THE IMMUNOPATHOLOGY OF LESIONS AND DRAINING LYMPH NODES IN BALB/c AND CBA/ca MICE INFECTED WITH *Leishmania major* AND *Leishmania mexicana*

by

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Caminante no hay camino se hace
camino al andar, paso a paso,
golpe a golpe ...

Antonio Machado 1875 - 1939.

ABSTRACT

This thesis presents the results of a study of the immunopathological events taking place in the cutaneous lesions and draining lymph nodes of BALB/c and CBA/ca mice infected with *Leishmania major* and *L. mexicana*, at various intervals after the inoculation of the parasites.

Four combinations of parasite and mice strains were used: CBA/ca mice infected with *L. major*; CBA/ca mice infected with *L. mexicana*; BALB/c mice infected with *L. major*; BALB/c mice infected with *L. mexicana*

In each of them, the clinical course of the infection and the immunopathological appearance of the lesions and their draining lymph nodes were followed for 18 weeks, attempting to correlate them with the changes in the humoral and cellular immune responses to the parasite.

The infection of CBA/ca mice with *L. major*, resulted in self-healing cutaneous lesions, while the infection with *L. mexicana* led to the development of non-healing lesions.

BALB/c mice developed non-healing lesions in response to the infection with either parasite.

The histopathological appearance of the skin lesions was different for each host-parasite combination, changing continuously during the course of infection, assuming patterns conforming to those described in BRYCESON's histological spectrum of leishmaniasis.

The changes in the cortical and paracortical areas of the lymph nodes and their infiltration by cells of the phagocytic

system are described and correlated with the clinical course of the infection and the humoral and cellular immune responses.

The immunopathological studies comprised a search for G and M immunoglobulins (IgG and IgM) and C3 complement factor by immunofluorescence techniques applied to cryostat tissue sections of both skin lesions and lymph nodes, and the identification of subpopulations among lymphocytes in tissue sections and disaggregates of cells, by determining the presence of IgG, IgM, and Lyt1 and Lyt2 surface markers in their cell surfaces. The attempt was made to assess the specificity of the staining by the use of FITC-*L. mexicana* antigen and the F(ab)'2 fraction of *L. mexicana* antibody.

Overall the same tissue structures and cells were stained in all infection models, but there were differences in the pattern and intensity of the fluorescence of Ig and C3 deposits both among infection models, and within the single infection models as infection evolved. Deposits of IgG and IgM were consistently detected in cutaneous lesions along the dermis-epidermis junction, vascular walls and muscles. Generally their temporal pattern of appearance corresponded to those of C3 deposits and of antigen detected by anti-*L. mexicana* antibody. The possible nature and origin of these deposits is discussed suggesting that they could be immune complexes.

Regarding the cells infiltrating the skin lesions, antigen, antibody and C3 were detected on the surface of macrophages and reticular shaped cells, suggesting that these cells may be presenting antigens. Antigen presenting cells were detected in

skin tissue from all host-parasite combinations with the exception of BALB/c mice infected with *L. mexicana*.

Polymorphonuclear eosinophils with their cytoplasm stained by FITC *L. mexicana* antigen and by anti-IgG sera were especially abundant in mice exhibiting non-healing lesions, the possible role of these cells in phagocytosing immune complexes, and their possible participation in leishmanicidal mechanisms is discussed.

Antigen, *Leishmania* antibody and C3 coexisted in intercellular deposits in cortical and paracortical areas of lymph nodes. These deposits seem to be associated with follicular dendritic cells and interdigitating cells in these areas. The sinus system was detected stained by the immunoreagents mentioned earlier plus anti-IgM and anti-IgG, this is interpreted as the result of the staining of macrophages lining the sinuses.

Anti-IgM and anti-IgG also stained the surface of small lymphocytes and the cytoplasm of immunoblasts and plasma cells, in follicular and medullary areas of CBA/ca mice infected with *L. major* or *L. mexicana*.

In both, the skin lesions and the lymph nodes, B cells bore predominantly IgG and stained with FITC-*L. mexicana* antigen. T cells exhibited the Lyt1+ phenotype.

The cellular response was measured by the delayed hypersensitivity response to the parasiteantigen, the results were as follows:

CBA/ca infected with *L.major* produced a response of the tuberculin-type when tested with both homologous and heterologous antigens.

CBA/ca and BALB/c mice infected with *L. mexicana* produce a DHR to homologous antigens only but the kinetics of the response was of the JONES-MOTE-type.

BALB/c mice infected with *L.major* did not develop DHR when challenged with the homologous antigen, although when they were challenged with the heterolgous antigen a response of lesser magnitude to these observed in mice from the rest of the experimental groups was observed.

Finally the humoral response was measured by determining circulating antibodies by direct agglutination and indirect immunofluorescent techniques, using homologous and heterologous antigens. The kinetics of the response using the agglutination technique showed an early rise of the antibody titres, reaching high levels after the first week and oscillating thereafter always at high values for the observed period; the immunofluorescent technique did not detect significant levels of IgG antibodies before the fourth week and then only at low titres, but increased steadily thereafter reaching levels comparable to those seen with the agglutination technique by the 16th week. Anti-IgM antibody oscillated at low levels during the whole period of the observations.

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CHAPTER I

LEISMANIASIS, AN OVERVIEW

I.1 INTRODUCTION

Leishmaniasis is a parasitic infection of animals and man, which occurs in many tropical and subtropical areas of the world (MAEKELT, 1972). As a disease it is, even today, a persistent public health problem and there are prophecies of its extension in future years (WILLIAMS & COELHO, 1978).

Leishmaniasis comprises a group of diseases caused by different species of the genus *Leishmania* (ROSS 1903) (Phylum-Protozoa: Order-Kinetoplastid a: Family-Trypanosomatidae). The infection is transmitted between vertebrate hosts by the bite of a phlebotomine sandfly vector.

The organism in the mammalian host is an obligate intracellular parasite; it lives inside macrophages as an amastigote lacking a free flagellum. When sandflies ingest the amastigotes from infected animal or human tissues, the parasites first transform into promastigotes (monoflagellate forms) in the insect midgut. The promastigotes then undergo a series of morphological variations in the sandfly's gut, where they multiply and migrate into the proboscis. From there, they are injected into the skin of the vertebrate host when the fly takes its blood meal. After inoculation into the mammalian host,

prosbocis-form promastigotes are engulfed by macrophages where they round up, lose their external flagellum and become amastigotes.

All species of the genus *Leishmania* are barely distinguishable morphologically by light microscopy in either the amastigote or the promastigote development stages, with the exception of *L.enriettii* which has a larger amastigote form than the other species (ADLER, 1964). However, *Leishmania* species can be differentiated by a combination of:

1- Geographical distribution and epidemiological features of the infection.

2- Distinct clinical pictures produced in man by different species.

3- Characteristics in culture and laboratory animals.

4- Serological and cross immunity tests (reviewed by BRAY, 1974; ZUCKERMAN, 1975).

5- Deoxyribonucleic acid (DNA) bouyant density (CHANCE et al. 1974, 1978; BARKER & BUTCHER, 1983).

6- Iso-enzyme identification (CHANCE et al.1978; ALJEBOORI & EVANS, 1980 a,1980 b; MILES et al. 1980; LAINSON et al. 1982).

7- Specific lectin binding (DWYER, 1977).

8- Monoclonal antibody specificity (MCMAHON & DAVID, 1981).

I.2 HUMAN LEISHMANIASIS. CLINICAL AND IMMUNOLOGICAL CHARACTERISTICS

Clinically leishmaniasis in man can be divided into three major groups:

1- Cutaneous leishmaniasis, caused by: *L.tropica* (syn: *L.tropica minor*), *L. major* (syn: *L.tropica major*) and *L. aethiopica* in the Old world, and *L. braziliensis guyanensis*, *L. braziliensis panamensis*, *L.mexicana mexicana*, *L. mexicana amazonensis*, *L. mexicana pifanoi*, *L. peruviana* and *L. garnhami* in the New world.

2- Cutaneous, leading to mucocutaneous leishmaniasis, caused by: *L. braziliensis braziliensis*.

3- Visceral leishmaniasis, caused by: *L. donovani donovani*, *L. donovani infantum* and *L. donovani chagasi*.

I.2.1 CUTANEOUS LEISHMANIASIS (a disease spectrum)

The concept of leishmaniasis as a polar or spectral disease, like leprosy, was proposed by DESTOMBES (1960) who pointed out that it was unnecessary to consider the condition of diffuse cutaneous leishmaniasis described in Venezuela (CONVIT, 1958) and Ethiopia (BALZER et al. 1960) as a separate disease entity, but rather as one polar form of cutaneous leishmaniasis, the features of which depended upon the host's response to the parasite.

However, the clinical spectra of leprosy and leishmaniasis differ markedly from each other: while in leprosy clinical forms can be ordered in a linear arrangement of increasing severity, corresponding to an spectrum of host responses ranging from high to low resistance. In leishmaniasis relatively benign infections producing lesions which cure either spontaneously or in response to chemotherapy are placed in the middle of an arrangement of clinical forms which increase in severity towards difuse cutaneous leishmaniasis (DCL) in one direction and towards lupoid or recidivans leishmaniasis in other. Benign forms cure and develop delayed hypersensitivity (DHR) response and immunity. Diffuse cutaneous leishmaniasis (DCL) is a condition characterized by the presence of abundant lesions rich in parasites and associated with a poor or negative DHR to parasite antigens. Lupoid or recidivans leishmaniasis, characterized by a unique lesion that never quite heals, or heals and relapses, and is associated with an exacerbation of the DHR to parasite antigens. It would appear that this disparity in the clinical spectra of the two diseases results from the fact that in leprosy the spectrum is the resultant from the interaction of an unique species of parasite and a range of host responses, while in leishmaniasis the multiplicity of parasite species complicates the interaction generating several clinical spectra.

Several lines of evidence support the suggestion of DESTOMBES (1960) that the host response to the parasite may

affect the outcome of the disease. The Ethiopian strains of *L. tropica* (now called *L. aethiopica*), isolated from patients with either self-healing lesions or DCL, were indistinguishable by haemagglutination (BRAY & BRYCESON, 1969).

CONVIT and coworkers (1972) in Venezuela showed: 1- That the parasite isolated from DCL cases did not cause DCL when inoculated into volunteers. 2- Epidemiologically, DCL appeared as isolated cases in areas where American cutaneous leishmaniasis caused by *L. braziliensis* or *L. mexicana spp.* is endemic. 3- A case occured in which two patients were living in the same house, one of whom had DCL and the other normal American cutaneous leishmaniasis.

On the other hand, BRYCESON (1970 a) suggested that the extreme ends of the spectrum are not as common as in leprosy and are only seen in certain geographical areas (Amazonian area and Ethiopia), which suggests that differences in host genetics, parasite strains and epidemiological events influencing host parasite contact may affect clinical patterns of the disease.

Protective immunity in many forms of cutaneous leishmaniasis seems to be predominantly of the cell mediated type (BRYCESON, 1970 a; MAEKELT, 1972; PRESTON & DUMONDE, 1975), as suggested by the early development of a strong and long lasting DHR in self-curing cutaneous disease patterns and low levels of circulating antibodies associated with a residual immunity. The non-healing forms of cutaneous leishmaniasis are accompanied by a defective immunological response which may be either stimulated

("allergic"), suggested by exacerbation of DHR to parasite antigens (lupoid leishmaniasis), or suppressed ("anergic") in which poor or negative DHR to the antigens is observed (DCL).

I.2.2 MUCOCUTANEOUS LEISHMANIASIS

This condition is characterized by the presence of an ulcerative skin lesion, whichmay cure or be cured, followed, perhaps many years later, by a metastasis to the mucous membrane and cartilages of the nasal septum and soft palate resulting in severe disfigurement (espundia). These destructive lesions are found in the presence of both a marked cell mediated reaction (DHR) and humoral antibodies (MAEKELT, 1972; PRESTON & DUMONDE, 1975).

I.2.3 VISCERAL LEISHMANIASIS OR KALA-AZAR

This disease is characterized by gross parasitization of systemic macrophages, notably in the bone-marrow, spleen and liver, and is fatal if not treated. DHR in this condition is absent until cure. Immunoglobulins are markedly elevated, probably due to non-specific B cell stimulation. Specific antibodies are present, but they represent only a small proportion of the total immunoglobulin. Therapeutic treatment of Kala-azar results in the ability to produce DHR and a fall in antibody titers (BRYCESON, 1970 a; MAEKELT, 1972; PRESTON & DUMONDE, 1975).

I.3 LABORATORY MODELS OF HUMAN LEISHMANIASIS

Guinea-pigs and mice have proved to be useful experimental models for human cutaneous infections.

L. enrietti produces in guinea pigs, and only in guinea pigs, cutaneous lesions similar to those observed in localized human leishmaniasis (BRYCESON et al. 1970, 1974).

Mice infected with *L. major* or *L.mexicana* and depending on their genetic background exhibit the self-healing and non-healing forms of the disease found in human cutaneous leishmaniasis (PRESTON et al.1978; PEREZ et al. 1979; HANDMAN et al. 1979; HOWARD et al. 1980 a, b). Mice and hamsters infected with *L, donovani* have been used as models of visceral leishmaniasis (BRADLEY & KIRKLEY, 1971, 1977; BRADLEY et al. 1979; BLACKWELL et al. 1980; BLACKWELL 1982).

I.3.1 *Leishmania enriettii* GUINEA PIG MODEL

As the pattern of infection in the guinea pig so closely follows simple sore infections in man, it has been used as a model for the study of the immunology and pathology of cutaneous leishmaniasis. The experiments carried out in this model demonstrate the importance of cell mediated immunity in the host response against the parasite as indicated by the appearance of DHR and the synthesis of macrophage inhibition factor (MIF) and mitogenic factor by immune lymphocytes in the presence of *Leishmania* antigen (BRYCESON et al.1970).

On the other hand, manipulations designed to suppress

the development or expression of CMI, such as treatment with anti-lymphocytic serum (ALS), infection with heavy inocula of amastigotes of the parasite and injection of soluble *Leishmania* antigen (PSA) during foetal life, increased the severity of the disease (TURK & BRYCESON 1971; BRYCESON et al. 1970; 1974). In these situations the pattern of infection in the guinea pig resembled that of DCL.

Stimulation of the immune response in infected animals, as determined by an increase of DHR to *Leishmania* antigens and an increase of specific parasite haemagglutinating antibody production, could be produced by prior regional injections of non-related antigens in adjuvant (*Mycobacterium tuberculosis* and *Corynebacterium parvum*) (BRYCESON et al. 1972). However, the animals so treated were more susceptible to *L. enriettii* infection, as judged by the more florid appearance of lesions and the increased incidence of metastasis. Animals given *Mycobacterium tuberculosis* adjuvant prior to *Leishmania* infection had increased DHR to *Leishmania* antigens, while those given *Corynebacterium parvum* had an increased specific anti-promastigote haemagglutinating antibody level. The pattern of infection after treatment with these adjuvants bore certain resemblances to leishmaniasis recidiva or mucocutaneous leishmaniasis in those animals given *M. tuberculosis* and to DCL in those treated with *C. parvum*. Stimulation by the homologous leishmanial antigen in Freund complete adjuvant protected guinea pigs against

consequent infection with *L. enrietti* (BRYCESON et al. 1970).

Circulating antibody, detected by passive cutaneous anaphylaxis, indirect haemagglutination (BRYCESON et al. 1970, 1972 and by indirect immunofluorescence (RADWANSKI et al.1974) has been determined in this model, but no clear association has as yet been found between anti-*Leishmania* antibody titer and the course of the infection (RADWANSKI et al. 1974).

Humoral immunity, however, cannot be completely ruled out as having a role in protective immunity as BRYCESON and coworkers (1974) have demonstrated that an immediate hypersensitivity develops in infected guinea pigs at times corresponding to the healing of primary lesions. POULTER (1980), on the other hand, showed that in the *L. enriettii* guinea-pig model antibody levels increased as healing commenced, being highest in convalescent animals and serum taken from recently challenged convalescent guinea-pigs was capable of transferring some resistance to naive recipients.

I.3.2 *Leishmania* MOUSE MODEL

Inbred mouse lines, following infection with *Leishmania*, display a spectrum of disease patterns similar to the spectrum of disease in humans.

L.major, when inoculated into CBA, C3H, and C57 brown strains of mice, produced self-healing lesions resulting in immunity to reinfection (PRESTON et al. 1978), but when inoculated into DBA, C57 black (PRESTON et

al.1978) and BALB/c (HANDMAN et al. 1979; NASSERI & MODABBER, 1979) produced non-healing persistent cutaneous lesions with eventual visceralization and death.

In *Leishmania mexicana* infected mice, strains of mice like BALB/c (PEREZ et al.1979; ARREDONDO & PEREZ, 1979) and CBA (ALEXANDER & PHILLIPS, 1978), when inoculated with this parasite, developed persistent cutaneous lesions, whereas mice strains like AKR and C57B1/6 produced self-healing lesions (Perez et al. 1979). In *L. donovani* infection of mice BRADLEY & KIRKLEY (1977) and BRADLEY (1977) showed that strains of mice varied in their response after intravenous inoculation of the parasite. They fell into two categories: 1. those in which *L. donovani* multiplied in the liver a 100 fold during the first 15 days of infection (NMRI, B10.D2 in-bred, and PO outbred), called susceptible, 2. those in which parasite multiplication was about 5 fold (C3H in-bred, and Ash outbred), called resistant. The acute susceptible mice varied in their response. Both NMR1 and B10.D2 recovered after three months of infection, while PO mantained an immense parasitic load for up to two years.

The genetic studies of BRADLEY and coworkers (1977), in *L. donovani* infection of mice, demonstrate that innate susceptibility to the parasite (measured over 2 to 4 weeks) is under the control of a single autosomal gene (*Lsh*) segregating for incompletely dominant resistant (r) and susceptible (s) alleles. This locus maps (BRADLEY et al. 1979) to a position between the centromere and ld-1 on chromosome 1 of the mouse. The gene acts at the level of

the resident tissue macrophage and does not appear to involve classical T cell mediated immune mechanisms (BRADLEY, 1980).

