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Development of mixed Th1/Th2-type immune response in mice following immunization with GP63 from *Leishmania donovani*

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Abstract : The M_r 63,000 glycoprotein (GP63) and lipophosphoglycan (LPG) of *Leishmania donovani* were evaluated as vaccine candidates against visceral leishmaniasis. Mice were immunized with liposome-encapsulated GP63 and/or LPG that were purified from the soluble extract of *L. donovani* promastigotes, and were challenged with virulent amastigotes. Mice immunized with GP63/LPG in liposomes plus BCG resulted in a 27.4% reduction of amastigotes in the liver compared to the control group (liposomes plus BCG), and mice immunized with liposome-GP63 plus BCG failed to induce a protective immune response against the challenge infection. Immunization of mice with GP63 fused to the *Schistosoma japonicum* glutathione S-transferase (GP63-GST) plus BCG also failed to elicit protective immunity. To analyze the cause of failure to induce protection, cytokine release from the spleen cells of immunized mice and *Leishmania*-specific serum antibodies were analyzed. Spleen cells from mice immunized with GP63-GST plus BCG that were exposed to soluble extract of *L. donovani in vitro* produced 10-fold greater quantities of IFN-gamma and 3-fold greater quantities of IL-5 than cells from mice receiving BCG only or saline. Western blot analysis revealed that sera from these mice had *Leishmania*-specific antibodies recognizing 1 to 3 antigens of *L. donovani* with M. W. of 60-65 kDa. Although immunization of mice with GP63-GST induced a strong Th1 response, this study indicated that GP63-GST simultaneously elicited the Th2 response of the CD4+ T-cell, which was known to abrogate the protective immune response conferred by the Th1 effector function.

Key words : *Leishmania donovani*, Th1, Th2, gp63, cytokine

Introduction

Parasites of the *Leishmania donovani* complex, a group of kinetoplastid protozoan parasites including *Leishmania donovani*, *L. infantum* and *L. chagasi*, are the causative agents of visceral leishmaniasis in dogs as well as in humans. Unlike cutaneous or mucocutaneous leishmaniasis, visceral leishmaniasis is often fatal[1]. Although chemotherapy is presently the preferred method for controlling the leishmaniasis in humans and animals, attention has been directed toward the use of immunological methods for the control of the disease.

Several preparations employing defined molecular antigens have been shown to stimulate protective resistance against cutaneous leishmaniasis in murine models. Examples of these include lipophosphoglycan (LPG)[2], M_r 63,000 glycoprotein (GP63)[3,4,5], GP46/M-2 of *L. amazonensis*[6], and amastigote cysteine proteinase (ACP)[7]. Of these, two parasite molecules, GP63 that binds to the complement receptor CR3 on macrophages[8] and LPG which selectively inhibits signal transduction in macrophages[9], are involved in the invasion of the parasites into macrophages in mice. Since GP63 and LPG are shared by both visceral and cutaneous species of *Leishmania*, the question arose whether these antigens would stimulate protective immunity against visceral leishmaniasis in mice. Although several gene products of *L. donovani* have shown to confer protection against experimental challenge[10,11,12], the use of GP63 and/or LPG from *L. donovani* as candidate molecules for protective immunization against visceral leishmaniasis has not been studied, and therefore was evaluated in this study.

The study of murine cutaneous leishmaniasis in resistant

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and susceptible inbred mice provided a model for comparison with human disease. Resistance against cutaneous *Leishmania* for both mice and humans was associated with a T-helper cell type 1 (Th1) CD4+ mediated response, and susceptibility with excessive T-helper cell type 2 (Th2) cytokine production[13]. It has also been reported that the protective immunization of mice against leishmaniasis has shown to require the stimulation of the Th1 lymphocyte population of the CD4+ T-cell subset[5]. However, previous report indicates that a Th1 response is sufficient to protect against cutaneous leishmaniasis, but the induction of a simultaneous Th2 response abrogates the Th1 effector function[14]. Therefore, this study compared the parasite burden in the spleen with the cytokine responses that represent the two T-helper cell types following immunization with GP63 and challenged with virulent promastigotes of *L. donovani*.

