DISCUSSIONS
DISCUSSION

The protozoan parasites of the genus *Leishmania* are the etiological agents of leishmaniasis which causes considerable morbidity and mortality in humans, the most serious of the many forms of leishmaniasis are the visceral types caused by the members of *L. donovani* complex. Visceral leishmaniasis is a debilitating, often fatal disease in man. The clinical sign may vary from one infected individual to another, according to the host’s ability to mount an effective immune defense response. For prophylactic purposes a few antileishmanial agents are commonly used in the medical practice but most of them are marked with relatively high toxicity, significant chemotherapeutic failures (TDR news, 1990) and immunosuppressive effects (Adinolli and Bonventre, 1990). More currently, significant chemotherapeutic advanced and continued effort is under progress to develop a more suitable immunotherapeutic agent, because immunotherapy is a low-cost, low risk alternative to chemotherapy (Convit et al., 1987).

Numerous investigations have so far been carried out to determine the immune aspects of the disease. The increasing evidences indicate that CMI mechanisms play a major role while humoral immune mechanisms only marginally help in protection through acquired immunity to visceral
leishmaniasis (Preston and Dumonde 1976b; Poulter 1980; Reed 1981). Since leishmanial infections are broadly related to depression of cell-mediated immunity, and it has long been clear that vaccines need to induce protective T-cell mediated immunity against the protozoan, therefore the aim is to prevent multiplication of the parasite within macrophages. Earlier work has already demonstrated that cytokines released by antigen-specific CD4+ T-cells enhance the antileishmanial activity of macrophages. Both oxygen-dependent and oxygen-independent leishmanicidal agents have been implicated (Playfair et al., 1990). Although, the leishmanicidal actions by different means have been fairly elucidated (Nacy et al. 1981; Ridel et al. 1987), but no vaccine against any form of leishmaniasis has been shown conclusively to be effective (Report of WHO Expert Committee, 1990).

Vaccination experiments in laboratory animals have been carried out using killed or attenuated parasites in combination with several immunoadjuvants. But complete protection of vaccinated animals has hardly been ever achieved. The use of different types of antigen preparations in such vaccination experiment was largely responsible for obtaining somewhat varying results (Holbrook and Cook 1983; Scott et al. 1990). Attempts have also been made towards immune enhancement of the host with the help of adjuvants for eliciting a more potent protective immunity. Killed
leishmania promastigotes together with live BCG were used for immunization of patients (Convit et al., 1987) where 94% protection was achieved. Earlier work of this nature in our laboratory has shown that whole, particulate and subcellular fractions of leishmania antigen in combination with glucan [(β-1,3) polyglucose, a derivative of inner cell wall of Sacchromyces cerevisiae], can induce protective immunity in golden hamsters (Obaid et al., 1989).

The present investigation describes the activity of Bacille Calmette-Guerin (BCG) as an immunoadjuvant for immunizing golden hamsters with whole, particulate and soluble fractions of L. donovani antigens. The antigenic stimulation provided to the animals with the help of BCG was assayed and characterized in detail. First of all, L. donovani whole antigen was prepared from laboratory cultures in Brain Heart Infusion Agar medium using HBSS as the liquid phase. The whole antigen was further fractionated into soluble and particulate antigen fractions. The antigens before immunization were partially characterized and immunizations were carried out either by the three antigen fractions only, or in combination with BCG in golden hamsters. The degree of protection afforded by these vaccine candidate antigens was assayed by estimating the humoral and CMI responses following immunization.
The induction of protective immunity, or the extent of immune protection achieved was studied by challenging the immunized animals with viable *L. donovani* promastigotes (1x $10^6$). The protection criterion was largely based on lowered organ parasite counts, rising antibody titres in ELISA and IHA tests, enhanced IgG and IgM levels in hyperimmune sera and, the appearance of delayed hypersensitivity response to leishmania antigen and increased macrophage migration inhibition in vaccinated animals. Haematological and pathological parameters were also taken into consideration for estimating the degree of protection. Also, the level of acid phosphatase was assayed in vaccinated animals in order to see the extent of protection.