Genetic studies on long term responses (BLACKWELL et al. 1980; BLACKWELL, 1982) in congeneic strains of mice susceptible to *L. donovani* infection demonstrated that they are largely controlled by a gene or genes in the K end of the H-2 histocompatibility complex. Three phenotype patterns of recovery have been described: early cure ($H\text{-}2^{a,r}$), cure ($H\text{-}2^{b}$), and non-cure ($H\text{-}2^{d,q,f}$), with cure behaving as a recesive trait in $H\text{-}2^{b/d}$. The experiments of DE TOLLA et al. (1980) in several strains of mice support those of BLACKWELL, and also suggest that other non H-2 linked genes (Ir-2 and H-11) influence the acquisition of the immune response. The ability of some susceptible strains of mice to effect self-cure on the other hand, is T cell mediated (SKOV AND TWOHY, 1974) and correlates with DHR (DE TOLLA et al. 1980).

Genetic studies in *L. major* infection, (HOWARD, 1980 b, and DE TOLLA et al. 1981) showed that resistance is controlled by a single non H-2 linked gene. Recent experiments of BLACKWELL et al. (1983) unpublished, quoted by BLACKWELL & ALEXANDER (1983) indicated that this gene maps on chromosome 8.

Although certain strains of mice have been classified by various workers as being resistant or susceptible, it has been shown that, individually, they can be manipulated to display the entire spectrum of cutaneous leishmaniasis.

PRESTON & DUMONDE (1976) and PRESTON et al. (1978) showed that *L.major* infections of CBA mice can be manipulated (varying the infecting inoculum or by treatment with *cyclosphosphamide*(CY)), to provide patterns of host response which mimic the sub-clinical, self-healing and allergic and anergic non-healing cutaneous forms of leishmaniasis in man.

The variations in the inoculum size in CBA mice gave rise to different clinical forms of the disease; 10^2 to 10^4 amastigotes produced subclinical infections, 10^5 to 10^6 self-healing lesions and inoculum over 10^7 anergic non-healing lesions.

Pretreatment of CBA mice with CY three weeks before infection with 10^5 parasites resulted in the development of allergic non-healing lesions.

PEREZ (1980) has described similar results in *L. mexicana* infected mice. Inoculum of 10^2 to 10^4 amastigotes of *L. mexicana* when injected in C57Bl/6 produced self healing lesions, while 10^6 amastigotes in the same strain led to the development of chronic lesions and 10^7 amastigotes caused an uncontrolled and fatal disease.

HOWARD et al. (1980 a, 1981), in experiments using BALB/c *L. major* infection model, however, showed that adult thymectomy, X irradiation and bone marrow reconstitution or pretreatment of the mice with a sublethal dose of irradiation resulted in resistance to the infection.

In all these reports, cell mediated immunity is

implicated and associated with the development of DHR.

PRESTON et al. (1972), in *L.major* infected CBA mice, have demonstrated that a thymus dependent cell population most probably plays an important role in the development of resistance to infection. Thymectomy of young mice produced immunosuppression revealed by depression of cell mediated reactions as measured by DHR and depressed blast cell reactivity in the paracortical area of lymph nodes; these changes were in turn reflected in the slower healing of the primary lesion. These experiments of PRESTON et al. (1972) were supported by the observations of HANDMAN et al. (1979) in which athymic nude mice of a genetic background identical to that of resistant mice were highly susceptible to the disease. Transfer of syngeneic T lymphocytes, on the other hand, confers resistance to infection in these highly susceptible mice (MITCHELL et al. 1980).

In order to assess directly the role of T cells in the resolution of cutaneous lesions induced by *L. major*, LOUIS et al. (1979, 1981, 1982, 1983) using enriched, homogeneous populations and clones of T lymphocytes specific for *L. major* antigens have demonstrated that this population of cells which exhibited the $Lyt1^{+}2^{-}$ phenotype were capable of: a. specific proliferative responses upon challenge in *vitro* with parasite antigens, b. specific helper activity for an in *vitro* antibody response in a hapten-carrier (DPN-*L. major*) system, c. transferring antigen specific DHR to normal mice and specific activation of parasitized

macrophages resulting in the destruction of intracellular parasites.

Studies by HOWARD et al. (1980 a), in BALB/c mice infected with *L. major*, indicated that the susceptibility exhibited by this strain of mouse to the parasite is correlated with specific suppression of DHR to parasite antigens during the course of experimental infection. This impairment of parasitic specific DHR was accompanied by the emergence of a T suppressor cell population which, when transferred to normal syngeneic mice, impaired the induction of DHR to leishmanial antigen. Cells mediating suppression were characterized as being T lymphocytes expressing the $Lyt1^{+}2^{-}$ phenotype (LIEW et al. 1982). It has been postulated (HOWARD et al. 1980 a) that the emergence of this T suppressor cell population could be due to a rapid amastigote (antigen) accumulation in macrophages expressing a primary genetic defect. Very recently LIEW (1983) isolated a clone of $Lyt1^{+}2^{-}$ $I\text{-}J^{-}$ T suppressor cells from BALB/c mice. This clone intensified the infection of *L. major* in BALB/c mice *in vivo*, suppressed T cell induced macrophage killing of *L. major in vitro* and suppressed the cell-transfer of DHR.

Involvement of antibody in protection in this model was suggested by enhancement of the protective capacity of the immune-peritoneal cells by immune-serum in transfer experiments (PRESTON & DUMONDE, 1976).

I.4 MECHANISMS OF ELIMINATION OF THE PARASITE

I. 4.1 CELL MEDIATED PROCESSES

Regarding the mechanisms involved in the elimination of the parasite, two cell mediated processes have been suggested:

1- Lymphocyte cytotoxicity against parasitized macrophages, and

2- Macrophage activation leading to digestion of the parasite.

In relation to the cytotoxicity mechanism, BRAY & BRYCESON (1968) and BRYCESON et al. (1970), have suggested that immune guinea-pig lymph node cells sensitized to *Leishmania* antigen may be endowed with activity against guinea-pig *L. enriettii* infected macrophages. However, (MAUEL et al. 1975) failed to corroborate the findings of the previous workers, concluding that the evidence provided by them could be the result of methodological artefacts. More recently, however, the same group of workers (MAUEL et al. 1981) have reported lysis of mouse macrophages infected with *L. enriettii* after culturing them with *Leishmania* immune syngenic spleen cells indicating that the direct cytolysis of parasitized macrophages by immune lymphocytes cannot be excluded as a possible mechanism in the elimination of the parasite.

Macrophage activation is defined as a process whereby the phagocytes acquire an enhanced capacity to phagocytosis

kill and digest microorganisms. At the biochemical level, activation is characterized by the production of a wave of metabolites of oxygen that are toxic for both intracellular pathogens (BUCHMÜLLER & MAUEL, 1979; MURRAY, 1981, 1982) and for extracellular targets (NATHAN et al. 1979). These metabolites appear to include the superoxide anion (O^-_2), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the hydroxyl radical (HO).

The activation is thought to be produced by sensitized T-lymphocytes in the presence of the sensitizing antigen (NORTH, 1973), this results in the elaboration of soluble factors (lymphokines) endowed with macrophage activating properties (BUCHMÜLLER & MAUEL, 1979; NACY et al. 1981)

The possible role of macrophage activation in the destruction of intracellular *Leishmania* has been investigated by several workers using different infection models. Non-specific stimulation of the mononuclear phagocyte system by BCG (WEINTRAUß & WEINBAUM, 1977) and glucan (COOK et al. 1980) has been shown to increase the resistance of mice against infection by *L. major* and *L. donovani* . However, *in vitro* studies have adduced the best evidence for the role of activated macrophages in the elimination of the parasite. For instance, MILLER & TWOHY (1969) found that macrophages from mice in the active period of infection with *L. donovani*, were able *in vitro* to inhibit the growth of the parasite and kill intracellular *Leishmania*.

In *L. enriettii* infected guinea pigs and *L. major* infected mice, MAUEL and his coworkers (1974, 1978,

1980) and BEHIN et al. (1979) demonstrated, that the incubation of *Leishmania* parasitized macrophages with specifically or non-specifically sensitized lymphoid cells was required for the activation of macrophages and consequent killing of the parasite. In these experiments macrophages, were able to destroy *Listeria monocytogenes* and the *Leishmania* species not normally infective for the macrophage donor. Moreover, macrophages from mice exhibiting different susceptibilities to *L. major*, differed in their ability to support parasite growth (HANDMAN et al. 1979; BEHIN et al. 1979). Activated macrophages from strains of mice resistant to infection with *L.major* were able to destroy *in vitro* ingested *Leishmania* . In contrast, activated macrophages obtained from a susceptible strain of mice were unable to kill the parasite. However, these macrophages were able to destroy the parasite *in vitro* if they were activated for a sustained period, suggesting that the threshold for activation necessary to kill *L. major* is higher in macrophages of susceptible mice (BEHIN et al. 1979).

On the other hand, the experiments of GORZINSKI & MACRAE (1982), have suggested that the differences in the ability of skin macrophages from resistant and susceptible mice to control parasite growth, is a function of a particular macrophage population.

The different ability of macrophages from various strains of mice to destroy ingested *Leishmania* organisms may be genetically determined. HOWARD et al. (1980 c) showed that irradiated and bone marrow

reconstituted chimeras, resistance and susceptibility was transferred with the donor bone marrow, suggesting that this may be a macrophage controlled response.

The implication of lymphokines in the macrophage activation mechanism has been suggested as soluble products from sensitized lymphocytes activated in various ways can activate macrophages, resulting in the destruction of intracellular *Leishmania* (BUCHMÜLLER & MAUEL, 1979; NACY et al. 1981).

That macrophage activation resulting from a cell mediated response against parasite antigen, may be an effector mechanism of protection against leishmanial infection in the recovering host is further supported by the demonstration that specific activation can be obtained *in vitro* by incubation of parasitized macrophages with lymphocytes from lymph nodes and spleen from recovered or immunized animal (LOUIS et al. 1981, 1982; MAUEL et al. 1981). Such activation may lead to destruction of the intracellular microorganisms, and further it is specific as shown by the fact that incubation of parasitized macrophages with lymphocytes sensitized against another antigen, such as ovalbumin, does not induce activation of the parasitized macrophages.

Susceptibility of *Leishmania* and other parasites to oxygen metabolites has recently been reviewed by NATHAN (1983). *Leishmania* promastigotes (*L. major* and *L. donovani*) are higly susceptible to lysis by H_2O_2 (MURRAY, 1981). Promastigotes effectively trigger the production of oxygen intermediate metabolites

when they are ingested by macrophages and are readily destroyed not only by activated macrophages but even by non-activated resident peritoneal macrophages (MURRAY, 1981). Unlike promastigotes, amastigotes were ingested without triggering substantial oxygen intermediate metabolites in resident macrophages or even macrophages non-specifically activated. In contrast, lymphokine activated macrophages simultaneously acquire the capacity to respond to ingestion of amastigotes by secreting oxygen intermediate metabolites and eliminating the parasite (MURRAY, 1982).

In addition to their microbicidal and degradative functions, macrophages also play a very important role in the initiation and regulation of lymphocyte proliferation (reviewed by UNANUE, 1981).

Foreign antigens are presented in association with histocompatibility antigens coded for by the major histocompatibility complex. Successful antigen presentation is therefore a function only of those macrophage subpopulations which bear Ia antigens.

The interaction between *Leishmania* antigen presenting cells and T lymphocytes was studied *in vitro* by LOUIS and coworkers (1979, 1981). These authors found, that *L. major* antigens induced a specific T cell proliferative response which was totally dependent on the presence of adherent cells bearing the Ia antigen.

There are two major pathways which may be important in determining whether or not macrophages become activated to kill parasites (BLACKWELL & ALEXANDER, 1983).

1- Presentation of antigen in association with the appropriate Ia antigen inducing the proliferation of those lymphocytes T_{DH} associated with both delayed hypersensitivity and the release of lymphokines.

2- Presentation of a parasite antigen inappropriately in association with the same or different Ia antigens may lead to the generation of suppressor T cells which can suppress lymphokine production and suppress macrophage activation.

Expression of *Leishmania* antigens on the surface membrane of infected macrophages *in vitro* has been reported by BERMAN & DWYER (1981) and HANDMAN et al. (1979). The latter authors found that macrophages from mice exhibiting different susceptibilities to *L. major* did not exhibit differences in their ability to present antigen as detected by the immunofluorescence technique. However, from the functional point of view, *Leishmania* infected BALB/c macrophages differed from infected CBA macrophages in being unable to sensitize syngeneic recipients for a delayed type hypersensitivity to that antigen. The authors associated this functional impairment of antigen presentation with a diminished expression of H-2 exo-antigens in the *L. major* BALB/c infected macrophages, which suggested that the parasite induced defect in antigen presentation is allowed by the genetic constitution of the BALB/c macrophages . However, this suggestion has been challenged by MAUEL and coworkers (personal communication).

GORZYNSKY & MACRAE (1982) studied macrophage subpopulations from the skin of strains of mice exhibiting

different susceptibilities to *L. major*. They showed that the same population of skin adherent cells from the relatively resistant CBA and the highly susceptible BALB/c mice differed in their ability to stimulate lymphocyte proliferation. Parasitized skin adherent cells from BALB/c mice failed to stimulate sensitized lymphocytes while parasitized cells from CBA mice did. However, the same macrophage subpopulation of cells from BALB/c mice when incubated with *L. major* excreted factor rather than live parasites, induced proliferation of sensitized lymphocytes, suggesting that the defect lay somewhere in the processing and presentation of the appropriate antigen from the live parasite (BLACKWELL & ALEXANDER, 1983). As suggested by GORZYNSKY & MACRAE (1982), a difference in the expression of antigen to the surface of parasitized resistant and susceptible macrophages could account for the differential triggering of T cell subsets.

I.4.2 ROLE OF THE HUMORAL RESPONSE IN THE ELIMINATION OF THE PARASITE

There are several observations that indicated the participation of humoral mechanisms in the elimination of the parasite. 1.-BRAY (unpublished) observed that, in the guinea pig infected with *L. enriettii*, the cytotoxic effect of sensitized lymphocytes was enhanced by the addition of antibodies or immune-complexes. 2.- MAUEL (1973) in personal communication to PRESTON & DUMONDE (1975), stated that the coating of *L. enriettii* amastigotes with immune guinea pig serum, in the presence

of complement, rendered the organism susceptible to destruction by normal guinea pig macrophages *in vitro*. 3.- Experiments by HERMAN, (1980) and BRAY (1983) have shown that the presence of cytophilic and opsonising antibody in mouse anti-*L. donovani* and anti-*L. mexicana* sera improved the binding and subsequent phagocytosis by macrophages. It may be, that such enhancement of intracellular parasitization *in vitro* may be the prelude to the destruction of the parasite that may occur through an antibody dependent cytotoxicity mechanism (HERMAN, 1980). In contrast, FARAH et al (1975), reported in anti-*L. major* sera the presence of an antibody with cytophilic, but not opsonising properties in relation to mouse and guinea pig macrophages. The latter authors speculated that cytophilic antibody might immobilize the parasites on macrophage cell surfaces, rendering them susceptible to the action of antibodies or to sensitized lymphocytes.

In conclusion, the evidence presented by previous studies shows that the principal features of self-healing, "allergic", and "anergic" leishmaniasis in man could be reproduced in mouse and guinea-pig models. Analysis of these experimental immunological models indicates that an integrated system of both cellular and humoral response were probably essential in the elimination of the parasites in *Leishmania* infections. However, a T cell response appeared to be the first and most important defence mechanism, and this response is correlated with DHR development.

The evidence indicates that the genetic composition of the host is critical in permitting an efficient cell mediated immune response to the infection which depends primarily on the ability of the activated macrophage to eliminate the parasite.

I.5 EXPERIMENTAL MODELS AND OUTLINE OF THE PROYECT

This thesis presents the progressive immunopathological events which take place in cutaneous lesions and draining lymph nodes, as well as the development of delayed hypersensitivity response (DHR) and the production of antibody in BALB/c and CBA/ca mice infected with *L. major* and *L. mexicana*. Four different experimental models were obtained as a result of infecting two strains of mice (BALB/c and CBA/ca) with two species of *Leishmania* parasites:

L. major-CBA/ca

L. mexicana-CBA/ca

L. major-BALB/c

L. mexicana-BALB/c

The immunopathological studies comprise a detailed histopathological study using routine histological techniques. The presence of immunoglobulins (M and G) and C3 complement factor was determined by immunofluorescence applied to cryostat cut tissue sections of skin lesions and draining lymph nodes.

Lymphocyte subpopulations were identified by determining the presence of G and M immunoglobulins on their cell surfaces and identifying the presence of Lyt1 and Lyt2 surface markers on cryostat cut sections and cellular suspensions from the same tissues.

An attempt was made to assess the specificity of the staining by using *L. mexicana* antigen conjugated to fluorescein isothiocyanate (FITC) and the $F(ab)'_2$ fraction of anti-*L. mexicana* IgG.

The cellular response to parasitic infection was determined by measuring the ability of the different experimental groups of mice to produce DHR when challenged with the homologous and heterologous parasite antigens. The humoral response was measured by determining circulating antibodies by direct agglutination and immunofluorescence techniques using promastigotes of *L. major* and *L. mexicana* as antigens.

As a result of the immunopathological studies, a more detailed account than heretofore published of the cellular development in the lesion of cutaneous leishmaniasis in healing and non-healing infections is presented.

An attempt to discuss the immunopathological findings in relation to the immune response, in the context of recent immunological knowledge, has been attempted.

It is important to point out that many authors erroneously name their parasite *L. tropica* when they are working with *L. major*. BRAY et al. (1973) have put forward reasons for treating *L. tropica major* as a full

species different from *L. tropica* and this has been very adequately confirmed by iso-enzyme analysis (CHANCE et al. 1978). Therefore, throughout this thesis I have substituted *L. major* for *L. tropica* (with apologies to the authors) where appropriate in order to standardise the terminology.

