection consisted of 1x10^5 promastigotes harvested on the 14th day of cultivation.

Mice - BALB/c and C57BL/10 mice were reared in the colonies maintained at the Institute of Biological Sciences, Federal University of Minas Gerais. Females between eight and 12 weeks of age were used.

Preparation of antigens - Promastigotes of each of the five species/strains of Leishmania were grown, separately, in LIT medium at 23°C for seven days. After centrifuging 1500g for 10 min, promastigotes were washed and resuspended three times in physiological saline. The final sediment was disrupted ultrasonically at 40 watts for 1 min, the process being repeated three times. The protein concentration was determined by the method of Lowry et al. (1951). Proteolytic inhibitors (2mM N-tosyl-L-phenylalanine chloromethyl ketone, 2mM phenylmethylsulfonyl fluoride, 2mM N-p-tosyl-L-lysine chloromethyl ketone), and merthiolate, at a concentration of 0.1 mg/ml were added to each suspension before storage at -70°C.

Production of monoclonal antibodies - Monoclonal antibodies (Mabs) were obtained by the method described by Galfre and Milstein (1981). Two 100µg doses of antigen extracts of each of the five species/strains of Leishmania, combined with 250µg of Corynebacterium parvum, were inoculated subcutaneously, with a seven day interval between doses, into the base of the tail of BALB/c mice. A booster of 10µg of antigen, without adjuvant, was given intravenously 35 days later. Fusions were carried out four days later, using spleen cells of immunized mice and SP2/0 myeloma cells. The fusion agent was polyethylene glycol (MW 1440, Sigma, USA).

Positive hybridomas were cloned by limiting dilution (Handman & Hocking 1982). To obtain large amounts of antibodies, cells were inoculated intraperitoneally into BALB/c mice and ascitic fluid was colleted two to three weeks later. Antibody production was assessed by ELISA on the supernatant of the culture and/or the ascitic fluid, using the method of Engval (1980) and the indirect immunofluorescence technique of Camargo (1966). For both reactions, the conjugates used were rabbit antibodies anti-mouse IgG and anti-mouse IgM, associated, with peroxidase (Sigma-USA) or fluorochrome (Cappel Laboratories).

The isotypic class of the Mabs was determined in the supernatants of cultures of each clone by double immunodiffusion in agarose, using rabbit antibodies anti-IgM of mice and anti-mouse subclasses of IgG (IgG1, IgG2a, IgG2b and IgG3 - Sigma-USA).

Characterization of monoclonal antibodies - Approximately 2x10^7 promastigotes of each strain/species of Leishmania were labelled with 250uci of Na_125^I, using iodogen (Sigma-USA), as described by Lepay et al. (1983). The labelled proteins were dissolved with Triton X-100. Culture supernatants (50µl) containing monoclonal antibodies were added to the marked antigen and kept at 4°C for 24 hr. Thereafter, 50µl of 50% Sepharose-4B was added and the mixture was incubated for 60 min at room temperature. The suspension was washed three times in PBS and resuspended in 30µl of sample buffer (0,125M Tris hydrochloride at pH 6.8, 4% sodium dodecyl sulphate, 10% 2-mercaptopoethanol, 20% glycerol). The samples were applied to 10% polyacrylamide gel which, after electrophoresis, was stained with Coomassie Blue, dried and autoradiographed (Laemmli 1970). The molecular weight of immunoprecipitated proteins was estimated by comparison with molecular weight standards (Sigma-USA).

Purification of GP63 glycoproteins - The antigen extract of each strain/species of Leishmania was purified by an affinity column prepared with CNBr-activated Sepharose CL-4B (Pharmacia-
USA) coupled to the homologous monoclonal anti-GP63. An eluate was made with 200mM of buffered glycine-HCl (pH 2.8). The resultant material was neutralized, lyophilized and then dialyzed against PBS (pH 7.2). The purity of the isolated antigen was assessed by electrophoresis in polyacrylamide gel, stained by the silver method of Wray et al. (1981) and by means of proteolytic activity, using the BApNA substrate method, as described by Erlanger et al. (1961).

**Immunization of mice** - Isogenic C57BL/10 mice were vaccinated by the method of Costa (1986). Each animal received two subcutaneous inoculations, separated by an interval of seven days, each dose containing 20µg of GP63 plus 250µg of C. parvum. Twenty-eight days after the second dose, the animals received a further 2µg of GP63, without adjuvant. Seven days after the booster, the animals were challenged with 1x10⁵ promastigotes of L. amazonensis, inoculated subcutaneously into the base of the tail. The animals were examined at two-week intervals for a total of 180 days after challenge to observe the appearance of lesions and their subsequent development.

**Controls** - The first control group consisted of C57BL/10 mice immunized with the basic vaccine (prepared from all five strains/species of Leishmania). The second included mice that received only C. parvum.

**Humoral immune response** - This was assessed by examining sera obtained 42 days after the first dose of GP63. ELISA was carried out using 2µg of protein/well of the antigens derived from the five strains/species of Leishmania and rabbit IgG, anti-IgG and anti-IgM associated with peroxidase (Sigma-USA).

