SEARCH FOR SURROGATE MARKER(S) OF IMMUNITY FOLLOWING VACCINATION WITH EXPERIMENTAL VACCINE (AUTOCLAVED *LEISHMANIA MAJOR* + BACILLE CALMETTE-GUÉRIN) IN HUMAN VOLUNTEERS

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Cutaneous leishmaniasis (CL) is usually a self-limiting lesion on the skin while visceral leishmaniasis is a progressive, systemic disease with high mortality even if treated. The problem associated with treatment and vector control justifies a search for an effective vaccine which seems to be the only practical means to control the disease. The aim of this study is to identify immunological surrogate marker(s) associated with protection against *Leishmania* infection. In a double blind randomized vaccine efficacy field trial, volunteers were injected with either autoclaved *Leishmania major* (ALM) mixed with *Bacille* Calmette Guerin (BCG) or BCG alone. Parasitologically proven cases of the disease in an early stage of infection from the following groups were selected: injected volunteers (ALM+BCG/BCG), leishmanin skin test (LST) positive individuals, non healing cases of CL and as controls: uninfected cases, cured individuals of CL and ALM+BCG/BCG injected individuals with no lesion.

The *in vivo* and *in vitro* immune responses against *Leishmania* antigens such as proliferation response of peripheral blood mononuclear cells (PBMCs) and cytokine production, Interferon-a and interleukin 4 against *Leishmania* antigen (SLA) were evaluated and compared with *in vivo* leishmanin skin test (LST).

The ALM+BCG injected cases exhibited higher proliferation response and interferon-a production compared to the BCG injected or uninfected group. Individuals with a history of CL and cases who were originally LST positive exhibited the strongest *in vitro* T cell response and interferon-a production compared to all other groups. Non-healing cases of CL showed the least proliferation response and interferon-a production with a relatively high level of interleukin-4.

The results indicate that a single dose of ALM+BCG induced Th1-like response but the level of such response is not sufficient for full protection. Accordingly, further evaluation of the vaccine is necessary other strategies multiple injections or changing the adjutant.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
ACL	anthroponotic cutaneous leishmaniasis
APC	antigen presenting cell
ACL	American cutaneous leishmaniasis
BCG	Bacillus Calmette Guerin
CL	Cutaneous leishmaniasis
DCL	Diffuse cutaneous leishmaniasis
DMSO	Dimethylsulfoxide
DTH	Delayed type hypersensitivity
FBC	Fetal bovine serum
FCS	Fetal calf serum
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp63	Glycoprotein 63
HBSS	Hank's balanced salt solution
i.d.	Intradermal
i.p.	Intraperitoneal
i.v.	Intravenous
IFN-a	Interferon gamma
IL	Interleukin
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
MHC	Major histocomatibility complex
MCL	Mucocutaneous leishmaniasis
mRNA	messenger RNA
NK	Natural killer
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PHA	Phytohemagglutinin
PKDL	Post kala-azar dermal leishmaniasis
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
s.c.	Subcutaneous
S.D.	Standard deviation
S.E.	Standard error
scid	Severe combined immune deficiency
SDS	Sodium dodecyl sulphate
SLA	Soluble *leishmania* antigen
TBS	Tris buffered saline
TCR	T cell receptor
TNF-α	Tumor necrosis factor alpha
TGF-β	Transforming growth factor beta
Th	T helper
T_s	Suppressor T cells
VL	Visceral leishmaniasis
ZCL	Zoonotic cutaneous leishmaniasis

CHAPTER 1

INTRODUCTION

Leishmaniasis is a parasitic disease with many distinctive manifestations ranging from a self healing cutaneous leishmaniasis to a lethal visceral form of infection. The disease is an important public health problem in more than 80 countries most of which are developing ones. Since neither vector nor reservoir control is cost effective or fully successful, treatment is expensive, painful and is not always feasible. Thus, it seems that the sole control strategy is to search for an effective vaccine. There are reasons to believe that an effective vaccine against leishmaniasis is feasible.

1. A protective immunity is usually induced following infection in an endemic area.

2. Often the disease is more severe in new-comers to an endemic area.

3. Natural infection can be prevented by leishmanization.

Under Worth Health Organization/Tropical Diseases Research (WHO/TDR) supervision, an autoclaved *Leishmania* vaccine (ALM) is currently being evaluated in endemic areas,

locally and abroad. Since field trials on this vaccine are currently underway in Iran, it was a very good opportunity to monitor the immune responses of the participants and search for immunological parameters which could be used as marker(s) for protection. This information would be valuable for further vaccine development programs.

Leishmaniasis

Leishmaniasis is caused by a protozoan parasite of the genus *Leishmania*. Infection occurs when a female sandfly transmits the promastigote stage of the parasite to a susceptible mammalian host. The parasite then invades macrophage, transforms to amastigote and divides, eventually rupturing the cell and invading other macrophages. This leads to a spectrum of clinical manifestations ranging from a self healing cutaneous lesion to a severe visceral form with very high mortality.

The disease is endemic in the tropical regions of America, Africa and the Indian sub-continent and in the sub-tropics of south-west Asia and the Mediterranean. The total number of infected people in the world is estimated to be 12 million with 350 million at risk, of whom 1.5 to 2 million will be infected annually (161).

Clinical forms

Leishmaniases depend upon the species involved and the host immune response presents a spectrum of manifestations including cutaneous, mucocutaneous, diffused cutaneous, visceral (kala azar) and post-kala-azar dermal leishmaniasis (137). Although the different spectrum of the diseases are not exclusive to any one species of the parasite, certain *Leishmania* parasite tend to be associated with a specific type of the disease.

Cutaneous leishmaniases

Cutaneous leishmaniasis (CL) is generally a mild localized lesion which heals spontaneously even with no treatment and often leaves a disfiguring scar. There are two types of CL: zoonotic cutaneous leishmaniasis (ZCL) which is normally passed on from animal reservoirs and is limited to areas where the reservoirs exist, dog, rat, fox, wolf, jackal, raccoon and other rodents have all been found to carry the parasites, and anthroponotic cutaneous leishmaniasis (ACL) in which the reservoir is human. In ZCL, incubation period and course of infection is shorter than in ACL.

CL is usually characterized by one or more lesions which might develop to a wide spectrum of skin involvement. New World CL is caused by members of the *L. braziliensis* and

L. mexicana complexes while Old World CL is caused by *L. major, L. tropica,* and *L. aetiopica* (4). *L. infantum* usually causes visceral leishmaniasis in infants and children but CL cases have been reported by this species in adults without visceral involvement, but the evolution of the lesion is slower and milder than those observed with *L. major* infections (23,78). The incubation period varies from a few weeks to several months. CL lesion begins as a small erythematous papule on an exposed areas of the body where infected sandfly vector have fed. After a few weeks the papule becomes a furunculoid lesion that ulcerates, but the ulcerated nodule may heal, without developing into other clinical forms and leave a slightly depressed scar. Certain diagnostic clinical features have been described such as volcanic nodules, satellite papules and subcutaneous nodules (137). The nodule usually ulcerates in a volcanic form, the ulcer may remain relatively dry (dry form) or it may exude seropurulent material (wet form, (163).

Mucocutaneous leishmaniasis (MCL)

Mucocutaneous leishmaniasis (MCL) is caused most commonly by members of *Leishmania* subgenus *Viannia,* particularly *L. V. braziliensis* and *L. V. panamensis.* MCL is reported with greatest frequency in Brazil, Bolivia, Ecuador, Peru, and other countries of northern and central

south America. The incubation period lasts from two weeks to many years and the lesion(s) involves the mucosa, soft tissue and cartilage of the upper respiratory tract most commonly the nasal septum (41). Virtually all patients with MCL have a history or a typical scar of previous cutaneous lesion. Mucosal involvement generally does not occur until the initial skin lesion(s) are healed often many years later. The average time between the cutaneous lesions and mucosal involvement has been reported to be between two to six years (95).

Visceral leishmaniasis (Kala azar)

Visceral leishmaniasis (V.) is a generalized form of *Leishmania* infection in which organisms multiply in the liver, spleen, bone marrow and lymph nodes. In India and Africa visceral leishmaniasis caused by *L. Donovan* is seen in all age groups (152), but in southern Europe, Iran and countries bordering the Mediterranean V. is caused by *L. infantum* and affects primarily young children and infants. In the western hemisphere V. is mainly a disease of very young children where the causative organism is *L. L. chagas* (5). The incubation period of visceral leishmaniasis is usually one to three months but can be as short as a few weeks. The disease is characterized by fever, anemia, hepatosplenomegaly, leukopenia which may be complicated by

serious infections and bleeding with high mortality even in treated subjects. V. is often accompanied by other diseases such as tuberculosis, pneumonia and diarrhoea (5). There are now well-documented instances in which unsuspected latent infections of unknown duration have been activated under conditions of immunosuppression. Kala azar is usually caused by *L. Donovan* complex but has also been associated with *Leishmania mexicana amazonensis* in the New World (foci in several areas of Brazil, Venezuela, and Colombia, and isolated cases in Central and South America), (152). *L. tropica* can also cause V. as reported in U.S. troops of Operation Desert Storm in Kuwait (51). The clinical presentations of kala azar are similar with different epidemiological features (163).

Diffuse Cutaneous Leishmaniasis (DCL)

Diffuse Cutaneous Leishmaniasis (DCL) is an uncommon presentation form of cutaneous leishmaniasis which occurs when the immune system fails to effectively respond to the parasite. The disease is caused most commonly by *L. aethopica* in the Old World (Ethiopia and contiguous countries) and *L. m. mexicana* in the New World (Venezuela, Guatemala, Brazil, the Dominican Republic and Mexico (24). DCL starts as a regular cutaneous lesion, but the nodule does not ulcerate and new nodules progress at the lesion

site, new nodules commonly develop on the face of the patient (Africa), or on the whole body (South America), the entire face could be covered by nodules similar to lepromatous leprosy. The lesions never heal without treatment, and the great majority of the cases relapse even after treatment (24).

Post Kala-azar Dermal Leishmaniasis

Post kala-azar dermal leishmaniasis (PKDL) is a known complication of visceral leishmaniasis in about 2-8% of V. patients from India, 10-12% from Kenya and 30-50% from Sudan. However, a number of cases has been reported in the absence of previous history of kala-azar (131,132). PKDL is characterized by the occurrence of macules, papules or nodules in the skin, mostly affecting the face, but it may involve other parts of the body (55). Some patients with PKDL heal spontaneously during the first six months, while other cases become chronic and the disease may continue for up to 10 years. Although some investigators believe that the development of PKDL is due to immunoregulatory mechanisms which occur in cured kala-azar patients but it is not clear how the immune responses influence the pathogenesis of the disease (91).

Parasite

There are over 20 known species of the *Leishmania* parasite and most of them are associated with various forms of leishmaniasis. The parasite has two main forms in its life cycle, an intracellular amastigote form within phagolysosomes of mammalian host monocyte-macrophages lineage and an extra-cellular promastigote form in the alimentary tract of the vector. In the gut of the sandfly vector, five developmental stages can be recognized namely procyclic promastigotes, nectomonad promastigotes, heptomonad promastigotes, paramastigotes and metacyclic promastigotes (155,100).

Life Cycle in the Insect Vector

The parasites are transmitted by approximately 70 of the 600 species of mosquito-like phlebotomine sandflies (*Phlebotomus* in the Old World, *Lutzomya* in the New World),(102). Female sandfly become carrier by feeding on infected mammalian host, the ingested amastigotes develop into promastigotes in the insect gut and then move to the salivary gland. When the female sandflies take a blood meal from an infected host, a minute amount of blood containing lymphocytes and infected macrophages are ingested, the number of amastigotes injected is thought to be extremely low even when the flies engorge directly on cutaneous lesion

(4). Engorgement is quickly followed by the production of peritrophic membrane which is secreted by the epithelial cells lining the midgut. This membrane retains the developing promastigotes during the first 72 hours and there is evidence that some promastigotes become embedded in it. As digestion proceeds the membranes break up and swimming promastigotes escape to initiate the establishment of infection in the midgut or in the *L. brazilensis* group in the hindgut. An association of the flagella with the microvilli of the midgut or an attachment to the cuticular entema of the hindgut (pylorus and ileum) are characteristic of the replicating forms of *Leishmania* in the sandfly. The initial establishment of the infection in both instances is followed by an anterior migration to the thoracic midgut, attachment to the esophageal valve and invasion of the mouthparts (esophagus, pharynx and proboscis). The growth and movement of promastigotes in the midgut are accompanied by their differentiation from a non-infective stage to a stage that is highly infective to a susceptible vertebrate host (168). The infective forms are referred to as "metacyclic" promastigote which are seen in the proboscis and are undoubtedly deposited in the skin when the sandfly bites. The saliva of sandflies has several potent pharmacologically active substances and has been reported to enhance the survival of inoculated promastigotes (186).

Life Cycle in the Mammalian Host

Humans usually acquire *Leishmania* infection when infected female sandfly takes a blood meal which is required for sandfly reproduction. During the sandfly bite infective stage "metacyclic" promastigotes are released and injected along with a potent vasodilator (157). Promastigotes bind to specific receptors on tissue histocytes which permit the entry in the cell. Within the host cells, promastigotes reside in a parasitophorous vacuole, which then fuses with secondary lysosome to form a phagolysosome where the organism survives, transforms and replicates by binary fission. *Leishmania* species are unique in that they allow fusion of phagosome with lysosomes to make phagolysosome where they can safely survive and divide. Transformation of promastigotes to dividing amastigotes is completed within 24 hours after phagocytosis and the parasites become rounded and lose their flagellum. Eventually the infected macrophages lyse, thereby releasing amastigotes which can infect additional macrophages. The life cycle is completed when the parasitized macrophages from the infected person or reservoir animal are ingested by the sandfly during feeding.

Genetic Regulation of Experimental Leishmaniasis

In the mammalian host *Leishmania* reside and multiply within macrophages and dendritic cells. Macrophage

activation leads to enhanced intracellular killing of the *Leishmania* parasite, in particular the inducible nitric oxide synthase gene (*Nos2*) induces the generation of a large amount of nitric oxide (NO) which appears to be the major effector mechanism in *Leishmania* infection (68,111). Given the broad spectrum of disease phenotypes in human leishmaniasis, one might predict that a genetic defect at a specific key point in the macrophage activation pathway leading to activation of different T-cell subsets, contributes to susceptibility and different clinical manifestations. This can be clearly demonstrated in murine models of leishmaniases, as it is shown in inbred and congenic mouse strains infected with *L. donovani*.

Several different mouse (host) genes control the infection of distinct species of *Leishmania*. In *L. donovani* infection, two stages of the disease have been identified with different genes involvement in regulation of each: **a.** Early phase of infection which is controlled by a non MHC-linked gene termed *Lsh* (21), susceptible mouse strains expressing Lsh^s allele are deficient in controlling initial parasite replication in the macrophages (42) while resistant mouse strains expressing Lsh^r allele are capable of controlling the initial phase of replication. **b.** Later phase of infection (infection that lasts for more than two weeks in mice with Lsh^s allele) which is controlled mainly

by MHC-associated genes (*H-2*) and also by genes at H-11 and Ir-2 positions (14,15,48). In murine experimental leishmaniasis with *L. major* and *L. mexicana*, the distinction between innate and later phase of genetic regulation is less clear. The susceptibility of inbred mice to *L. major*, infection is controlled by *Scl-1*, *Scl-2* and *H-11* linked genes. So in considering disease pattern in both *(L. donovani* and *L. major)* infection, it is possible to map the susceptibility genes into five different regions of the genome (129,164).

***Lsh/Ity/Beg* Gene/Mouse chromosome 1**

The murine macrophage resistance gene, *Ity/Lsh/Beg*, was described for its role in the early control of *Salmonella typhimuriumin*, termed *"Ity"*, Mycobacterial infection including *Mycobacterium bovis*, termed *"Beg"* and *Leishmania donovani*, termed *"Lsh"* (22,158,178). This gene provided the most clear-cut demonstration of single gene Mendelian inheritance of susceptibility to infection. Mouse strains which are Lsh^s have a single base pair change from the Lsh^r gene found in resistant mice. The expression of natural resistance associated macrophage protein (designated *Nramp1*) depend on the same gene. The gene has been cloned (191), and functional studies indicate that *Nramp1* regulates early pathway of macrophage priming and activation for

antimicrobial activity. Therefore, the gene has many effects on macrophage function including regulation of the early response gene *KC* which is a neutrophil chemoattractant belonging to the C-X-C family of small cytokines as well as TNF-α, IL1-β, iNOS and MHC class II expression. This upregulation leads to a higher level in macrophage resistant (16,18). Although *Lsh* gene control is important in *L. donovani* infection, it is not involved in the genetic control of *L. major* infection, as both Lsh^r and Lsh^s strains are found to be equally susceptible to *L. major* infection (128). It should be mentioned that studies with *L. donovani* in mice were done by *i.v.* injection of amastigotes, which is not a natural route of infection. *L. major* studies were done using promastigotes injected by *i.d.* route which is more like natural infection, and this could account for differences seen (Modabber, F., WHO/TDR, personal communication).

MHC (*H-2 linked*) Gene/Mouse chromosome 17

The later phase of murine *L. donovani* infection in homozygous recessive Lsh^s mouse strains is controlled mostly by MHC-associated (*H-2 linked*) genes (14). With respect to the parasite load in the liver three different disease patterns are detected in Lsh^s mice: mice with $H\text{-}2^s$ or $H\text{-}2^r$ haplotype cure disease early, mice with $H\text{-}2^b$ haplotype cure

eventually, while mice with H-2^d, H-2^q or H-2^f haplotype do not cure. The genes controlling these responses map to I-E and a subregion to the left presumably I-A, therefore polymorphism in class II MHC molecules is directly responsible for disease manifestation (14).

Th-2 cluster (*H-11 linked*) Gene/Mouse chromosome 11

Studies with congenic strain of B10 mouse (B10.129) which carries alternative alleles at the more proximal region of mouse chromosome 11 revealed that the normal cure phenotype of B10 mouse had converted to a non-cure phenotype (15). This region which controls the later phases (>15 days) of infection contains a cluster of T helper 2-related cytokine genes including IL-4, IL-5, IL-9. These genes have not been cloned yet, however studies in man have identified polymorphism in IL-4 and IL-9 genes associated with susceptibility to asthma (165). Unlike *Lsh*, expression of the *H-11 linked* genes has a similar effect on both *L. donovani* and *L. major* infection (15).

***Scl-1* Gene/Mouse chromosome 11**

Infection of inbred mouse strains with *L. major* revealed that BALB/c mouse strain is extremely susceptible to the infection and ultimately succumb. This extreme susceptibility maps to a single autosomal gene which is not

linked to *H-2* and referred to as the "susceptibility to cutaneous leishmaniasis" gene *(Scl-1)*. The *Scl-1* gene has been mapped to the distal region of mouse chromosome 11 (129,165). Expression of *Scl-1* results in a primary macrophage defect, allowing increased parasite multiplication *in vitro* in skin macrophages from susceptible mice and differences in the ability of these macrophages to process and present antigen (165). This region carries the inducible nitric oxide synthase (*Nos2*) gene, *Nos2* is known to be important in generating NO for anti-*Leishmania* activity in activated macrophages (129).

Scl-2 Gene/Mouse chromosome 4

Scl-2 gene is associated with the phenotype of DBA/2 mice which are resistant to infection with *L. mexicana* and is found on chromosome 4 as a gene controlling "no lesion growth" phenotype (164). The gene for Janus tyrosine kinases (JAK) which play a key role in the IFN-γ-mediated macrophage activation pathway, is located in the same region in mouse and man (129).

Immune Response to Experimental Leishmaniasis

The murine model of leishmaniasis using inbred mice which either are resistant (such as C57BL/6 or C3H/HeJ) or are susceptible (such as BALB/c and P/J) to infection has

provided an excellent opportunity to investigate disease resistance and susceptibility. When the susceptible strain of mice is infected with *L. major,* a primary lesion develops and disease progresses to multiple lesions and systemic *Leishmania* infection and eventually kills the animal. This type of infection is similar to human visceral leishmaniasis (kala-Azar), while injection with *L. major* of the resistant strain of mice results in a cutaneous leishmaniasis which heals spontaneously and animals are resistant to reinfection similar to human cutaneous leishmaniasis (74). Further studies showed that the outcome of the disease in the murine model depends on the predominant T-helper cell (Th) subset (138). In C57BL/6 strain which is resistant to *L. major* infection, the outcome of the disease has been shown to be correlated with the proliferation of $CD4^+$ Th1 lymphocytes which produce IL-2, IFN-γ and stimulates cell mediated immunity. In susceptible strain of mice (BALB/c) infection with *L. major* results in the generation of $CD4^+$ Th2 lymphocytes which produce IL-4, IL-5, IL-10 and stimulates humoral immune response particularly production of IgE and IgG1 (80,133).

Humoral Responses *vs.* Cell-Mediated Immunity

The generation of cell-mediated immunity has been shown to be essential in protection against *Leishmania* infection

while humoral response is not considered to play an important role.

Anti *Leishmania* antibodies participate in complement mediated lysis of *L. donovani* promastigotes *in vitro* (154), and enhance parasite uptake by macrophages *in vitro* (84). Monoclonal F_{ab} fragments of anti-lipophosphoglycan (LPG) which binds to parasite surfaces have been shown to prevent the establishment of *L. major* infection in susceptible BALB/c mice when co-administered with a low number of parasite. The role of these antibodies *in vivo* is unclear. It is suggested that the monoclonal antibody fragments to the surface molecules on the promastigotes may prevent attachment of parasites to the macrophages and thus hinder the infection. In this experiment increasing the number of parasites or using a lower antibody titer reduces the effect of antibody treatment (75). In addition, protective immunity induced by vaccination against gp46 (a promastigote surface protein) was shown to be correlated with levels of anti-gp46 antibody suggesting a possible role for antibody in the generation of protective immunity (30). While these data suggest a possible protective role for humoral response against leishmaniasis, the following data suggest a lesser role for antibody in protection immunity against cutaneous leishmaniasis. The antibody titer or isotype differences in murine cutaneous leishmaniasis in different mouse strains

does not correlate with resistance to disease (153). Biozzi AB/L mice (deficient in humoral immune response) are resistant to *L. major* infection (73). Depletion of B-cells in *Leishmania* infected BABL/c mice reduced the lesion size suggesting that B-cells in fact are counter protective in this model (167). Passive transfer of immune serum or antibody fractions do not affect the course of infection in BALB/c mice (88). While antibody is not critical in determining the outcome of the infection, generation of cell mediated immune response has been shown to be the major host defense mechanism against invasion of the parasite *Leishmania*.