I.6 EXPERIMENTAL DESIGN

120 female CBA/ca mice and 120 female BALB/c mice, 8 to 10 weeks old, were used for the experiments. 60 CBA/ca mice were infected with *L. major* and 60 CBA/ca mice with *L. mexicana* . Similarly 60 BALB/c mice were infected with *L. major* and the other 60 with *L. mexicana* .

After inoculation of 10^5 amastigotes in the foot pad, the state of the lesions was assessed by their appearance and by measuring their thickness weekly up to the sixteenth week after the inoculation of the parasite.

Three animals from each group were bled and sacrificed weekly until the sixth week and then every other week up to the eighteenth week after the inoculation of the parasite.

Skin lesions and draining lymph nodes were removed. The biopsied tissues were then divided into two samples; one sample was fixed in Carnoy's and the other frozen in liquid nitrogen.

The tissues fixed in Carnoy's were embedded in wax and sections cut for histopathology studies by conventional techniques (haematoxylin and eosin, methyl-green pyronin, Giemsa colphonium and Van Gieson staining). The frozen

tissues on the other hand, after cryostat sectioning, were used in the determination of fat deposits by oil-red staining, non-specific esterase determinations and immunofluorescence studies (outlined above). Histopathologic studies were done in samples taken weekly up to the sixth week and then biweekly up to the eighteenth week after infection. The immunofluorescence studies were performed on samples taken at biweekly intervals up to the eighteenth week after infection.

Experiments using cellular disaggregates from skin lesions and lymph nodes in order to characterize B and T lymphocyte populations were performed at six and twelve weeks after infection. Two separate experiments employing three mice from each experimental group at each time were made.

The cellular immune response was estimated by measuring the DHR to *L. major* and *L. mexicana* antigens in three to six animals from each experimental group at 2, 4, 8, 12 and 16 weeks after infection.

The presence and titre of circulating antibodies was determined in the sera of three mice from each experimental group of mice by direct agglutination and immunofluorescence technique at 2, 4, 8, 12, 16 and sometimes 18 weeks after infection using *L. major* and *L.mexicana* promastigotes as antigen.

CHAPTER II

CLINICAL EVOLUTION AND HISTOPATHOLOGY OF SKIN LESIONS AND DRAINING LYMPH NODES IN CBA/ca AND BALB/c MICE INFECTED WITH L. major AND L. mexicana

II.1 INTRODUCTION

As has been said, cutaneous leishmaniasis can be thought of as a spectrum of disease presentation similar to that seen in leprosy (TURK & BRYCESON, 1971), ranging from the hypersensitive to the anergic forms, but unlike leprosy, in which the spectrum goes from high to low resistance forms, in leishmaniasis the healing forms of the disease occupy the middle of the spectrum and the non-healing forms are located at the extremes (see Chapter I). The evidence shows that the clinical features of the disease are influenced not only by the species of parasite, but also by the immunological response of the host (Bryceson,1970 a). There is in fact a precise parallel between the histological spectrums in leprosy and leishmaniasis which led BRYCESON (1969) to propose a classification of leishmaniasis forms based, as in leprosy, on histopathological features and likewise associated to varying cellular immune responsiveness to the parasite.

This classification includes:

MM; macrophage form, an anergic form similar to lepromatous leprosy where the macrophage is the predominant cell in the lesion.

MI; macrophage-intermediate.

II; intermediate, analogous to border line leprosy where the lesion consists of infected macrophages and sensitized lymphocytes.

IT; intermediate tuberculoid.

TT; tuberculoid with the appearance of some epithelioid cells.

RIDLEY (1979), on the other hand, in reviewing the histology of cutaneous leishmaniasis in man, suggested that the spectrum was more complex and more varied than that of leprosy, and that although the histological counterpart of delayed hypersensitivity could be observed in some cases, the most important and constant mechanism leading to the elimination of parasites appeared to be an immunologically induced necrosis of the amastigote infected macrophages; a feature which was not taken into account in BRYCESON's classification. Ridley and his colleages proposed a new, if tentative, classification based on the appearance of the inflammatory response, categorized as: cellular (C), fibrinoid (F) and mixed (M), and correlated to the parasite load of the lesion (RIDLEY et al. 1980). This classification, originally intended only for Brazilian cutaneous leishmaniasis, was later extended to Central American and Old World forms, but as RIDLEY himself pointed

out, it requires further clinical evaluation and development (RIDLEY, 1980).

Self-healing and non-healing forms of cutaneous leishmaniasis can be modelled by infecting different inbred strains of mice with the same species of parasite (PRESTON et al. 1978; PEREZ et al. 1979) (see Chapter I). This chapter describes a study of the histology of the lesion and draining lymph nodes throughout the evolution of the infection in the four experimental models of murine leishmaniasis mentioned above, which provides a basis for an immunopathological comparison of models of self-healing and non-healing forms of leishmaniasis. The histopathological study consisted of conventional staining of sections of lesions and draining lymph nodes by haematoxylin and eosin, in order to assess the general picture of the structure, by Giemsa colophonium for the identification of the *Leishmania* amastigotes in the sections, by methyl green pyronin for the investigation of pyroninophilic cells and by Van Gieson stain (trichromic) for the study of collagen. In addition, oil-red staining for neutral fats was performed on cryostat sections.

II.2 MATERIALS AND METHODS

II.2.1 PARASITES

The strains of parasite used were:

Leishmania mexicana mexicana strain code LV4, Liverpool school of Tropical Medicine, isolated from *Nyctomis sumichrasti* in Belize.

Leishmania major strain code: LV39 Liverpool school of Tropical Medicine isolated from *Rhombomys opimus* in Soviet Asia, and recently confirmed at the London School of Hygiene and Tropical Medicine.

Both strains of parasites have been maintained by serial passage into the foot pad of young female BALB/c mice. When promastigotes were required they were grown in a protein-free dialysed medium (NAKAMURA, 1967) at 26° C, from amastigotes of the infection in BALB/c mice.

II.2.2 MICE

CBA/ca and BALB/c female mice 8 to 10 weeks old (Olac 76, Shaw Farm Blackthorn, Oxon) were used in all the experiments.

II.2.3 INFECTION

L.mexicana and *L. major* amastigotes were obtained from foot pad lesions of infected BALB/C mice. The nodules were dissected out aseptically, washed in HANK's balanced saline (BSS) containing 10 µg/ml gentamicin,

finely teased, and ground in a glass homogenizer containing cold BSS. The resulting suspensions were allowed to rest for ten minutes for the larger aggregates to settle. The supernatants were then removed, and washed 3 times in tissue culture medium 199 (199) at 1200 g for 10 minutes at 4°C. Finally the amastigotes were resuspended in 199 and samples of the suspensions counted in a haemocytometer. The viability of the amastigotes was determined by the trypan blue exclusion technique: a 0.4 % stock trypan blue solution in phosphate buffered saline (PBS) is mixed with a sample of the test suspension, to a final dye dilution of to 0.2 % v/v, and allowed to rest for 5 minutes. After this time, dead parasites can be recognized by their inability to exclude the dye. The number of live parasites is then estimated by counting only unstained organisms. The final concentration of parasites was adjusted to 10^{6} per 200 µl and injected intradermally into the left hind foot pad.

II.2.4 CLINICAL EVALUATION

A sample of the inoculated animals from each experimental group was examined every week for 16 weeks, paying special attention to the appearance of the ulcers and metastasis. The thickness of the foot pad was measured with a Pocotest micrometer gauge and the lesion size was calculated as the difference in thickness between the infected foot and the uninfected contralateral foot of the same animal. If a metastasis appeared in the contalateral foot, the lesion in the animal in question was not measured.

II.2.5 TISSUE EMBEDDING, SECTIONING AND STAINING

Embedding

Every week after infection up to the 8th and every two weeks from the 8th to the 18th week, three animals from each experimental group were sacrificed by ether anaesthesia, bled and the skin lesions and draining lymph nodes removed. Samples of each tissue were then either fixed in Carnoy's fixative for two to three hours, or frozen in liquid Nitrogen for subsequent cryostat sectioning. The samples fixed in CARNOY's fluid were later rinsed in 90 % alcohol and dehydrated by one hour incubation in alcohols, ascending in strength from 90 to 100 %, changed every hour. The samples were then cleared in cedar wood oil and xylene and embedded in paraffin wax at 56°C.

Sectioning

The embedded samples were sectioned at 5 - 7 microns thickness, using a Reichert microtome and ribbons of sections placed on albuminised slides, flooded with distilled water and kept at 60° on a hot plate to dry and stretch.

The frozen samples were sectioned in a SLEE cryostat to a thickness of 4 to 5 microns. The cryostat sectioning procedures are described in detail in Chapter III.

Staining

The sections were deparaffinised by quick flaming and

xylene rinsing, then hydrated by passing them through descending strengths of alcohols to distilled water. Sample sections from each kind of tissue were stained by each of the following techniques: a - Gomory's haematoxylin and eosin (GURR, 1958), b - methyl green pyronin TREVAN & SHARROCK's method (CULLING, 1963), c - Van Gieson acid fuchsin for collagen (CULLING, 1963) and d - Giemsa colophonium (BRAY & GARNHAM, 1962).

The cryostat sections were stained with oil-red using LILLIE's method (PEARSE, 1968) in order to demonstrate neutral fats.

II.3 RESULTS

II.3.1 CLINICAL EVOLUTION

The clinical manifestations of *L. mexicana* and *L. major* infection followed different patterns in the different strains of mice (see Table and Figure 1). By the second week after infection, CBA/ca and BALB/c mice infected with *L. major* developed a visible and palpable nodule at the site of inoculation, a nodule of the same size was noticeable in CBA/ca and BALB/c mice infected with *L. mexicana* by the third to the fourth week after infection.

As long as the infection progressed, CBA/ca mice infected with *L. major* developed small non-ulcerating lesions which reached their maximum by the fourth week and

then declined progressively, showing a complete regression by the twelfth week after infection. BALB/c mice infected with *L. major* had lesions which, by four weeks, were more than twice the size of those of the CBA/ca mice infected with the same parasite. After four weeks of infection, the lesions in BALB/c mice infected with *L. major* started to ulcerate, thereafter the lesions continued develop gradually and by the fifth and sixth week after infection 75 % of the lesions had punched out ulcers, which often were covered by a crust. These ulcerated lesions persisted until the end of the observations. Metastasis were observed in one mouse 14 weeks after infection. The metastasis were located in the skin surrounding the eye and on the foot contralateral to the lesion. In three cases, by the sixteenth week after the infection, the lesions developed to an stage in which they threatened to amputate the foot; these animals were sacrificed.

CBA/ca mice infected with *L. mexicana* developed non-ulcerating nodules, which gradually increased in size and persisted until the end of the observations. This group showed the smallest lesions among the groups exhibiting non-healing lesions. Metastasis were not observed.

In BALB/c mice infected with *L. mexicana*, the size of the lesion increase rapidly throughout the whole period of observation. Seven weeks after infection, 23 % of the lesions were partially covered by a black coloured crispy epidermis which gradually became a crust, exudation liquid was seen leaking from those regions. No punched out ulcers

TABLE 1

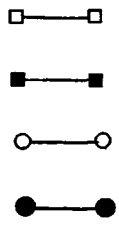
DIFFERENCE IN SIZE BETWEEN INOCULATED AND NON INOCULATED FOOT PAD (MEAN & STANDARD ERROR) IN CBA/ca AND BALB/c MICE FOLLOWING INFECTION WITH L.major AND L.mexicana.

	CBA/ca MICE						BALB/c MICE					
	INFECTED WITH L. major			INFECTED WITH L. mexicana			INFECTED WITH L. major			INFECTED WITH L. mexicana		
WEEKS	MEAN	S.E.	n	MEAN	S.E.	n	MEAN	S.E.	n	MEAN	S.E.	n
1	0.24	0.03	27	0.12	0.02	27	0.23	0.04	25	0.18	0.02	24
2	0.56	0.03	24	0.09	0.02	24	0.50	0.05	20	0.17	0.03	19
3	0.64	0.05	24	0.28	0.03	24	1.57	0.10	20	0.28	0.03	20
4	0.79	0.06	24	0.50	0.04	24	2.29	0.14	19	0.68	0.08	20
5	0.57	0.07	21	0.62	0.06	21	2.09	0.16	18	1.03	0.11	18
6	0.47	0.04	21	0.79	0.10	21	2.19	0.20	18	1.66	0.13	17
7	0.28	0.04	21	0.86	0.09	21	2.36	0.22	18	2.38	0.16	17
8	0.23	0.04	21	0.94	0.14	21	2.41	0.30	17	3.01	0.19	17
9	0.16	0.04	17	1.26	0.18	17	2.59	0.37	15	3.61	0.20	14
10	0.14	0.04	11	1.35	0.27	11	2.42	0.36	10	4.80	6.26	19
11	0.12	0.03	10	1.63	0.32	11	2.51	0.42	10	5.27	0.26	9
12	0.09	0.02	16	1.49	0.23	16	2.87	0.40	13	5.23	0.36	12
13	0.07	0.01	12	1.64	6.30	12	3.15	0.49	11	5.94	0.42	8
14	0.03	0.02	12	1.80	0.38	12	2.67	0.61	7	6.84	0.47	7
15	0.00	0.00	8	2.33	0.42	7	3.03	0.56	4	6.80	0.80	5
16	0.00	0.00	6	2.18	0.56	5	3.40	0.66	3	7.50	0.94	5

S. E. : standard errors. n : number of mice used in the experiment.

FIGURE 1

Course of the infection in CBA/ca and BALB/c mice infected in the foot pad with 10^5 amastigotes of *L. major* or *L.mexicana*.



□——□ CBA/ca mice infected with *L. major*.

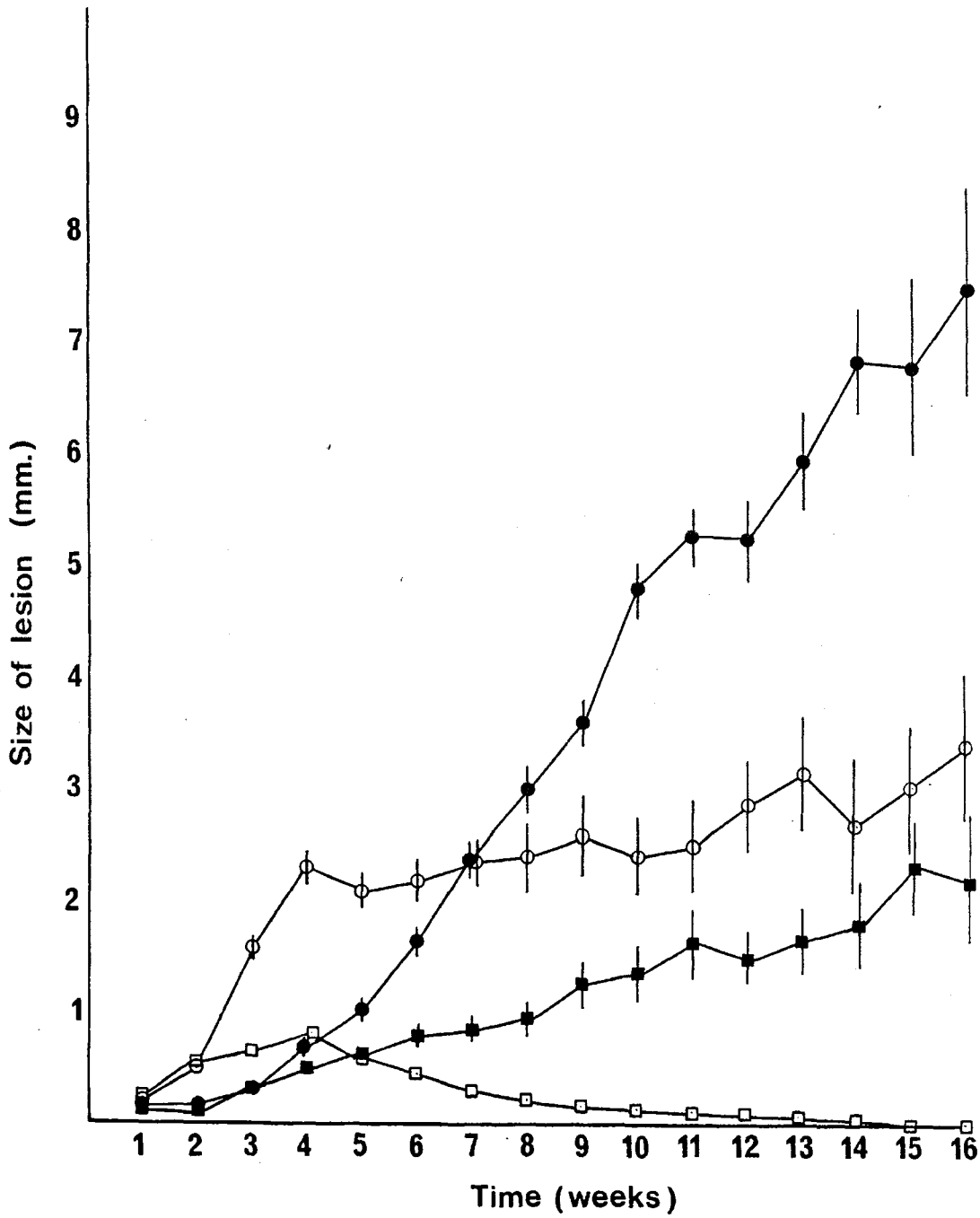
■——■ CBA/ca mice infected with *L. mexicana*.

○——○ BALB/c mice infected with *L. major*.

●——● BALB/c mice infected with *L. mexicana*.

Vertical bars represent standard errors.

Figure 1


Size of lesion (mm.)
9
8
7
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (weeks)

were seen.

II.3.2 HISTOLOGICAL STUDIES

In the course of this study the terms immature and mature macrophages are used as described by ADAMS (1974). Immature macrophages are small round cells with a centrally slightly vesiculated round nucleus, located at the centre of the cell and displaying small nucleoli. The cytoplasm is eosinophilic, slightly foamy and sparse with distinct borders. Mature macrophages are large polygonal cells with large, eccentric oval nuclei with open, vesicular and slightly marginated chromatin and large eosinophilic nucleoli; these cells touch one another lightly and the cytoplasmic borders are slightly blurred.