**Assay for cytokines** - Levels of γ-IFN and IL-4 were determined by immunoenzimatic method in the supernatants of 72-hr cultures of spleen cells (5x10⁶ cells/ml) stimulated, by an antigenic extract of L. amazonensis. For γ-IFN, we used the method described by Scott et al. (1987). For IL-4 quantification, monoclonal antibody anti-IL4 adsorbed on a microplate (2µg/ml - Pharmingen-USA) was used, with 100µl of each sample. Samples of IL-4 remained in the plates for 4 hr at 25°C. The material was washed with saline containing 5% normal calf serum and then incubated for 45 min with 100µl of anti-IL-4 monoclonal antibody biotinylated at a concentration of 1 µg/ml (Pharmingen-USA). Finally, the conjugate avidin-peroxidase (Pharmingen-USA) was added and the preparation was held at 25°C for 30 min. The reaction was revealed by the addition of a buffered substrate containing 150mg/500ml of ABTS [2,2′ Azino-Bis-3-ethylbenzthiazoline-6-sulphonic acid] (Sigma) and hydrogen peroxide, for 10-30 min at 25°C. Absorbance was measured at 405nm.

For IL-2, culture supernatants were examined after 24 hr. Assessments were made using a commercially available kit (Intertest:Mouse Interleukin-2Elisa kit, Genzyme).

**Statistical analysis** - Statistical significance was determined by Student t test, analyzing at least two determinations.

**RESULTS**

**Monoclonal antibodies** - In fusions with extracts from the different strains/species of Leishmania, 196 hybrids were obtained, and these provided 85 clones secreting antibodies. The clones showing the greater reactivity by ELISA were characterized by immunoprecipitation (Fig. 1). 95% of monoclonal antibodies from 85 clones recognized a single antigen with a molecular weight of approximately 63 kDa and 45% of this were specific to homologous strain and for each strain, a single monoclonal antibody was selected. The antibodies, when tested in RIFI against antigens of the different strains of Leishmania, only reacted with the homologous strain (Table I). When we made the crossed immunoprecipitation with the different monoclonal antibodies against the antigen extracte of each of five Leishmania species/strains, all of five monoclonal antibodies reacted only with the homologous GP63.

![Fig. 1: immunoprecipitation of anti-GP63 monoclonal antibodies derived from five species/strains of Leishmania](image-url)
Isolation of GP63 from the different strains of *Leishmania* - The chosen monoclonal antibodies proved to be efficient in isolating different antigens of high purity. Fig. 2 shows the single band for the different purified GP63 of each of the constituent species/strains of the vaccine. The mean return obtained in affinity columns was 60µg of purified antigen for 750mg of applied antigen extract. When we assayed the proteolytic activity of each purified GP63 after they had been incubated on a BApNA substrate, (N\(^{α}\) benzoyl-L-arginine p-nitroanilide chloridrate), with or without a metaloprotease inhibitor (ortho-phenanthrone), we demonstrated that the isolated glycoproteins displayed an intense proteolytic activity that was blocked by the action of ortho-phenanthrone.

Humoral immune response - Fig. 3 shows the levels of IgM and IgG antibodies on the 42nd day after the first immunizing dose given to C57BL/10 mice. In all the groups of vaccinated animals, the levels of IgM antibodies were higher than those of IgG.

Fig. 4 shows the results of cross reactions, by ELISA, between the sera of mice immunized with the purified GP63s and the different strains of *Leishmania*. Analysis of these results reveals a specific humoral response to the tested antigens; in each case, the reaction to the homologous strain was statistically significant (P < 0.05).

Cellular immune response - Table II shows that spleen cells of mice immunized with one of the five different GP63s produced γ-IFN and IL-2, when stimulated in vitro with a total extract of *L. amazonensis*. IL-4 was not detected.
DISCUSSION

Mayrink et al. (1978) showed that extracts of killed promastigotes of *Leishmania*, when injected into human subjects, can induce changes in cutaneous reactions to *Leishmania* antigens. Mayrink et al. (1985) and Antunes et al. (1986) established that the changes in cutaneous responses to *Leishmania* are correlated with the development of immunoprotection in man. Comparable results were obtained in experiments with mice (Costa 1986, Costa et al. 1992). Other studies (Nascimento et al. 1990) showed that the vaccine of Mayrink et al. (1979) induces a humoral response characterized by low levels of IgG but elevation of IgM. In relation to cellular immunity, it has been shown that cutaneous hypersensitivity is due to lymphocyte proliferation following *Leishmania* antigen stimulation and the presence of IL-2 and γ-IFN in the supernatants of tissue cultures of vaccinated individuals (Mendonça et al. 1993).

Analysis of the phenotype of T lymphocytes has revealed a predominance of LT CD8+ in vaccinated individuals but a predominance of LT CD4+ in patients with active lesions (Mendonça et al. 1993).

The vaccine against ACL developed by Mayrink et al. (1979) was composed of five strains/species of *Leishmania* isolated from different parts of Brazil. The strains/species isolated from human subjects were each associated with different clinical forms of leishmaniasis.