Since complete protection was not achieved in this study, therefore, a small dose of Stibogluconate was used intramuscularly to render the vaccinated animals completely protected. The protection studies were largely based on estimating the parasite burden in the liver and spleen by organ impression smears.

The parasites from different Leishmania species are almost indistinguishable morphologically, but the results of molecular and immunological analysis such as electrophoretic patterns of isoenzymes (Miles et al, 1980), buoyant density of K-DNA and nuclear DNA (Shaw, 1982), serology of excreted factors (Schnur et al, 1972), reactions using monoclonal...
antibodies (McMahon-Pratt et al, 1982) and antigen preparations have helped to solve the different intricate problems. Since purification of parasite antigen needs large number of parasites free from contaminants therefore a large number of media has been devised for the cultivation of L. donovani, in vitro. However, the majority of such media are not entirely satisfactory for large scale cultivation of parasites, especially for the purpose of antigen isolation and for carrying out a large number of other immunological studies.

In this study Brain Heart Infusion Agar medium using Hank's Balanced Salt Solution (HBSS) as a liquid phase, was used for large scale cultivation of L. donovani. This medium was found to give maximum growth in our cultures. This medium was also supplemented with citrated rabbit blood. The Brain Heart Infusion Agar Medium supplemented with citrated rabbit blood is still used by a number of workers (Jalees et al, 1979) for in vitro cultivation of this parasite. It has been claimed to be very suitable for antigen preparation. The HBSS used in this medium as overlay contained 0.25 percent glucose instead of 0.1 per cent. This overlay was proved to be more superior and better than Locke's solution. The initial inoculum in our study was $5.5 \times 10^5$ cells/ml at a 0 hr reading and maximum growth was obtained at 168 hr reading where the rate of multiplication at 168 hr reading was found
to be $6 \times 10^5$/hr. Here the number of parasites at 168 hr reading was $1.0 \times 10^9$ cells/ml.

During the course of earlier studies in our laboratory by Obaid et al. (1985) brain heart infusion agar medium was supplemented with 50% egg homogenate for in vitro cultivation of *L. donovani* in which maximum growth was obtained in cultures. The starting inoculum was $1.7 \times 10^6$ cells/ml, that gave a yield at $1.2 \times 10^9$ cells/ml at 168 hr reading. Our results using brain heart infusion agar medium supplemented with citrated rabbit blood are similar to that of Obaid et al., 1985. Ray and Ghosh (1980) have used a liquid culture medium (LDLC-II) for bulk cultivation of *L. donovani* promastigotes. The maximum growth in this medium was obtained between 10-13 days after which the parasite count declined.

Whole antigen preparation was made from 6th day old cultures following repeated washing in PBS (pH = 7.2) to remove the extra material and the organisms were resuspended at $1.2 \times 10^9$ parasite/ml in 100 mM Tris HCL and 1mM EDTA (pH 8) which were incubated at 4°C for 10 min. The parasites were then subjected to ultrasonication at 30 MHz for 1 min burst (X5) at 4°C, and viewed microscopically to ensure complete parasite disruption. The parasite suspension was then centrifuged at 105,000 X g for 1 hr in order to get soluble and particulate antigen fraction. The above
operations were carried out according to the method of Jalees et al (1981) with slight modification.

Cook and Holbrook (1983) have used another method for extraction of parasite antigen. They used alternate freezing and thawing for obtaining soluble and particulate antigen fractions. The preparation was further fractionated by sonication, followed by centrifugation at 11,000 x g for 20 min. Leon et al (1986) have used yet another method for antigen purification. Cells obtained from bulk growth were harvested on the seventh day, washed 6 time with 0.15 mol/1 phosphate-buffered saline at pH 7.2, and disrupted by sonication at 95 W, five times (3 min each). This sonicated material was ultracentrifuged at 100,000 x g for 30 min and the supernatant was filtered through an 0.2 um millipore membrane. In order to minimize proteolytic degradation, all the procedures were carried out at 4°C.