II.3.2.I CBA/ca MICE INFECTED WITH *L. major*

II.3.2.1.1 SKIN LESIONS

During the first week after infection, the developing lesion was confined to the dermis.

The subepidermis area showed fibrocytes, mast-cells and dendritic or spindle shaped cells, some of which were laden with pigment. These cells were placed among bundles of slightly oedematous and basophilic collagen.

Deeper layers of the dermis showed scattered monocytes and immature macrophages. Deeper still, there were a few compact inflammatory foci made up of macrophages, eosinophils, mast-cells and scanty amastigotes inside macrophages.

Small blood vessels, although present throughout the infiltrate, were more abundant towards the surface of the skin, where they contained mononuclear and red blood cells.

The second week marked the appearance of changes in the epidermis, which became acanthotic and hyperkeratotic in some areas. Meanwhile, in the dermis the infiltrate extended towards the deeper zones (Plate I, Fig. 1)[1], becoming more compact, surrounding blood vessels and nerves, and reaching the muscle layer.

The infiltrate consisted of monocytes and immature and mature macrophages. Some of the macrophages were parasitized, while others showed vacuolization of the cytoplasm and nucleus. They were closely packed together making small groups or sheets, accompanied by groups of monovacuolated cells resembling fat cells.

Arterioles and capillaries showed oedematous endothelial cells whose nuclei protruded into the lumen. Occasional infiltration of the adventitia of the vessels and of the perineurum of the nerves by inflammatory cells was also observed.

Poorly demarcated hyaline collections were seen near the muscles. These collections consisted of an amorphous mass, containing dark structures similar to muscle cell nuclei. The outer limits of these masses showed faint striations

1 - I regret the inappropriate use of word "plate" to describe my sets of photographs, but as I believe that the arrangement used is better suited for the study of groups of related figures than the alternate serial numeration, I shall, in absence of a better solution, refer to such sets of photographs as plates.

which disappeared towards the central region. The overall histological appearance of these masses strongly suggests they originate from muscular degeneration.

During the third week, lymphocytes, epithelioid cells and scanty giant cells were detected. Mast cells degranulated and eosinophils increased in numbers. Most of the nerves and blood vessels were tumefacient and infiltrated. Some nerves, muscles and vessel walls were vacuolated. Small necrotic areas were observed near the hyaline collections.

By the fourth week, cytoplasmic vacuolization and nuclear constriction of the cells of the prickle layer, along with fragmentation of the rete ridges, joined the acanthosis and hyperkeratosis of the epidermis. Some epidermal areas were also infiltrated by cells coming from the dermis.

One biopsy showed intraepidermal necrosis consisting of an amorphous hyaline substance, mononuclear cells, eosinophils and cellular debris.

In the subepidermis, some areas showed great disruption of the collagen with necrosis; in others there were abundant fibroblasts and collagen infiltrating deeper areas of the skin giving the infiltrate a looser appearance.

Small blood vessels full of red blood cells proliferated throughout the infiltrate, especially in the upper areas.

In the infiltrate lymphocytes became abundant, eosinophils and mast-cells decreased and the macrophages remained unchanged.

From this point onwards, the changes in the epidermis started to regress and the repair processes began. By the fifth week, only some acanthotic areas remained and these gradually disappeared over the next weeks, so that by the 8th to the 10th week, the epidermis had recovered its normal appearance.

In the dermis, the resolving process progressed between the fifth and tenth week. Collagen, fibroblast and blood vessels increased greatly, necrotic areas and hyaline collections gradually disappeared and the number of infiltrating macrophages declined slowly.

The intracellular parasites that were seen up to the fourth week, were no longer detected, lymphocytes and epithelioid cells become predominant, and by the sixth week, small numbers of plasma cells were detected for the first time.

By the eighth week, eosinophils showed a slight increase and mast-cells appeared laden with granules.

By the tenth week, the infiltrate consisted of fibroblasts, lymphocytes, some epithelioid cells and a few eosinophils and mast-cells.

From the 12th week onwards, the skin recovered its normal appearance.

II.3.2.1.2 LYMPH NODES

One week after the infection, the cortical area showed slight hyperplasia accompanying the appearance of a few, poorly developed, secondary follicles which increased in number and developed fully during the second and third

week.

The germinal centres of these follicles were pale areas containing cleaved and large lymphocytes, pyroninophilic cells and macrophages laden with cellular debris.

Between the 4th and the 6th week, the typical structure of the follicles was no longer discernible, due to the infiltration of the cortical area by histiocytes which became arranged as a sheet lining the subcapsular area. A high power examination did reveal areas still identifiable as part of the germinal centres.

The paracortical area showed the greatest development (Plate II. Fig. 1). Moderately hyperplasic areas appeared during the first week. These areas consisted of small and large lymphocytes, pyroninophilic cells and epithelioid or nearly epithelioid cells.

During the second and third week, the paracortex appeared as a well developed, eosinophilic area, due to the infiltration of clusters of histiocytes with epithelioid appearance, which adopted a concentric arrangement and were clearly demarcated from the surrounding lymphocytes (Plate II. Fig. 4). These clusters varied in size and some times coalesced into big masses of cells, displacing a significant proportion of the lymphoid tissue.

Four to six weeks after infection, the cell clusters grew even bigger, and some cells showed pigment and cellular debris inside.

Six weeks after the infection, almost all the tissues from the capsular to the medulla were infiltrated by masses of epithelioid cells. Most of these cells showed increasing

vacuolization and started to disappear thereafter, although some clusters persisted without vacuolization. By the eighth week, aggregates of small lymphocytes appeared in the paracortex, along with some pyroninophilic cells and eosinophils. Eosinophils were also seen in the subcapsular area. By this time well-formed and developed germinal centers were again seen, such centers remaining apparent to the end of the observations.

By the tenth week, lymphocytes were seen forming a narrow rim along the subcapsular region with projections into the rest of the organ breaking up the masses of histiocytes.

In the medullary cords, plasma cells were detected from the second week of the infection and continued to be detected until the end of the observations, showing a marked increase during the 12th week.

The marginal sinus was enlarged since the first week, and its continuity with the rest of the node sinuses was very apparent.

During the second and third week, the sinuses exhibited the maximum dilation, especially in the medulla; this dilation persisted up to 14th week.

Plasma cells, pyroninophilic cellsand macrophages were seen inside the sinuses. In the marginal sinus some of the macrophages were laden with pigment.

Blood vessels were apparent throughout the structure between the first and the 12th weeks of the observations. They were especially conspicuous in the cortex and medulla, and during the 6th to 8th weeks also in between the medulla

and the paracortex. The vessels were filled with mononuclear and abundant red blood cells; some blood cells were also seen scattered in the tissue and inside macrophages.

By the 12th week, lymphoid cells were occupying most of the lymph node again, and the number of epithelioid cells had decreased significantly.

Between the 14th and the 18th weeks, very few epithelioid cell clusters remained, plasma cells could still be detected in medullar cords, and the structure looked almost normal.

The parasites were seen occasionally in the cortex as amastigotes inside macrophages up to third week, but only after careful search. They were not detected thereafter.

II.3.2.2 CBA/ca MICE INFECTED WITH *L. mexicana*

II.3.2.2.1 SKIN LESIONS

During the first two weeks after infection, the epidermis remained intact.

By the first week of infection, small inflamatory foci, consisting of parasitized macrophages were detected in the deep dermis. Melanin granules, either free or incorporated into superficial dendritic shaped cells, were seen in the subepidermis. Mast-cells, immature macrophages and monocytes were scattered throughout the whole of the dermis.

By the second week, the infiltrate was bigger. Immature macrophages increased in numbers and eosinophils appeared close to them.

During the third week, the epidermis showed acanthosis in some areas and the cells of the prickle layer exhibited cytoplasm vacuolization.

In the dermis the infiltrate showed degranulating mast-cells, and although continuing to be diffuse, it was more dense in the deeper zones. In the subepidermis there was abundant collagen, but it was oedematous and basophilic.

Collections of hyaline material, similar to those previously described in this study, in the *L. major* CBA/ca model were observed close to the muscular strands.

During the fourth and the fifth weeks, the acanthosis of the epidermis increased; the rete ridges became disorganized and the layer was invaded by monocytes, eosinophils and parasitized macrophages from the dermal infiltrate.

At the same time, in the dermis a distinct area of infiltrate consisting of bundles of collagen, parasitized macrophages, degranulating mast-cells and congested blood vessels occupied the outer part of the lesion. Further inwards, the infiltrate adopted several patterns: in some areas it consisted predominantly of heavily parasitized macrophages, in other areas monocytes, macrophages, eosinophils, epithelioid cells, some lymphocytes and occasional giant cells, adopted a compact and concentric

arrangement, surrounding blood vessels and nerves. In still other areas, the macrophages were vacuolated, some having several small vacuoles, in others these seemed to have coalesced into a large single vacuole occupying most of the cytoplasm, displacing the nucleus to one side and giving the appearance of a ring. Some of these big, monovacuolated cells were parasitized.

Patches of necrosis, characterized by fibrinoid material containing degenerated macrophages, amastigotes, cellular debris and eosinophils, were observed in the central dermis scattered within the infiltrated areas.

From the sixth to the tenth week, the epidermis started to show areas of necrosis with conspicuous clusters of eosinophils within them.

Between the sixth and the eighth week, most of the central and deep dermis was occupied by an extensive loose granuloma of parasitized macrophages, infiltrated by abundant eosinophils, degranulated mast-cells, lymphocytes and scanty neutrophils. Within the granuloma, especially in the deeper areas, necrotic foci, hyaline collections and pink patches of epithelioid cells were observed.

By the tenth week, the granuloma was growing towards the surface, the majority of the macrophages were vacuolated and parasitized. Epithelioid cells were arranged in concentric sheets, and scattered degranulated mast-cells, lymphocytes and eosinophils disrupted the uniform pattern of vacuolated macrophages. A band of collagen remaining in the subepidermal area seemed to be holding up the advance of the infiltrate. In the area of the infiltrate close to

this band, the macrophages were parasitized, but not vacuolated, and abundant eosinophils were seen close to them (Plate I Fig.2).

Towards the deep dermis, the infiltrate had invaded blood vessels, nerves and muscles, disrupting their structures.

In the midst of the infiltrate there were areas of haemorrhage and fibrinoid necrosis, surrounded by lymphocytes, monocytes and neutrophils. Lymphocytes were abundant in the deep infiltrate, they were seen forming clusters and tended to congregate around the blood vessels.

By the twelfth week, abscesses appeared in the epidermis. The alterations were conspicuously focal, the abscessed areas alternated with areas of thickening and areas where the epidermis was thin and atrophic due to pressure of the nearby infiltrate. This picture persisted until the end of the observations.

At this time the dermal lesion was a huge granuloma of parasitized and vacuolated macrophages, limited on the surface by a thin layer of collagen and at the bottom by lymphocytes and eosinophils. The necrotic areas had diminished in size and in numbers, and abundant eosinophils were seen in between the macrophages.

From the fourteenth to the eighteenth week, the granuloma continued to grow upwards and the layer of collagen limiting it became progressively thinner. Well-preserved nerves and blood vessel, surrounded by clusters of lymphocytes and eosinophils, were occasionally seen within the granuloma.

II.3.2.2.1 LYMPH NODES

Development of the cortical area was apparent right from the beginning of the observations in the first week after infection, when secondary follicles with small germinal centres were seen. These follicles reached their maximum development by the sixth and eighth weeks, at which time they consisted of plasma cells, macrophages containing either parasites or cellular debris (but never both) and cluster of cells with some epithelioid appearance.

In the subcapsular area a band of histiocytes was detected by the first week after infection. This band increased in thickness during the following weeks, and from the second week, projections of it penetrated deeply to the rest of the node. The appearance of the histiocytes varied from that seen in the *L. major* L CBA/ca model; in this case the cells were less epithelioid, exhibiting a less elongated and eosinophilic cytoplasm and a heavily stained nucleus. By the fourth week after infection, multivacuolated macrophages laden with parasites were observed in this area.

The paracortical area showed little development. The postcapillary venules became conspicuous from the first week, showing transformed lymphocytes and macrophages in their lumen. Lymphocytes undergoing transformation were also seen. After the first week, cellular proliferation increased steadily, and by the fourth and sixth weeks, histiocyte clusters appeared along with transformed lymphocytes and vacuolated macrophages containing

amastigotes.

In the medulla, some pyroninophilic cells were seen at the beginning of the observations; by the third week they had transformed into plasma cells and were particularly abundant. They remained so until the end of the observations by the 18th week.

The medullary sinuses were conspicuously dilated by the third week, the dilation continued steadily and by the 14th week the complete sinus system from the marginal sinuses to the medulla was markedly widened, containing macrophages with amastigotes, plasma cells and eosinophils. The latter were particularly abundant in medullary cords during the fifth week.

Blood vessels were conspicuous in the medulla and cortex from the second week after infection, by the tenth week they were tumefacient and haemorrhagic.

Considering the organ as a whole, the lymphoid tissue was progressively replaced by masses of vacuolated and parasitized macrophages from the eighth week. By the tenth week, extensive haemorrhagic zones, containing abundant eosinophils and clusters of histiocytes, were present. In some of the clusters, the nuclei of the histiocytes were displaced towards the periphery, resembling giant cells of the Langerhans type (Plate II, Fig. 7); in others the nuclei concentrated in the center, resembling the giant cell type seen in foreign body granulomata.

From the fourteenth week onwards, the typical structure of the organ was unrecognisable. The lymph node consisted of a thin rim of lymphocytes, enveloping a loose mass of

vacuolated and parasitized macrophages and interspaced histiocytes, eosinophils and smaller proportions of lymphocytes and pyroninophilic cells (Plate II, Fig. 8). Nevertheless, towards the periphery of the organ, a few germinal centers remained, and in the area corresponding to the medulla, abundant plasma cells were observed.

II.3.2.3 BALB/c MICE INFECTED WITH *L. major*

II.3.2.3.1 SKIN LESIONS

The epidermal changes in this infection model were more pronounced than in the previous ones, and led to ulceration and crust formation.

The sequence of events up to ulceration appeared to be as follows:

Slight acanthosis was seen during the first three weeks after infection, and was followed from the fourth week on by enlargement, lengthening and fusion of the rete ridges. Many of the prickle cells in these areas had vacuolated cytoplasm and condensed nuclei. In other areas, the epidermis was flat.

By the fifth and sixth weeks, hyperkeratosis and parakeratosis appeared, the cornified layer showed an exaggeratedly waved pattern. The epidermal cells, especially those of the prickle layer, were tumefacient and clear. They had lost contact with one another, giving this area a vesiculate appearance. Near the basal membrane, the alterations were less marked. The prickle cells, although

hypertrophic, remained in contact.

In the granulous area, some cells had vacuolated cytoplasm and others contained large amounts of a keratohyaline material.

By the eighth week, the areas of vesicular appearance had become collections of hyaline, haemorrhagic material and cellular debris, by the fourtenth to the sixteenth weeks, these areas achieved their maximum development, constituting abscesses which sloughed off. The lesions, however, did not progress at the same rate in all areas; in some areas smaller abscesses developed faster and sloughed off by the fifth week.

The dermal alterations were, to a certain degree, similar to those described in the model CBA/ca mice infected with *L. mexicana*. The changes, however, took place more quickly, so that the dermis exhibited a dense infiltrate from the first week of infection. This infiltrate consisted not only of cells of the macrophage-phagocytic system (ranging from monocytes to macrophages and epitheliod cells), eosinophils and neutrophils, but also, even at this early stage, lymphocytes, degranulating mast-cells, macrophages laden with amastigotes, epithelioid cells and streaks containing dark nuclei, probably resulting from the destruction of the parasitized macrophages.

Blood vessels were congested and filled with numerous leucocytes. In the nerves, the perineurum was thickened and infiltrated by cells, and the central nervous bundles were

displaced to one side. Hyaline collections, similar to those described before as the probable result of muscular degeneration, were seen near the muscular layer (Plate I, Fig. 3).

The distribution pattern of the infiltrate was also similar to those of the other infection models.

By the second week, the cells of the infiltrate aggregated compactly around the blood vessels and nerves. Eosinophils increased considerably in numbers, neutrophils became scanty and a few giant cells were detected. Some areas had parasitized macrophages; some of these were heavily parasitized with a foamy appearance and others had few parasites, with no or very few vacuoles. Small collections of hyaline material were seen scattered throughout the central dermis.

From the third week onwards, the infiltrate grew upwards, and the subepidermal area became thinner, showing a basophilic, oedematous and disrupted collagen and numerous blood vessels filled with erythrocytes. Epitheliod cells, arranged concentrically or in sheets, were seen immediately under this area.

The granuloma showed poorly demarcated areas of necrosis consisting of fibrinoid material, cellular debris, degenerating parasitized macrophages and free amastigotes surrounded by eosinophils and neutrophils. The fibrinoid material had spread to the areas adjacent to the necrotic centres. Towards the bottom of the infiltrate, numerous lymphocytes were seen in between muscle strands (Plate I, Fig. 4) and surrounding blood vessels and nerves, which

showed hyperplasia, oedema and mononuclear infiltration of their walls (Plate I, Fig 5).

Between the fifth and the eighth weeks,the changes intensified; the necrotic areas grew. Blood vessels and nerves appeared severely damaged, showing vacuolization of the walls. Mast-cells increased in numbers, especially in the subepidermis and central dermis. Plasma cells were seen occasionally by the fifth week and increased thereafter, becoming abundant by the eighth week. Lymphocytes also increased after the fifth week.

By the tenth to the twelfth week, some areas in the upper granuloma appeared as collections of heavily parasitized and vacuolated macrophages, surrounded by strands of collagen (Plate I. Fig. 5). This collagen enveloped aggregates of macrophages, which eventually evolved into areas of necrosis containing hyaline material, free amastigotes and cellular debris towards the end of the observations at eighteen weeks. The progression of these collagen bounded structures was not simultaneous and even from the twelfth week they could be seen in all the described stages mentioned in the pattern of development.

By the fourteenth week, mast-cells were seen once again to be degranulating and this was followed by a subsequent increase of eosinophils.