Materials and Methods

Parasites and animals

L. donovani MHOM/AF/43/Kh (WR378) was routinely maintained by serial passage in Syrian golden hamsters, *Mesocricetus auratus*, and promastigotes were grown in Schneider's *Drosophila* medium (GIBCO, Grand Island, NY, USA) as previously described[15]. Female DBA-2N and C3H mice (Harlan Sprague Dawley Co., Madison, WI) were housed in the animal resources facility. They were 10 to 15 weeks old when used for experiments.

Vaccine preparations

GP63 and LPG from promastigotes of *L. donovani* were isolated by biochemical methods and reconstituted into liposomes as previously described[3]. Expression of *L. donovani* GP63 protein fused to *Schistosoma japonicum* glutathione S-transferase (GST) in pGEX cloning vector and the purification of the GP63 fusion protein (GP63-GST) was described previously[4]. Viable bacille Calmette Guérin (BCG) was kindly provided by Dr. Barry R. Bloom (Albert Einstein College of Medicine, Bronx, NY).

Vaccination with GP63 and/or LPG and evaluation of resistance to challenge infection

Each experimental group consisted of five female DBA-2N mice. GP63 or LPG, separately or in combination, was incorporated into liposomes as previously described[3]. Twenty micrograms of GP63 and/or LPG in liposomes were administered to each mouse with 2×10^4 particles of live BCG in a final volume of 0.2 m*l* PBS. The experimental groups were inoculated with the following formulae: GP63 liposomes plus BCG (GLB); LPG liposomes plus BCG (LLB); and GP63/LPG liposomes plus BCG (GLLB). Control groups consisted of mice inoculated with empty liposomes plus BCG (LB), or BCG only, or a comparable volume of PBS (vehicle control). Two immunizing injections, total volume 0.2 m*l*/mouse/injection, were administered *via* the intraperitoneal route, three weeks apart. Mice were challenged *via* the intracardial route with 5×10^6 splenic amastigotes of virulent *L. donovani* three weeks after the last immunizing injection. Mice were killed 3 weeks after challenge and the total numbers of amastigotes in the livers were counted as described previously[15,16].

Vaccination with GP63-GST and evaluation of resistance to challenge infection

Experimental groups consisted of ten 10-week-old female C3H mice obtained from Harlan Sprague Dawley (Madison, WI). Each mouse received 50 μg of GP63-GST plus 2×10^4 particles of live BCG in 0.2 m*l* of PBS in each immunizing injection (GGB). Control groups consisted of mice inoculated with BCG only in PBS, or PBS alone. Three immunizing injections, total volume of 0.2 m*l*/mouse/injection, were administered *via* the intraperitoneal route, two weeks apart. Five mice from each group were challenged *via* the intracardial route with 5×10^6 splenic amastigotes of virulent *L. donovani* four weeks after the last immunization. These mice were killed 3 weeks after the challenge and the total numbers of amastigotes in the livers were counted[15,16].

Antigens and mitogens for *in vitro* studies

Promastigotes of *L. donovani* harvested from Schneider's *Drosophila* medium containing 20% fetal bovine serum and 50 μg/m*l* gentamicin were used as the source of leishmanial antigens. Promastigotes were harvested from culture by centrifugation at $2000\times g$ for 10 minutes at 4°C, washed three times in PBS, and were resuspended at 5×10^8 cells in 10 mM Tris and 2 mM EDTA. Parasites were sonicated (Fisher Sonic Dismembrator, Pittsburgh, PA, USA) 10 times on ice for 30-s at 60% microwave settings with 30-s rests in between sonications, then centrifuged at $10{,}000\times g$ for 1 hour at 4°C. Total protein content was measured by BCA protein assay[17] (Pierce, Rockford, Illinois, USA); the soluble promastigote

antigens from the supernatant were subsequently diluted in RPMI 1640 medium to a concentration of 1 mg/m*l* before used. Concanavalin A was purchased from Sigma (St. Louis, MO, USA) and was diluted in RPMI 1640 medium to a concentration of 1 mg/m*l* before used.