Thymectomized irradiated resistant mice display reduced ability to resolve the *Leishmania* infection (159). Protective immunity can be transferred to syngeneic naive mice by T-cells from resistant mice that have cured of a primary infection (160). T-cell deficient mice were unable to control *Leishmania* infection (74,159). Athymic nude mice (nu/nu) which are T-cell deficient, are susceptible to *L. major* infection even in a resistant CBA or C57BL/6 background (74). Adaptive transfer of syngeneic T cells to these mice restores the ability to resist infection (127). Similarly in susceptible mice, T-cells from immunized mice can transfer protection to naive recipients (107). The exacerbation of infection in resistant C3H mice by anti-μ

antibody treatment is reversed by transfer of T cells alone (174).

Role of $CD4^+$ T Cells

The immunity in leishmaniasis is dependent on T cell response and the outcome of the disease is related to $CD4^+$ subset generation. Reconstitution of T-cell deficient mice with $CD4^+$ T cells restored resistance to infection (127). Transfer of $CD4^+$ T cells from resistant strains of mice that have resolved the infection could also transfer protective immunity to syngeneic recipients (107,160). Similarly, $CD4^+$ T cells from susceptible BALB/c mice that have been immunized against infection can transfer this immunity to naive BALB/c mice (107).

Injection of killed *L. major* parasites in BALB/c mice via intravenous or intraperitoneal routes induces immune responses which protects the mice from challenge infection (87). However, the injection of the same antigen preparation via subcutaneous route induced an exacerbation of the disease which resulted from challenge infection (108). T cell transfer from such immunized mice could also suppress the protective immunity induced in mice immunized intravenously or intraperitoneally (109). Further experiments showed that the cells mediating this anti-protective effect are $CD4^+$ T cells. These cells showed

ability to mount a Jones Mote type of DTH response upon subcutaneous injection of parasite antigens, this type of reaction peaks around 12-15 hour rather than the classical DTH which peaks about 24-48 hour (109). Thus both susceptibility and resistance are determined by $CD4^+$ T cells.

Th_1 and Th_2 subsets

$CD4^+$ T cells are divided into at least two subsets according to lymphokine secretion. Th_1 cells secrete IL-2, IFN-γ and TNF-β (lymphotoxin) upon antigen or mitogen stimulation. Th_2 cells release IL-4, IL-5, IL-10 and IL-6 when stimulated. IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF) and TNF-α are secreted by both subsets of T cell clone (138,139). Th_1 subset induces cellular immune response such as DTH (34) while Th_2 cells provide help for antibody production (35). Th_1 cells participate in the provision of B-cell help for the generation of Ab, but Th_2 cells are much more efficient in this regard (35,181). Th_1 cell responses induce $IgG2_a$, whereas Th_2 cells induce production of IgG1 isotypes and IgE (35,181).

Th_1 and Th_2 cells regulate induction/proliferation of each others. *In vitro* IFN-γ inhibits the proliferation of Th_2 cells in response to IL-2 or IL-4, but does not affect

Th_1 cells. This is thought to be due to the differential expression of the IFN-γ receptor β-subunit on the surface of the cells, allowing Th_2 cells to receive negative signals from IFN- γ (58,64,156).

Th_2 cells secrete IL-10 which inhibits the synthesis of cytokines by Th_1 cells and also limit Th_1 cell proliferation by indirect action on antigen presenting cells. Thus, cytokines produced by Th_2 cells inhibit Th_1 cell function, while Th_1 cell cytokines have a similar effect on Th_2 cells (61,140).

In vitro Spleen or lymph node cells from recovered C57BL/6 mice or susceptible BALB/c mice immunized intravenously with killed *L. major* produced IFN-γ when stimulated with *Leishmania* antigens. In contrast, cells from susceptible mice with progressive disease or from immunized mice subcutaneously, produced little or no IFN-γ when similarly stimulated *in vitro* (110,171). Spleen or Lymph node $CD4^+$ T cells from infected resistant C57BL/6 mice showed elevated levels of mRNA for INF-γ and IL-2 and undetectable levels of Th_2 cytokines (IL-4 and IL-10). Conversely, $CD4^+$ T cells from infected susceptible BALB/c mice produced IL-4 and IL-10 mRNA in the spleen and lymph nodes (80).

The existence of polarized Th_1 and Th_2 *in vitro* was followed by data implicating these in the cure or

progression of murine experimental *L. major* infection. Transfer of T-cell lines that responded to *L. major* antigen *in vitro* to secrete IL-2 and IFN-γ, to BALB/c mice, resulted in protection of recipients. Similarly, transfer of T-cell lines that secrete IL-4 in response to *L. major* antigen, to BALB/c mice exacerbate the infection (175). The same results were obtained when severe combined immunodeficient (SCID) mice which are highly susceptible to *L. major* infection were reconstituted with either Th_1 or Th_2 cell line which leads to protection or exacerbation respectively (86).

Role of $CD8^+$ T Cells

Although $CD8^+$ T cells play a key role in the determination of resistance to primary infection, there are reports which indicate that parasite specific $CD8^+$ T cells are triggered during infection with *Leishmania* and these cells play a role in immunity to infection with either *L. major* or *L. donovani*.

Three weeks after infection with *L. major* an increased number of lymph node $CD8^+$ T cells occurs which correlates chronologically with lesion healing in resistant CBA mice (126). Treatment of these mice with anti-$CD8^+$ monoclonal antibody resulted in lesion exacerbation in both resistant and susceptible strains of mice and a greater number of parasites are found in the lesions compared to untreated

infected controls (185). Although the $CD8^+$ T cells were severely depleted the pattern of the disease in the mice model remained unchanged as the resistant strain eventually exhibited lesion healing and susceptible mice failed to resolve the lesions. Secondary infection in resistant CBA mice, which were depleted of $CD4^+$ and $CD8^+$ T cells, exacerbated the disease markedly, whereas depletion of either subset alone had no effect (142). The same effect was observed in sublethal irradiated BALB/c mice which was reconstituted with $CD4^+$ T-cell syngeneic bone marrow cells. The purpose of this experiment was to explore the protective role of $CD8^+$ T cells subset (85,141). $CD8^+$ T cells have been demonstrated to be able to mediate parasite killing *in vitro* via the production of IFN-γ and subsequent macrophage activation which produce a higher amount of nitric oxide (NO) (179).

These data suggest a clear role for $CD8^+$ T cells in the control of *Leishmania* infection, however the transfer of $CD8^+$ T cells to nude mice of both resistant and susceptible strains, is insufficient to transfer protective immunity (134), whereas in these experiments transfer of $CD4^+$ T cells completely restored immunity to *L. major* infection. $CD8^+$ T-cells produce IFN-γ as well as TNF-α (47,62). Both IFN-γ and TNF-α are known to be important in macrophage activation to kill *Leishmania*, therefore $CD4^+$ and $CD8^+$ T cells may

interact to mediate parasite killing, both by production of cytokines and by direct lysis of infected macrophages. Further experiments with $CD8^+$ knockout (ko) DBA/2j mice and $CD4^+$ and $CD8^+$ knockout C57BL/6 mice (B6) was shown that although the wild type B6 mice are resistant to *L. panamensis* infection, the $CD4^+$ ko and $CD8^+$ ko mice are susceptible to *L. panamensis* infection. $CD8^+$ ko mice initially developed the lesion similarly to the wild type mice but stabilized the lesion 80 days onward, while the wild type cure the lesion (67).

Th_1 and Th_2 Cytokines:

Interferon gamma (IFN-γ) and Interleukin 2 (IL-2)

IFN-γ, a glycoprotein, is a major histocompatibility complex (MHC) class II antigen inducer, an important factor in macrophage activation, and an essential element in antibody production and function. IFN-γ is mainly produced by both the helper/inducer or $Th_1(CD4^+)$ and the suppressor/cytotoxic or Ts/c ($CD8^+$) T lymphocytes upon stimulation with antigen or mitogen. Also non-T and non-B lymphocytes are able to synthesize IFN-γ.

In vitro, interferon γ is required to activate macrophages to kill effectively *Leishmania* species (143,144). *In vivo*, treatment of C3H/HeN mice, which normally resolve *L. major* infection, with anti IFN-γ

antibody, resulted in non-heating and ultimately death (9). This state is achieved with even a single injection of anti-IFN-γ, which has to be administrated within a week of infection. A similar result was obtained by infection of resistant mice with targeted disruption in the IFN-γ gene, these mice are unable to cure lesion after *L. major* infection indicating the IFN-γ requirement to resolve the *L. major* infection in resistant mice. In these experiments the animals developed a Th_2 response to infection, suggesting that Th_2 responses in these mice can occur but are regulated by IFN-γ (193).

The role of another Th_1-derived cytokine, IL-2 in protection and susceptibility to infection has been examined. Spleen and lymph node cells of either resistant or susceptible mice during *L. major* infection produced a comparable level of mRNA for IL-2 (117), but much of this appears to be derived from B cells (80). $CD4^+$ T cells from resistant mice also contained detectable levels of mRNA for IL-2 compared to the $CD4^+$ cells from susceptible mice (80). The requirement of IL-2 in the development of a Th_2 response has been supported *in vitro* and *in vivo* experiments (10,105). Neutralization of IL-2 in susceptible BALB/c mice at an early stage of infection is sufficient to prevent progressive disease (81). The neutralization of IL-2 is associated with increased IFN-γ and decreased IL-4 mRNA in

the draining lymph node cells, thus the protection mediated by anti-IL-2 treatment may be due to the control of IL-4 production at an early stage of infection. Conversely, anti IL-2 therapy in resistant C57BL/6 mice had no effect on lesion development or cure from disease (81), suggesting that IL-2 has little effect on the development of Th_1 response in resistant mice.

Interleukin 4 (IL-4)

IL-4 is considered to be a critical cytokine for the development of Th_2 response. Susceptible BALB/c mice are cured of disease by administration of an anti-IL-4 monoclonal antibodies during early infection. This cure had the characteristics of a Th_1-like cytokine (high levels of IFN-γ, low levels of IL-4) and resistance to reinfection (172). Administration of recombinant IL-4 into the lesions of BALB/c mice, early stage of infection, resulted in exacerbation of the infection (106). Although injection of IL-4 after lesion development limited parasite growth and lesion size, this effect was observed with both *L. major* and *L. mexicana* (27). In contrast, the treatment of resistant C3H/HeN mice with recombinant IL-4, at the time of infection, has little effect on the course of infection (32). Further evidence for the importance of IL-4 in disease exacerbation has been obtained from IL-4 transgenic

mice on a resistant background, which over-express IL-4 in the B-cell compartment. Spleen cells from these mice produced a significantly high level of IL-4 but a low level of IFN-γ when stimulated *in vitro* with specific antigen, compared with normal controls (104). Thus, Th_2 cell development or non-healing response requires continuous presence of IL-4 in experimental leishmaniasis. In a study of several mouse strains with various degrees of resistance to *L. major* infection, the expression of IL-4 mRNA correlates well with severity of the disease, while the expression of IFN-γ mRNA in response to infection did not correlate with the state of resistance (136). Thus, the production of Th_2 cytokine or IL-4 is more efficient than is IFN-γ to control the disease.

Interleukin-10 (IL-10)

The role of IL-10, previously called cytokine synthesis inhibitory factor, in leishmaniasis is indicated by the detection of IL-10 mRNA in the draining lymph node $CD4^+$ T cells of infected susceptible BALB/c but not resistant C57BL/6 mice, (80). IL-10 was found to be produced by other cells, including $Ly\text{-}1^+$ β cells, mast cells and under certain conditions macrophages (89). It is likely that IL-10 inhibits IFN-γ production by Th_1 and NK cells. Administration of anti IL-10 Ab at the time of infection did

not alter the disease progression or alter the Th response (36). IL-10 seems to be a potent inhibitor of IFN-γ induced macrophage microbicidal activity against both intracellular and extracellular parasites (65). This blockade is mediated through the suppression of an arginine-dependent pathway leading to the production of the toxic effector molecule, nitric oxide (NO) which appears to be the major effector mechanism in *Leishmania* infection (43). IL-10 has been found to down-regulate a number of different macrophage functions, including cytokine production [Tumor necrosis factor α, IL-1, IL-6] and respiratory burst (61).

Interleukin-12 (IL-12)

IL-12 is 70kDa heterodimeric cytokine composed of covalently linked 35kDa (p^{35}) and 40kDa (p^{40}) subunits (101). Neither subunit alone showed to have any biological activity. Constitutive production of IL-12 p^{35} mRNA has been observed in many cell types, including B cells, myeloid cells and T cells. In contrast, production of IL-12 p_{40} is restricted to B cells, macrophages and monocytes (46). The level of IL-12 p_{40} production and biologically active IL-12 appears to be correlated (46,101).

IL-12 stimulates growth of T cells and NK cells and induces synthesis of IFN-γ by these cells (118,176). IL-12 is produced by macrophages and certain B-cell lines in

response to inflammatory stimuli (82). The most prominent function of IL-12 is its stimulator effect on the cytotoxicity of and IFN-γ production by NK cells as well as on the development of Th_1 cells (188).

In several models of parasitic infection, *in vivo* treatment of recombinant IL-12, induces strong IFN-γ production and reciprocally inhibits Th_2 cytokine production (59). Treatment of BALB/c mice with recombinant IL-12 is sufficient to cure disease when it is administrated at an early stage of infection with *L. major* (184). Resistant C57BL/6 mice treated with anti-IL-12 lgG developed progressive disease upon infection with *L. major*. The lymph node cells of these mice showed a diminished capacity to synthesize IFN-γ *in vitro* and *in vivo* (83). As mentioned above, in susceptible BALB/c mice, administration of IFN-γ alone is not sufficient to cure the animal of the disease, suggesting that IL-12 mediates its protective effects partly through the induction of IFN-γ. The protective action of IL-12 may also be due to its effect on IL-4 synthesis, as the lymph node cells of anti-IL-12 treated mice consistently produced increased amounts of IL-4 and IL-10 through at least 6 weeks of infection (83). These results are consistent with other findings which demonstrated that IL-12 treatment in susceptible BALB/c mice reduced IL-4 production in *L. major* infection (184). Thus, IL-12 confers its

protective effect not only by induction of IFN-γ but also by both down-regulation of IL-4 production and suppression of Th_2 differentiation. This suppression of Th_2 differentiation is not dependent on the availability of IFN-γ, as shown in IFN-γ ko mice, as IL-12 is able to inhibit the induction of IL-4 mRNA in *L. major* infection (104). For this reason, IL-12 may prove useful as a component of cytokine-based therapies and Th_1-selective vaccines. BALB/c mice immunized with soluble leishmanial antigen (SLA) plus IL-12 completely protected against disease. The lesion size in the mice was as small as that produced in the resistant C3H/HeN mice. Cytokine analysis in these animals showed a low level of IL-4 and a high level of IFN-γ comparable to those observed in resistant C3H/HeN mice (1). Further experiments indicated that, daily administration of small doses of recombinant murine IL-12 (down to 0.2 μg per day) for the first week of infection cured cutaneous disease in normally susceptible BALB/c mice; however, IL-12 therapy after seven days of infection was ineffective to alter either the disease or immunological outcome (184). Further experiments showed that, IL-12 immunotherapy of established infection required concomitant neutralization of IL-12 antagonists (such as TGF-β, IL-10 and IL-4) (90).

Transforming Growth Factor β (TGF-β)

TGF-β can inhibit the intracellular killing of *L. major* by activated macrophages (147), through the inhibition of the production of the effector molecule nitric oxide (NO) (151). TGF-β diminishes the induction of NO synthesis in macrophages activated by MIF (44). Thus TGF-β may play a major role in inhibiting the protective immune responses involved in murine cutaneous leishmaniasis.

Effector Mechanisms

Nitric Oxide (NO)

Nitric oxide is a short-lived radical gas that is generated by activated macrophage. NO is cytotoxic for a variety of pathogens, including *Leishmania major, Plasmodium falciparum, Schistosoma mansoni, Trypanosoma cruzi and Toxoplasma gondi* (94,115). The NADPH-dependent enzyme, nitric oxide synthase (NOS), oxidatively generates NO from the terminal guanidino nitrogen atom(s) of L-arginine. L-citrulline is produced as a by-product (182). NOS enzymes are of two biochemical types, a constitutive type termed cNOS, which is dormant until activated by calcium ions and produces the physiological concentration of NO needed for house-keeping, and an inducible type [iNOS], which is calcium-independent and detected in murine macrophages which is expressed only after transcription induction so a lag

phase of several hours is required before NO is generated (120). Production of iNOS is inducible in a number of cell types including macrophages, hepatocytes, neutrophils, and endothelium cells by different immunological stimuli (13,120). It has been reported by several laboratories that control of leishmaniasis in murine model is NO dependent. *In vitro* macrophages from resistant mice express significantly higher level of NOS, and produce higher amounts of NO compared to the susceptible mice (115). Furthermore, bone marrow derived macrophages from Lsh^r mice, which are resistant to *L. donovani* infection, produce high levels of NO compared to macrophages from Lsh^s mice in response to IFN-γ and LPS (63). The convincing evidence of the role of NO in the control of *Leishmania* infection came from the development of iNOS ko mice, which are highly susceptible to *L. major* infection and developed visceral disease at day 70 post-infection (195).

In vitro, leishmanicidal activity of macrophages is inhibited by the arginine analogue, L-N^G monomethyl arginine [L-NMMA], which is a NOS competitive inhibitor (68). *In vivo,* resistant mice injected with L-NMMA developed significantly larger lesions with a higher parasite load compared with the control group which were vaccinated with D-NMMA (an inactive enantomer) or phosphate buffer saline (PBS). Thus resistance to *L. major* infection

correlated with the ability of macrophages to produce NO (111).

The Regulation of Nitric Oxide Synthesis by Cytokines

The mechanism(s) by which the NOS of macrophages is regulated by cytokine(s) is intriguing. *In vitro* it has been demonstrated that parasitized macrophages are activated when exposed to IFN-γ to kill *L. major and L. donovani* (143,146). IFN-γ induces minimal iNOS activation but a second stimulus (such as bacterial LPS or muramyl dipeptide from the cell wall of mycobacteria) synergistically induces high production of NO. This increased NO level is associated with the development of potent leishmanicidal activity (53,182). In experimental leishmaniasis, it is thought that endogenous production of TNF-α by macrophage acts as a second stimulus to synergize with IFN-γ in the production of NO in response to *L. major* infection (69). The synergistic effect of IFN-γ and TNF-α results in high levels of No_2^- and enhanced leishmanicidal activity (113). The importance of TNF-α in mediating parasite destruction and resistance to infection confirmed *in vitro* by demonstration that lymph node cells from resistant mice produced larger amounts of TNF-α following stimulation, compared to the cells from susceptible mice which produced little or no TNF-α (187). Moreover, treatment of infected mice with

recombinant TNF-α reduced lesion progression, while anti-TNF-α treatment caused an exacerbation, although the outcome of the disease was not altered (114,187). TNF-α, in the absence of IFN-γ, did not activate murine macrophages to clear infection, and thus it played a secondary role to IFN-γ (19).

Th_2 cytokines, such as IL-4 and IL-10, can down-regulate macrophage activation and so inhibit parasite killing. Macrophages from genetically resistant CBA mice were exposed to IL-4 before activation with IFN-γ and LPS, followed by infection with *L. major*. The leishmanicidal activity of these macrophages was significantly inhibited and this correlated with NO production (116). IL-10 down-regulates iNOS induction, caused by IFN-γ stimulation, and inhibits the anti-microbial effect of activated macrophages against *Toxoplasma gondi* via this mechanism (65). Cytokines such as TGF-β can also inhibit NO synthesis in activated macrophages and prevent intracellular killing of *L. major* (50). The role of such mediators in experimental leishmaniasis is not clear, thus Th_2 cytokines can prevent macrophage activation caused by Th_1 cytokine (IFN-γ) and so restrict parasite killing by inhibiting induction of iNOS of macrophage.

Vaccine and Prophylaxis in Leishmaniasis

Prevention of leishmaniasis is based largely on avoiding contact with the vector, a method not always feasible because of the way the disease is transmitted. In cutaneous leishmaniasis, the complex epidemiology of the disease combined with the sylvatic nature of both the vectors (many sandfly species have been identified as vectors) and reservoirs (most of them, still not identified), (103), effective prophylactic measures are rarely useful in this form of leishmaniasis (130). Measures, such as insecticide spraying and elimination of the reserviors, are virtually unfeasible. In addition, the possibility of insecticide resistant sandfly species has also to be taken into consideration. Thus, with the fact that individuals who have recovered from leishmaniasis are refractory to further infection, vaccination remains one of the most acceptable, safe, and practical prophylactic measures against cutaneous leishmaniasis.

Efforts have been made over the years, both in the Old World (Iran, Israel, Pakistan, Sudan and Tunis) and New World (Brazil and Venezuela), in order to develop a vaccine against the cutaneous forms of leishmaniasis and a number of vaccination procedures have been used:

1. Leishmanization

This procedure involves the inoculation of live

parasites into a non-exposed area of the body, usually the deltoid area of the arm, of people living in regions of high incidence of cutaneous leishmaniasis to produce a self healing lesion that are associated with a state of protection against natural infection. This method of protection induces resistance in a high percentage of the individuals. Apart from the technical difficulties of using live parasites for mass leishmanization, there are clinical questions involved in the use of a live vaccine. In addition leishmanization can produce a large long-lasting lesion which in some cases takes 3-5 years to heal (130) and can result in a depressed immune response to other vaccines (71), and thus is not recommended by World Health Organization (WHO).