The final appearance of the lesion by the eighteenth week, was that of a granuloma, limited in its uppermost zone by a band of collagen and in its lowermost region by palisading lymphocytes. The granuloma itself contained the

collagen bound collections of macrophages mentioned earlier, still in different stages of evolution, free parasitized macrophages and, scattered between them, mast-cells, eosinophils and epithelioid cells. The necrotic areas were extensive.

LYMPH NODES

At the beginning, the most conspicuous alterations in the lymph nodes were the dilation of the sinus system and the development of the cortical area (Plate II, Fig. 2, 3).

The dilation of the sinus system was apparent from the first week of infection, and the sinuses were seen to be filled with histiocytes and pyroninophilic cells, later on by plasma cells and lymphocytes which had joined the other cells and, by the end of the observations, the entire system was clogged with cells. The histiocytes were seen passing down from the afferent lymphatics to the marginal sinus and accumulating as a band in the subcapsular area. The appearance of these histiocytes was intermediate between epithelioid and foamy or vacuolated.

The capillaries in the cortex and postcapillary venules in the paracortex were conspicuous from the first week, and during the fourth to the eighth week, they were prominent in the paracortex and medulla.

In the cortex, germinal centers started to develop from the first week and reached their maximum development by the fourth to the eighth week (Plate II, Fig. 2), when they were also infiltrated by histiocytes, some of which were parasitized. From the tenth week onwards, the structure of

the follicles was masked by an infiltration of histiocytes, however, a few well developed germinal centers remained visible throughout the observation period.

The paracortex was the least developed area, the main alteration being heavy infiltration by aggregates of histiocytes from the beginning of the observations. During the fourth and the eighth weeks of infection, areas of the paracortex were deprived of lymphoid cells and contained reticular-like cells, neutrophils, eosinophils and parasitized macrophages. By this time, the histiocytes in the aggregates varied in appearance from intermediate between epithelioid and foamy to foamy monovacuolated and some times syncitial. In some aggregates, the cells contained amastigotes or cellular debris, while in others the cells displayed the appearance of giant cell (Plate II, Fig. 6).

The medulla was seen to contain plasma cells from the second week. By the tenth week, they were the most abundant cells. Eosinophils, macrophages and red blood cells were also seen.

Thereafter, the infiltration by histiocytes continued to increase, and by the end of the observations the organ appeared as a mass of histiocytes, interspersed with strands of lymphocytes and pyroninophilic cells, all surrounded by a thin rim of lymphocytes.

By the eighteenth week large haemorrhagic zones (Plate II, Fig. 6) appeared in the whole of the organ.

II. 3.2.4 BALB/c MICE INFECTED WITH *L. mexicana*

II.3.2.4.1 SKIN LESIONS

The epidermis showed no abnormalities during the first three weeks, from the fourth week onwards the layer started to be invaded by parasitized macrophages and eosinophils from the dermal infiltrate. The infiltration of the epidermis was more apparent by the fifth and sixth weeks, when it was joined by acanthosis and fragmentation of the rete ridges and by alterations of the cells of the prickle cell layer, similar to those described earlier for the previous infection models.

Hyperkeratosis and parakeratosis appeared during the eighth week. Intra-epidermal necrosis was noted from the twelfth week onwards along with superficial pustules, encroaching upon the parakeratotic areas. These lesions persisted until the end of the observations, alternating with areas of thinned epidermis.

In the dermis, during the first week, the infiltrate was light and not very compact, consisting of cells of the macrophage-phagocytic system, ranging from monocytes to macrophages, degranulating mast-cells and eosinophils. Towards the deep dermis, there were small foci of cells containing amastigotes.

By the second week, macrophages had increased in number and some of them were laden with amastigotes. By the third week, monovacuolated macrophages appeared in some areas,

some of which were parasitized. All during the first three weeks, blood vessels were filled with white cells.

During the fourth week the infiltrate became heavier, macrophages continued to increase in numbers and most of them were vacuolated and parasitized. Abundant eosinophils and some lymphocytes and neutrophils were seen scattered among the macrophages, forming clusters or surrounding blood vessels and nerves. Small necrotic areas were also seen sparsely in the infiltrate. Collagen was very scanty in the infiltrated areas, but subepidermal areas contained a fair amount of it.

Some nerves showed constriction of the central nervous bundles and their walls were invaded by parasitized macrophages.

During the fifth and sixth weeks, the granuloma expanded towards the epidermis, the necrotic areas became more numerous and larger. Towards the deep dermis, the infiltrate invaded and fragmented muscle strands, blood vessels were tumefacient and oedematous, filled with red cells, white cells and occasionally parasitized macrophages.

From the sixth week on, the granuloma continued to grow; in its central area eosinophils increased in numbers to become the second most abundant cell (Plate I, Fig. 6). Blood vessels and nerves were infiltrated and disrupted by parasitized macrophages. The necrotic areas were reduced in size and number and eventually disappeared by the eighth week.

By the end of the observations, a fully developed

histiocytoma (consisting of monovacuolated heavily parasitized macrophages) occupied the whole of the dermis. In some areas of the subepidermis though, strands of collagen persisted and there were still eosinophils and the parasitized macrophages which were not yet vacuolated. These areas also contained congested blood vessels and haemorrhagic zones (Plate I, Fig. 7).

II.3.2.4.2 LYMPH NODES

In the cortex, secondary follicles were apparent from the first week, but fully developed germinal centres were only observed from the fourth week.

In the subcapsular area, macrophages were present from the third and fourth weeks; some of them were parasitized and vacuolated. In some areas they coalesced into syncytia which appeared as clear areas. Some eosinophils were seen close to the syncytia.

By the fifth and sixth week, parasitized macrophages were seen inside the germinal centres.

In the paracortex, clusters of histiocytes, without foamy or epitheliod appearance, appeared during the second week. During the third week, the postcapillary venules were conspicuous and pyroninophilic cells appeared.

In the medullary area, the sinuses were dilated and the blood vessels were hypertrophic from the first week. By the fourth week, all the blood vessels of the the organ were dilated. In medullary cords, large transformed lymphocytes, macrophages and some eosinophils were seen from the very beginning.

By the eighth week, the cortex and paracortex were completely infiltrated by parasitized and vacuolated macrophages and by eosinophils. Histiocyte clusters were less abundant, and some of their cells were parasitized. In other clusters these aggregates of cells coalesced into giant cells.

By the tenth week, the lymph node consisted of a mass of parasitized macrophages and eosinophils, amidst which small and scanty areas of lymphocytes persisted. The blood vessels were congested and some scattered haemorrhages were seen.

By the twelfth to the fourteenth week, medullary sinuses, filled with lymphocytes, plasma cells and eosinophils, were the only recognizable structure of the organ.

By the sixteenth to the eighteenth week the node consisted of a histiocytoma the center of which was occupied by a huge area of necrosis, consisted of dead macrophages, cellular debris and parasites (Plate II, Fig. 9). Eosinophils were seen inside and outside the necrotic area. Haemorrhages were seen around the necrotic area, and plasma cells were detected in the periphery of the organs.

PLATE I

SKIN LESIONS. DERMAL INFILTRATE IN CBA/ca AND BALB/c MICE INFECTED WITH *L.major* AND *L.mexicana*. FIGURES 1 TO 7.

FIGURE 1

CBA/ca mouse, 2 weeks infected with *L. major*. Dermal infiltrate in central dermis consisting of cells of the phagocytic system ranging from monocytes to macrophages. Some macrophages are parasitized. (v)= vessel. Section, Giemsa colophonium. X 80.

(cntd)

FIGURE. 1

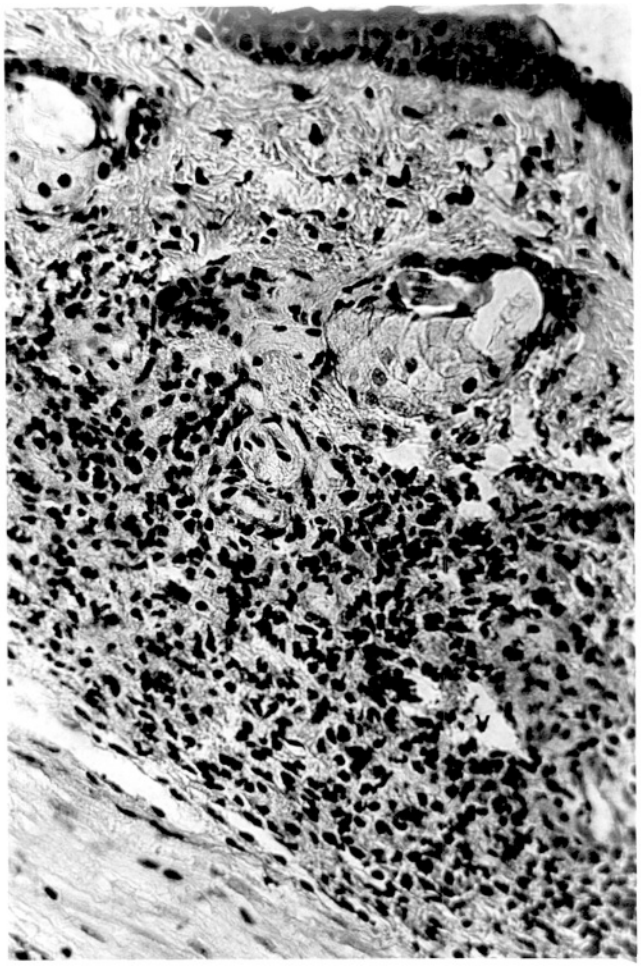


PLATE I (cntd)

FIGURE. 2

CBA/ca mouse, 10 weeks infected with *L. mexicana*. Dermal infiltrate showing masses of heavily parasitized macrophages most of them vacuolated. Towards the dermal surface bands of collagen eosinophils, lymphocytes and little vacuolated but heavily parasitized macrophages limit the infiltrate. Section, H & E. X 50.

(cntd)

PLATE I (cntd)

FIGURE 3

BALB/c mouse, 14 weeks *L. major* infected. Infiltrate in the deep dermis shuwing hyaline degeneration of muscular structures (m). Oedematous collagen (c) is present in between the cells of the infiltrate, Section staining Van Gieson. X 80.

FIGURE 4

BALB/c mouse 4 weeks infected with *L. major*. Observe numerous lymphocytes infiltrating in between muscular strands in the deep dermis. Section staining Giemsa-colophonium. X 50.

FIGURE 3

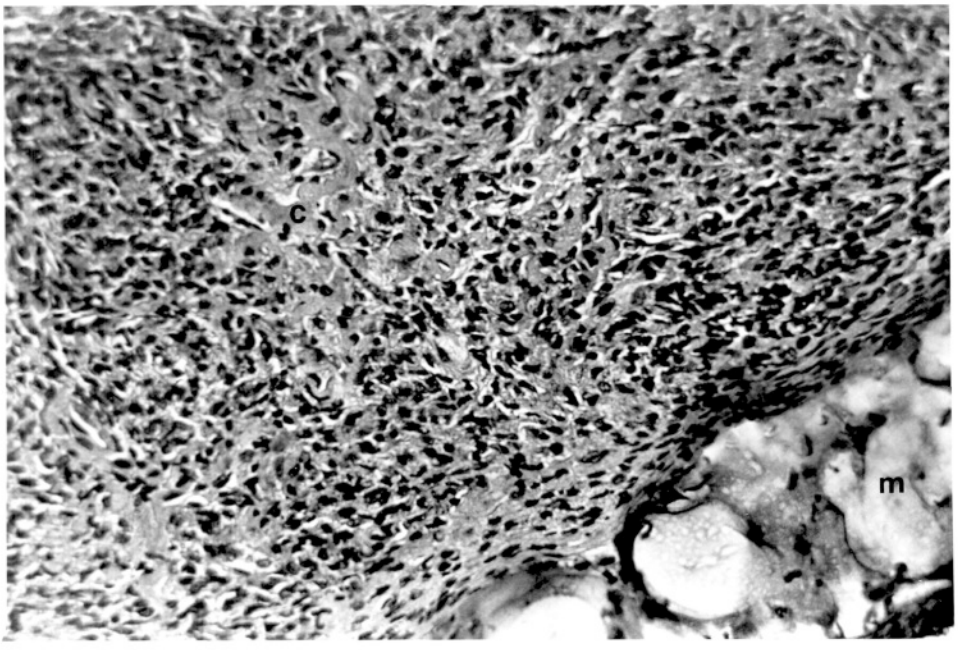


FIGURE 4

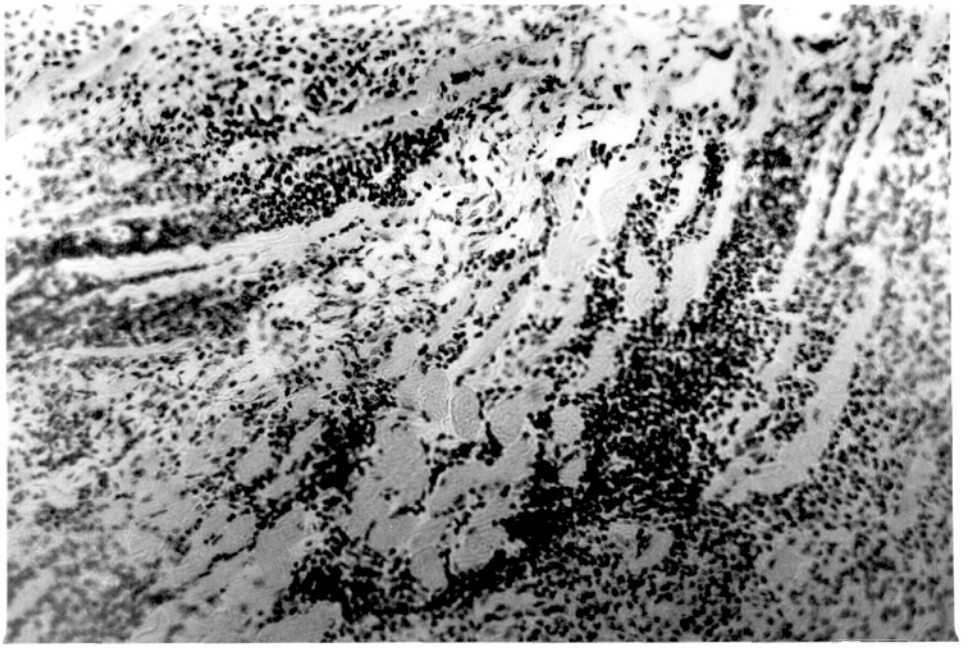


PLATE I (cntd)

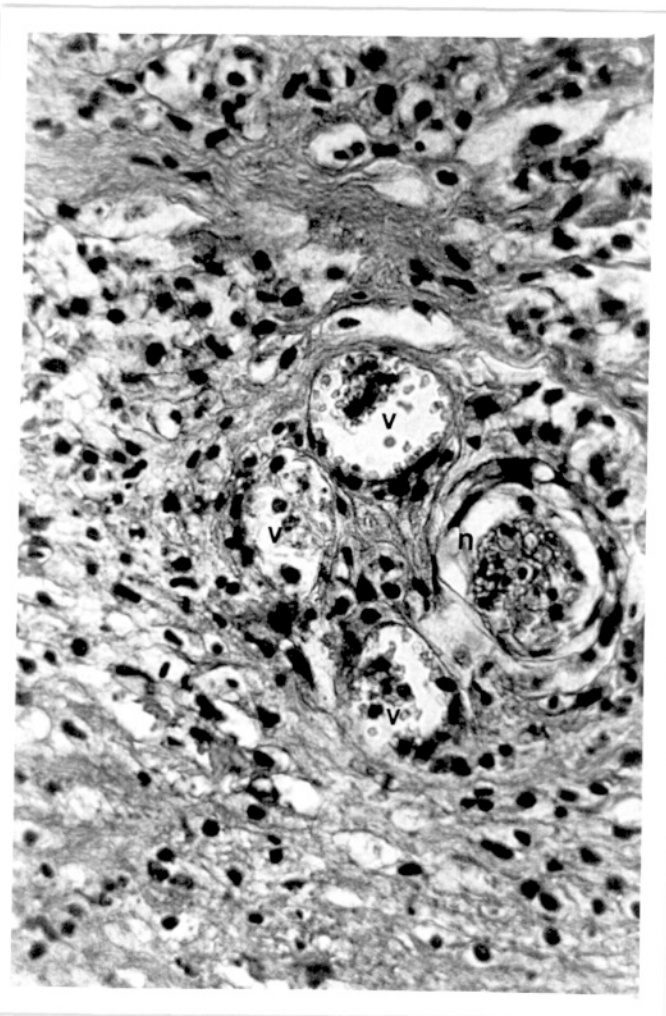


FIGURE 5

BALB/c mouse, 12 weeks infected with *L.major*.Dermal infiltrate showing infiltration of blood vessels (v) and nerve (n) by inflammatory cells, macrophages multi-and monovacuolated some of them showing amastigotes inside. Note the abundance of collagen fibers between and surrounding the cells and structures of the infiltrate.
Section, H & E. X 126.

(cntd)

PLATE I (cntd).

Fig. 6

BALB/c mouse, 6 weeks *L. mexicana* infected . Dermal infiltrate showing a histiocytoma (monovacuolated macrophages laden with amastigotes). Note polymorphonuclear eosinophils sparcely distributed in the infiltrate. Section, H & E. X 60.

Fig. 7

BALB/c mouse, 18 weeks infected with *L. mexicana*. Dermal infiltrate showing congested blood vessel containing abundant erythrocytes and polymorphonuclear eosinophils in the lumen. In dermis a mass of eosinophils is seen surrounding the vessel. Monovacuolated heavily parasitized macrophages surround the rest of the infiltrate. Section, H & E. X 50.