For immunization purposes, Scott et al (1987) have used the following procedure for obtaining soluble antigen preparation. The parasites were grown in medium 199 with 20% fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin G-potassium, 100 μg/ml streptomycin sulphate and 25 mM HEPES at 27°C. Log growth phase promastigotes were used for antigen preparation, because in preliminary experiments these promastigotes proved better for immunizing the animals than stationary growth phase organism. Promastigotes were
radiation-attenuated by exposure to 50,000 rad-irradiation from a cesium (137Cs) source, or were heat killed by inoculation at 56°C for 1 hr. To prepare soluble leishmania antigen, promastigotes were washed four times in cold phosphate buffered saline and were resuspended at 1.2 x 10^9 parasite/ml in 100 mM Tris-HCL 1mM EDTA (pH-8) with 50 μg/ml leupeptin, 50 μg/ml γ-2-macroglobulin, and 1.6 mM phenylmethylsulfonyl fluoride and were incubated at 4°C for 10 min. The parasites were then subjected to sonication at 4°C with 2-20 sec blasts and were viewed microscopically to ensure that all the parasites had been disrupted. The parasite suspensions were then centrifuged at 27000 x g for 20 min, and the supernatant was collected and recentrifuged at 100,000 x g for 4 hr. The supernatant was then harvested and dialyzed against PBS, and was sterilized by passage through a 0.22 μm filter.

Chemical characterization of parasite antigen showed that protein-carbohydrate ratio in the whole, particulate and soluble antigen was 2.14:1, 2.73:1 and 10.2:1, respectively. The results clearly suggest that the various antigenic fractions of L. donovani antigen are both of protein and carbohydrate nature. A small amount of DNA was also found in these antigenic preparations. Some workers in this area suggested a protein-polysaccharidic nature of L. donovani promastigote antigen (Ghosh and Roy, 1983). Moreover, many
other workers have prepared a carbohydrate rich factor which reacted strongly with homologous rabbit antiserum raised against promastigotes. These components alternatively labelled as excretion factor, exometabolite, or antigenically active glycoproteins have been used in serotyping of leishmania strains (Schnur et al, 1972).

Whole antigen preparation in SDS PAGE resolved into nineteen bands. One band having the molecular weight of more than 94,000, two bands in between 94,000 and 67,000, five bands in between 67,000 and 43,000 five bands in between 43,000 and 30,000, five bands in between 30,000 and 20,100 and one diffused band in between 20,100 and 14,400 daltons were observed. In particulate antigen fourteen bands were observed. One band having the molecular weight of more than 94,000, two bands in between 94,000 and 67,000, four bands in between 67,000 and 43,000, one diffused band in between 43,000 and 30,000, five bands in between 30,000 and 20,100 and one diffused band in between 20,100 and 14,400 daltons were detected. In soluble antigen seventeen bands were observed: two bands in between 94,000 and 67,000, five band in between 67,000 and 43,000, three bands in between 30,000 and 20,100 and four bands in between 20,100 and 14,400 daltons. Ramasamy et al (1981) have shown the presence of one band of 53,000 dalton on the outer surface membrane of L. donovani promastigotes. Four labelled proteins with molecular
weight values of 65,000, 60,000, 50,000 and 26,000 were detected in both *L. donovani* and *L. chagasi* extracts (Lamesre et al, 1985).