2. Vaccines based on Crude Extract of Parasite

The variety of epidemiology of different forms of the disease in the world makes it impossible to apply a single control measure universally, except possibly, a cross-protective vaccine. The over-whelming cross-reactive antigen against different species (132) justify striving for development of a single vaccine. In addition unlike some other parasites, *Leishmania* can be grown in cell-free media and large quantities can be obtained with ease. This, plus the use in humans for the past several decades of killed parasites as skin test antigens for diagnosis (leishmanin),

makes it possible to try killed organisms with or without adjuvants as vaccine or immunotherapy in clinical studies (3,123).

Studies of using killed parasites as an immunizing antigen against cutaneous leishmaniasis date back to 1939 when Salles- Gomes and Prssoa attempted to immunize the local population in Sao Paulo state in Brazil with this type of preparation (66). Mayrink and his colleagues (121) used a vaccine of whole and sonicated promastigotes from four or five strains of *Leishmania* injected intramuscularly. Although a positive skin test was induced and there appeared to be no side effects, but the disease disappeared from the area, making an evaluation of long term protection impossible. However, the protection in those who converted to positive skin test as a result of the vaccination was only about 50% in one trail. The related studies were crucial in opening the road to further developments in the use of killed parasites as vaccine candidates, since no unwanted side effects were detectable in the several thousands who received the vaccine and who were followed for 2-3 years post-injection. Other groups in Iran and Venezuela are using a killed *Leishmania* vaccine with BCG for additional immunotherapy (7,40,51). The first trail in Iran was conducted in a nonendemic area (Yazd Povilence) in a preliminary study using killed *Leishmania major*. However, no

major side effect was observed the study was not conclusive since it covered a limited number of volunteers. In another study, a limited number of volunteers from Bahar Medical Laboratories were divided into two groups, based on tuberculin skin test (PPD) reaction. PPD-positive volunteers received 62 µg LmV alone and PPD-negative volunteers received 62 µg LmV mixed with a full dose of BCG in an open uncontrolled trail. Follow-up of volunteers indicated that the side effects noted were related to BCG and not LmV (6). The next safety trail of the killed *Leishmania major* was a randomized, double-blind (BCG as placebo) trail, in nonendemic area (51).

The efficacy and safety of an autoclaved *Leishmania* vaccine (ALM) with BCG is currently being tested in endemic areas in a randomized, doubled-blind [placebo (BCG)], controlled trails against zoonotic cutaneous leishmaniasis in Iran and Pakistan and against visceral disease in Sudan.

3. Vaccination with Fractions from *Leishmania* sp Extracts

This approach mostly restricted to experimental models, following antigenic analysis of parasite and experimental vaccination in mice, has identified a number of membrane antigens, particularly lipophosphoglycan (LPG) and two major surface glycoprotein of promastigotes, gp63(kDa) and gp46(kDa), as subunit vaccines. Also molecular vaccines have been developed by using expression of the genes of the

Leishmania antigens (recombinant protein) or removing specific genes from pathogenic *Leishmania sp* (*Leishmania* mutants).

Lipophosphoglycan (LPG)

LPG is found on all *Leishmania* species and is the most abundant surface component (76). LPG is expressed in distinct forms on amastigote and promastigote which consists of phosphorylated saccharides linked by a carbohydrate core, to unique lipid anchor (189). LPG enhances the survival of the parasite within the macrophage (31). This molecule is highly immunogenic, complete or partial protection was achieved in susceptible mice with purified *L. major* LPG (75) or resistant and susceptible mice with *L. mexicana* LPG (166). It is not clear if LPG itself contains any T cell epitopes or if they are expressed on a tightly associated protein component. Because proteinase treated LPG was unable to stimulate a T cell response (31). Thus the T cell reactivity and possibly protection by LPG are induced by proteins tightly bound to LPG.

Glycoprotein 63 (gp63)

The promastigote surface protease (PSP) or gp63 is a major surface glycoprotein, expressed on both promastigotes and amastigotes of all *Leishmania* species examined (20,33)

and can represent over 1% of the total cellular protein content, depending on species and strains. This membrane protease is anchored to the plasma membrane by means of covalently attached glycosyl-phosphate idylinositol, similar to those of other glycolipid-anchored proteins (173). The genes encoding *Leishmania major* GP63 have been cloned and characterized (25). There are multiple copies of GP63 genes in all *leishmania* species examined and in *L. major*. These genes are tandemly linked at a single chromosomal locus (26). The DNA sequence for the gene copy which maps to the 5' end of the locus was determined (25). The translated protein sequence predicts that gp63 protein is synthesized as a precursor protein containing a leader peptide of 39 residues (pre region) followed by a region of 61 residues (pro region) that is removed from the amino terminus prior to expression of gp63 on the promastigote cell surface (25). Gp63 isolated from promastigotes also exhibits enzymatic activity as it functions as a metalloprotease with a broad range of substrate specificity.

The molecular and biochemical characterization of gp63, together with its abundance, has led to its use in several vaccine trails, alone or in conjugation with other antigens. Gp63 can elicit a T cell response in patients with leishmaniasis (93). Gp63 reconstituted into liposomes induces a strong protection in both susceptible, BALB/c, and

resistant, CBA/Ca, mice against challenge with *L. mexicana mexicana* without any exacerbation of the disease (166). Although, the protection in susceptible BALB/c mice was less complete. In this protection, the route of administration was critical, significant protection was achieved if the gp63-liposome preparation was administrated intravenously (96).

Molecular Vaccines

Mutant Leishmania

Using homologous recombination to remove specific genes from *Leishmania* strains, developed by Beverley and his colleagues, Cruz, et al., produced a stable *L. major* clone which lacks the gene for the enzyme, dihydrofolate reductase-thmidilate synthetase (DHFR-TS) which is essential for parasite growth (it allows thymidine to be synthesized from simple organic molecules). They deleted both copies of the gene completely without introducing selective markers such as neomycin (NEO). This *dhfr-ts*-Knockout parasite is auxotrophic on thymidine (11). *In vitro* infection of macrophage with this mutant, the parasite failed to grow although it differentiated successfully into amastigotes. In mice the parasite failed to grow although it persisted for up to 2 months, disappearing steadily with a half-life of 2 to 3 days (11). No evidence of disease was seen in

either BALB/c or nude mice. Vaccination of BALB/c and CBA mice showed excellent protection when given intravenously. Good protection was seen in CBA mice vaccinated intramuscularly, or subcutaneously. These parasites are currently being tested for safety and immunogenicity in non-human primates in Nairobi and Rio de Janeiro.

Recombinant protein of gp63

The major surface glycoprotein of both promastigotes and amastigotes of all *Leishmania* species is gp63, this glycoprotein with proteinase activity is responsible for initiation of the infection in the macrophage and is highly conserved across species of the parasite, so it has been considered to be the suitable candidate vaccine. The gene for *L. major* gp63 was transformed into highly attenuated mutant of *Salmonella typhimurium* (SL 3261) and the resulting construct, which stably expressed the gp63 antigen *in vitro* was used to immunize resistant CBA mice by the oral route (196), the immunized mice developed significant protection against a challenge infection with *L. major.* Spleen cells from these mice were mainly $CD4^+$ T cell and secreted IL-2 and IFN-γ but not IL-4. Also gp63 gene from *L. major* was cloned into a mycobacterial expression vector (BCG mutant) and expressed in E. coli. This recombinant form of gp63 conferred partial protection by oral immunization against *L. major* challenge.

CHAPTER 2

MATERIALS AND METHODS

The *Leishmania* vaccine efficacy trial was a randomized double blind study.

Volunteers:

Individuals, older than 5 years with no history of cutaneous leishmanioses based on physical examination were interviewed (each individual) and a questionnaire form (form 1) was completed. The volunteers were selected according to the following criteria:

* Age over 5 years.
* No history of leishmanioses or leishmanization.
* No response to leishmanin skin test.
* PPD reaction <15 mm or between 15-20 mm with the presence of BCG scar.
* No evidence of current illness according to the physical examination.
* No history of recent hospitalization.
* No history of allergy.
* Not being pregnant or nursing at the time of vaccination.

* Willing to participate in the trial and sign the consent form.

In case of children the consent form was signed by the guardians.

Based on the above criteria, the volunteers were selected and a questionnaire form (form 2) was completed for each volunteer.

Vaccine

Leishmania vaccine produced under good manufacturing practices (GMP) at Razi Vaccine and Serum Research Institute, Hesark, Iran (77). Razi Vaccine and Serum Research Institute is a worldwide well known institute in research and production of different biological products including human and life stock vaccine and antisera specially anti-venom sera. *Leishmania* vaccine produced from *L. major* (MRHO/IR/76/ER) which was originally isolated by Dr. Nadim *et al.*, This stain has been studied extensively and used to leishmanized over two million individuals in Iran. Briefly, *L. major* was passaged in Balb/c mice to maintain its pathogenicity, the organisms were isolated from the mouse lesions and cultured in NNN diphasic medium and then transferred to 50-200 mL volumes in RPMI-1640 (Gibco, Grand Island, NY, USA) with 15% inactivated fetal calf serum (Sigma, St. Louis, MO, USA) at 25 °C, in Roux bottles. Fresh

medium was gradually added on different days to reach 200 mL. Promastigotes were harvested in stationary phase. Stationary phase was determined by daily enumeration of the parasite and when in two consecutive days the number of parasites was constant or decreased the parasites were harvested. The promastigotes were washed thoroughly five times with pyrogen free phosphate buffer saline (PBS) pH 7-7.2 and stored at -70°C. Twelve harvests were pooled to constitute a batch used throughout these studies (Phase I-III). Samples from each harvest and the final lot were assayed for sterility, innocuity, cell count and protein concentration. The protein content was determined by Lowry method (119).

Phase I-II safety and immunogenicity of the vaccine was performed in a series of stepwise studies designated as SAF I-V. In SAF-V different preparations and doses of killed *Leishmania major* were used. In one preparation (designated as KLM) a sample of the final lot was diluted to desired concentration (44.44 mg/mL and 11.11 mg/mL) with PBS then freeze-thawed five times and then treated with thimerosal 0.01% and kept at -70°C until being used. In the other preparation (designated as ALM) a sample of the final lot was diluted to two concentrations (44.44 mg/mL and 11.11 mg/mL) with PBS and aliquots were added to small vials and autoclaved for 15 minutes at 121°C, (15PSI) and kept at 4°C.

In phase I-II volunteers from non endemic area were selected and two doses of two preparations alone or mixed with 10^{th} of normal dose of BCG (final *Leishmania* protein concentration was 1 mg per dose for low dose and 4 mg per dose for high dose) were injected intradermally at the base of left deltoid. The vaccinees were followed up for any possible side effect, and the immune response of those vaccinated were evaluated *in vivo* and *in vitro*. The safety results indicate that both preparations and doses are safe with acceptable ranges of side effects which is mainly due to BCG and not to the killed *Leishmania* alone. Based on the *in vivo* test (leishmanin skin test conversion) and *in vitro* tests (IFN-γ production) and feasibility it was decided to use ALM 1 mg/dose mixed with 10^{th} of normal BCG in efficacy field trials (8,51).

Location

Isfahan is a province in central part of Iran with more than two million population. The area north east of Isfahan is endemic for zoonotic cutaneous leishmaniasis. Air Base county with a population of more than 20,000 was selected from this area because of high incidence rate of cutaneous leishmaniasis (approximately 10%) and the migration of susceptible individuals from non endemic areas to reside there for at least two years. The disease and complications

are well known to the people and authorities so they are highly motivated and cooperative.

Bacillus Calmette Guerin (BCG)

BCG was produced at Pasteur Institute of Iran, BCG vial was reconstituted exactly before vaccination according to the manufacture instruction. Prior to the phase III trials in a stepwise study various doses of BCG were used and the results showed that 10^{th} of the normal human dose produced the least side-effects and induced the same reactions as full BCG dose. Thus, in phase III trials 10^{th} of the normal human BCG dose was used. 0.3 ml of the prepared BCG was added to two types of identical vials with a volume of 2.7 ml of either ALM or BCG diluent, mixed well and used within 2 hours. The final injected dose of BCG is a 10th of normal dose of BCG (roughly 5×10^4 CFUs).

Purified Protein Derivative (PPD)

PPD used in this study was produced at Razi Vaccine and Serum Research Institute. Each volunteer was injected intradermally with 5 units of PPD on the left forearm and the result was measured 48-72 hours later as the leishmanin skin test.

Leishmanin

Leishmanin was produced at the Pasteur Institute, Tehran, Iran. The same isolate of *Leishmania major* (MRHO/IR/76/ER) that was used for vaccine production was used (2). Briefly, the parasites were harvested at the beginning of stationary phase, collected by centrifugation, washed three times with PBS, and then selected for counting and total protein concentration after which they were preserved at 2-8 °C. The concentrated suspension was mixed with an equal volume of PBS plus 0.05% thimerosal, then it was diluted with PBS containing 0.01% thimerosal (or 0.5% phenol for preparation of phenol leishmanin), to reach a concentration of 6 X 10^6 promastigotes /ml. The preparations were aliquoted in 1.2 ml or 2.1 ml volumes in small vials. A volume of 0.1 ml of this preparation was injected intradermally on the forearm. The reactions including erythema and induration at the site of injection was measured according to the ball point pen method of Sokal (180) at 48-72 hours post injection.

Soluble *Leishmania* Antigen (SLA)

Leishmania soluble antigen (SLA) was kindly provided by David Sacks, National Institute of Health, Bethesda, MD, USA.

Vaccination

The selected volunteers who were willing to participate in vaccine trial and follow up were injected with 0.1 ml of the vaccine vial content which is either ALM (1 mg/dose) mixed with BCG or BCG alone intradermally at the base of left deltoid.

Follow up

The volunteers were checked for safety of vaccine on days 0, 1, 3, 7, 30 and 80. Each volunteer was visited, physically examined and the reaction at the site of vaccination was measured and recorded in every visit. At day 80 post vaccination a leishmanin skin test was performed and the results were recorded at 48-72 hours post injection.

Case finding was performed through passive and active follow up to now 30 months post vaccination. In passive follow up a *Leishmania* clinic was established and local physicians were trained to be able to identify suspected cases and follow up the cases. Also dermatologists attended the clinic and visited the patients regularly. Active

follow up was performed every three months and each vaccinee was visited and checked for any suspected lesion. Any suspected lesion was confirmed by direct smear or culture. If the organism was not isolated from the suspected lesion in first try, the second and third sample was taken. Proven cases of cutaneous leishmanioses were treated with a standard protocol, it was originally proposed to treat all cases uniformly but during the trial some of the cases were not willing to follow exactly the therapeutic protocol so in these cases other types of WHO/TDR approved treatment were offered free of charge. Such cases were visited regularly every two weeks and the status of every lesion was recorded precisely. A questionnaire form for each visit was completed and any possible information in regard to cases such as the time of onset, number, location, size and evolution of the lesions, the type of treatment and the response to the treatment were recorded. This information was recorded to use for any partial protection which vaccination might induced.

Study subjects:

Adult proven cases who were willing to donate blood sample were selected, interviewed and a questionnaire form was completed for each volunteer. A blood sample was taken as early as possible to rule out the effect of infection in

the immune response. A total of 157 blood samples were taken in first year follow up and 50 blood samples were taken in second year follow up. Also out of the field trail, 11 individuals with history of self healing cutaneous leishmaniasis and 14 non-injected cases were selected and blood samples were taken as positive control, seven blood samples were taken from individuals with no history of cutaneous leishmaniasis and used as negative control as well, and 18 blood samples were taken from cases with chronic cutaneous leishmaniasis (Non-healing cases) these cases which were either leishmanized or naturally infected (Table 3 and 4).

Data Analysis

EPI-INFO (Version 6.02) and Hardvard Graphics (Version 3) were used for production of questionnaire, graphs and data analysis.

Isolation of Peripheral blood Mononuclear Cells

Required materials:

1. Hanks balanced salt solution (HBSS):

Kcl	(400.0 mg)	Merck
Na_2HPO_4	(47.5 mg)	Merck
KH_2PO_4	(60 mg)	Merck
$NaHCO_3$	(350 mg)	Merck

$CaCl_2$, $2H_2O$	(185 mg)	Merck
$MgSO_4$, $7H_2O$	(200 mg)	Merck
NaCl	(8000 mg)	Merck
D-glucose	(1000 mg)	Merck

Autoclaved distilled water (pyrogen-free) up to 1 liter, adjusted to pH 7.2, sterilized by filtration through a 0.22 µm nitrocellulose filter.

2. Phosphate-buffer saline (PBS):

NaH_2PO_4 (anhydrous)	0.23 g (1.9mM)
Na_2HPO_4 (anhydrous)	1.15 g (8.1mM)
NaCl	9.0 g (154mM)

Autoclaved distilled water (pyrogen-free) up to 900 ml, and adjusted to pH 7.2 (using 1 M NaOH or 1 M Hcl). Autoclaved distilled water was added to 1000ml. Sterilized by filtration through a 0.22 µm nitrocellulose filter.

3. Lymphocyte separation medium (LSM; Histopaque-1077 from Sigma

Diagnostics, St. Louis, MO. USA)

4. Complete RPMI medium which was RPMI-1640 medium containing:

L-glutamine	2mM (0.3 g/100 ml)	Gibco
Penicillin	100 U/ml	Biofluids
Streptomycin	100 µg/ml	Biofluids
HEPES[N-2 hydroxyethylpiperazine-N-2-ethanesulfonic acid]	10mM	Biofluids

Heat-inactivated, fetal calf serum (FCS), 5% Sigma

Sodium bicarbonate 2 g/100ml Merck

Sterilized by filtration through a 0.22 µm nitrocellulose filter.

5. Freezing media which includes:

Fetal bovine serum	84 ml/100 ml	Sigma
Complete RPMI medium	8 ml/100ml	Sigma
Dimethyl sulfoxide (DMSO)	8 ml/100ml	Merck

6. 50ml Polypropylene Conical tubes Cat# 2070 Falcon
7. 15ml Polypropylene Conical tubes Cat# 2097. Falcon
8. 1.2ml Cryovials, NALGENE, USA

Principle:

Heparinized blood samples were overlaid onto a lymphocyte separation medium (e.g. Histopaque). This medium is a solution of polysucrose and sodium diatrizoate having a specific gravity of 1.077 ± 0.001 which facilitates rapid recovery of viable mononuclear cells from blood. During centrifugation erythrocytes and granolocytes are aggregated by polysucrose and rapidly sediment, whereas lymphocytes and other mononuclear cells remain at the plasma-Histopaque interface. Most platelets were removed by low speed centrifugation during the washing steps.

Procedure:

Heparinized peripheral blood samples (12 to 20 ml) were obtained from volunteers at the trial site clinic and transferred to the Al Zahra hospital, Isfahan University of Medical Sciences located 40 kilometers from the trial field. The blood samples were diluted 1:1 with Hanks Balanced Salt Solution (HBSS). The diluted samples were overlaid gradually with the same volume of Histopaque and then the peripheral mononuclear cells (PBMCs) were separated by centrifugation gradient at 580xg for 30 minutes at room temperature. After centrifugation 0.5 to 1 ml of the upper layer was transferred to an Eppendorf tube for antibody titration. The opaque interface was aspirated by a Pasteur pipet and transferred to a 15 ml disposable conical centrifuge tube. The interface containing mononuclear cells was washed two times with Phosphate Buffer Saline solution (PBS) and once with RPMI-1640 at 460xg centrifugation for 10 minutes at 5 °C. The cell number of each sample was counted with Neuber hemacytometer and the cell pellet was resuspended in 0.5 ml RPMI-1640 and stored in freezing medium. These aliquots of cells were kept frozen in cryofreezing tubes in 1 ml volume. Freezing the cells was done gradually, first the cells were stored at -30 °C for 24 hours then transferred to the steam of liquid nitrogen for few hours followed by storage in liquid nitrogen.

***In vitro* Lymphocyte Proliferation Assay**

Required materials:

1. RPMI-1640 medium supplemented with 5% heat inactivated human, type AB positive, serum.
2. Phytohemagglutinin (M form) 25 µg/ml Gibco
3. *Leishmania* soluble antigen (SLA) 25 µg/ml
   (Kindly provided by Dr. David Sacks, National Institute of Health, Bethesda, MD.)
4. U-bottomed, 96-well tissue culture plates Costar (cat#3799)
5. ^{3}[H] Thymidine 1.0 µC*i*/well Amersham, UK
6. Fiberglass filter Cat# 1822 849 Whatman, UK
7. Scintillation fluid:
   POPOP [1,4-bis (5-Phenyloxazol-2-yl) benzen] 0.25 g/l Sigma
   POP [2, 5-Diphenyloxazol 6.5 g/l Sigma
   Toluene up to 1 l Merck
8. Cell harvester system LKB
9. β-Counter system Pharmacia

Procedure:

The cells were thawed rapidly, washed with and resuspended in RPMI-1640, examined for viability with trypan blue, if the percent of viable cell was higher than 30 then the sample was used for further steps. The cells were

washed twice more with RPMI-1640. The cells were then counted with a Neubar hemacytometer slide, resuspended in complete culture medium, RPMI-1640 with 5% human type AB positive serum. The cell number was adjusted to a final concentration of 1 X 10^6 cells per ml. The cells were cultured in U-bottom 96 well culture plates, 2 X 10^5 cells at 200 µl volume per well was used. The cells were either cultured alone as control or stimulated with PHA (5 µg/well) or *Leishmania* soluble antigen (4 µg/well). The cells were incubated in a humidified atmosphere at 37 °C and 5% CO_2 for 5 days and pulsed during the last 20 hours with 1.0 µC *i* ^{3}[H] thymidine per well. The cells were then harvested onto fiberglass filters and ^{3}[H] thymidine incorporation was determined by liquid scintillation counting. All tests were performed in triplicate. Proliferation response are expressed as the stimulation indices (SI), which represent the ratio of mean proliferation of stimulated cells in triplicate wells to the mean proliferation of cells in medium controls.

Cytokine assay:

Culture supernates were collected at 4 days post stimulation for both antigen treated and PHA treated wells.

100 µl per well were collected carefully and each triplicate was pooled together. The supernatants were kept at -20 °C until used for cytokine titration.