(cntd)

FIGURE 6

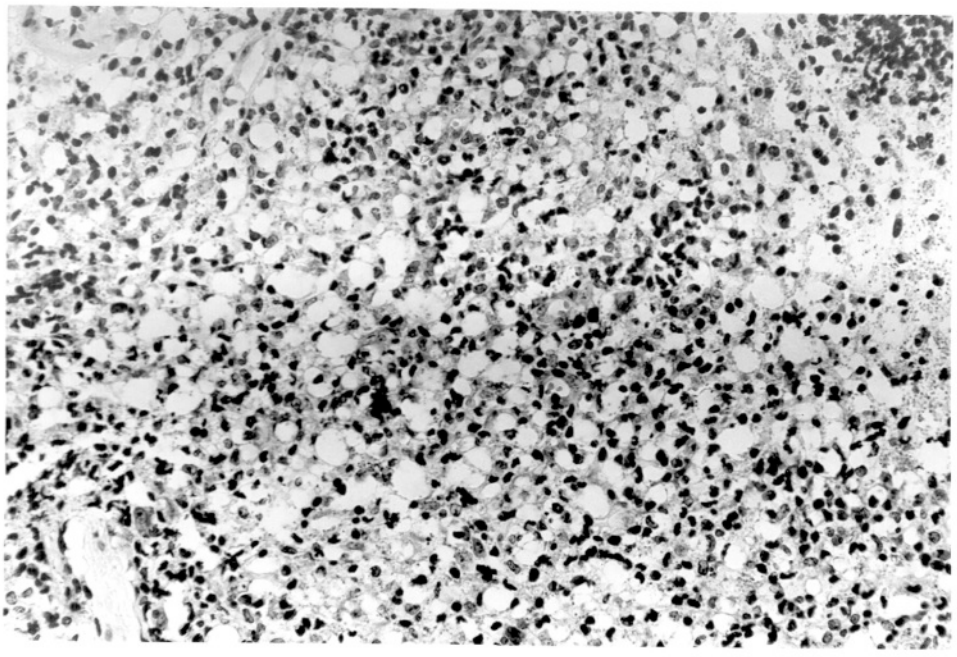


FIGURE 7

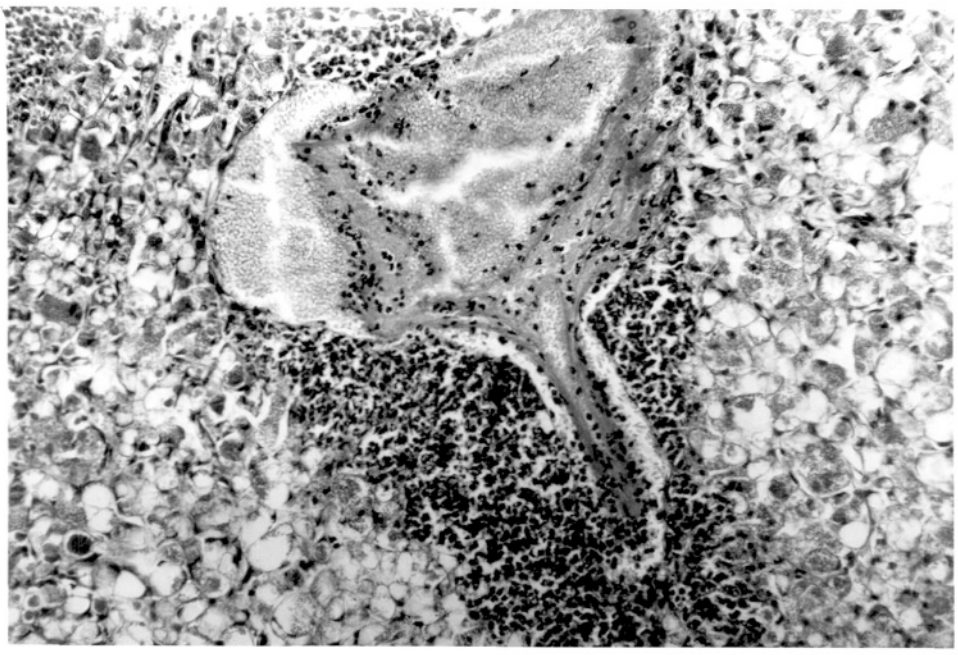


PLATE II

DRAINING LYMPH NODES. HISTOPATHOLOGY IN CBA/ca AND BALB/c MICE INFECTED WITH *L. major*, OR *L. mexicana*. Figures 1 to 9.

FIGURE 1

CBA/ca mouse, 8 weeks infected with *L. major*. Draining lymph node showing extensive development of the paracortical area. Section, Giemsa-colophonium. X 20.

FIGURE 2

BALB/c mouse, 4 weeks *L. major* infected. Draining lymph node demonstrating enlarged and active germinal centers. Section, H & E. X 20.

(cntd)

FIGURE 1

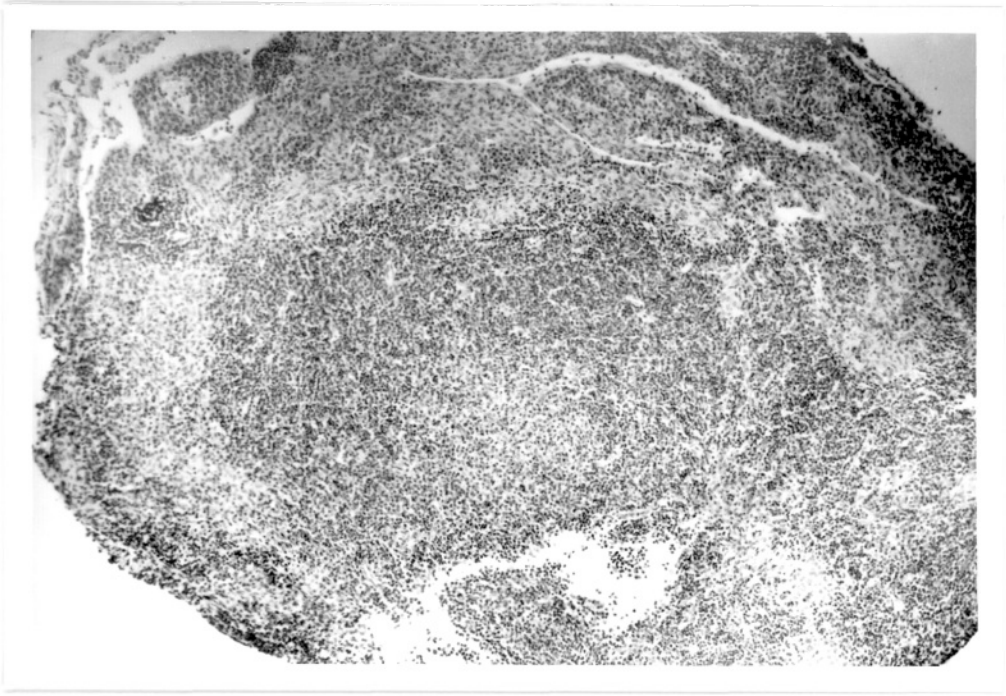


FIGURE 2

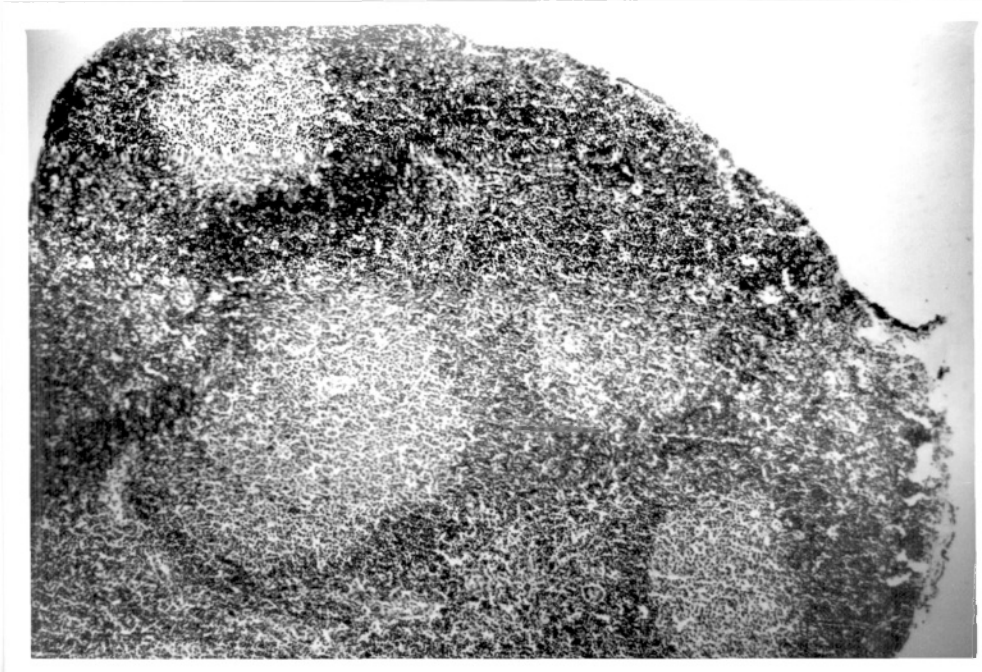


PLATE II (cntd)

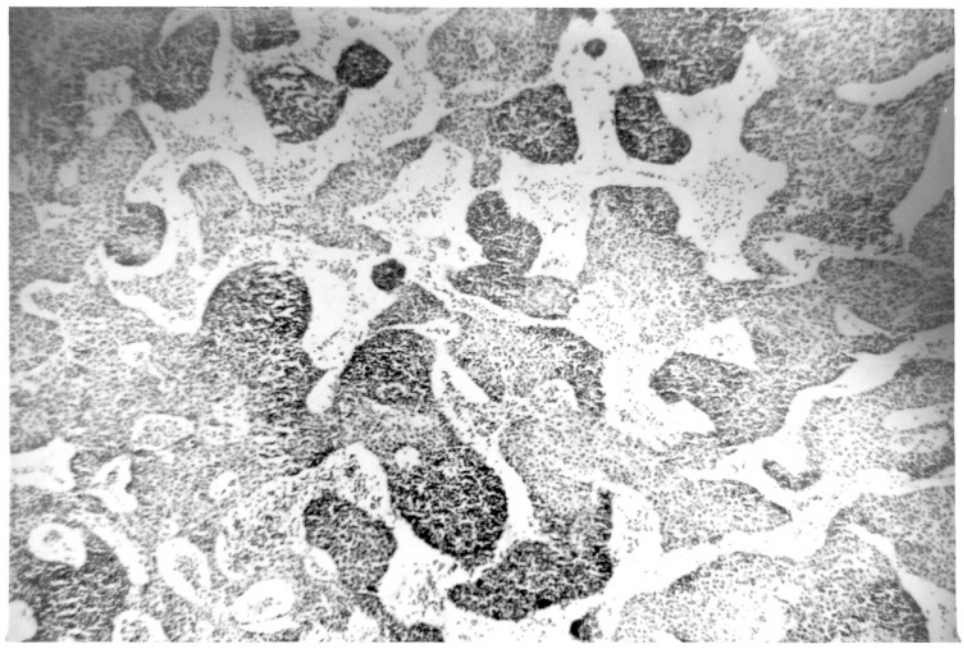


FIGURE 3

BALB/c mouse, 4 weeks *L. major* infected. Draining lymph node showing heavily dilated medullary sinuses. Section, H & E. X 20.

(cntd)

PLATE II (cntd)

FIGURE 4

CBA/ca mouse, 3 weeks infected with *L. major*. Draining lymph node showing clusters of histiocytes with epithelioid appearance infiltrating in the paracortical area. Section, H & E. X 100.

FIGURE 5

BALB/c mouse, 4 weeks infected with *L. major*. Draining lymph node showing in the paracortical area infiltration by clusters of cells of the histiocyte phagocyte system. Note that some of the cells making up the clusters have an intermediate appearance between epithelioid and foamy, others (the majority) are foamy in appearance and sometimes syncitial. Section, H & E. X 100.

(cntd)

FIGURE 4

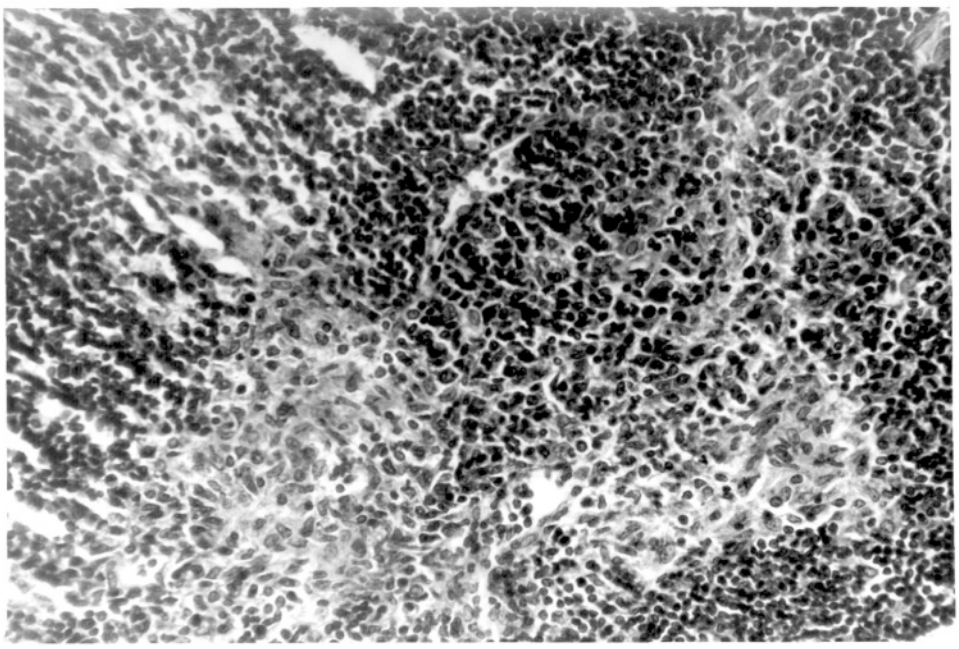


FIGURE 5

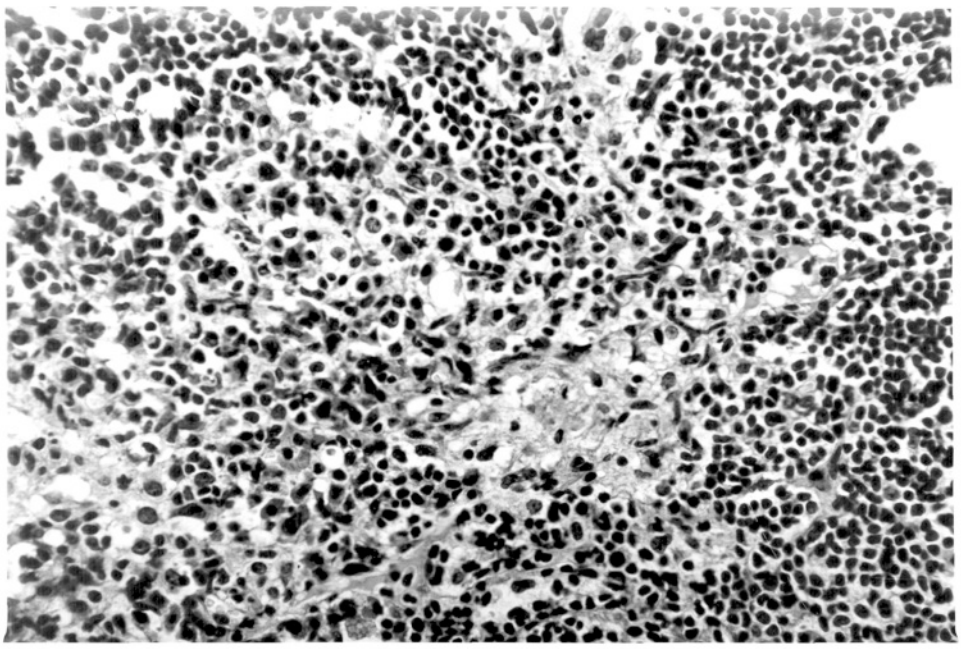


PLATE II (cntd)

FIGURE 6

BALB/c mouse, 18 weeks *L. major* infected. Draining lymph node showing haemorrhage (h) and giant cells, Langerhans (⟶) and foreign body type cells (⟶). Section, H & E. X 120.

FIGURE 7

CBA/ca mouse, 10 weeks infected with *L. mexicana*. Draining lymph node showing clusters of cells of the phagocytic system in the paracortical area adopting giant cell configuration (Langerhans type). Section, Giemsa-colophonium. X 120.