Media and chemicals

The medium (complete medium) for spleen cell cultures was RPMI 1640 (GIBCO) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 50 g/m*l* gentamicin, 10^{-5} M 2-mercaptoethanol, 20 mM HEPES buffer, and 10% fetal bovine serum (Sigma).

Lymphokines and antibodies

Murine recombinant IFN-gamma for ELISA standards was purchased from Amgen (Thousand Oaks, CA, USA). The myeloma cell line producing anti-mouse-IFN-gamma mAb R4-6A2 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The R4-6A2 and polyclonal rabbit-anti-mouse-IFN-gamma were produced as described by Harlow and Lane[18]. Rat anti-mIL-5 mAbs TRFK 4-peroxidase conjugate, TRFK 5 and mIL-5 standards were kindly provided by Dr. Robert L. Coffman (DNAX Research Institute, Palo Alto, CA, USA).

***In vitro* production of lymphokines**

Spleen cells were obtained from five mice two weeks after the last immunization, and a cell suspension was prepared by mincing spleens with sterile forceps, which was then followed by passage through a 23-gauge needle. After lysis of erythrocytes by hypotonic shock, spleen cells were washed 3 times and were resuspended in complete medium. Spleen cells were then cultured at 5×10^6/m*l*, 2 m*l* per well in 12-well culture plates (Costar, Cambridge, MA, USA) at 37°C, 5% CO_2 in the presence of 5 μg/m*l* of Con A, or 10 μg/m*l* of soluble extract of *L. donovani* promastigote. Supernatants were collected after 3 days and aliquots were stored at -80°C until used.

Determination of murine IL-5 concentration

Murine IL-5 from the supernatants of spleen cells was measured by ELISA using two monoclonal antibodies against the murine IL-5: TRFK-5 and TRFK-4-peroxidase conjugate as previously described[19].

Determination of murine IFN-gamma concentration

The IFN-gamma ELISA used was a modification of a procedure previously described[20]. Standards and samples were diluted in RPMI 1640 containing 2.5% fetal calf serum (FCS, Sigma) before use, and all samples were assayed in triplicate. Individual wells of 96-well plates (Immulon 2®, Dynatek, Alexandria, VI, USA) were coated with 50 μ*l* of a 2 μg/m*l* preparation of the anti-IFN-gamma mAb R4-6A2 overnight at 4°C. After washing 3 times with PBS containing 0.5% Tween-20, plates were blocked with 1% bovine serum albumin (BSA) in PBS. IFN-gamma standards and test supernatants were then added at 50 μ*l* per well. After a 2-h incubation at room temperature (RT), plates were washed and 50 μ*l* of a 1/2000 dilution of polyclonal rabbit-anti-IFN-gamma was added. After a 2-hour incubation at RT, plates were washed, and 50 μ*l* of a 1/1000 dilution of goat-anti-rabbit-IgG-peroxidase-conjugated antibody (Kirkegaard & Perry, Gaithersburg, MD, USA) was added to the wells, and an one-hour incubation at RT followed. Plates were washed 3 times, and 50 μ*l* of 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS, Kirkegaard & Perry) was added, and plates were read after 30 minutes on an ELISA reader (BioRad EIA Reader, model 2550) at 405 nm. The limit of sensitivity of the ELISA was 0.5 to 1 U/m*l*.