IFN-γ measurement by an enzyme-linked immunosorbent assay (ELISA):

Principle:

IFN-γ is captured from the sample fluid by a solid-phase monoclonal antibody specific for human IFN-γ and then detected using monoclonal anti-human IFN-γ. The detection signal is amplified by reaction with enzyme-linked polyclonal anti-immunoglobulin reagents and is quantitated by monitoring of the conversion of a substrate into a chromogenic product.

The required materials for IFN-γ measurement:

1. Monoclonal antibody to human IFN-γ (capture Mab), Batch # 3420-3C3 Endotell. Sweden

2. Biotinylated monoclonal antibody to human IFN-γ (detecting Mab), Batch # 3420-6C3 Endotell. Sweden

3. Human, Recombinant IFN-γ, Lyophilized form, Expressed in *E. coli* Product No, I-1520, Sigma USA

4. Avidin conjugate (streptoavidine-horseradish peroxidase): This product was examined at two different dilution, 1:500 or 1:100 in PBS (containing 1% BSA, 0.05% Tween), GIBCO, BRL

5. Substrate: Sigma fast O-phenylenediamine dihydrochloride (OPD):GIBCO, BRL

This product was diluted 1:10 (0.5 ml 10x OPD, 4.5 ml sodium citrate buffer and 6 µl H_2O_2)

6. 96-well microtiter ELISA plates, Maxisorp surface NUNC, Denmark

7. ELISA reader

8. Buffer solutions:

a. Coating buffer:

Na_2HPO_4, $2H_2O$	1.42 g
KH_2PO_4	0.2 g
Kcl	0.2 g
NaCl	8 g

Distilled water up to 1 liter, adjusted to pH 7.2

b. Washing solution:

NaCl	9 g
Tween 20	1 ml

Distilled water up to 1 liter

adjusted to pH 7.2

c. Phosphate-buffer:

Na_2HPO_4, $2H_2O$	1.42 g
NaCl	8.0 g

KCl	0.2 g
KH_2PO_4	0.2 g
BSA	5 g
Tween 20	1 ml

Distilled water up to 1 liter adjusted to pH 7.2

d. Blocking solution:

Tween 20	0.05%
BSA	5%
PBS	94.95%

e. Developer solution:

0.5 ml OPD 10x, 4.5 ml sterile sodium citrate buffer (0.1 M itric acid, 0.1 M Na_2HPO_4, $2H_2O$, pH 4.5) 6 µl H_2O2. This solution was made immediately before use.

f. Stop solution: 4N H_2SO_4

Procedure:

1. Anti human IFN-ɣ (capture Mab) diluted 1 µg/ml in PBS.
2. Each well of ELISA plate was coated with 50 µl of diluted capture Mab.
3. The plates were incubated in moist chamber at 4^0 C overnight.
4. The capture Ab of each well was removed completely
5. 100 µl of blocking solution was added to each well.
6. The plates were incubated at 37^0 C for 2 hours (or at 4^0 C overnight).

7. The blocking solution of each well was aspirated.

8. 50 µl of test supernatant was added in duplicate wells, 50 µl of serial dilutions of human IFN-γ for standard curve was added in separate wells of each plate in duplicate. Duplicate wells had 50 µl of only standard diluent as controls.

9. The plates were incubated at 4^0 C overnight.

10. The plates were washed 3 times with washing buffer then they were tapped on paper towel.

11. 50 µl of diluted biotinylated Mab to human IFN-γ (0.5 µg/ml in PBS containing 1% BSA and 0.05% Tween 20) was added to each well as detecting Ab.

12. The plates were incubated at 37^0 C for 2 hours.

13. The plates were washed 3 times with washing buffer, then tapped on paper towel.

14. Streptoavidine peroxidase conjugate diluted 1:500 in 1% BSA, 0.05% Tween/PBS buffer.

15. 50 µl of diluted streptoavidine peroxidase was added to each well.

16. The plates were incubated at 37^0 C for 2 hours.

17. The plates were washed 3 times with washing buffer then tapped on paper towel.

18. 50 µl of substrate solution (0.5 ml of 10x OPD, 4.5 ml sodium citrate buffer and 6 µl H_2O_2) was added to each well.

19. The plates were incubated at 37^0 C (in dark) until color develops.
20. The reaction was stopped by adding 50 µl of stop solution in each well.
21. The plates were read at 792 nm in ELISA reader.
The procedure of ELISA was determined by positioning the mean absorbance of each duplicate wells on the standard curve. By drawing a vertical line to intersect the X axis, the cytokine concentration of each sample was determined.

Standard curve

On a separate plate duplicate serial dilutions (log 2 dilution) of standard IFN-γ were made, from 10 ng/ml down 80 pq/ml in standard diluent, 50 µl of each dilution were added to the precoated plates and all the steps were done in the same way as in the assay test including two wells with diluent alone as controls.

IL-4 measurement by a competitive enzyme immunoassay (EIA) method

Principle

With this assay system, precoated goat anti-rabbit antibody are used to capture a specific cytokine complex in each sample consisting of cytokine antibody, standard or unknown, and biotinylated cytokine. Biotinylated cytokine

conjugate (competitive ligand) and sample or standard form a competition reaction in which the samples or standards compete for antibody binding sites with the biotinylated cytokine. Therefore, as the concentration of cytokine in the sample increases, the amount of biotinylated cytokine captured by the antibody decreases, resulting in an inverse relationship between OD and concentration.

With the addition of streptavidine-conjugated alkaline phosphatase, substrate solution and Amplifier solution the amount of biotinylated cytokine is detected.

Required material:

All materials were purchased from Chemicone, Inc. CA, USA.

1. Pre-coated goat anti-rabbit ab 96-Well plates (Cat# CTY01501)
2. Interleukin-4 Antibody (Cat# CYT015c), bottle of lyophilized IL-4 Antibody was reconstitute with 1 ml of Assay Diluent before use.
3. Interleukin-4 Conjugate, Biotinylated (Cat# CYT015e), bottle of lyophilized IL-4 Conjugate was reconstituted with 1 ml of Assay Diluent before use then diluted 1:6 with Assay Diluent.
4. Streptavidine-Alkaline Phosphatase (Cat# CYT015k), diluted 1:30 with Assay Diluent before use.

5. Substrate (Cat# CYT015g), Lyophilized form which was reconstituted with 13 ml of substrate diluent 10 minutes before use.

6. IL-4 Standard (Cat# CYT015d), lyophilized form which was reconstituted with 625 µl of serum diluent (made a concentration of 800 ng/ml).

7. Amplifier (Cat# CYT015i), lyophilized form, reconstituted with 8 ml of Amplifier Diluent before use.

8. Washing Buffer (Cat# CYT015a) and Assay Diluent (Cat# CYT015b).

Procedure:

1. 100 µl of test supernatant was added in duplicate wells of pre-coated plates.

2. 100 µl of serial dilutions of the standard for standard curve was added in separate wells of each plate in duplicate.

3. Interleukin-4 Antibody diluted 1:5 in assay diluent.

4. 25 µl of diluted Interleukin-4 Antibody was dispensed into each well.

5. The plates were sealed with Acetate Plate Sealer to prevent evaporation and incubated at room temperature for 3 hours.

6. The sealer was removed, 25 µl of IL-4 conjugate was

added into each well and incubated at room temperature for 30 minutes.

7. The fluid of each well was removed by flicking the plates over the sink, the plates were washed 5 times with washing buffer, after the last wash, the excess fluid was removed by aspiration.

8. 50 µl of diluted Streptoavidine-alkaline phosphatase was added into each well. The plates were incubated for 30 minutes at room temperature.

9. The plates were washed 5 times with washing buffer then they were tapped on paper towel.

10. 50 µl of prepared substrate solution was added into each well and incubated for 20 minutes at room temperature.

11. 50 µl of prepared Amplifier solution was added into each well, then incubated at room temperature.

12. The plates were read at 490 nm in ELISA reader within 5 minutes, after this initial reading the plates were read at 5 minutes intervals until the OD for the "0 dose" of standard wells reached between 1.5 to 2.0

Calculation of results for IL-4 measurement

Known concentration of IL-4 on semi-log paper were plotted on the X-axis and the corresponding OD on the Y-axis. The standard curve showed a sigmoid shape that indicates the inverse relationship between IL-4

concentration and the corresponding OD's (absorbance). By positioning the mean absorbance of each duplicate wells on the standard curve and drawing a vertical line to intersect the X axis, the IL-4 concentration of each sample was determined.

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The vaccine injection started on February 1995. The transmission cycle started from April to September and first year cases were identified from the beginning of August, 1995 (7 months post vaccination) to mid December, 1995. In the second year of the case finding follow up, blood samples were collected from September to the end of November 1996. The blood samples were taken as soon as a case was identified. During the first year case finding follow up a total of 215 parasitologically proven cases of cutaneous leishmaniasis were identified in injected groups: 112 cases among ALM+BCG injected and 103 cases among BCG injected volunteers (Table 2) and also 41 cases were identified among eligible non injected group. Among the injected and non injected cases hundred and two of them were adult and eligible for bleeding according to the protocol. Sixty two of them agreed to donate blood samples. As control blood samples were collected from the following groups: fourteen cases from non injected group, fifty four individuals who were injected (either with ALM + BCG or with BCG alone) and did not develop *Leishmania* lesion, fourteen cases who were not eligible for vaccination due to original positive LST, six suspected cases (parasitologically not proved) and four non-healing cases. As positive controls, three individuals with history of cutaneous leishmaniasis or a high level of IFN-γ induction (Table 3) were chosen.

During the second year follow up a total of 200 cases were identified (99 among ALM+BCG injected and 101 among BCG injected volunteers), eighty three of them were adult and eligible for bleeding, fifty of them agreed to donate blood samples. As control blood samples were also taken from non injected cases as well as the following groups: non-healing cases, recovered individuals from the disease and normal control from non-endemic area (these groups were out of the trail and brought to the study either for evaluation or as control), (Table 4).

Table 2. Distribution of cases among injected volunteers in two years follow up.

Injected	Number	Cases		Total
		1st	2nd	
ALM+BCG	1188	112	99	211
BCG	1122	103	101	204
Total	2310	215	200	415

Although a standard unique procedure was used and necessary precautions were implemented it was noticed that some of the subjects'PBMCs lost their viability during the process of shipping and recovery. The PBMCs with low viability were excluded and only ones with enough numbers of viable cells were used, as shown in table 3.

Table 3. First year study subjects

Volunteer	Description	Subject groups	Number of participants	Number of recovered (Percent of recovery)
Eligible for the trial	Selected for injection	Cases	62	35 (56%)
		Non-cases	54	34 (63%)
	Non-injected	Cases	14	7 (50%)
Control out of the trial		Non-healing cases	4	2 (50%)
		Cases with previous positive LST	14	8 (57%)
		Suspected cases, not parasito-logically proved	6	3 (50%)
		Individuals with history of the disease	3	2 (66%)
Total			157	91

Table 4. Second year study subjects.

Description	Subject groups	Number of participants	Number of recovered (Percent of recovery)
Eligible from the trial	Injected cases	50	15 (30%)
Out of the trial	Non injected Cases	14	7 (50%)
	Individuals with history of CL	11	10 (91%)
	Non-healing cases	18	17 (94%)
	Non-exposed individuals to *Leishmania*	5	5 (100%)
Total	98		52

Leishmanin skin test (LST)**

LST was used as one of the main inclusion/exclusion criteria in the *Leishmania* vaccine field trials (phase I to

phase III). Individuals with no reaction to leishmanin skin test were selected for the trials. During the trial volunteers also were tested with leishmanin two times, at day eighty and one year post vaccination. Leishmanin skin test (LST) conversion was significantly higher in volunteers who received ALM+BCG than the group of volunteers who received BCG alone (LST conversion at day eighty: 36.2% *vs.* 7.9% and at one year: 37.8% *vs.* 24.7 %). Also the injection of ALM+BCG produced significantly higher magnitude of LST response in volunteers compared to control either at day 80: Mean reactions±SD = 3.4±3 mm for ALM+BCG injected and 1.05±2 for BCG injected volunteers ($p<0.001$) or one year post injection. The magnitude of LST reaction in ALM+BCG injected cases compared to BCG injected at day 80 was (mean±SD) 2.38±2.46 *vs.* 1.23±1.98 mm and at one year post injection was (mean±SD) 6.73±4.98 *vs.* 5.92±5.14 mm (Table 9).

(**) All the experiments have been completed and the results analyzed before the vaccine codes were opened by World Health Organization/Tropical Diseases Research (WHO/TDR) authorities.

Lymphocyte proliferation Response and IFN-γ Production by Stimulated PBMCs of Injected Volunteers who Developed Lesion(s)

Data from studies of 28 different subject cases who were injected with either ALM+BCG or BCG are shown in figure 1, the pattern of lymphocyte proliferation response of PBMCs among these cases was heterogeneous. Ten cases had strong *Leishmania*-specific proliferative response within a stimulation indices range from 5 to 20, (mean±SD: 4.11±4.16). There is no correlation between proliferation response of the PBMCs with the magnitude of leishmanin skin test conversion at day eighty post injection (Fig. 3), but correlation exists between these two values when the injected volunteers were skin tested one year after injection (Fig. 4). PBMCs from some cases which proliferate in response to SLA produced high level of IFN-γ [Figs. 1, 5 and table 9]. From the second year cases, data from eleven subjects are shown in figure 6, the stimulation indices of PBMCs from these cases compare to control group were higher. Virtually all the cells of these cases had a lower stimulation index in response to SLA compared to that of the cases from the first year transmission cycle (mean±SD: 1.79±0.69 *vs.* 4.12±4.16) and the producers of IFN-γ were among the cases with high proliferation response (Figs. 1 and 6).

Proliferative Response and IFN-γ Production by Stimulated PBMCs of Injected Volunteers with no Lesion

The pattern of lymphocyte proliferation response by stimulated PBMCs with SLA from 24 different injected (either with ALM+BCG or BCG) subjects without development any lesion is shown in figure 7. An increase in stimulation index is observed in few subjects of this group within a range from 2 to 15, but compared to that of injected cases from the same year is lower (mean±SD: 2.17±2.55 *vs.* 4.12±4.16). There is no correlation between proliferation response of the PBMCs in injected group with the magnitude of leishmanin skin test conversion either at day eighty or one year post injection (Figs. 8 and 9). The mean production of IFN-γ by the stimulated cells with SLA was also lower compared to that of injected cases (mean±SD: 63.5±119.55 *vs.* 101.75±229.78 pq/ml), (Table 8).

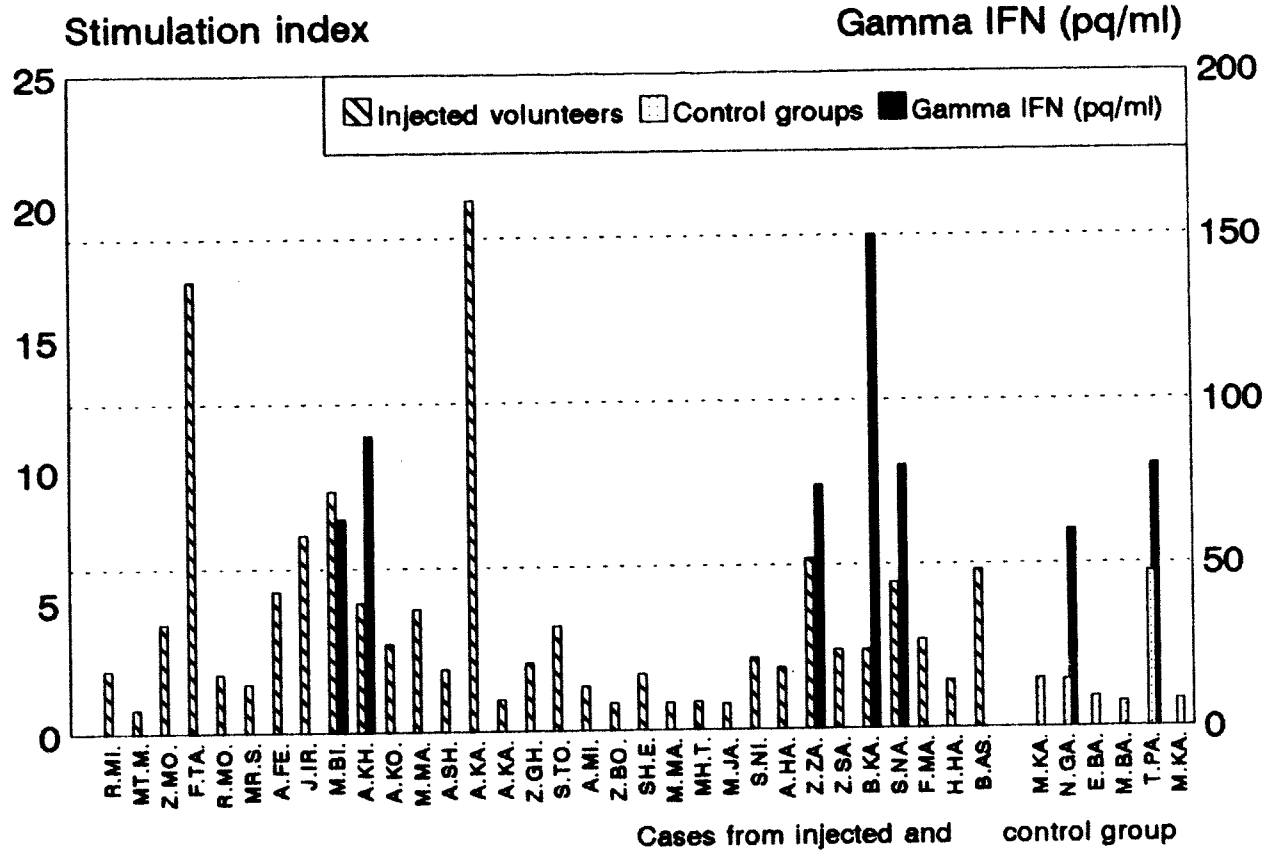

Figure 1. Lymphocyte proliferation response and interferon gamma production of cultured PBMCs from first year cases who were injected with either ALM+BCG or BCG and non-injected control group.

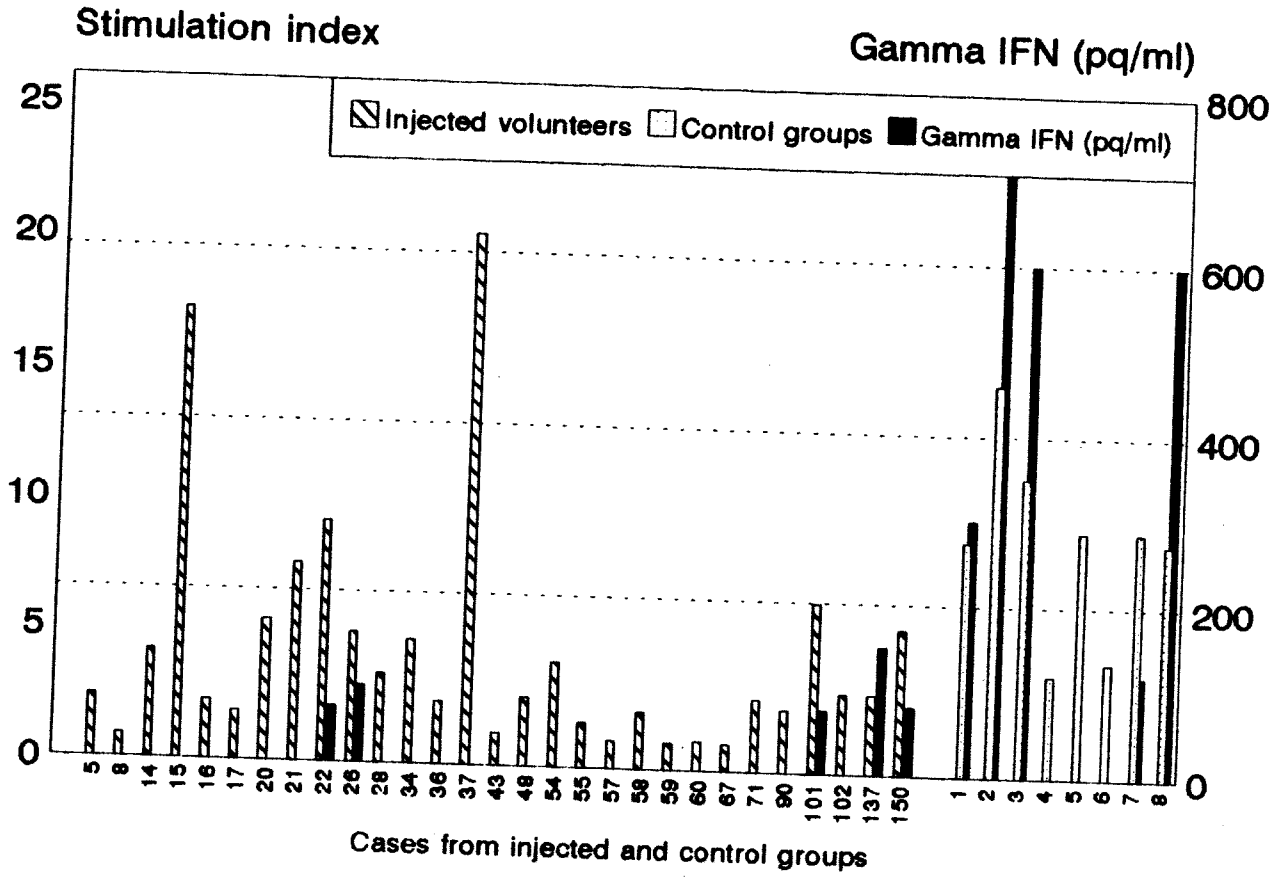

Figure 2. Lymphocyte proliferation response and interferon gamma production of cultured PBMCs from cases who were injected with either ALM+BCG or BCG and recovered individuals from the disease.