Definitive diagnosis of visceral leishmaniasis depends on the microscopic demonstration of Leishman-Donovan (LD) bodies in the bone marrow/spleen biopsy or by culture of aspirated fluid. But the patients where the demonstration of LD bodies is not possible, the diagnosis is only presumptive (Srivastava, 1989). With the development of sensitive and specific antigens a number of tests have now become available for diagnosis of the disease. They include indirect haemagglutination (IHA) test (Bray and Lainson, 1967), indirect fluorescent antibody (IFA) test (Quilci et al, 1968; Shaw and Volter, 1964) and enzyme linked immunosorbant assay (ELISA), (Hommel et al, 1978). Among the different techniques which have been used for diagnosis of kala-azar, micro ELISA was found to be more sensitive than other tests like IFA, IHA, and ELISA (Srivastava, 1989). Other serological tests including immunofluorescence, were also used by numerous workers for the detection of specific antibodies to *L. donovani*. These tests are passive haemagglutination, gel precipitation and complement fixation (Kagan, 1975; Pappas et al, 1985). ELISA was found more practical than indirect fluorescence antibody test in large scale seroepidemiological surveys for kala-azar (Srivastava 1989,
Choughdry et al., 1990; Gupta et al., 1993). The first application of this technique (ELISA) in the detection of Leishmania antibodies by Hommel (1976) and Voller et al (1976) had shown promising results. Antibody to *L. donovani* was also detected with IHA test in patients with kala-azar and post kala-azar dermal leishmaniasis in India (Mukherjee et al, 1968; Sen Gupta 1968). More recently Evans et al (1990) have also used IFA and ELISA in Brazilian Dogs infected with *L. donovani chagasi*. Of 405 dogs; 8% were positive by IFA obtained from blood collected by drying onto filter paper followed by elution, 17% were positive by IFA against appropriate sera, while 38% were positive in ELISA using the same sera. The ELISA recognized all the dogs with proven infection but IFA detected 10 to 12 only. In this study IFA tests were also performed in order to see the antibody titres. Pearson and Roberts (1990) have also used IFA technique to demonstrate immunoglobulin bound to the surface of hamster spleen infected with amastigotes of *L. donovani*.

Earlier workers in our laboratory have used ELISA and IHA to see the antibody titres in the golden hamsters immunized with leishmania antigen in association with glucan (Obaid et al, 1989). ELISA and IHA tests were successfully employed in this study where higher antibody titres were found in the animals immunized with parasite antigen only. But, animals immunized with whole antigen plus BCG showed a
higher antibody titres than those obtained with individual antigen fractions. Next in the order, were the antibody titres obtained from animals which are given particulate antigen fraction in association with BCG and soluble antigen fraction plus BCG.

The appearance of cell-mediated immune responses in the immunized animals was detected in vivo, by means of delayed type hypersensitivity (DTH) responsiveness and macrophage migration inhibition test (MMIT). This test has been widely used in studies on the epidemiology of kala-azar in Kenya (Manson-Bahr 1961) and of mucocutaneous leishmaniasis in South American countries (Pessoa and Pereire Barretto 1948). Costa et al (1988) have used DTH responsiveness to leishmania antigen in their vaccination studies of mice as one of the parameters to see the extent of protection. The leishmanian test was found slightly more specific for leishmaniasis, although it is not species specific (Manson-Bahr 1961). However, in cases of cutaneous leishmaniasis a very strong DTH reaction to leishmanin was observed 14 years after living in a non-endemic area (Modabber 1990). This strongly suggests that antigen or parasites persisted for a very long time after recovery from the infection.
In the present investigation, the DTH, as measured by footpad swelling in response to animal challenge by phenol suspensions of promastigotes, was negative in all the hamsters immunized with leishmania antigen with or without BCG. The skin testing for CMI was carried out on day 30, 45 and 60 post immunization. Our results showed that cell-mediated immune response developed 30 days after challenge with viable *L. donovani* promastigotes. These tests were positive only in animals immunized with leishmania antigen in association with BCG. But the animals, receiving antigen only were not able to generate such delayed type reactions. In order of magnitude, the DTH response was higher in animals receiving soluble antigen in association with BCG; followed by animals receiving particulate antigen with BCG and, those given whole antigen with BCG.