Western blot analysis

Serum for Western blot (WB) analysis was collected from each mouse at three weeks following challenge infection when mice were killed for evaluation of the parasite burden. A 10% resolution and 4% stacking gel was cast basically following the method of Laemmli (1970). The same soluble extract of *L. donovani* promastigotes prepared for the *in vitro* studies was electrophoresed at 200 V for approximately 50 minutes (Bio-Rad Mini Protean Electrophoresis Cell). After electrophoresis, proteins were transferred onto a nitrocellulose (NC) membrane as described previously[21], and the immunoblot was performed as described elsewhere[22] with a slight modification. Briefly, the NC membrane was washed in distilled H_2O and blocked in Blotto[23]. Strips cut from the NC membrane were incubated for 1 hour at RT on a shaker with sera from mice at 1:250 dilution in Blotto. The strips were washed and incubated for 1 hour at RT with a 1:1000 dilution of affinity-purified, peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry) in Blotto. The strips were again washed and reacted at RT with 3,3'-diaminobenzidine tetrahydrochloride (Sigma).

Statistical analysis

Liver amastigote burdens of mice and ELISA data obtained were analysed by an unpaired, two-tailed Student's t-test. Data that resulted in $p<0.05$ were considered significant. Data were expressed as mean ± standard error.

Results

Effect of immunization of mice with GLB and LLB on number of amastigotes

Although DBA-2N mice vaccinated with either the GP63 liposome plus BCG (GLB) or LPG liposome plus BCG (LLB) contained a somewhat lower number of amastigotes in the liver than the empty liposomes plus BCG (LB), a significant reduction in liver amastigotes was only observed in mice immunized with the combination of GP63 and LPG liposome plus BCG (GLLB) when compared to LB (Fig 1, $p = 0.038$). The reduction, however, was marginal in that only 27.5% reduction was observed. The LLB or GLB did not elicit significant reduction of parasites compared to LB. Since the mean number of amastigotes in the livers of mice vaccinated with LB was not significantly different from the number of liver amastigotes in the mice vaccinated with BCG only, liposome incorporation of vaccines was omitted in the subsequent experiment using GP63-GST.

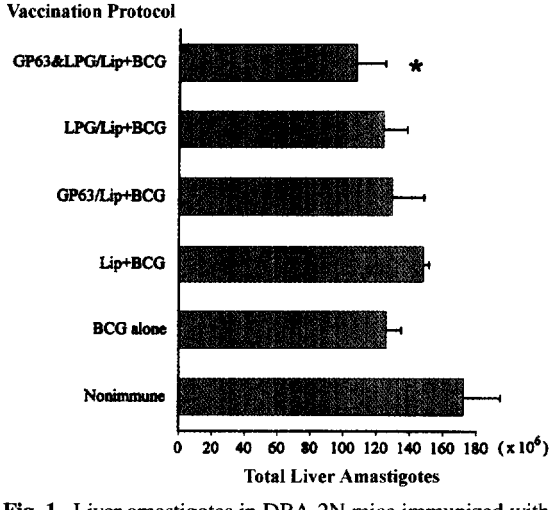

Fig 1. Liver amastigotes in DBA-2N mice immunized with the biochemically purified GP63 and/or LPG incorporated into liposomes plus BCG. GLLB: GP63&LPG in liposomes +BCG, LLB: LPG in liposomes + BCG, GLB: GP63 in liposomes + BCG, LB: Empty liposomes + BCG.
*: Significant reduction in liver amastigote burden compared to Lip + BCG. *Error bar* = 1 standard error, n = five to six mice.

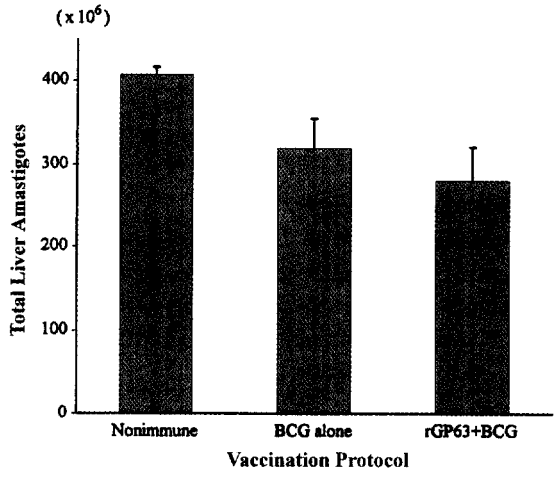

Fig 2. Liver amastigotes in C3H mice immunized with GP63-GST plus BCG (GGB). *Error bar* = 1 standard error, $n = 5$ mice. GGB: GP63-GST + BCG.