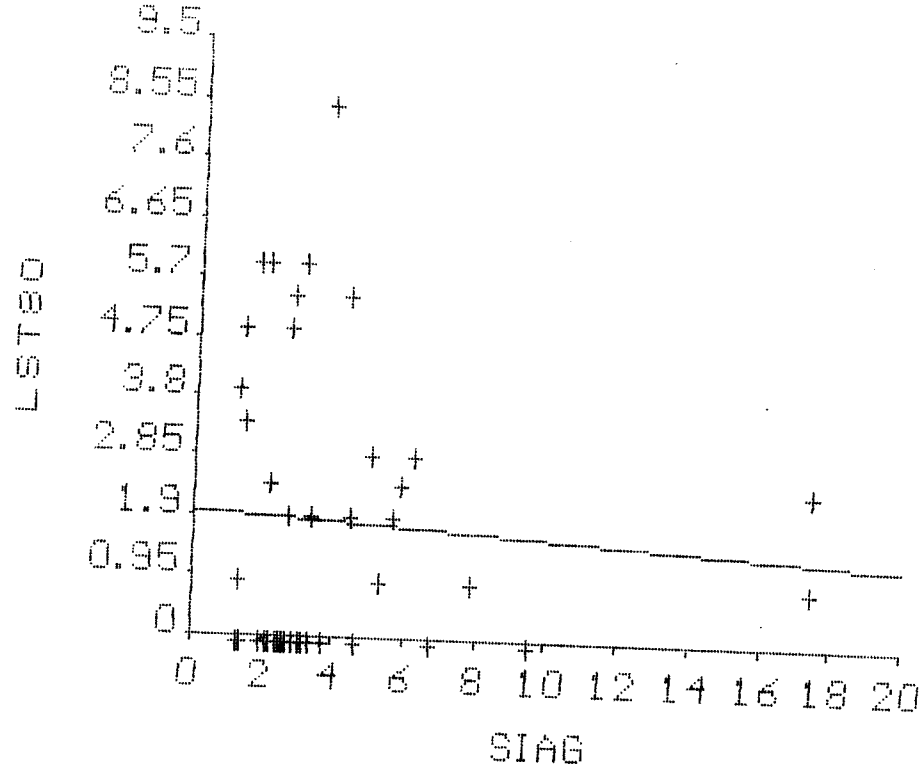

Figure 3. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* magnitude of LST induration (tested at day 80 post vaccination) of injected volunteers (with either ALM+BCG or BCG) who developed lesion .

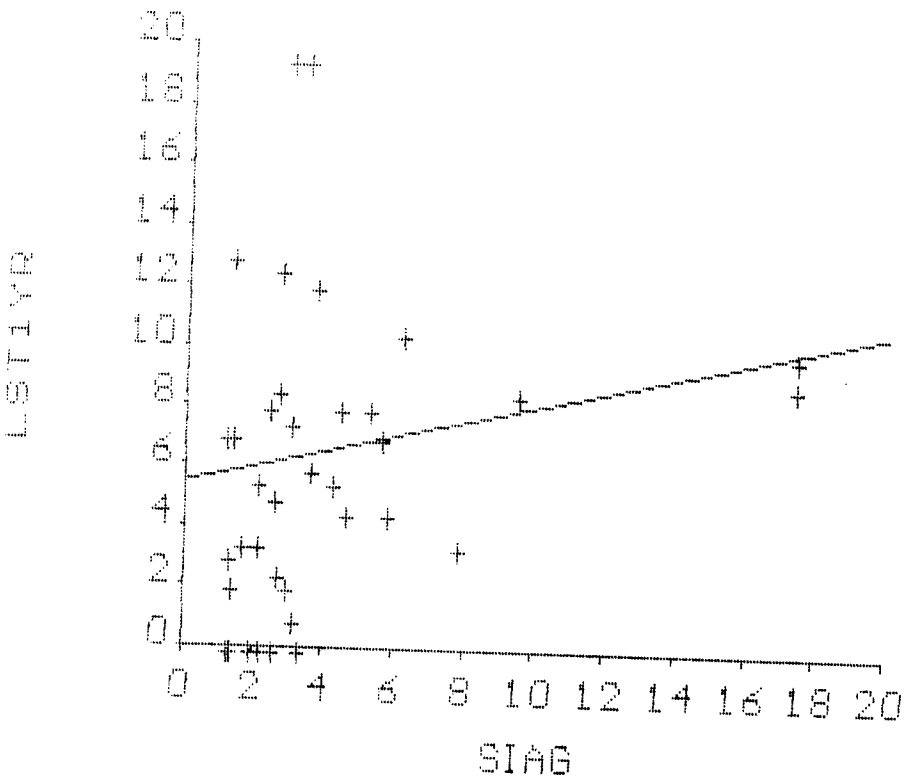

Figure 4. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* magnitude of LST induration (tested one year post vaccination) of injected volunteers (with either ALM+BCG or BCG) who developed lesion .

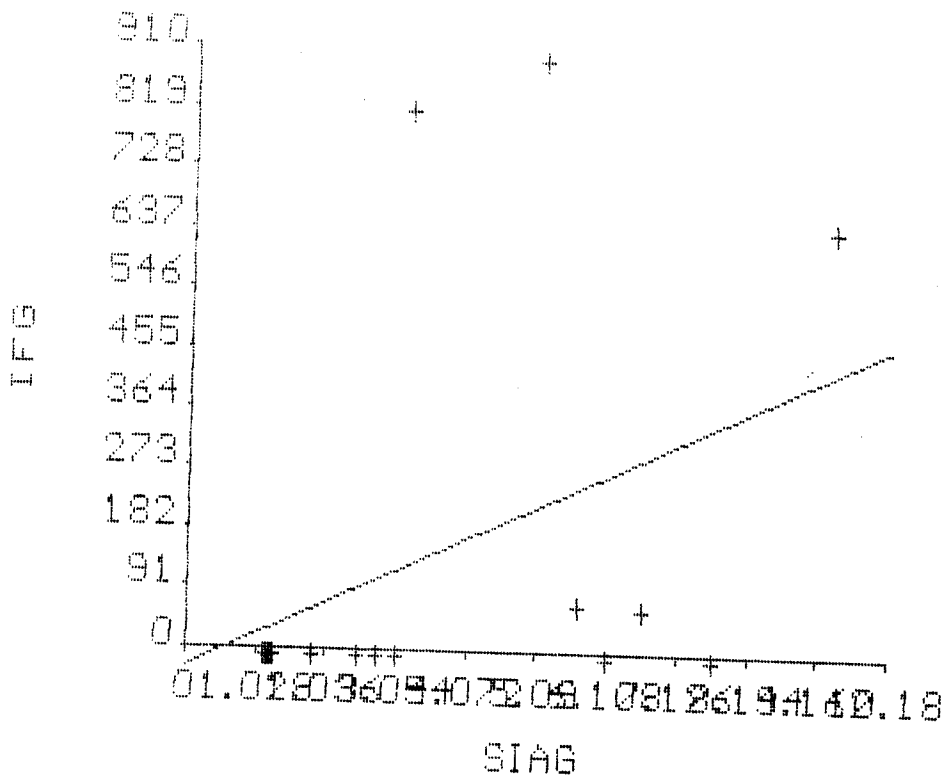

Figure 5. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* IFN-γ production by stimulated PBMCs with SLA from subject cases injected with either ALM+BCG or BCG.

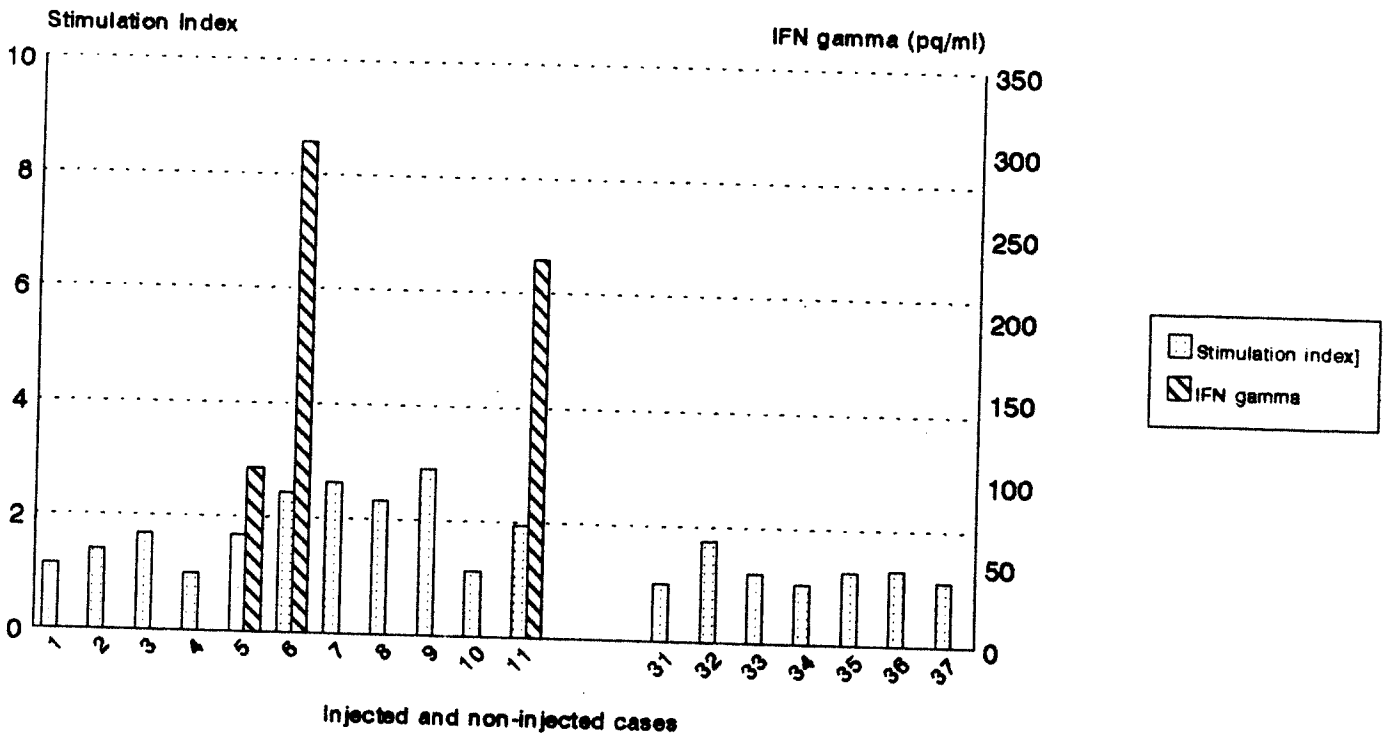

Figure 6. In vitro lymphocyte proliferation response and gamma interferon production of cases wo years post injection with either ALM+BCG or BCG and non-injected control cases.

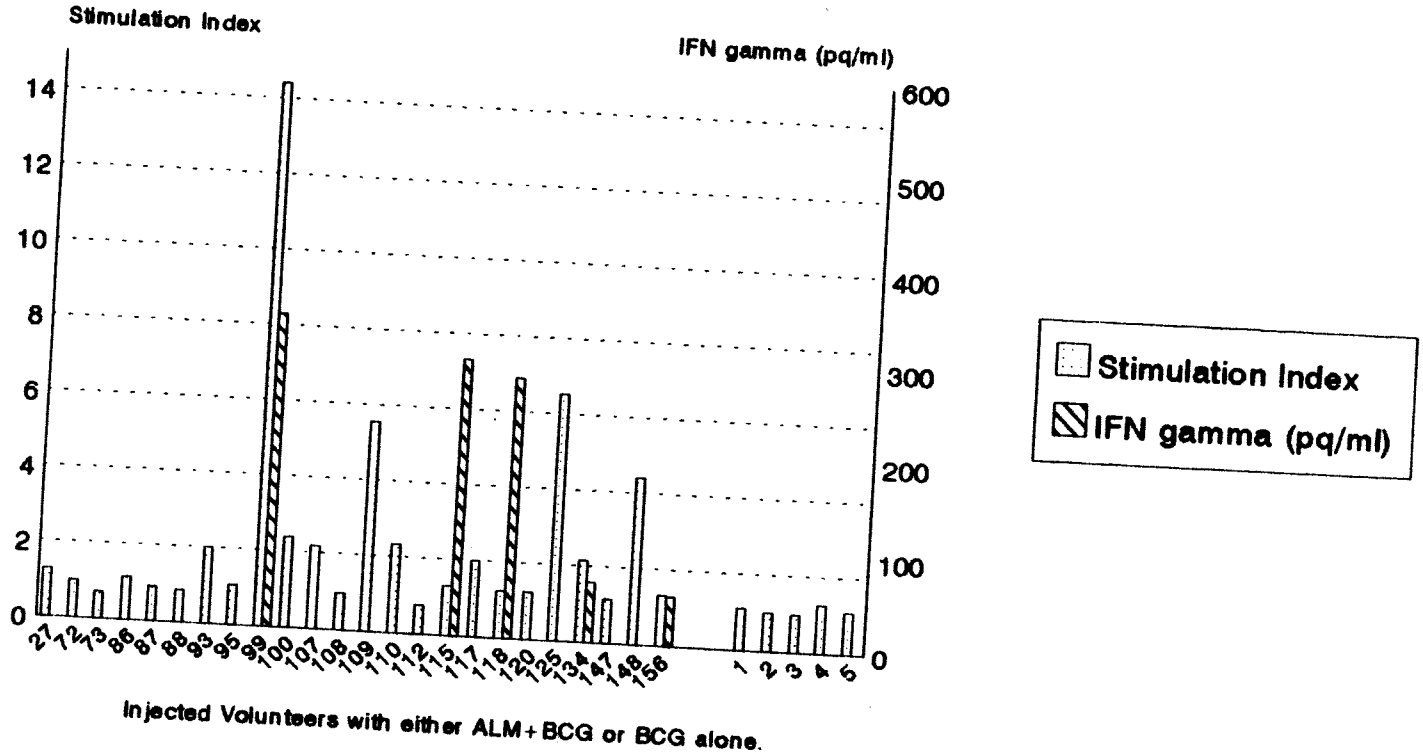

Figure 7. Lymphocyte proliferation response and interferon gamma production of cultured PBMCs from injected volunteers with either ALM+BCG or BCG alone and non-injected individuals.

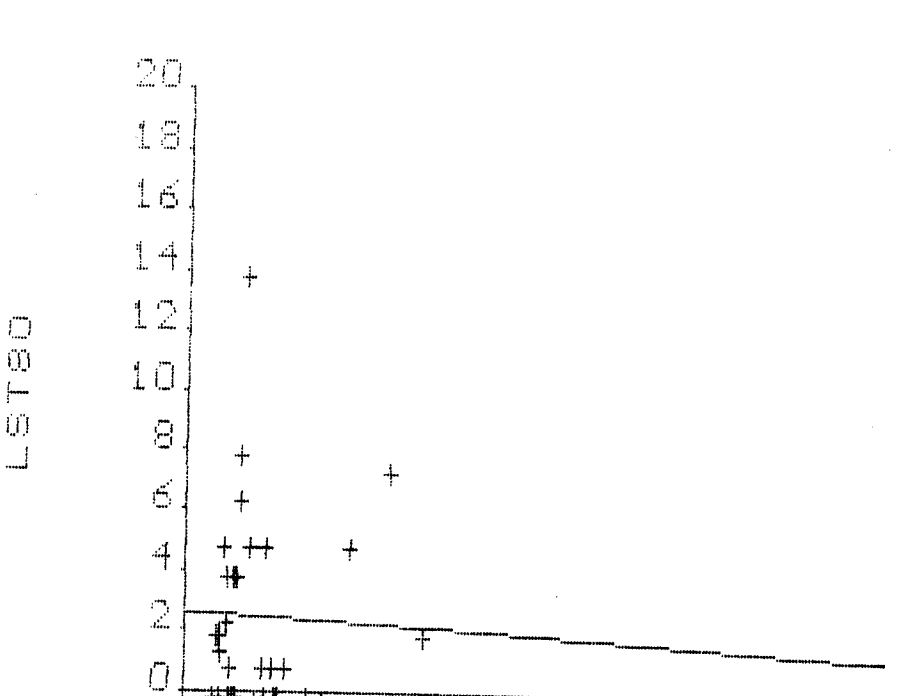

Figure 8. ***In vitro*** **lymphocyte proliferation response (expressed as SI_{Ag})** ***vs.*** **magnitude of LST induration (tested at day 80 post vaccination) of injected volunteers with no lesion with either ALM+BCG or BCG.**

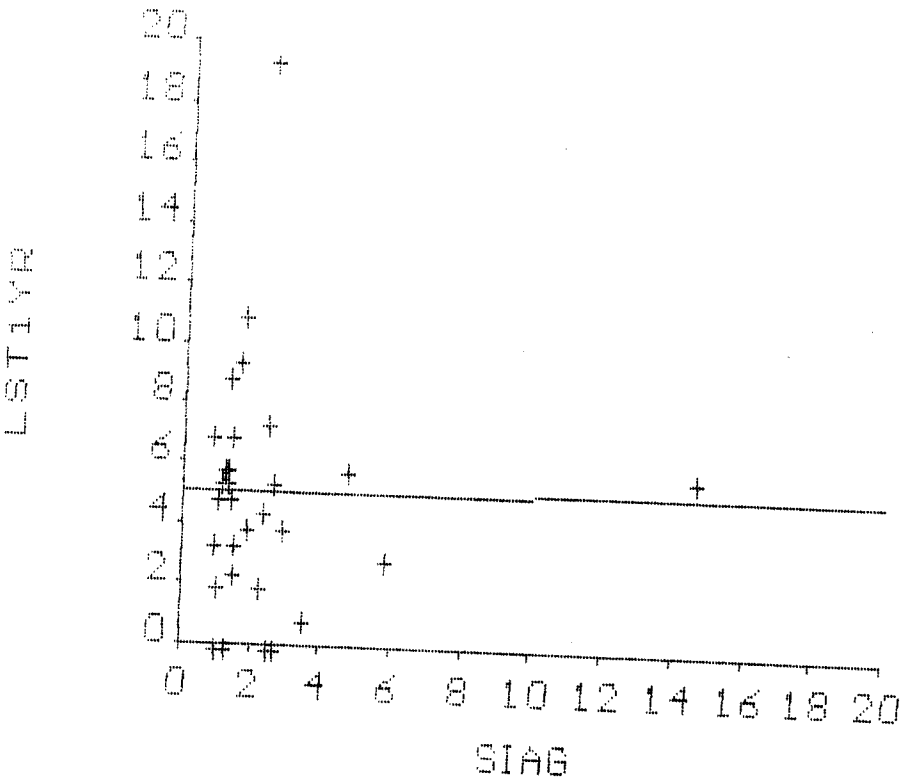

Figure 9. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* magnitude of LST induration (tested one year after vaccination) of injected volunteers with no lesion with either ALM+BCG or BCG.

Proliferative responses and the Pattern of IFN-γ production of PBMCs from leishmanin-positive cases

Virtually most of the leishmanin-positive cases demonstrated greater LST reaction (mean±SD, 4.36±2.32 mm) on screening day whereas any other subject groups or the controls showed no reaction before the injection (of ALM+BCG or BCG), (Tables 5 and 7). The stimulated PBMCs by SLA from these cases showed significantly higher stimulation indices (mean±SD: 8.13±3.90) compared to those of the other groups and the controls (P <0.001), however the mean of this value was lower than that in individuals with history of cutaneous leishmaniasis (8.13±3.90 *vs.* 9.26±3.5). IFN-γ production in supernatants of stimulated PBMCs from these patients was higher (mean±SD, 406.66±317.65) than that observed in BCG injected (30.66±53.11) or non injected (28.0±38.98) cases (Fig. 10 and tables 7 and 9).

TABLE 5

In vitro lymphocyte proliferative responses (LTT) of PBMCs from injected volunteers with either ALM+BCG or BCG and the magnitude of leishmanin skin test (LST) reaction (mm) to crude *L. major* antigen on screening day and 80 days post injection.

Groups	LST(Screeing day)**	LST(Day 80)**	SI**
Cases from injected group (35)*	0	1.87±2.31	4.11±4.16
Cases from third arm control group (7)	0	ND	3.08±3.15
Injected volunteers with no lesion (34)	0	2.42±3.19	2.17±2.54
Cases with pervious positive leishmanin skin test (7)	4.36±2.32	ND	8.13±3.90
Non-healing cases (17)	6.20±5.96	ND	1.93±1.32

* Number of subjects studied

** Mean±Std Dev values of LST and SI (Stimulation index)

TABLE 6

Leishmanin skin test (LST) reaction (mm) to crude *L. major* antigen of injected cases with either ALM+BCG or BCG at day 80 and one year post injection.

Groups	LST (Day 80)	LST (1 year)
Cases from injected group (38)*	1.87±2.31 **	6.33±5.01
Cases from third arm control group (7)	ND	4.37±2.68
Control from injected volunteers with no lesion (34)	2.42±3.19	5.03±3.95

* Number of subjects studied

** Mean±Std Dev values of LST and SI (Stimulation index)

Table 7

In vitro lymphocyte proliferation response and gamma IFN production of stimulated PBMCs with SLA from injected (with either ALM+BCG or BCG) and non-injected cases and the magnitude of LST reaction (mm) at day 80 and 1 year post injection.

Groups	Age(year) (Mean±SD)	LST on screening day	LST at day 80 post injection (Mean±SD)	Proliferative response (Mean of SI±SD)		IFN-γ production (pq/ml) (Mean±SD)
				PHA	Ag	
1 (*)	28.14± 10.86	0	1.87±2.31	84.0± 101.55	4.12± 4.16	101.75± 229.78
2	37.57±18	0	ND	114.96± 172.66	3.08± 3.15	28.0± 38.98
3	24.48± 10.66	0	2.42±3.19	62.02± 70.55	2.17± 2.55	63.5± 119.55
4	27.58±10.1	ND	ND	42.06± 26.17	1.93± 1.32	21.64± 51.27
5	34.5± 19.35	4.36±2.32	ND	73.04± 64.62	8.13± 3.90	406.66± 317.65

(*) Number of volunteers

(1) Cases from injected group (35)

(2) Cases from third arm control group (7)

(3) Control from injected volunteers with no lesion (34)

(4) Non-healing cases (17)

(5) Cases with pervious positive leishmanin skin test (7)

Lymphocyte proliferation response and cytokine production (IFN-γ and IL-4) of the cultured PBMCs from vaccinated cases

The lymphocyte proliferation response of the culture PBMCs of ALM+BCG injected cases to soluble *Leishmania* antigen (SLA) showed an increase in proliferation response of cultured PBMCs from ALM+BCG injected cases (mean±SD: 4.33±4.15) compared to BCG injected (3.35±3.75) or non-injected control groups (3.08±3.15), (Figs. 15, 16 and table 9). The measurement of IFN-γ production in supernatants of cultured PBMCs in response to *Leishmania* antigen indicated that the cases receiving ALM+BCG, produced higher level of this cytokine (280.44±388.73) compared to the cells from BCG injected cases (30.66±53.11) or non injected control group (28.0±38.98) and the high responders of this cytokine were among cases with high proliferation response (Table 9 and fig. 16). The measurement of IL-4 in the culture supernatants of PBMCs from ALM+BCG injected cases stimulated with SLA showed undetectable level of IL-4 and as shows in figure 8a there is no correlation between PBMC proliferation response in ALM+BCG injected group with the magnitude of leishmanin skin test conversion at day eighty post vaccination, but relatively some correlation exists between these two values when the vaccinees were skin tested one year after injection (Fig. 12). Also there is association

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TABLE 8

Leishmanin skin test (LST) conversion of vaccinees and BCG injected cases at day 80 and one year post injection.