Promastigotes of *L. donovani* are immobilized and killed by normal human serum, whereas the amastigotes formed after multiplication in macrophages are not affected. The killing has been shown to be due to IgG and IgM binding followed by activation of the classical complement pathway (Pearson and Steigbigel 1980). Considerable increase in serum γ-globulins (primarily IgG) are reported in human visceral leishmaniasis (Manson-Bahr 1971; Bray 1972; Ghose et al, 1980). The increase in γ-globulin levels in squirrel monkeys challenged with *L. donovani* was due primarily to IgG and to a lesser
extent to IgM. The factors contributing to the increase in γ-globulin levels in visceral leishmaniasis are not clearly known, but the increase is thought to result from a polyclonal B-cell activation of immunoglobulin secreting cells (Campos-Neto and Bunn-Morenso 1982; Belehun et al, 1980), leading to increased synthesis of IgG (Ghose et al, 1980).

In our study the animals immunized with antigen in association with BCG showed higher immunoglobulin levels compared to animals immunized with antigen only. Among the antigen-BCG group the level of immunoglobulin was highest in the animals immunized with soluble antigen in association with BCG. The level of IgG was every time higher in each group compared to IgM level. The animals which were challenged after immunization with $1 \times 10^6$ promastigotes also showed higher IgG and IgM levels compared to immunized groups. Again the animals immunized with antigen in association with BCG on challenge showed higher IgG and IgM level compared to animals immunized with antigen in association with BCG. The soluble antigen-BCG immunized group on challenge showed higher IgG and IgM levels compared to all other groups. Furthermore, the animals immunized with whole and particulate antigen showed lowest IgG and IgM levels compared to all the groups. The animals immunized with whole antigen and particulate antigen in association with
BCG showed somewhat higher levels of IgG and IgM compared to animals immunized with antigen only. Our studies are similar to that of Dennis et al (1986) who carried out experiments on monkeys. They infected monkeys shown IgG levels which were higher than IgM levels.

Leishmania infections are broadly related to depression of cell-mediated immunity, and it has long been clear that vaccines need to induce protective T-cell mediated immunity against protozoa. The aim is to prevent multiplication of the parasite within macrophages. More recently, Sypek and Wyler (1990) have demonstrated using lymph node lymphocytes from L. major infected mice and cloned T-cell hybridoma that macrophages can be in vitro activated to exert antileishmanial defense. The clone 1D5, produced lymphokines (including gamma interferon) that induced these affects. Production of macrophage activating lymphokines and the protective effect of 1D5 were enhanced by the addition of Cyclosporin A to the cultures. In our studies on CMI, the macrophage migration inhibition test (MMIT) was maximally positive in animals receiving soluble antigen in association with BCG. Animals which received particulate antigen with BCG, and those which received whole antigen with BCG, also showed higher values. Animals which were given BCG alone were almost negative in these tests. The results from skin testing and MMIT experiments were in agreement with each other. Our
results are in agreement with other workers who have used leishmania antigen in association with glucan as an adjuvant for experimental immunization (Cook and Holbrook 1983; Obaid et al., 1989).

The mechanism whereby leishmania amastigotes survive in mononuclear phagocytes in the presence of lysosomal enzymes is not known. Alexander and Vickerman (1975) suggested that the inhibition of host lysosomal enzymes by amastigotes might account for their survival in the macrophage. The studies of El-On et al (1980) supports this hypothesis, suggesting that this inhibition might be mediated by excreted factors.

In our study, a depletion in acid phosphatase activity was observed in macrophages infected with *L. donovani*. The animal groups immunized with antigen only showed more depletion in acid phosphatase activity when challenged with *L. donovani* promastigotes compared to animal groups which received antigen in association with BCG. The animals which were immunized with antigen in association with BCG showed less depletion in acid phosphatase activity when challenged with *L. donovani* promastigotes. The animals immunized with soluble antigen in association with BCG showed lowest depletion in acid phosphatase activity when challenged with *L. donovani* promastigotes. Moreover, animals immunized with particulate antigen and whole antigen in association with BCG
when challenged showed more acid phosphatase depletion compared with soluble antigen-BCG immunized group.