Although immunization of mice with the GP63-GST plus BCG (GGB) resulted in a 31.5% reduction in the liver amasitgote numbers compared to the vehicle control (Fig 2, $p = 0.01$), the reduction was not statistically significant when compared to the BCG control. BCG alone elicited a 21.7% reduction ($p = 0.02$) compared to the vehicle control, which received PBS alone. The mean liver amastigote burden of mice receiving GGB, BCG alone, and vehicle alone were 278.8± 43.6, 318.5 ± 38.1, and 406.7± 10.7× 10^6 amastigotes/liver, respectively.

Effect of immunization with GGB on IFN-gamma and IL-5 production

The failure of the defined molecular antigens to induce protection from the challenge infection with *L. donovani* led us to examine whether the administration of the molecular antigens stimulated the immune system. This was done by measuring lymphokine production from the spleen cells and the serum antibodies of mice immunized with GGB.

In the absence of any stimulus *in vitro*, the supernatant from the spleen cells of mice immunized with GGB contained significantly higher amounts of IFN-gamma ($p<0.01$) than those from mice receiving BCG alone or PBS (Fig 3A). The cultured supernatant of the spleen cells from mice immunized with GGB in the presence

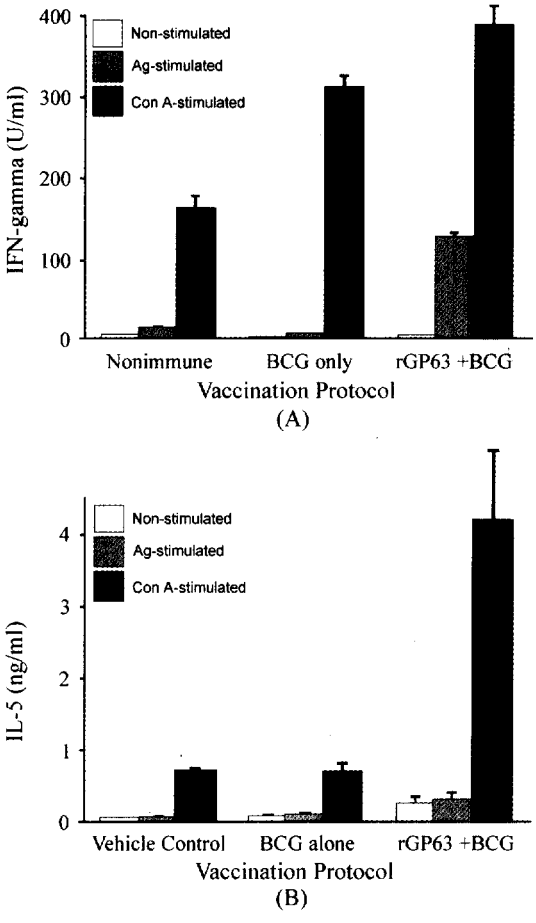

Fig 3. Secretion of IFN-gamma (A) and IL-5 (B) by spleen cells of C3H mice immunized with GP63-GST and/or BCG. Vertical bars represent standard error in ELISA assay. GGB: GP63-GST + BCG.