Description		LST(At day 80)	LST(At one year)
Injected with lesion(s) development	ALM+BCG (24)	2.37±2.64	6.73±4.98
	BCG (19)	1.23±1.98	5.92±5.1
Injected without lesion development	ALM+BCG (18)	2.97±2.73	5.03±4.64
	BCG (14)	2.03±3.79	4.85±3.33

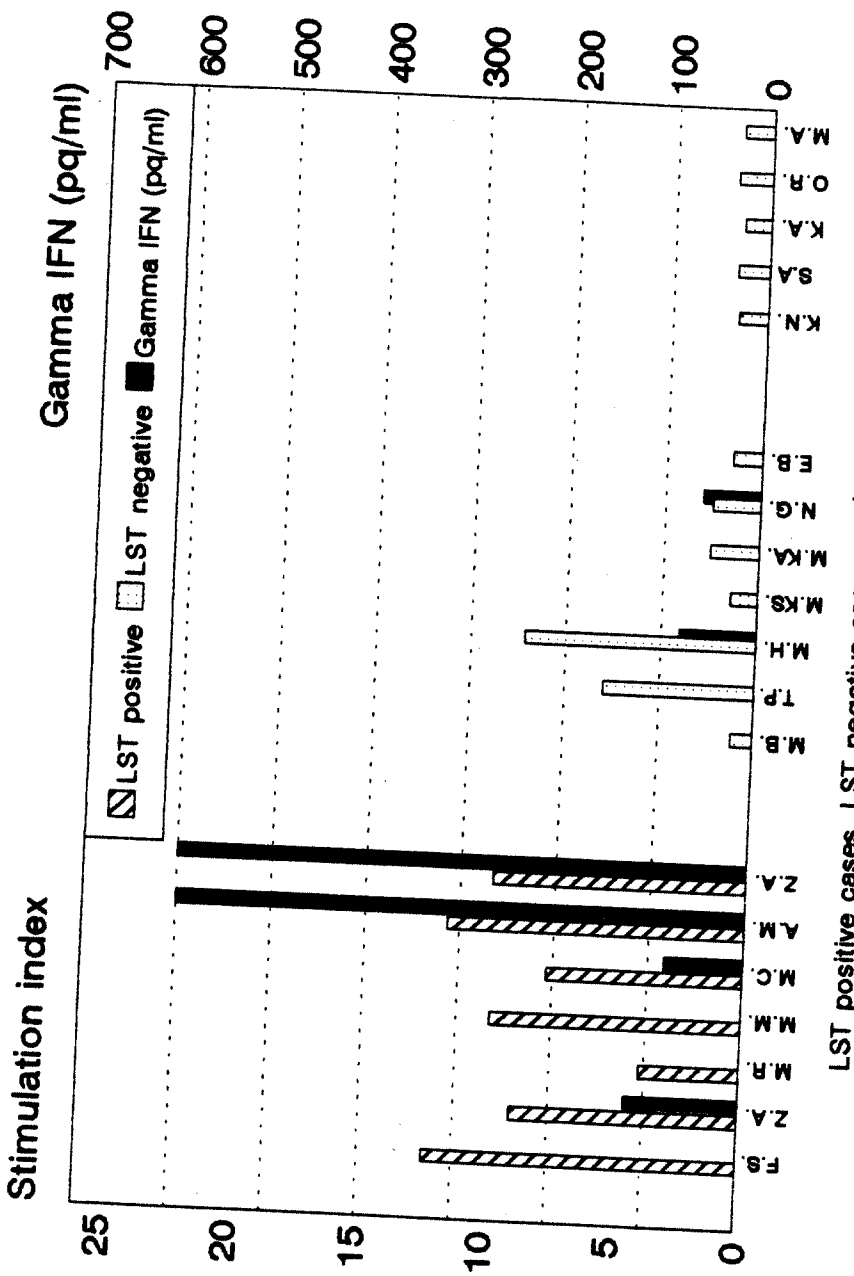

Figure 10. Lymphocyte proliferation response and interferon gamma production of cultured PBMCs from leishmanin positive cases, leishmanin negative cases and normal control individuals

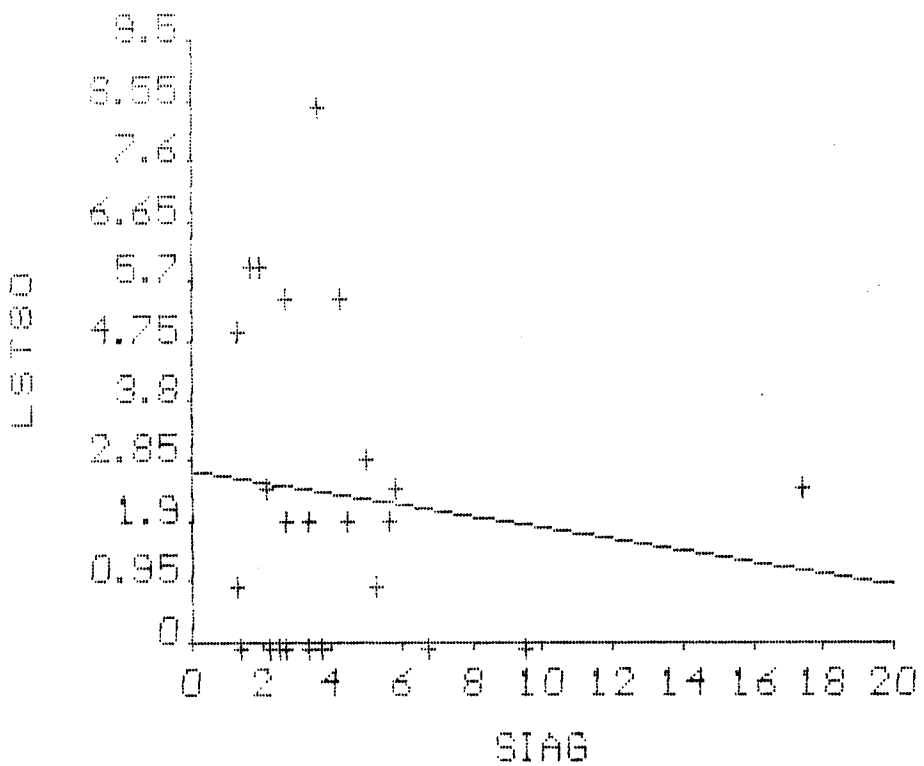

Figure 11. *In vitro* **lymphocyte proliferation response (expressed as SI_{kg}) *vs.* magnitude of LST induration (tested at day 80 post vaccination) of injected cases with ALM+BCG.**

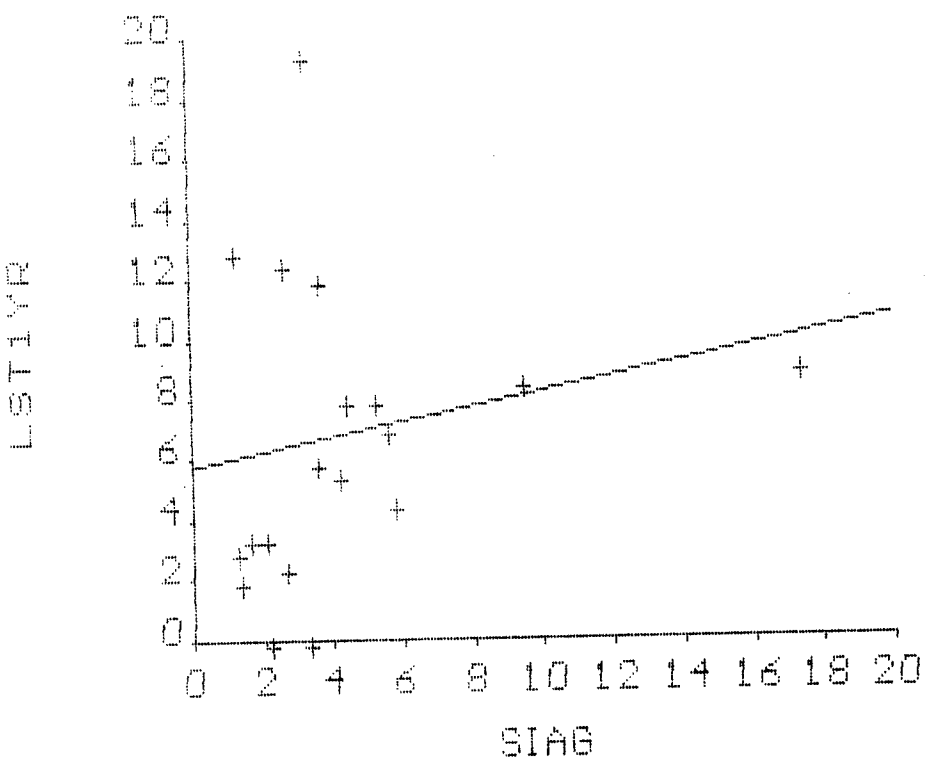

Figure 12. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* magnitude of LST induration (tested one year post-vaccination) of 20 injected cases with ALM+BCG.

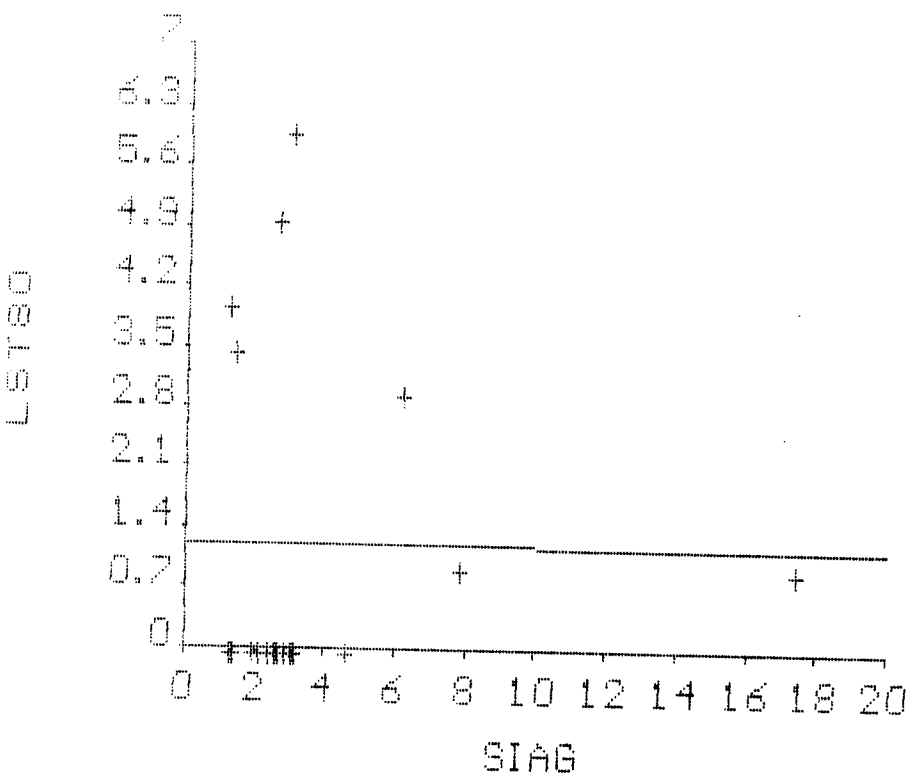

Figure 13. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* magnitude of LST induration (tested at day 80 post vaccination) of injected cases with BCG alone (control group).

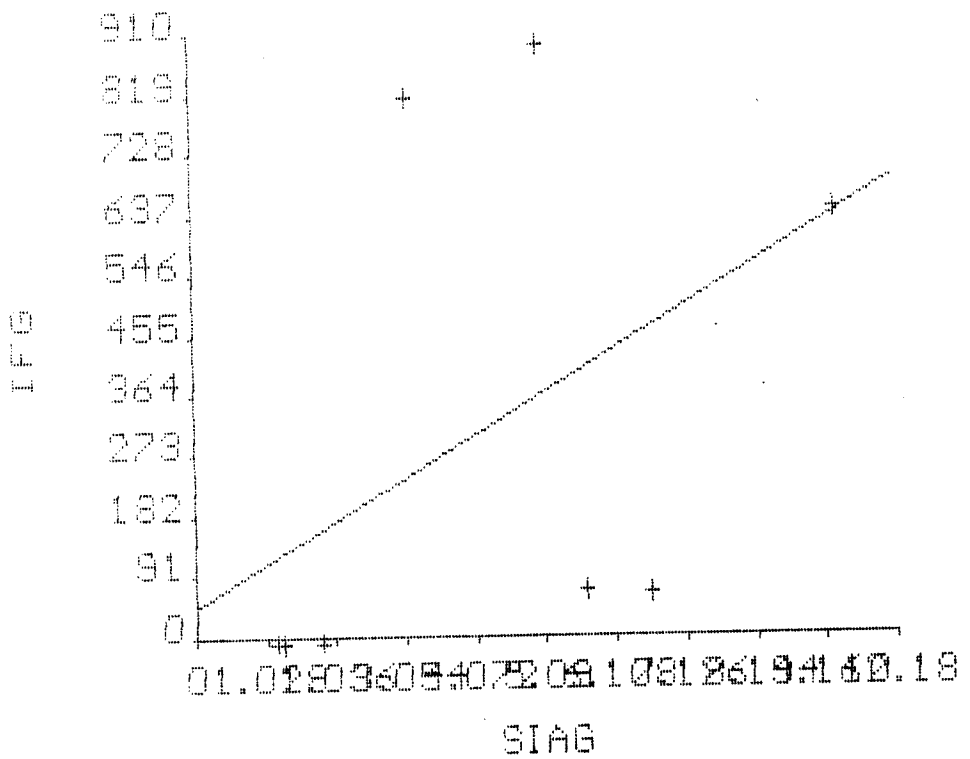

Figure 14. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* IFN-γ production by stimulated PBMCs with SLA from subject cases injected with ALM+BCG.

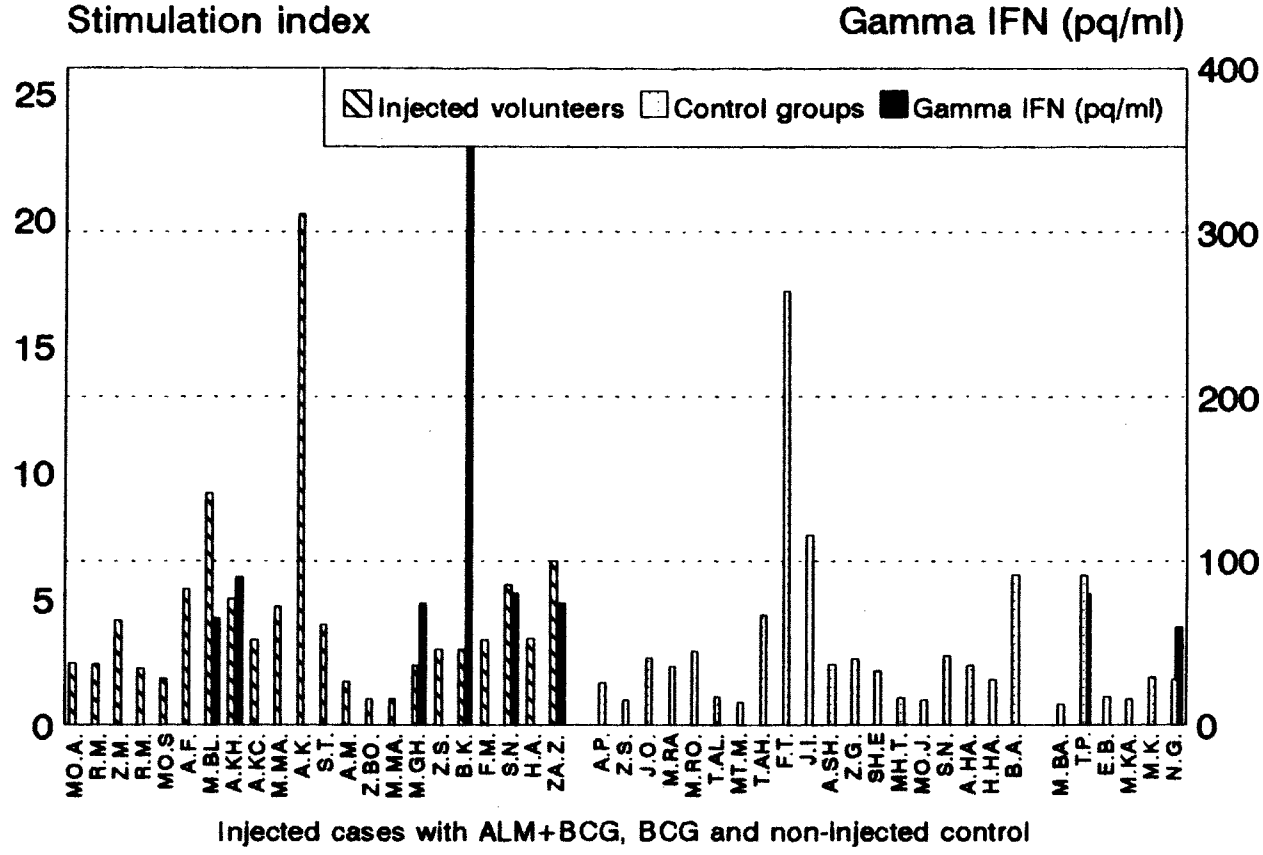

Figure 15. Lymphocyte proliferation response and interferon gamma production of cultured PBMCs from injected cases with ALM+BCG and control groups (BCG injected and non-injected cases).

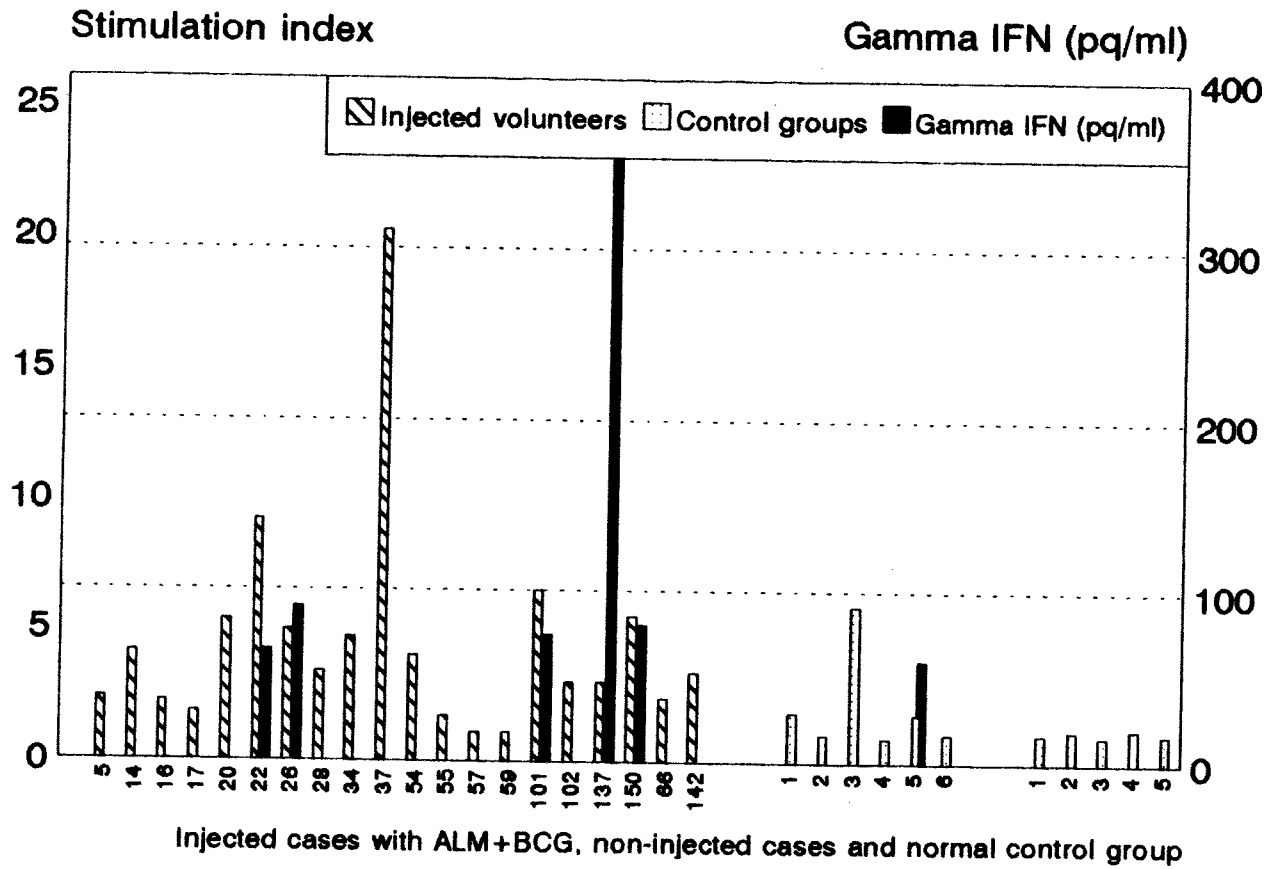

Figure 16. Lymphocyte proliferation response and interferon gamma production of cultured PBMCs from injected cases with ALM+BCG, non-injected cases and normal control group.