(cntd)

FIGURE 6

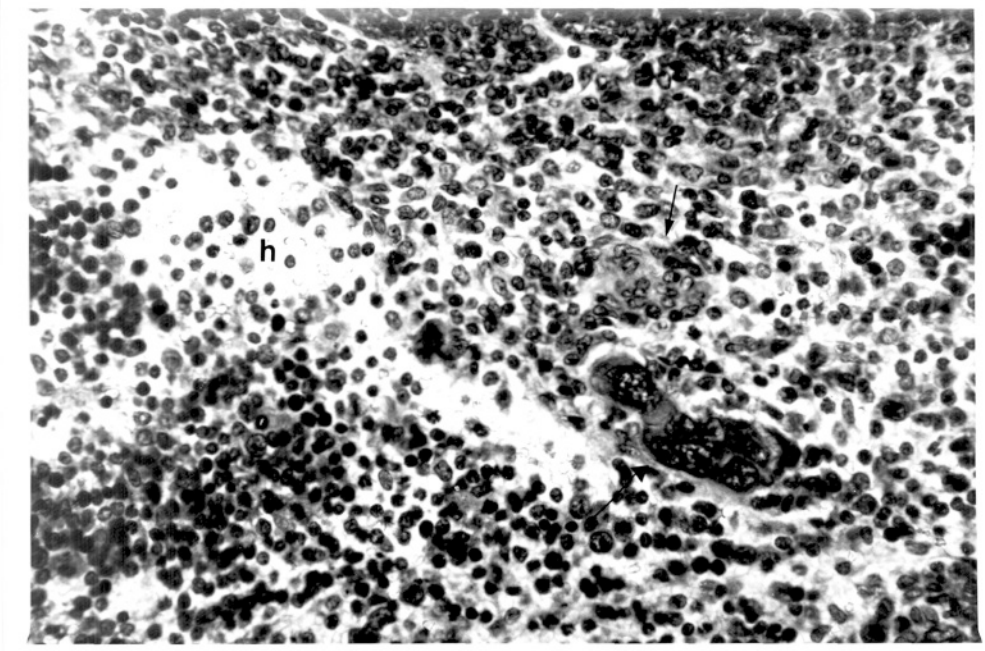


FIGURE 7

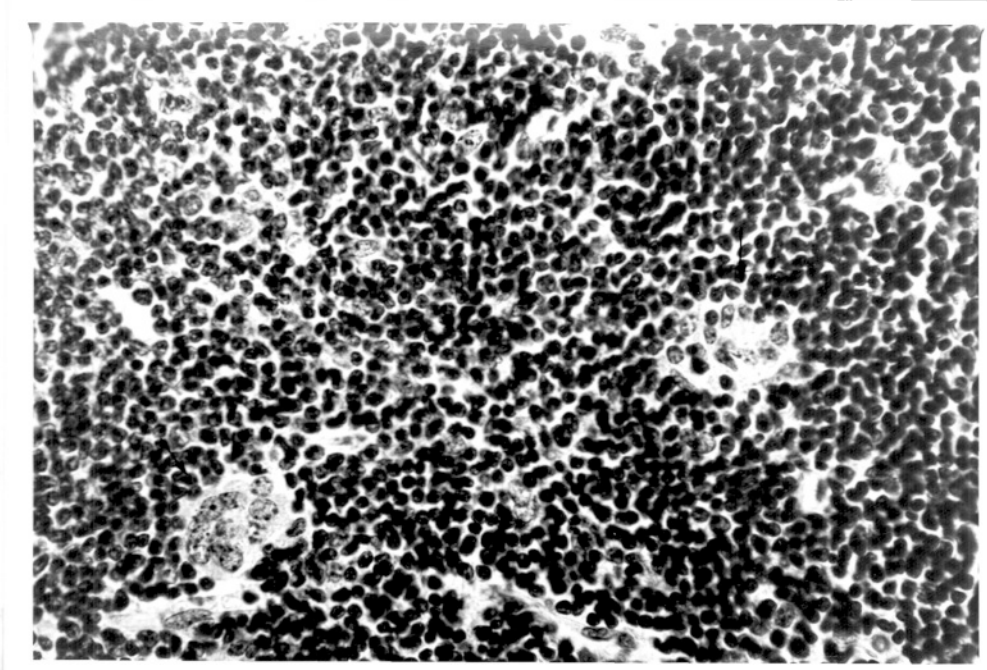


PLATE II (cntd)

FIGURE 8

CBA/ca mouse, 16 weeks infected with *L. mexicana*. Draining lymph node showing infiltration by masses of heavily parasitized macrophages and scattered eosinophils replacing the lymphoid tissue. A rim of lymphocytes is observed in the upper area. Section, H & E. X 80.

FIGURE 9

BALB/c mouse, 16 weeks *L. mexicana* infected. Draining lymph node, normal structure has been totally replaced by a histiocytoma (h). Note area of haemorrhage and necrosis (n). Section, H & E. X 80.

(cntd)

FIGURE 8

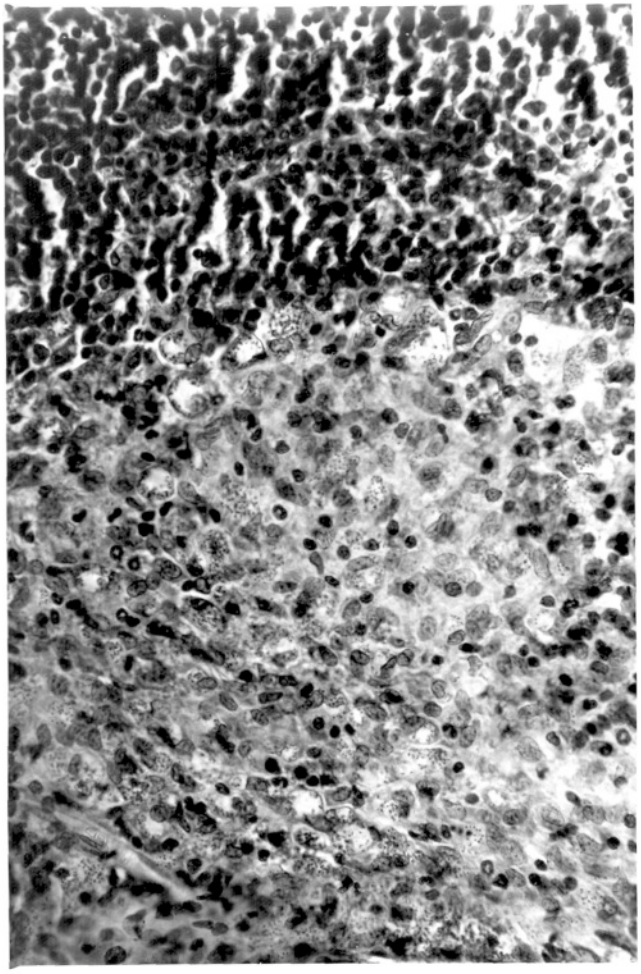
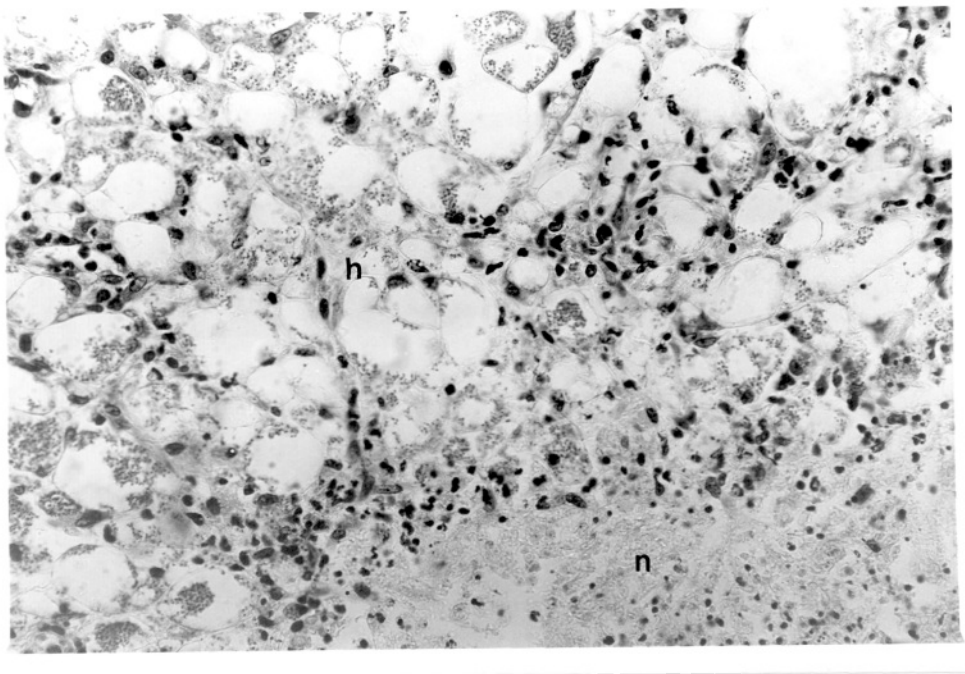


FIGURE 9



II.4 DISCUSSION

II.4.1 SKIN LESIONS

Clinical examination of the skin lesions showed that their appearance varied with time, strain of mice and species of *Leishmania*. Thus, while CBA/ca mice infected with *L. major* developed a non-ulcerating self-healing lesion, BALB/c mice infected either with *L. major* or *L. mexicana* and CBA/ca mice infected with *L. mexicana* developed non-healing lesions.

The different disease patterns produced by *Leishmania* infections in different strains of mice (PRESTON et al. 1978; PEREZ et al. 1979, HOWARD et al. 1980 a) confirmed the existence of a genetic base underlining the immune response of the mice to cutaneous infections with *Leishmania* (see Chapter I).

The histological study of the lesions produced by *Leishmania* infection in the different experimental groups reported here, can be related to BRYCESON's classification. Thus, at the peak of the infection, CBA/ca mice infected with *L.major* conformed to the IT type, although the epidermis did not ulcerate. BALB/c mice infected with *L. major* and CBA/ca mice infected with *L. mexicana* conformed to the MI type. BALB/c mice infected with *L. mexicana* conformed to the MM type.

Intradermal and epidermal foci of necrosis were observed in all the infection models. Necrosis, apart from ulceration, did not figure in BRYCESON's classification,

however, it was the basic element in RIDLEY's classification. RIDLEY (1980), in a histopathological study of human cutaneous leishmaniasis, reported an association between marked fibrinoid changes and the forms of disease in which the parasites were never numerous, while, conversely, there was an association between slight connective tissue involvement and forms of the disease in which parasites were numerous and freely tolerated.

Looking at the necrotizing process reported in this study in that context, an inverse correlation between parasite numbers and the involvement of the connective tissue was observed in established lesions in all the experimental groups except BALB/c mice infected with *L. major*.

BALB/c and CBA/ca mice infected with *L. mexicana* exhibited lesions in which, after the twelfth week of infection, association between numerous freely tolerated parasites and only a slight connective tissue involvement was observed. Necrotic areas, however, were detected during the fourth and twelfth weeks after infection and they coincided with the period in which a delayed hypersensitivity response (DHR) was detected (see Chapter V). After the twelfth week of infection, necrotic areas were no longer seen and the infiltrate comprised masses of heavily parasitized macrophages without any involment of the connective tissue. Ulceration was observed in BALB/c, but not in CBA/c mice infected with *L. mexicana*.

In BALB/c mice infected with *L. mexicana*, ulceration took place in a thin epidermis which cracked, exuded serous

fluid and finally became covered by a crust. This type of lesion is similar to that seen in human diffuse cutaneous leishmaniasis, in which ulceration is described as a consequence of trauma on areas of the skin made thin by the upward growth of the infiltrate (BRYCESON, 1969). The lack of ulceration in the lesions produced in CBA/ca mice by the infection with *L. mexicana*, as opposed to the ulceration of the lesions of BALB/c mice infected with the same parasite, would appear to support BRYCESON's suggestion about the traumatic origin of the ulcerations since lesions in this strain of mice were always smaller than the size at which BALB/c mice lesions started to ulcerate.

In CBA/ca mice infected with *L. major*, the parasite was poorly tolerated by the host and the parasite did not reach such large numbers as did *L. mexicana*. Necrotic areas were observed by the third to fifth week of infection, after which the process of healing started. In this experimental model, DHR was detected by the second week of infection and remained evident after healing and to the end of the observations. Ulceration of the epidermis, a phenomenon widely reported in this infection model (PRESTON et al. 1972, 1978, PRESTON & DUMONDE, 1976), was not observed in this study. PRESTON & DUMONDE (1976) demonstrated in the CBA *L. major* model that the pattern of infection depended upon the number of parasites comprising the initial infection. Thus, low infection doses (below 10^4) produced non-ulcerative healing lesions, while high doses (above 10^5) produced ulcerative

lesions.

In the BALB/c *L. major* infection model, in which non-healing lesions occured and parasites were abundant, the inverse relationship between connective tissue involvement and parasite load was not observed. On the contrary, the abundance of heavily parasitized macrophages coexisted with large dermal and epidermal areas of necrosis that sloughed off, leaving ulcers which exhibited a punched out appearance. No association was observed between necrosis and DHR.

It has been suggested that necrosis is the most potent means of eliminating the parasite in human infections and that its presence is indicative of a good prognosis for the patient in human infections (RIDLEY, 1979; RIDLEY et al. 1980). However, this suggestion patently does not apply to *L. major* infected BALB/c mice, where necrosis and the healing mechanisms were dissociated.

It is important to point out that in the experimental groups exhibiting non-healing lesions, the zones of the infiltrate undergoing necrosis were associated with those areas in which macrophages were moderatedly parasitized and exhibited at most only a slight vacuolization of their cytoplasm. Generally, these areas were situated towards the periphery of the infiltrate, surrounding masses of monovacuolated heavily parasitized macrophages in which necrosis was rarely seen.

Necrotic areas in the epidermis and dermis in all the experimental groups contained an intense deposition of fat demonstrated by oil red stainig.

Eosinophils and mast-cells were detected in all the experimental groups, but they were particularly abundant in *L. mexicana* infections. These cells were detected in all the experimental groups singly, scattered and as clusters of cells appearing close to macrophages and the necrotic areas of the infiltrate. The abundance of eosinophils in *L.mexicana* infections in mice has been reported by GRIMALDI et al. (1980), who found that in these infections the mononuclear infiltrate was accompanied by neutrophils and eosinophils in 45 % of the lesions.

The phagocytic capacity of eosinophils for a variety of particles, including bacteria, erythrocytes and inert material, has been demonstrated (reviewed by BERRETY & CORMANE, 1978; SAMTER & CZARNY, 1971). Interactions, *in vitro*, between *Trypanosome dionisii* and human eosinophils (THORNE et al. 1979), *T.cruzi* and rat eosinophils (SANDERSON & DE SOUZA, 1979) and *T. cruzi* and mouse eosinophils (KIPNIS et al. 1981) have shown that phagocytosis by these granulocytes was antibody dependent and led to the destruction of the parasites. CHANG (1981 a) reported phagocytosis and destruction of *L. donovani* amastigotes without specific opzonization, by human eosinophils *in vitro*. However, it is currently believed that these granulocytes possess a preferential affinity for immune complexes (LITT, 1964 a, b; ISHIKAWA et al. 1974; reviewed by HOUBA et al. 1976).

Studies on the possible mechanisms whereby eosinophils migrate into tissues demonstrate that immune complexes play an important role in the attraction of these cells (LITT,

1961, 1964 a, b). Further studies have revealed the existence of a complement dependent eosinophil chemotactic factor (ECF-C) resulting from antigen antibody reactions with the activation of complement (WARD, 1969; KAY, 1971; HOUBA et al. 1976).

PEARSON & STEIGBIGEL (1981) have demonstrated that *L. donovani* promastigotes are able to activate complement via the classical pathway. BRAY (1983) reported that promastigotes and amastigotes of *L. mexicana* activated complement by the alternate pathway. In the immunopathology study reported here (see next chapter) the presence of the C3 complement factor on tissue structures and cells of skin and lymph nodes is reported. The presence of C3 was coincident with the presence of antigen and antibody on skin structures and on cells of the infiltrate. This strongly suggests the presence of immune complexes, which could be involved in the activation of complement and subsequent attraction of eosinophils to the tissues.

Mast-cell degranulation was a feature observed preceeding the increase of eosinophils in the infiltrate. The release of factors from degranulating mast-cells had been considered chemotactic for eosinophils (reviewed by ASKENASE, 1980).

During the course of this study, it seemed unlikely that eosinophils were involved in phagocytosing amastigotes, rather, they appeared to be ingesting immune complexes as suggested by immunofluorescence studies (see Chapter III).

Another possibility to consider is the participation of eosinophils in the mechanism of killing the parasite. The

possibility that these cells may provide mature macrophages with the enzymes necessary for efficient killing of intracellular parasites has been suggested by NOGUEIRA et al. (1982), who showed that trypomastigotes of *T. cruzi*, coated with eosinophil peroxidase, became sensitized to killing by normal macrophages. The parasiticidal mechanism of killing seems to be attributable to the peroxidase dependent H_2O_2 system and oxygen metabolites generated by the phagocytosis induced respiratory burst of the macrophages. These authors also speculated on the possibility that, *in vivo*, peroxidase, released by degranulation and leakage of eosinophills at the inflamatory foci, may sensitize extracellular organisms for killing by normal macrophages, or it is also possible that the extracellular enzyme could be taken up by macrophages from the extracellular medium (STEIMAN & COHN, 1972) and be used to kill the parasite intracellularly by active oxygen metabolites.

II.4.2 LYMPH NODE

The histological appearance of the lymphatic tissue varies with variation of the antigenic stimulation. In general, antigens that are associated with antibody production cause development of lymphoid follicles and germinal centres, while those that stimulated cell mediated immune response cause activation of paracortical areas (OORT & TURK, 1965).

In the present study of *Leishmania* infection, the histopathology of the lymph nodes was examined at the same periods of time as the skin lesions. It was found that the histological appearance of the draining lymph nodes reflected the changes in the humoral and cellular response in the different infection models used (see Chapter V). Throughout the course of the experiment, changes in the cortical, paracortical and medullary areas of the lymph nodes were observed in all the experimental models, however, these changes varied in intensity. In the model *L. major* CBA/ca, in which self-healing lesions occured, the paracortical area showed marked hyperplasia and the cortical area showed relatively moderate development. In the experimental models exhibiting non-healing lesions (*L. major* BALB/c, *L. mexicana* BALB/c and *L. mexicana* CBA/ca) on the other hand, the paracortical area showed slight to moderate development, while the cortical area was markedly hyperplasic. A striking characteristic in all the groups was the infiltration of paracortical and cortical areas by cells of the macrophage histiocytic serie, which gradually replaced the lymphocytes. Similar infiltrations have been reported in the paracortical areas of the lymph nodes of *Leishmania* infected animals in which cell mediated immunity has been impaired or prevented, e.g., in the *L. enriettii*-guinea pig model, by treatment with anti-lymphocytic serum (ALS), or by administration of large doses of soluble antigen (BRYCESON et al. 1970; TURK

& BRYCESON, 1971) and in the *L. major*-CBA/ca model, by thymectomy of the animals previous to *Leishmania* infection (PRESTON et al. 1972).

However, the infiltration of lymph nodes by these cells does not necessarily indicate a failure of CMI, as such changes have also been observed in the lymph node draining the site of the intradermal inoculation of BCG vaccine in guinea pigs, where a high level of CMI against *Mycobacterium tuberculosis* exists (GAAFAR & TURK, 1970; BRYCESON & TURK, 1971). In leprosy, on the other hand, infiltrating histiocytes in lymph nodes have been observed in patients presenting the different clinical forms of the disease (TURK & WATERS, 1971). These authors also studied the characteristics and evolution of the infiltrating histiocytes throughout the spectrum of leprosy and found that at the lepromatous end of the spectrum, the lymphocytes of the paracortical area were replaced by foamy, sometimes sincytial histiocytes, which failed to eliminate *Mycobacterium*. As resistance to the infection increased along the leprosy spectrum towards the TT end, histiocytes became more differentiated and in polar tuberculoid leprosy, they appeared typically epithelioid.