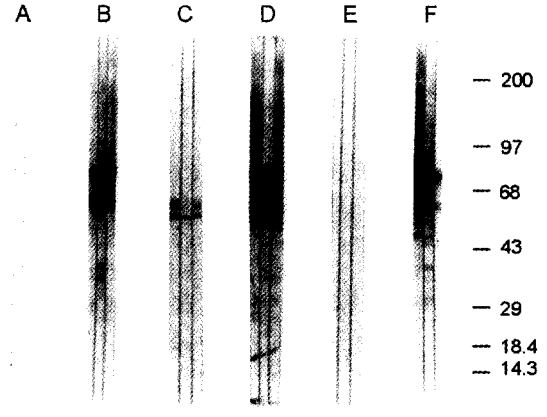

Fig 4. Antibody responses following immunization of mice with GP63-GST plus BCG. Soluble extract of the *L. donovani* promastigote was employed as antigenic protein. Lane A: Non-immunized, non-infected; B: non-immunized, challenged; C: immunized with GP63-GST/BCG, non-challenged; D: immunized with GP63-GST/BCG, challenged; E: immunized with BCG alone, non-challenged; F: immunized with BCG alone, challenged. Three representitive mice per group were shown.

of *L. donovani* sonicate promastigote antigens (10 μg/ml) contained more than 10 times IFN-gamma than those receiving the BCG or PBS ($p<0.01$). Non-specific activation of spleen cells producing IFN-gamma in the presence of Con A at 5 μg/ml was also the highest in spleen cells from mice immunized with GGB, compared to those of mice immunized with BCG or vehicle alone ($p<0.02$).

As shown in Fig 3B, supernatants from the spleen cell cultures also contained slightly increased levels of IL-5. Without any stimulation *in vitro*, supernatants of spleen cells from mice immunized with GGB contained approximately four times as much IL-5 as those from mice receiving either the the BCG alone ($p=0.03$) or vehicle only ($p=0.02$). In the presence of sonicate promastigote antigens, the results indicate a three-fold increase in the IL-5 production in cells obtained from mice receiving GGB when compared to IL-5 production by those receiving the BCG alone ($p=0.04$) or vehicle only ($p=0.02$). The potential of spleen cells to produce IL-5, as demonstrated by Con A stimulation of spleen cells, was six times higher in the cells from the group receiving GGB than the groups receiving BCG alone ($p<0.01$). Examined together, results indicated that spleen cells of the mice receiving GGB released significantly greater amounts of IL-5 into the medium than cells from those mice receiving BCG alone or vehicle only.

Antibody response of mice immunized with molecular antigens

Sera from mice immunized with GGB obtained prior to challenge with virulent amastigotes contained antibodies that recognized several soluble promastigote antigens of *L. donovani*, as illustrated by immunoblotting (Fig 4). Specific antibodies to *Leishmania* promastigote antigens from sera of mice immunized with BCG alone were not recognized. However, sera from the mice immunized with the GGB contained *Leishmania*-specific antibodies responding to 1 - 3 promastigote antigens at 60 - 65 kDa. As expected, sera from all mice immunized with BCG and/or GP63-GST contained antibodies to numerous

antigens of the promastigotes subsequent to challenge with the virulent parasites.

Discussion

The use of GP63, GP63-GST, or LPG from the promastigotes of *L. donovani* as vaccines against visceral leishmaniasis in mice was evaluated in this study. The stimulation of the immune system by these vaccines was monitored by Western blotting of serum antibodies, production of IFN-gamma and IL-5 from the spleen cells of immunized mice. While both GP63 and GP63-GST inoculated with BCG elicited only marginal resistance upon challenge, our results indicated that the immunization procedure resulted in increases of both IFN-gamma and IL-5.

Unlike observations with visceral leishmaniasis presented in this study, some previous workers have reported that in cutaneous leishmaniasis, biochemically purified GP63 induced protective immunity against *L. mexicana* infection in mice[3] and LPG obtained from *L. major*-induced resistance against *L. major* infection in mice when given with killed *Corynebacterium parvum* as an adjuvant[2]. The GP63 gene of *L. major* transformed into the AroA$^-$ vaccine strain of *Salmonella typhimurium* stably expressed the GP63 antigen (SL3261-GP63) *in vitro*, and the construct conferred significant resistance against a challenge *L. major* infection to mice[5]. On the other hand, some previous workers have not been successful in the immunization of mice with molecular antigens of *Leishmania*. For example, attempted immunization with recombinant GP63 of *L. major* with *C. parvum* as an adjuvant failed to protect mice against challenge infection[4].