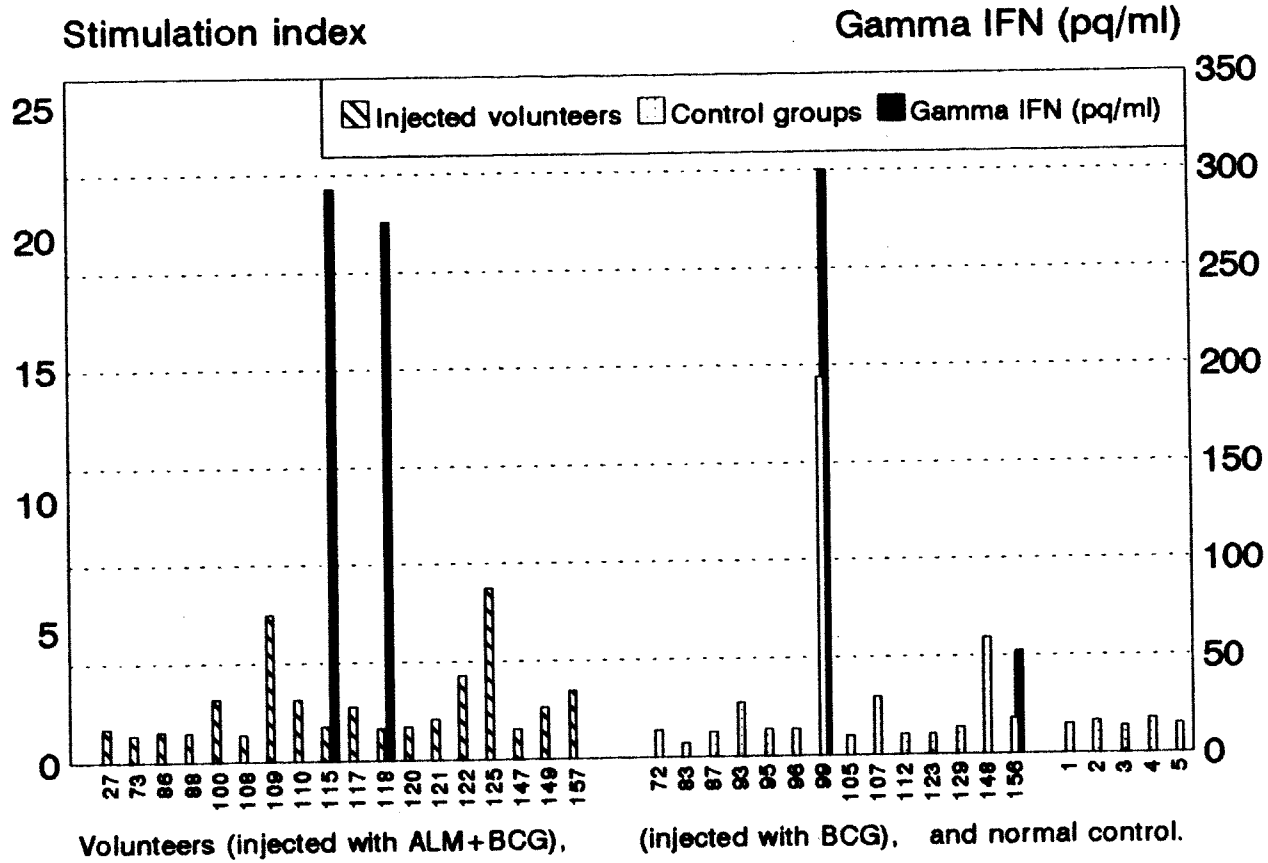

Figure 17. Lymphocyte proliferation response and interferon gamma production of cultured PBMCs from non-case injected voulunteers with either ALM+BCG or BCG and non-injected control group.

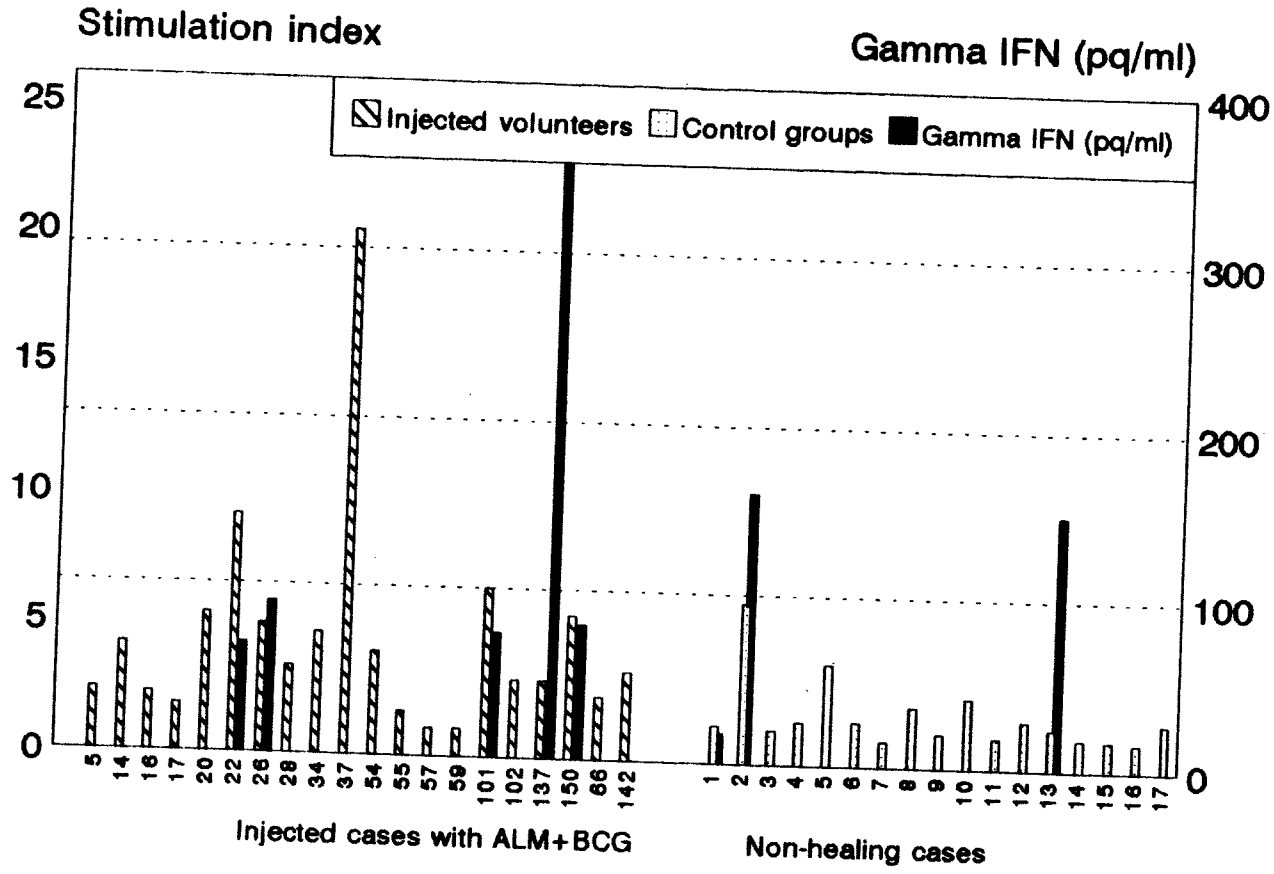

Figure 18. Lymphocyte proliferation response and interferon gamma production of cultured PBMCs from injected cases with ALM+BCG and non-healing cases.

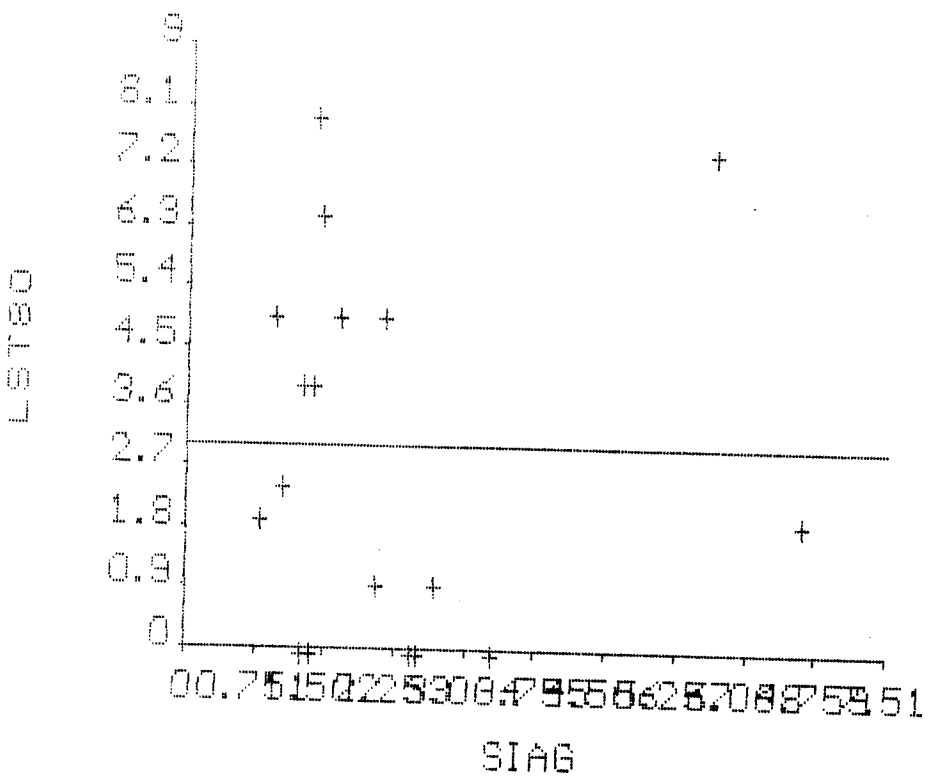

Figure 19. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* magnitude of LST induration (tested at day 80 post vaccination) of injected volunteers with no lesion with ALM+BCG.

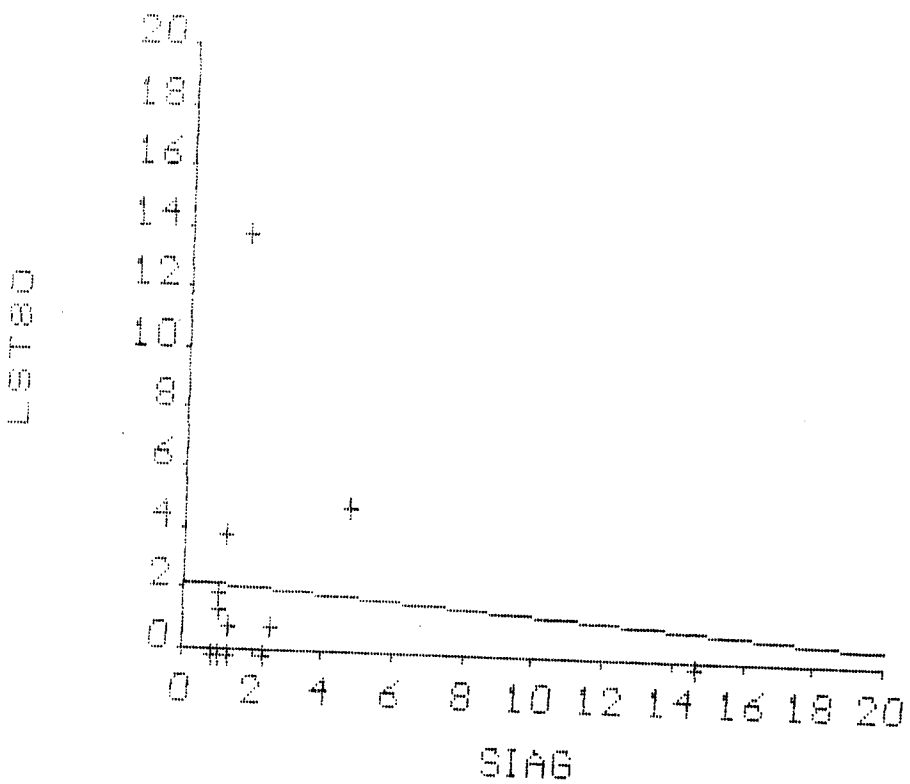

Figure 20. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* magnitude of LST induration (tested at day 80 post vaccination) of injected volunteers with no lesion with BCG.

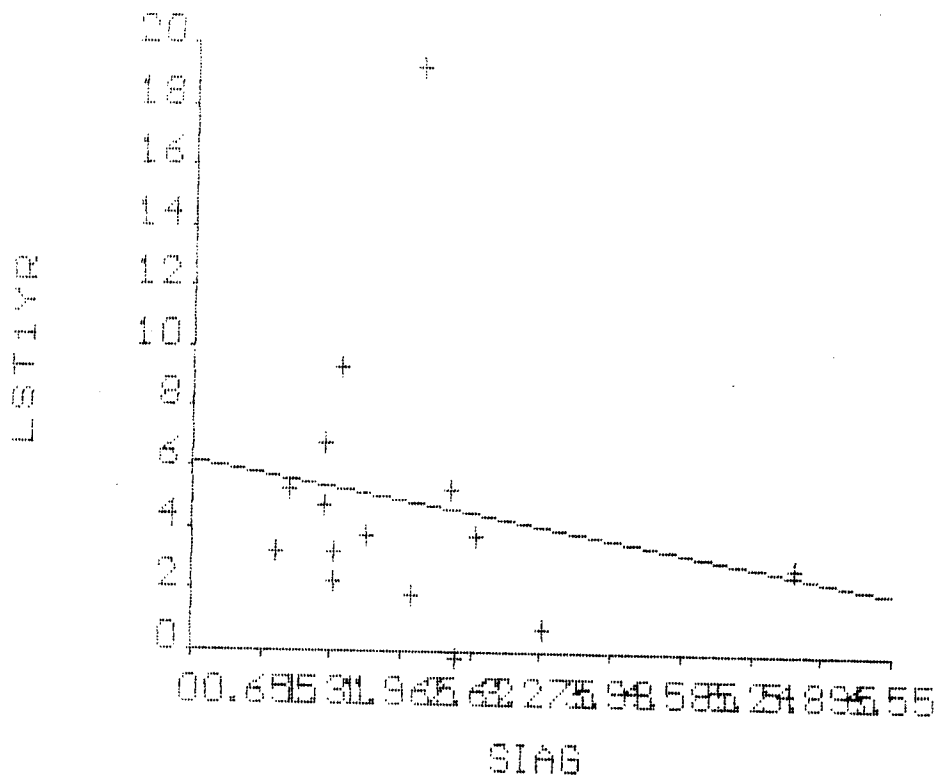

Figure 21. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* magnitude of LST induration (tested one year post-vaccination) of injected volunteers without development lesion with ALM+BCG.

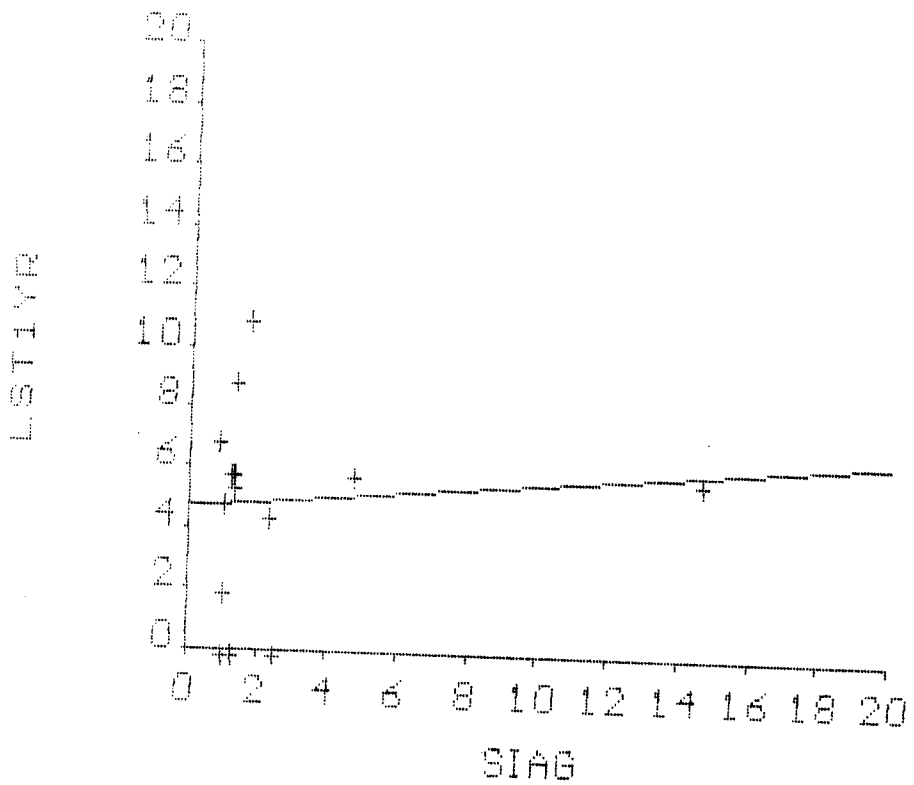

Figure 22. *In vitro* lymphocyte proliferation response (expressed as SI_{Ag}) *vs.* magnitude of LST induration (tested one year post-vaccination) of injected volunteers without development lesion with BCG.

The immunological profiles of cultured PBMCs stimulated with SLA from non-healing patients of cutaneous leishmaniasis and cured individuals from the disease

The lymphocyte proliferation responses and IFN-γ production in cell cultured from 17 patients with non-healing lesion(s) are shown in figure 23. The mean ^{3}[H]thymidine uptake in cultures stimulated by SLA was not statistically different from that observed in unstimulated cultures, reflecting the low stimulation index in these patients (mean±SD, 1.93±1.32). But PBMCs from individuals with history of cutaneous leishmaniasis exhibited a strong lymphocyte proliferation response (mean±SD: 9.26±3.5) when stimulated with SLA (Fig. 24 and table 9) and this is significantly higher compared to any other groups ($P<0.0001$). Absent or low IFN-γ production (ranging from less than 8 to 150 pq/ml) was observed in supernatants of PBMC cultures in response to SLA from non-healing patients, in contrast, when cultured mononuclear cells of individuals with history of CL stimulated with SLA, high level of IFN-γ are measured (mean±SD, 603.33±726.68 pq/ml) and correlate well with lymphocyte proliferation response (Fig. 24 and table 9).

The measurement of IL-4 in the cultured supernatants of PBMCs from non-healing cases stimulated with SLA showed relatively high level of this cytokine (Mean±SD: 751.25±797.32 pq/ml) compared to that in non exposed individuals (39.2±53.67 pq/ml), however there is a variation in IL-4 production among the patients, ranging from <49 to 2000 pq/ml (Fig. 25). No IL-4 level was detected in PBMC culture of individuals with history of CL in response to *Leishmania* antigen.

Table 9

In vitro IFN-γ production and lymphocyte proliferation response of PBMCs from injected volunteers with either ALM+BCG or BCG and different subject groups as well as leishmanin skin test reaction (mm) at day 80 and 1 year post injection.

Groups		LST80(mm)**	LST1YR(mm)	SI_{Ag}	IFN-γ (pq/ml)
Cases from injected group	ALM+BCG (21)*	2.38±2.46	6.73±4.98	4.33±4.15	280.44±388.73
	BCG (19)	1.23±1.98	5.92±5.14	3.35±3.75	30.66±53.11
Control from injected volunteers with no lesion	ALM+BCG (18)	2.97±2.73	5.03±4.64	2.28±3.62	189.67±164.4
	BCG (14)	2.03±3.79	4.85±3.33	2.1±1.58	42.66±109.85
Cases from third arm control group	(7)	ND	4.37±2.68	3.08±3.15	28.0±38.98
Cases with previous positive LST	(7)	ND	ND	8.13±3.90	406.66±317.65
Non-healing cases	(17)	ND	ND	1.93±1.32	21.64±51.27
Recovered individuals	(9)	ND	ND	9.26±3.5	603.33±726.68

* Number of subjects studied

** Mean±Std div values of LST response, SI and INF-γ production

ND Not determined

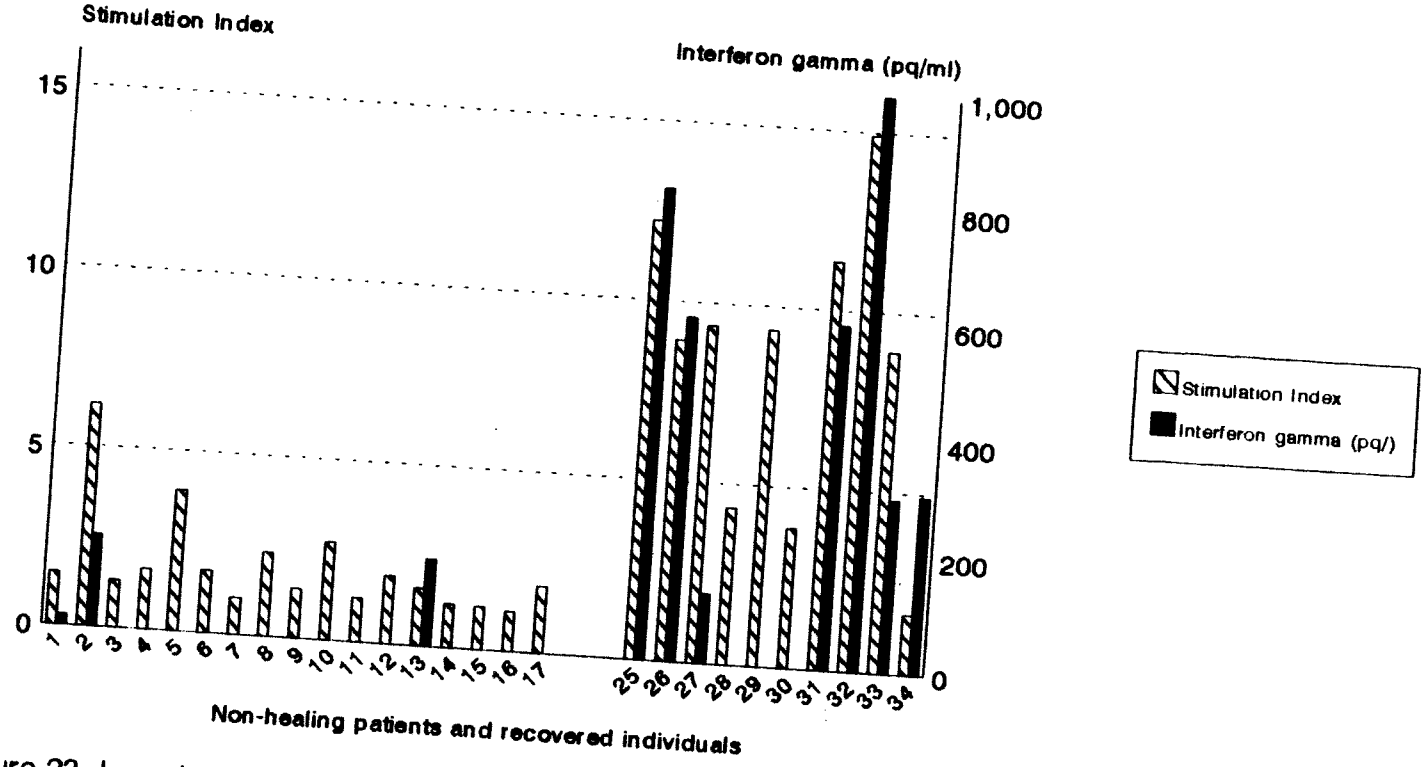

Figure 23. Lymphocyte proliferation response and interferon gamma production of SLA stimulated PBMCs from non-healing patients and recovered individuals from cutaneous leishmaniasis.