The clinical sign may vary from one infected individual to another, according to the host’s ability to mount an effective immune defense. Increasing evidences indicate that CMI mechanisms play a major role and humoral immune mechanisms a minor role in acquired immunity to visceral leishmaniasis (Preston and Dumonde 1976; Poulter 1980; Reed 1981). In the active stage of visceral leishmaniasis, the CMI is absent, or not prominent, therefore an increasing number of patients with acquired immunodeficiency syndrome (AIDS) or other forms of immune defeciency are being reported to suffer recrudescent leishmaniasis (Bodaro et al, 1986; Franco-Vicario et al, 1987; Mulliez et al, 1987). This may be one of the reasons why hypersensitivity reactions and leishmania skin tests become positive only after 6-8 weeks of treatment.

Excellent studies in rodents suggest that T-cells (Skov and Twohy 1974), natural killer cells (Kirkpatrik and Fareell 1982) and soluble mediators (Murray et al, 1982) are possible mechanisms responsible for acquired resistance in visceral leishmaniasis. However, antibodies may also play a role in resistance, since humans completely cured of visceral leishmaniasis develop a strong transient Arthus reaction at the site of reinoculation with \textit{L. donovani}, and parasites are not recovered at the site of the challenge inoculum
(Manson-Bahr 1961). In the present study, challenged animals which received parasite antigens in association with BCG showed higher antibody titres compared to those given parasite antigen alone. Moreover, the antibody titres in animals immunized with soluble antigen in association with BCG were highest compared to animals immunized with particulate antigen and whole antigen in association with BCG.

Following immunization, higher values in total leukocyte count (TLC) were observed in animals immunized with parasite antigen in association with BCG. The animals which were given only parasite antigen showed slight changes in TLC compared to control animals. Animals which received only BCG also showed an increase in TLC values. In addition, the percentage of polymorphonuclear leukocytes was higher in the antigen-BCG groups than the animal groups immunized with antigen alone. The TLC values were highest in animals immunized with antigen in association with BCG compared to animals immunized with particulate and whole antigen in association with BCG. It has been shown that some decline occurs in the parasite burden of spleen and liver of the BALB/c mice inoculated with BCG (Smrkovski and Lainson 1977).

Moreover, complement fixing antibodies produced by visceral leishmaniasis patients are best demonstrated with the use of Mycobacterium antigens (Lainson and Bray 1966).
Even though these antibodies have no detectable role in immunity against the disease, the use of *Mycobacterium* antigen in the complement fixation test for visceral leishmaniasis indicates a specific cross reactivity between members of protozoan genus, *Leishmania*, and the genus *Mycobacterium*.

Following challenge, all the control animals and those which were immunized with antigen alone showed leukopenia accompanied with reduction of granulocytes and a corresponding increase in lymphocyte and monocyte counts. But the animals immunized with antigen in association with BCG or BCG only, showed higher TLC values, with a slight increase in the number of lymphocytes and monocyte counts. Many workers have shown that usual feature of kala-azar is a marked reduction in the number of all forms of granulocytes, leading to leukopenia with relative lymphocytosis (Aikat et al, 1979; Manson-Bahr 1971; Srivastava et al, 1983). In the present investigation, most of the control animals showed amastigotes in the peripheral blood smears, while the immunized animals did not show any parasite in the blood. Parasitaemia with *L. donovani* is reported to be a common feature in visceral leishmaniasis. Chulay et al (1985) have shown that *L. donovani* parasitaemia is common in patients with visceral leishmaniasis in Kenya. The level of parasitaemia was found
to be correlated with the density of parasites in spleen aspirate smears.

The parasite burden from different organs of challenged animals revealed that animals immunized with leishmania antigen in association with BCG had significantly lower levels of spleen and liver parasitization compared to animals which were immunized with antigen alone, or control animals. However the animals which received soluble antigen in association with BCG had fewer liver and spleen parasites than other animals. These results were very much similar to that of Cook and Holbrook, 1983; who have performed immunization against *L. donovani* in experimental animals using glucan as an adjuvant.