Since GP63 and LPG appear to be present in all species of *Leishmania*, the reasons for some reported immunization successes with these molecules as well as some failures is not known. Several factors are probably involved such as the method of preparation of the molecular antigens, the adjuvant used, quantity of antigen administered, and route of administration of antigen. Although little evidence of protective immunity was obtained in this study, the data indicate that the vaccination procedures with GGB stimulated both Th1- and Th2-like responses since an increase in the *in vitro* production of both IFN-gamma and IL-5 by spleen cells obtained from experimental mice was noted four weeks after the last immunization. These observations are of some interest since immunization with synthetic peptides derived from GP63 or recombinant GP63 of *L. major*, which elicited significant resistance against homologous challenge infection preferentially induced the Th1-derived cytokines (IL-2 and IFN-gamma, but no IL-4 production) from spleen cells of immunized mice prior to challenge[5,24].

The Th1 subset of T cells have been strongly associated with protective immunity against cutaneous leishmaniasis by a number of investigators[13,25,26], and previous reports indicate a significant role for these cells in protective immunity to visceral leishmaniasis[27]. However, the induction of a simultaneous Th2 response abrogated the Th1 effector function in an experimental vaccination with plasmid DNA encoding the host-protective *Leishmania major* parasite surface Ag-2 primed for an essentially exclusive Th1 response that protected mice against *L. major* infection[14]. Therefore, it is likely that the co-stimulation of both Th1 and Th2 responses, as shown in this study, appears to have caused the failure of protection against *L. donovani* infection in the immunized mice.

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내장리슈만편모충 유래 GP63 항원을 마우스에 접종한 후 관찰되는 Th1/Th2-type 복합 면역반응

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국문초록 : 병원성 내장리슈만편모충(*Leishmania donovani*)에서 추출한 GP63 또는 LPG 항원을 liposome으로 캡슐화하고 보강제로서 BCG를 조합하여 DBA-2N 마우스에 면역접종을 한 후, 내장리슈만편모충의 병원성 amastigote를 접종하여 이들 물질의 방어면역 효과를 관찰하였다. 그 결과 GP63과 LPG, 그리고 BCG를 모두 첨가하여 접종한 마우스의 간 조직에서 유의성 있는 내장리슈만편모충의 감소가 관찰되었으나 감소율은 27.4%에 불과하였다. 실험적 피부리슈만편모충증에 대하여 성공적인 방어면역성을 나타낸 GP63이 내장리슈만편모충 감염에 대하여 방어면역성을 상실한 원인을 분석하기 위한 실험에서 C3H 마우스에 GP63-GST 단백질과 BCG를 혼합하여 면역접종하고 내장리슈만편모충의 병원성 amastigote로 접종한 후, 혈청 내 특이항체와 비장세포에서의 감마인터페론 및 IL-5의 생산을 관찰하였다. 그 결과 GP63-GST와 BCG를 혼합하여 면역 접종한 마우스의 간 조직에서도 유의성 있는 amastigote의 감소는 관찰되지 않았다. 한편 이들 마우스의 비장세포에서는 BCG 만을 접종한 군에 비해 10배 이상의 감마인터페론과 3배의 IL-5가 생산되었다. 이와 같은 사실은 GP63-GST 단백질과 BCG를 혼합하여 접종한 마우스에서 Th1 및 Th2 타입 면역반응이 모두 활성화되었음을 시사하며, Th1 뿐만 아니라 Th2 타입 면역 반응도 함께 활성화된 것이 실험적 내장리슈만편모충 감염에 대한 방어면역에 실패한 원인 중의 하나일 것으로 사료되었다.

Key words : *Leishmania donovani*, Th1, Th2, gp63, cytokine