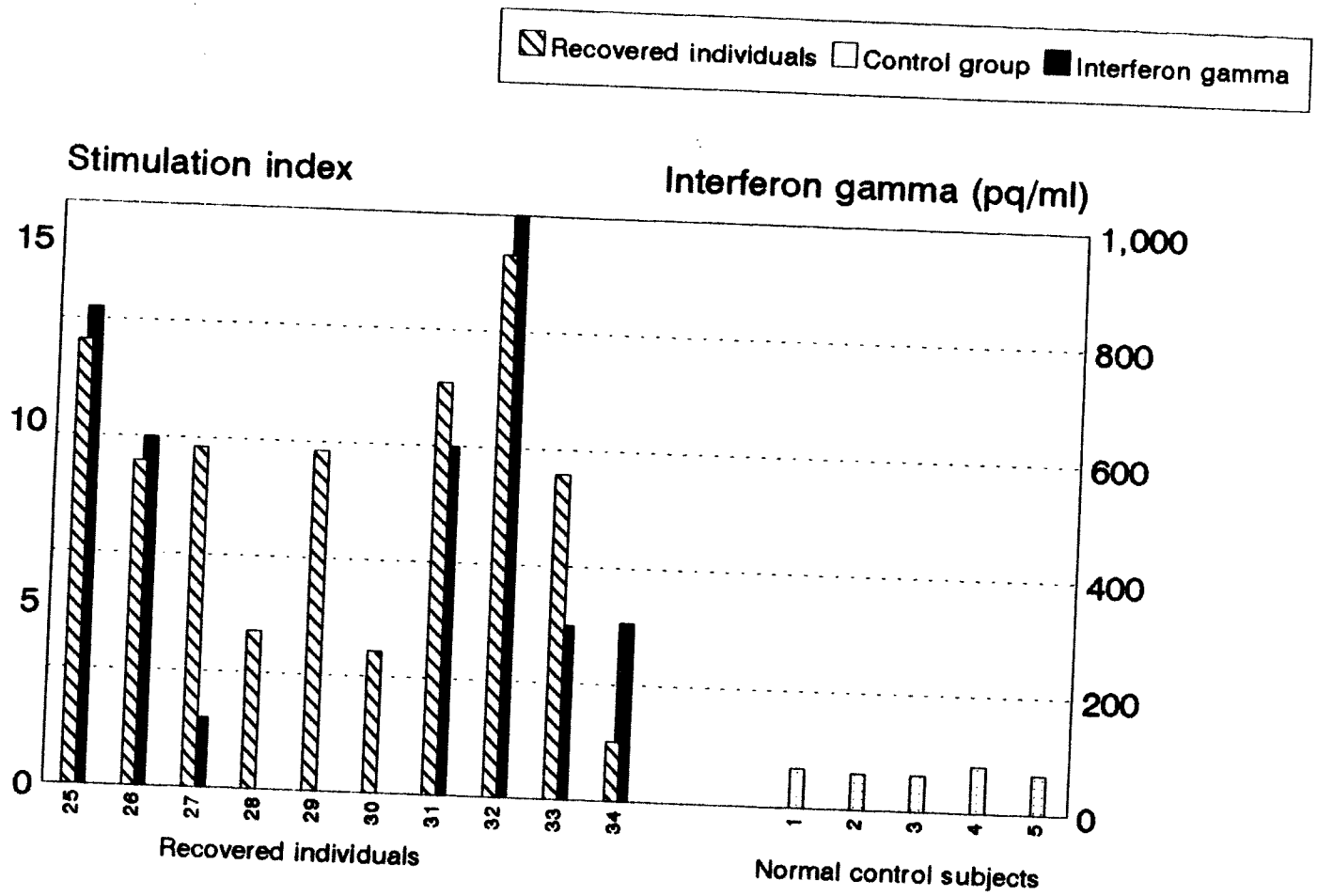

Figure 24. Lymphocyte proliferation response and interferon gamma production of cultured PBMCs from individuals with history of cutaneous leishmaniasis and normal control.

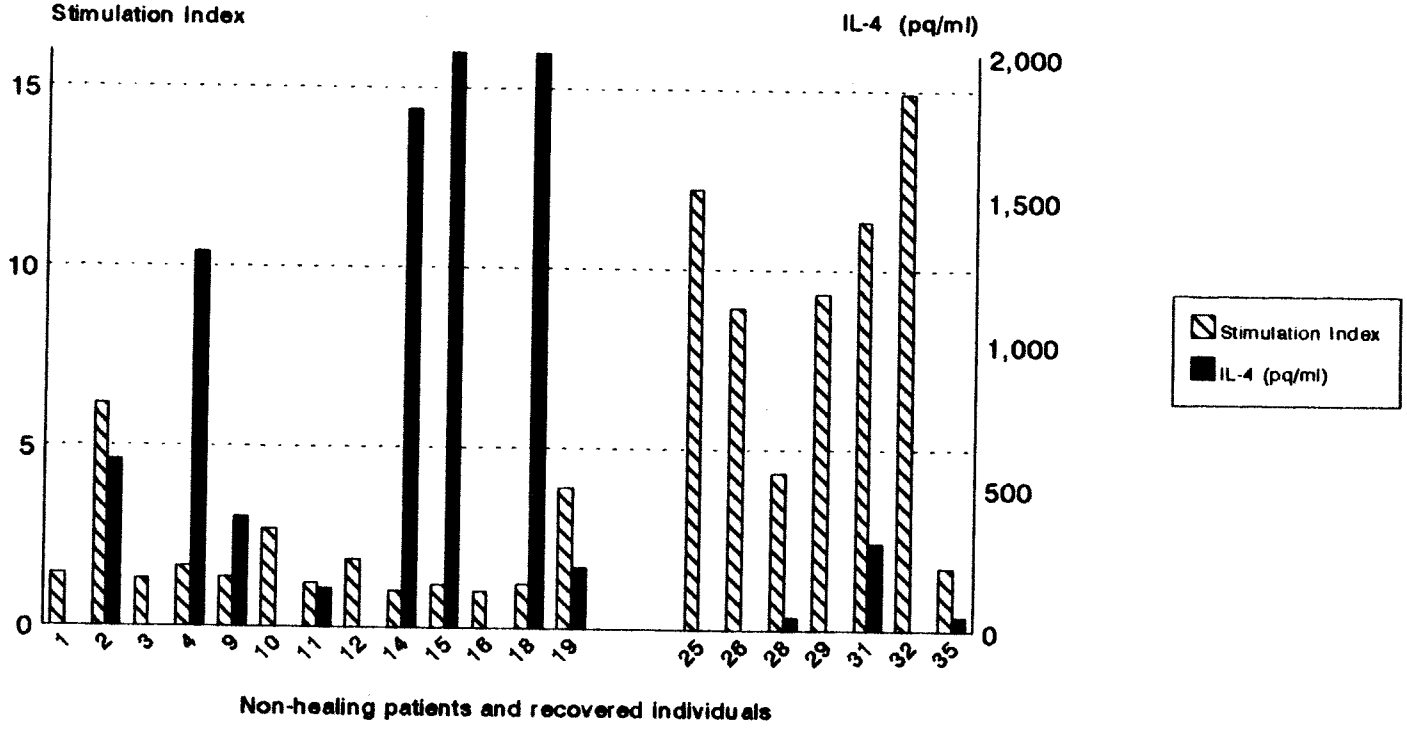

Figure 25. Lymphocyte proliferation response and IL-4 production of SLA stimulated cultured PBMCs from non-healing patients and individuals with history of cutaneous leishmaniasis.

CHAPTER 4

DISCUSSION

Leishmaniases present a group of diseases with different clinical manifestations ranging from a self limiting cutaneous lesion to a severe, progressive, systemic disease with a high mortality even in treated cases. The disease is caused by over 20 different species of *Leishmania*. The epidemiological diversity of different forms of the disease makes it impossible to control leishmaniasis with single control measure. Vector control and destruction of reservoir colonies are not cost effective or always achievable, it seems that an effective vaccine is the sole practical means to control the disease.

Different preparations of a crude *Leishmania major* vaccine have been evaluated in phase I-II trials. Based on the phase II results an autoclaved *Leishmania* vaccine mixed with BCG is used in phase III efficacy field trials locally and abroad (8,51). The aim of the this study is to search for surrogate marker(s) related to protection against *Leishmania* infection in vaccinated volunteers. In this regard the immune responses of the vaccinees and control groups were evaluated to determine if a single injection of

Leishmania vaccine mixed with BCG sensitized the vaccinees and as a result the vaccinees immune system induces a Th1 response upon natural infection via sandfly bites. All the experiments have been completed, data have been analyzed before the vaccine codes being opened by WHO/TDR authorities.

The volunteers who were willing to participate and donate blood samples were selected for this study. Blood samples were taken from the following groups of volunteers: Parasitologically proven cases of cutaneous leishmaniasis who received either ALM + BCG or BCG alone, leishmanin-skin-test-positive individuals who developed a *Leishmania* lesion, volunteers who were injected with either ALM + BCG or BCG alone and did not develop *Leishmania* lesion, non healing cases of cutaneous leishmaniasis. The recovered cases of cutaneous leishmaniasis were used as a positive control (Tables 3 and 4).

Leishmanin skin test (LST) was used as one of the main inclusion/exclusion criteria in the efficacy field trials of *Leishmania* vaccine. In the current efficacy field trials individuals with no reaction to leishmanin antigen were selected. LST positive individuals were excluded mainly due to the following reasons. It was thought that

LST positive reaction induced due to *Leishmania* sub-clinical infection and so they are resistant to re-infection. It was also thought that it is not safe to inject LST positive individuals and more over no safety data was available at the time. Active follow up of LST-positive individuals in current field trials in Isfahan and Bam, Iran, showed that LST positive individuals are not resistant and cases of cutaneous leishmaniasis among LST positive individuals were identified (The mean magnitude of LST reactions±SD: 4.36±2.32 mm). Further safety trials proved that LST positive individuals could be included in efficacy field trials (99). LST was performed in injected volunteers twice post vaccination (at day 80 and at one year post vaccination). LST conversion is significantly higher in volunteers who received ALM + BCG than in the group of volunteers who received BCG alone (LST conversion at day 80 post injection, 36.2% *vs*.7.9% and at one year, 37.8% *vs*.24.7%). The magnitude of LST response is also significantly higher in ALM + BCG injected group than BCG injected control group, at both occasions: day 80 and one year post vaccination, [mean±SD: LST80=3.4±3 mm for vaccinees and 1.05±2 for BCG injected volunteers ($p<0.001$)]. Also among the cases, as shown in tables 8 and 9, the magnitude of LST reaction is higher in the ALM + BCG injected group

than BCG injected group (mean±SD: LST80=2.38±2.46 mm *vs.* 1.23±1.98 mm and LST1yer=6.73±4.98 mm *vs.* 5.92±5.14 mm).

In an approach to produce an effective vaccine against New World cutaneous leishmaniasis, a vaccine consisted of killed promastigotes of five American dermotropic *Leishmania* strains (Leish vaccine 5) was used in the trial. The vaccinees were skin tested with leishmanin approximately 40 days after receiving the second vaccine dose and the result showed 74% leishmanin skin test conversion (124), which is consistent with our results in the current field trail.

Lymphocyte proliferation response to soluble *Leishmania* antigen (SLA) is higher in ALM + BCG injected cases (mean±SD: 4.33±4.15) compared to BCG injected (3.35±3.75) or cases from non injected control group (3.08±3.15), (Table 9 and fig. 10). ALM + BCG injected volunteers who did not develop any lesion had a lower stimulation index compared to the cases (2.10±1.58 *vs.* 4.33±4.15). This result is consistent with the result shown in the efficacy field trial performed by Mendonca *et al.*, who vaccinated the volunteers with two doses of composed vaccine (Leish vaccine 5) and the results showed a significant enhancement (124). In another study which was conducted in Brazil (149), killed promastigotes of five or six *Leishmania* strains (Leish vaccine 5 or Leish

vaccine 6) alone or mixed with *Corynebacterium parvum* as an adjuvant was used and the results showed that stimulation indices are significantly higher in the vaccinated group compared to the placebo injected control group ($P<0.001$).

The stimulation indices of LST positive individuals are significantly higher (8.13±3.90) than any other groups tested ($P<0.001$). However individuals with a history of cutaneous leishmaniasis showed the highest stimulation indices to SLA (mean reactions±SD: 9.26±3.5; $P<0.0001$), (Figs. 23, 24 and table 9). Non-healing cases of cutaneous leishmaniasis showed the least proliferation responses (1.93±1.32) against SLA (Figs. 18 and 23).

Kemp *et al.*, in 1994 showed that PBMCs from Sudanese individuals with a history of cutaneous leishmaniasis proliferated vigorously and produced IFN-γ after stimulation with either a crude preparation of *L. major* antigens or the major surface protease (gp63) of the parasite. The individuals with positive LST and no history of cutaneous leishmaniasis was also included in the above study and the results showed a high proliferation in LST positive subjects compared to the cases from non injected control group (98).

Carvalho *et al.*, in 1995 showed that lymphocyte proliferation response is significantly higher ($P<0.01$)

in individuals with a history of cutaneous leishmaniasis compared to the cases with active lesions (29). These reports are consistent with the current data obtained from the recovered individuals of CL and leishmanin positive cases and demonstrated that the T cell response to *Leishmania* antigens in humans who were cured from the disease or are LST-positive, possibly due to previous subclinical *L. major* infection, is a Th1-like response.

It is well documented in murine model of cutaneous leishmaniasis that the outcome of the infection is dependent upon the class of T-helper cell response induced. If a Th1 cell response generated, with the production of IL-2, IFN-γ, TNF-β and lymphotoxin, then the outcome of the disease is a self limiting lesion similar to human cutaneous leishmaniasis. While the induction of Th2 response, with IL-4, IL-5, IL-6 and IL-10 production, has been characterized with susceptibility and a progressive disease which eventually kill the animal (80).

IFN-γ provides the necessary signals for macrophage activation to kill the parasite (143). Infection of resistant mice, that have a targeted disruption in IFN-γ gene, with *L. major* results the development of lesion, indicating that IFN-γ is necessary for cure of experimental leishmaniasis (194). In human visceral leishmaniasis, lack of IFN-γ production by *in vitro*

stimulation of the PBMCs with SLA has been demonstrated (28). In the current study the level of IFN-γ production was assessed *in vitro* in PBMC culture as an indication of protection. The results indicate that the cases who received ALM+BCG produced a higher level of this cytokine (280.44±388.73) compared to the cells from BCG injected cases (30.66±53.11) or non injected control group (28.0±38.98), while a higher production of IFN-γ was observed in cases with high proliferation response (Figs. 1, 5 and table 9). Similar results was observed for injected volunteers with no lesion (189.67±164.47 *vs.* 42.66±109.85). The highest level of IFN-γ production was produced by the cells from LST positive cases (406.66±317.65 pq/ml) and the cells from individuals with history of cutaneous leishmaniasis (603.33±726.68 pq/ml).

IFN-γ and IL-4 were measured in the current study based on the evidence that comes from the study of Kemp *et al.*, which shows a Th1/Th2 polarization in human leishmaniases (97,98). No IL-4 was detected in the supernatants of SLA-stimulated PBMC culture of ALM + BCG injected cases, in contrast, a relatively high level of this cytokine was detected in the SLA stimulated PBMC culture of non-healing cases. The IL-4 level is higher in non-healing cases (Mean±SD: 751.25±797.32 pq/ml) compared to that in non exposed individuals (39.2±53.67 pq/ml),

however there is a variation in IL-4 production among the patients, ranging from <49 to 2000 pq/ml(Fig. 18) which might be a reflection of the course of infection. No IL-4 level was detected in PBMC culture of individuals with history of cutaneous leishmaniasis.

There is no association between PBMC proliferation response or IFN-γ production in ALM + BCG injected group with the magnitude of LST conversion which was seen at day eighty post vaccination (Fig. 11). One of the reason might be that the LST was performed at day 80 which was before the transmission cycle starts and PBMC proliferation response and IFN-γ production was measured when a *Leishmania* lesion developed, since correlation exist between these two values when the vaccinees were skin tested one year post injection (Fig. 12). Our data corresponds with the results of phase II studies of the same vaccine, in phase I-II study the *in vivo* (LST) and *in vitro* (PBMC proliferation response and IFN-γ production) was evaluated simultaneously (8). Also lack of correlation between stimulation indices or IFN-γ production and the magnitude of LST of the vaccinated cases is consistent with the results of a field trail conducted by Nascimento *et al.*, who performed leishmanin skin test five months post booster. The current results are also correspond with the data presented by Mendonca *et al.*, who carried out LST

in forty days post booster (124,149).

Strong proliferation response and IFN-γ production in ALM+BCG injected cases is an indication of Th1 response and also an evidence of protection against *Leishmania* infection.

There were two efficacy field trials ongoing in Iran simultaneously, one in all ages against zoonotic cutaneous leishmaniasis which the current study carried out in adult (vaccinated and control groups) and the second trial was performed against anthroponotic cutaneous leishmaniasis in school children. No immunological study was performed in school children due to ethical consideration. The codes were revealed after all experiments were completed and the data were analyzed. The data were analyzed by several centers independently, the results show that always a difference exists between ALM + BCG injected volunteers and BCG injected group. The incidence rate was lower in ALM+BCG injected group in both trials. Similar to other killed vaccines it was not expected to induce full protection with single dose of *Leishmania* vaccine but the study was started with single dose due to safety considerations. The *in vitro* results of our experiments also correspond with the results of incidence rate. *In vivo, in vitro* and epidemiological results altogether indicate that more than one dose of killed *Leishmania*

vaccine is needed. So phase I-II trial with multiple doses has completed and based on the results several multiple doses have started locally and abroad.

The results obtained from non-healing cases showed that PBMCs did not proliferate or produce IFN-γ in response to *Leishmania* antigens but produce a high level of IL-4. The lack of IFN-γ production and a high level of IL-4 seems to be a Th2 responses in these patients. These results could be used for future chemotherapy and immunotherapy in chronic or non-healing cases of cutaneous leishmaniasis.

CHAPTER 5

CONCLUSION

The type of immune response induced is depend upon several factors such as kind of antigen, weather or not mixed with an adjuvant, the type of adjuvant used, the route and the number of injection. The aim of *Leishmania* vaccine development is to search for a vaccine which induce a Th1 response and as a result protect the vaccinees.

In this study the immune responses of the following groups were evaluated: a group of volunteers who received a single dose of autoclaved promastigots of *Leishmania major* (ALM) mixed with Bacillus Calmette Guerin (BCG), the individuals with history of cutaneous leishmaniasis (CL), cases of CL who were originally leishmanin skin test (LST) positive and patients with non-healing lesion(s) of CL. The following results were obtained:

The volunteers who were injected with ALM+BCG exhibited significantly higher leishmanin skin test (LST) conversion, also these individuals showed higher *in vitro* proliferation response and interferon gamma (IFN-γ) production than the group of volunteers who received BCG alone. The peripheral blood mononuclear cells (PBMCs) of these volunteers did not

produce any detectable level of interleukin 4 (IL-4).

The individuals with history of CL who are refractory to the disease exhibit the highest magnitude of LST reaction, significantly higher *in vitro* proliferation response and IFN-γ production than the control group. These values were even higher than in the volunteers received ALM + BCG. Lack of IL-4 production in the cell culture of these individuals was observed.

The *in vitro* responses of the PBMCs of non-healing patients of CL showed that the cells from these cases do not proliferate or produce IFN-γ in response to *Leishmania* antigens but produce a high level of IL-4. Based on the results it is concluded that:

1. The use of a single dose of ALM + BCG is safe and dose not induce any immunological response which might exacerbate the natural infection.
2. A single dose of ALM + BCG induced Th1-like response but the level of such response is not sufficient enough for full protection. These results suggest to consider further evaluation of the vaccine with following strategies:

a. Multiple injection of ALM + BCG might results the induction of sufficient Th1 response. Safety field trails of three injection of ALM + BCG have been completed and the efficacy field trails of this protocol is currently underway against cutaneous and visceral leishmaniasis.

b. Changing the adjuvant which could result in good protection. In this regard Alum precipitated mixed with BCG is a candidate and primate studies is initiated in National Institute of Health (NIH), Bethesda, MD. and in India.

CHAPTER 6

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SEARCH FOR SURROGATE MARKER(S) OF IMMUNITY FOLLOWING VACCINATION WITH EXPERIMENTAL VACCINE (AUTOCLAVED *LEISHMANIA MAJOR* + BACILLE CALMETTE-GUÉRIN) IN HUMAN VOLUNTEERS

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Majid Mahmoodi, B.S., M.S.P.H.

Denton, Texas

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