Other workers also reported the development of footpad lesions in hamsters following *L. donovani* infection (Jalees et al, 1981). The appearance of such post kala-azar dermal lesions is common in India (Heyneman 1971). They are known to be caused by identical serotypes of *L. donovani* (Bray et al, 1973). The reticuloendothelial hyperplasia that follows infection with *L. donovani* affects the spleen, liver, mucosa of the small intestine, bone marrow, lymph nodes and other lymphoid tissues. Many of these cells are heavily parasitized. Lymphocyte infiltration is scanty. In the spleen and other lymphoid organs, there may be atrophy of
paracortical areas (white pulp), but plasma cells are numerous. The life span of leukocytes and erythrocytes is reduced, causing granulocytopenia and anaemia. Liver function is normal, and few hepatocytes are invaded, later, prothrombin production decreases. Together with thrombocytopenia, the prothrombin depletion may result in severe mucosal haemorrhages. Hypoalbuminaemia is associated with oedema and other features of malnutrition. Diarrhoea may occur as a result of intestinal parasitization and ulceration of secondary enteritis. In the advanced stage, intercurrent infections are frequent, especially pneumonia, dysentery and tuberculosis and these are common causes of death (Report of WHO Expert Committee 1990).

In our study hamsters which are immunized with parasite antigen only were also seen to develop granulomatous foci, hyperplasia of kupffer cells and atrophy of hepatocytes. The liver architecture was distorted. These tissue reactions were less obvious in antigen-BCG immunized groups, in which the liver architecture was maintained and the LD bodies were rarely seen in macrophages after 60 days of challenge. The splenic tissue of control animals showed accumulation of parasitized macrophages in red pulp, with an accompanying hyperplasia of the white pulp. After 60 days of infection, the white pulp was invaded by parasitized macrophages, in addition to many foci of tissue necrosis. Similar changes
were observed in animals which were immunized with antigen only, although they did not show tissue necrosis. In the antigen-BCG group such tissue reactions were minimal compared to other immunized groups. The fibrous reaction was obvious and the LD bodies were rarely seen in proliferated macrophages.

BCG is a well known immunotherapeutic agent in human as well as in animal tumour systems. A non-specific BCG induced regression has been documented in several tumour types (Lemond 1973). BCG as an adjuvant has also been used in several vaccination studies against leishmaniasis (Costa et al, 1988) in mice model system. A one hundred percent protection against challenge (1 x 10^6 amastigotes) by purified L. mexicana amazonensis amastigotes was observed in mice immunized with microsomal preparation (P01-F) of L. m. amazonensis with BCG. Convit et al (1987) have carried out immunotherapy versus chemotherapy experiments in localized cutaneous leishmaniasis. The study revealed a similar cure rate (94%) when a combination of vaccine consisting of live BCG together with killed leishmania promastigotes was used to compare the results with a standard antimonial drug (meglumine antimonate). gene for control of this response is a human homologue to the murine resistance gene (Lsh/Bcg/Ity) which regulates macrophage priming/activation and acesory cell function, resulting in the rapid generation of a cell-
mediated immune response to leishmanias, mycobacteria, and salmonellas (Blackwell, 1989).

Finally, the results of this study indicate that vaccination of golden hamsters with soluble leishmania antigen in association with BCG was adequate enough to elicit considerable immune protection (95%) against *L. donovani* challenge (1x10^6 promastigotes). This protection was confirmed by estimated lower parasite burdens in liver and spleen compared to control animals. In addition, these animals showed other parameters of immunity such as positive skin test responsiveness after challenge, increased antibody titres and high IgG and IgM levels, and high total leukocyte counts. Scott et al (1987) have also demonstrated that BALB/c mice can be protected against a fatal infection (challenged with 1 x 10^6) stationary growth phase of *L. major* promastigotes) by i.p. immunization with a soluble leishmania antigen preparation in conjunction with the adjuvant, *Corynebacterium parvum*. The soluble leishmania antigen was further separated into nine distinct fractions by anion exchange liquid chromatography, and the fractions were analyzed for their ability: to stimulate T-cells from immunised mice, to be able to recognize vaccine-induced antibodies and, to induce protective immunity. Only fraction 9 stimulated significant immunity (76% protection).