Monoclonal antibodies against each of the five components of the originally described vaccine were developed in the present study. The monoclonal antibodies selected for study are those that recognize GP63, known to be an immunogenic protein (Tavares et al. 1990, Burns et al. 1991, Russo et al. 1991, Yang et al. 1991), present in the membrane of promastigotes (Etges et al. 1985, 1986, Chaudhuri & Chang 1988); it is involved in the interaction between promastigotes and macrophages (Chang & Chang 1986, Russell & Wilhelm 1986), and promotes the development of immunoprotection in mice (Costa et al. 1988, Russell & Alexander 1988, Tavares et al. 1990, Yang et al. 1991).

The monoclonal antibodies were tested against promastigote antigens of homologous and heterologous strains by means of indirect immunofluorescence reactions. Those showing marked specificity, that reacted only to the homologous strain, were selected for the isolation of the GP63s of each of the five strains. Isolates with a high level of purity were obtained by affinity chromatography and confirmed by polyacrylamide gel electrophoresis using silver nitrate as the stain. Biochemical
analysis of the purified glycoproteins showed that intense proteolytic activity was due to a metalloprotease, a characteristic of GP63 (Etges et al. 1986, Chaudhuri & Chang 1988, Chaudhuri et al. 1989).

Immunization of C57BL/10 mice with the different GP63 proteins led to the production of IgG and IgM antibodies, especially the latter. A similar observation was made by Nascimento et al. (1990) in studies on the sera of individuals immunized with the vaccine of Mayrink et al. (1978, 1979). In the present study, antibodies in the sera of immunized mice, when assessed by immunoenzymatic methods, showed specific responses to the GP63 of the homologous strain. This is an additional evidence of the high level of purification attainable by the methods used to isolate the antigens.

An important feature in characterizing the responses induced by immunization with purified glycoproteins was the detection of γ-IFN and IL-2 in the supernatants of tissue cultures of spleen cells from immunized mice, after stimulation by extracts of L. amazonensis promastigotes. The cellular immune response to promastigote antigens of L. amazonensis was observed in animals immunized with glycoproteins other than those of L. amazonensis, thus contrasting with the specific responses in humoral immunity.

Convit et al. (1972), Mitchell (1984) and Mendonça et al. (1986) considered that the mechanisms involved in the cellular immune response are of fundamental importance in establishing protective immunity against ACL. The present results confirm the findings of Nascimento et al. (1990) and Russo et al. (1991) by showing that GP63 is the important protein in the protective process and that the greatest part of the cellular immune response to the parasite is undoubtedly against GP63. Despite the evidence of Steinkrauss and Langer (1992) that GP63 is coded by different sequences in different species of Leishmania, the present results reveal that the cellular immune response (at least in C57BL/10 mice) against the glycoprotein is not predominantly directed towards species-specific epitopes.

This is not surprising as GP63 is highly conserved among the species of Leishmania and Russo et al. (1991), Button et al. (1991) have previously reported cross-reactive T cell responses to the complete protein, although cross-reacting epitopes had not been previously defined. This variability at the sites of dominant T cell epitopes may be one mechanism of parasite survival. Although the significant finding that cross-reactive epitopes existed in GP63, it was possible that dominant species-specific epitopes also exist (Russo et al. 1993). This situation has been shown in murine models (Jardim et al. 1990, Yang et al. 1991). In addition, human T cells from individuals with cutaneous, mucosal or cured visceral leishmaniasis respond in vitro to certain peptides of GP63. Antigenic and immunogenic GP63 peptide sequences have been defined, some appearing to be conserved among Leishmania species and others to be species-specific (Russo et al. 1993). When crossed immunoprecipitation was performed, each monoclonal antibody only recognized the homologous GP63, showing that, in fact, species-specific epitopes are conserved among the different GP63 of Leishmania species.

When animals immunized with different GP63s were challenged with promastigotes of L. amazonensis, 50% of the animals, irrespective of the immunizing agent, failed to develop lesions in 180 days. This level of protection is similar to that observed by Costa et al. (1992) after animals had been immunized with the complex vaccine: that is, with a “cocktail” of five strains.

The studies of Champsi and McMahon-Pratt (1988), Lohman et al. (1990) and Tavares et al. (1990) on purified molecules of extracts of promastigotes have shown the existence of other antigens that induce immunoprotection against cutaneous leishmaniasis in mice. Tavares et al. (1990), using an association of 63 kDa and 97 kDa isolated from the vaccine of Mayrink et al. (1979), recorded a higher degree of protection than that obtained in mice immunized with the complete vaccine, when promastigotes of L. amazonensis were used as the challenge.

It can be concluded that purified antigens induce protective immunity against cutaneous leishmaniasis in C57BL/10 mice, that the antigen GP63 is of importance in the development of the immune process, and that there is cross immunity induced by this glycoprotein derived from different species/strains of New World Leishmania species causing human ACL.

ACKNOWLEDGEMENTS

To Dr Rosângela Barbosa de Deus for technical assistance with the GP63 proteolytic activity determination and to Dr Phillip Scott for providing compounds used in γ-IFN analysis.

REFERENCES


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