A reciprocal relationship between the epithelioid cells and macrophages phagocytosing has been reported by SPECTOR (1980) in BCG infection in mice. The greater the number of bacilli-laden macrophages, the fewer the number of epithelioid cells. This reciprocal relationship, between histiocytes with epithelioid aspect and phagocytosing macrophages, seems to be related to the immunological

status, specially to CMI (SPECTOR, 1980). In lepromatous leprosy, epithelioid cells are uncommon, macrophages are numerous and cell mediated immunity is low; in tuberculoid leprosy, there are many epithelioid cells and cell mediated immunity is high. This suggestion was supported by the observations of RIDLEY &WATERS (1969), who pointed out that the histological appearance of histiocytes in lepromatous leprosy changes towards the epithelioid form during a reversal reaction in which patient's CMI is restored.

In this study, a relationship was found between the histological appearance of the infiltrating histiocytes and the immunological status of the host. In the *.L. major*-CBA/ model, in which, an effective immune response occured and lesions healed, the infiltrating histiocytes had a typical epithelioid appearance and were arranged in concentric patterns, constituting whorls which were well demarcated from the surrounding lymphocytes. As long as the infection progressed, the infiltrate whorls coalesced giving rise to masses of epithelioid cells, which, by the sixth week after infection, infiltrated the paracortical and cortical areas of the lymph node. From then onwards, the infiltrating cells started to decline, coinciding with the appearance of aggregates of small lymphocytes in the paracortical area. The lymph node gradually recovered its normal appearance but some clusters of histiocytes remained even after clinical and histological cure of the lesions (12 weeks) and up to end of the observations (18 weeks). In this context, TURK & WATERS (1971) stated that

the histiocytic infiltration of the paracortical areas of lymph nodes in patients with lepromatous leprosy could persist for at least ten years after the beginning of anti-leprosy treatment. This suggests an incomplete clearance of the organism, resulting in a persistent subclinical infection capable of producing histiocytic infiltration of the lymph node (TURK & WATERS, 1971). The presence of epithelioid clusters in the *L. major*-CBA/ca infection model, after the healing of the lesion, may therefore be related to the existence of a premunition state. The question of whether immunity in leishmaniasis is of the premunition, or non-sterile protection type, or a true residual (sterile) immunity has not been solved yet. Long term persistence of the agent in *Leishmania* infections in man is attested by:

1 - The finding of living organisms in human cutaneous lesions up to 13 years after cure (reviewed by HEYNEMAN, 1971).

2 - The clinical history of espundia which demonstrates long parasite survival and eventual metastasis, presumably from hidden foci in the skin and nasal mucosae.

3 - The frequent development of post-kala-azar dermal leishmanioid several years after the apparent cure of visceral leishmaniasis.

4 - The relapses after apparent cure in lupoid or recidivans leishmaniasis.

Recently, LECLERC et al. (1981), isolated viable organisms from the spleen of strains of mice resistent to *L. major*, even after the recovery from their cutaneous

lesions. If immunity to *Leishmania* is due to premunition, it would be expected that under conditions of severe immuno-suppression, a relapse of systemic leishmaniasis would occur (LECLERC et al. 1981). The reports of HAUTEVILLE et al.(1980), and GASTAUT et al. (1981), who observed a visceral leishmaniasis during chemotherapy for leukaemia, support the idea of premunition in human leishmaniasis. Nearly all recent cases of Kala-azar in France in recent years have been in immuno-suppressed kidney transplant patients (BRAY, personal communication).

In the infection models exhibiting non-healing lesions (CBA/ca and BALB/c mice infected with *L. mexicana* and BALB/c mice infected with *L. major*), the appearance of infiltrating histiocytes varied with the evolution of the infection. During the first eight weeks after infection, when the development of the cortical area was very apparent and paracortical areas showed slight to moderate development, infiltration of histiocytes was observed as a band in the subcapsular area and as aggregates of these cells in the paracortical and cortical areas. The appearance of these histiocytes differed from those described in the *L. major*-CBA/ca model; in the BALB/c-*L. major* model, the boundaries of these aggregates were not so well defined from the surrounding cells as in the previous model and the appearance of histiocytes, per se, was intermediate between those of epithelioid and foamy cells. Some of the cells contained parasites or cell debris.

In the *L. mexicana* infection models, on the other hand, histiocytes exhibiting intermediate appearance were mixed with moderate to heavily parasitized macrophages. Sometimes the histiocytes were seen within aggregates, at other times they were spread between lymphocytes in the cortical and paracortical areas. Giant cells were also observed.

After the eighth week of infection, in the *L. major*-BALB/c model clear areas consisted of vacuolated parasitized macrophages and reticular shaped cells were detected in the paracortical area. Reticular shaped cells in the paracortical area have been reported by BRYCESON & TURK (1971) in animals treated with anti-lymphocytic serum and by PARROT et al. (1966) in the lymph node of thymectomized mice. The significance of these cells has not been established.

The rest of the lymphoid structure in the *L. major* BALB/c model was infiltrated by masses of histiocytes, eosinophils, giant cells and extensive areas of haemorrhages. In the *L. mexicana* infection models, the histologic changes after the eighth week of infection resembled those occurring in skin lesions; the lymph node progressively became invaded by heavily parasitized macrophages. By the end of the observations, the lymphoid tissue was almost completely replaced by a granuloma similar to that found in the skin, presenting areas in which eosinophils, pyroninophilic cells, giant cells and zones of haemorrhage were interspersed between the masses of the parasitized macrophages.

The histopathologic features observed in the lymph node of the different experimental models reported here reflected the changes in the immune response (Chapter V). Thus, in the infection model exhibiting self-healing lesions, the cortical and paracortical areas of the lymph node were well and clearly developed, cell mediated immunity (CMI) determined by delayed hypersensitivity response (DHR) to *Leishmania* antigens was present and the humoral response measured by the levels of circulating antibodies were apparent during all the period of observations.

In the infection models exhibiting non-healing lesions, on the other hand, the humoral response was evident during the whole period of observation, which in CBA/ca mice infected with *L. mexicana* and BALB/c infected with *L. major*, coincided with the presence of germinal centers detected in cortical areas up to the end of the observations. In BALB/c mice infected with *L. mexicana*, the lymph nodes were almost completely replaced by a histiocytoma from the tenth week after infection. However, this fact is not incompatible with the high levels of antibodies observed in this experimental model, since some plasma cells were observed in the distorted lymph node up to the end of the observations, and MITCHELL (1979) showed that a single plasma cell can produce thousands of immunoglobulin molecules per second.

The paracortical area in all three infection models exhibiting non-healing lesions presented a moderated and transient development, which coincided with the detection

of a transient DHR to *Leishmania* antigen.

BRYCESON (1970 b) hypothesised that a compromised lymphatic drainage may be among the possible causes of the immunological paralysis in disseminated cutaneous leishmaniasis (DCL). KADIVAR & SOULSBY (1975) demonstrated in the *L. enriettii*-guinea pig model that a disease resembling DCL could be induced by interrupting the lymphatic draining of the site of the infection. POULTER & PANDOLPH (1982) showed that the excision of the mouse popliteal lymph node, prior to infection with *L.major,* in the foot pad of the same limb produced a faster developing and more severe infection in both the susceptible BALB/c and the resistant B6D2 mice. The excision of the lymph nodes also resulted in an initial blockage of the DHR, which was maintained in the BALB/c mice but not in the B6D2 (in which it emerged later). Differences in the clinical course of infection resulting from different sites of inoculation (POULTER & PANDOLPH, 1982), or different routes of inoculation (POULTER & PEARCE, 1980) could be a reflection of variations in the lymphatic drainage from these sites. All these observations appeared to reinforce BRYCESON's hypothesis that an efficient local drainage is important for the establishment of an adequate cell mediated response. Any natural variation, or acquired alteration in the lymphatics, or in the local draining node, may lead to a delay of the manifestation of the immune response, allowing the parasite to grow more rapidly and produce an excessive antigenic load, which may be responsible for a continuing and

maintained immunosuppression.

II.5 CONCLUSIONS

the results presented in this Chapter show that the *Leishmania*-infection models used mimicked some of the forms of human cutaneous leishmaniasis. The models also showed the correlation between clinical patterns, histopathology of the lesion and the draining lymph node and cellular and humoral immune response which have been recognized in the human disease.

The CBA/ca *L. major* model, for instance, mimicked self healing cutaneous leishmaniasis, while the infection of BALB/c and CBA/ca mice with *L. mexicana* and BALB/c infected with *L. major* produced lesions resembling human diffuse cutaneous leishmaniasis.

CHAPTER III

IMMUNOPATHOLOGICAL STUDY OF SKIN LESIONS AND DRAINING LYMPH NODES IN CBA/ca AND BALB/C MICE INFECTED WITH L. major AND L. mexicana

III.1 INTRODUCTION

The histopathology of the skin occupies an important place in general Pathology and Immunology. The accessibility of the skin makes it a relatively easily available material which can be biopsied safely, allowing histological diagnosis and enabling one to follow the progress of a disease (HABER & SYMMERS, 1980).

While conventional histopathological methods provide a base in the analysis of tissue disorders, the use of immunological techniques can establish the involvement of immune-mechanisms that might operate in different diseases. This has opened up new approaches towards the understanding of histopathological changes and has become a valuable diagnostic tool in some dermal disorders in human and experimental animals.

The immunofluorescence technique, among other immunological techniques, has been employed to recognize the different structures and the nature of infiltrating cells involved in cutaneous disorders. Specific

immunoreactants (antigen (Ag), antibody (Ab), and Ag-Ab complexes), inflammatory mediators (complement and fibrinogen), and components of the connective tissue (collagen, fibronectin, laminin) have been demonstrated using this technique.

The detection of infiltrating dermal cells, the nature of which are often difficult to determine with certainty by classical histological methods unless electronmicroscopic studies are used, is made relatively easy by the immmunofluorescence technique (IFT) using conjugates of antibodies to surface markers. The availability of monoclonal antibodies to T and B lymphocytes is of great importance in clarifying the pattern of the cellular infiltrates seen in dermal lesions.

Immunofluorescence is now established as a very useful means of diagnosis in a number of important diseases that involve the skin, e.g., Lupus erythematosus, Pemphigus vulgaris, Dermatitis herpetiforme (BEUTNER et al. 1973). The application of IFT to studies of dermal disorders caused by infective agents, as in dermato-mycoses (SOHNLE & KIRPATRICK, 1976) and Leprosy (QISMORIO et al. 1975; WAALER et al. 1971; WALLACH et al. 1979), has helped towards a better understanding of the immune mechanisms involved in these diseases.

In leishmaniasis, RADWANSKI et al. (1974) reported an immunopathological study of the lesion and the draining lymph nodes in the *L. enriettii* guinea-pig model, and MORIEARTY et al. (1982) applied IFT to Brazilian cases of cutaneous leishmaniasis in man with the purpose of

identifying the class of immunoglobulin (Ig) produced by intralesional plasma cells.

In this Chapter, a study of the immunopathology of the skin lesions and draining lymph nodes in murine cutaneous leishmaniasis is detailed.

The study comprised a search for Ig (M and G), C3 factor of complement and T lymphocyte subpopulations in frozen sections of the tissues of mice from the four host parasite combinations detailed in the last chapter.

The specificity of staining was assesed using FITC-*L.mexicana* antigen and the $F(ab)'_2$ fraction of anti- *L. mexicana* IgG.

III.2 MATERIALS AND METHODS

III.2.1 TEMPERATURE

Except where otherwise stated, all manipulations were carried out at room temperature.

III.2.2 PREPARATION OF *Leishmania* ANTIGENS AND ANTIBODIES TO BE USED IN THE IMMUNOFLUORESCENCE TECHNIQUE

III.2.2.1 Organisms and antigenic extracts

The maintenance procedures used for *L. major* and *L. mexicana* have been described in ChapterII.

Leishmania antigens were prepared following the BRYCESON et al. (1970) technique slightly modified. Promastigotes of both species of *Leishmania* were grown in bulk in a protein free dialysate medium (NAKAMURA, 1967) in 1 litre conical flasks kept at 26°C. The organisms were harvested after 8 to 10 days growth and washed 3 times by centrifugation at 1200 g for 10 min at 4°C in cold phosphate buffer saline pH 7.2 (PBS).

The pellet was resuspended in 10 ml of cold PBS, chilled to 0°C and sonicated cold at 12 µm peak to peak excursion (RHZ) for five intervals of one minute each. The material was then observed under phase compound microscope to see that the organisms were fully disintegrated.

Urea was added to the sonicated mixture to a final concentration of 8 M, the resulting suspension was

incubated for two hours at 37°C, then dialysed against 40 times its volume of 0.05 M ammonium bicarbonate and buffered to pH 7.4. by acetic acid. The dialysis proceded for 48 hours with 6 to 8 changes of the dialysing fluid with continuous stirring, at 5°C. The dialysate was then centrifuged at 105.000 g for one hour at 4°C obtaining two antigenic fractions: the deposit, called purified insoluble antigen (PIA) and the supernatant, called purified soluble antigen (PSA). The PSA was either freeze-dried (to be used as antigen in testing hypersensitivity, or in the preparation of immune-serum), or concentrated by negative pressure dialysis (to prepare FITC-conjugates). Protein concentrations were determined by the Biuret method.

III.2.2.2 Preparation of antiserum to *Leishmania* antigens

III.2.2.2.1 Preparation and inoculation of the antigen

10 mg of freeze-dried PSA of *L. mexicana* or *L. major* were dissolved in 0.1 ml of Freund's complete adjuvant (DIFCO) by forcing the mixture through a fine neddle and then emulsified with an equal volume of 2 % Tween 80 in saline solution.

The resulting preparation was inoculated into adult New Zealand white rabbits 10 to 20 weeks old (Cheshire rabbit

farm, Huxley). Each animal received 2 weekly injections of the antigen into the popliteal and axillary lymph nodes of both front and back legs.

III.2.2.2.2 Determination of anti-*Leishmania* Ig levels in the immune rabbit serum

Samples of blood were taken 10 days after the last inoculation, the serum separated and the antibody levels titrated by the indirect immunofluorescence technique (IIFT). The general procedure of the technique is described in detail in ChapterIV. For the titration of rabbit antisera, smears of *L. major* and *L.mexicana* promastigotes, fixed in acetone were used as the antigen. Serially diluted immune serum to *L.major* or *L.mexicana* was added to the homologous antigen (first layer), after washing with PBS, fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit Ig (NORDIC LABORATORIES, MAIDENHEAD, ENGLAND), at a working dilution of 1:40, was added to the antigen (second layer). This titration showed that a third boosting injection was necessary to achieve good antibody levels. This injection was given after a resting period of 1 to 2 weeks. Positive titers to both antigens were 1:3200.

III.2.2.2.3 Preparation of serum immunoglobulins

The rabbit was bled from the marginal vein of the ear 10 days after the third injection of antigen, following the technique described by HERBERT (1978). Normally, 35 to 40 ml of blood were obtained, yielding 15 to 20 ml of serum.

The serum immunoglobulins were precipitated using a 50 % saturated ammonium sulphate solution at pH 7.2.

The antiserum to be precipitated was first diluted 1:1 in PBS pH 7.2, and then an equal volume of 50 % saturated ammonium sulphate solution was added dropwise, with constant stirring to the diluted antisera maintained in an ice bath. The precipitate was kept at 4°C overnight and then centrifuged at 9000 rpm for 15 min at the same temperature; the supernatant was decanted and the pellet resuspended in PBS to the original volume of the serum. The same volume of saturated ammonium solution was added and centrifuged again, this resuspension and spinning procedure was repeated two more times. The final immunoglobulin precipitate was redissolved in a minimum volume of PBS and the residual ammonium sulphate eliminated by either dialysis against PBS or by Sephadex G 25 filtration.

III.2.2.2.4 Desalting of the immunoglobulin precipitate

Dialysis

The precipitated immunoglobulins were dialysed against PBS pH 7.2 at 4°C with continuous stirring; the dialysing fluid was replaced 3 to 5 times daily and the dialysis continued until ammonium sulphate as detected by Barium precipitation was no longer detected in the dialysing PBS.

After dialysis, any remaining precipitate was spun out and discarded.

Sephadex G 25 filtration

In other instances, the desalting was achieved by passing the Ig precipitate, previously equilibrated by dialysis with 0.01 M phosphate buffer, through a column of Sephadex G 25 pre-equilibrated with 0.01 M phosphate buffer pH 8. The void volume of the column was determined previously by Dextran blue exclusion.

III.2.2.2.5 Sample concentration

When necessary, the samples were concentrated using negative pressure dialysis. The sample was placed into a section of dialysis tubing (DICE INTERNATIONAL 1-8/32") closed by a knot at one end, which was then placed inside a one liter filter flask, filled up to 3 cm from its bottom with the same fluid in which the sample was contained. The flask mouth was sealed with a bored stopper, through which a section of glass tubing was passed to allow the refilling of the dialysis tubing with further amounts of the sample.

The assembly was checked for leaks in the sealing and vacuum was applied with continuous stirring of the fluid until no bubbles were visible. The vacuum tube was then sealed and the system transferred to 4°C where the vacuum was allowed to act until the desired concentration was reached. The sample's protein content was then determined as described elsewhere.

III.2.2.2.6 Purification of Immunoglobulin G (IgG)

IgG was purified by ion exchange chromatography using diethyl aminoethyl (DEAE) cellulose. Both the batch and the column method were used .

Batch method

DEAE cellulose (Whatman DE52) was first equilibrated with 0.01M phosphate buffer (pH 8.0). To 100 g. of DE52, 550 ml of 0.01M phosphate buffer pH 8.0 were added, the mixture was titrated back with 1M HCl, the slurry allowed to settle for 30 min and the supernatant removed. This cycle of settling, decantation and resuspension was repeated 10 times, after which the slurry was poured into a Buchner funnel containing two layers of Whatman Nº 1 filter paper and the cellulose sucked dry by vacuum for 30 secs.

The rabbit immunoglobulin (Ig) suspension to be purified using this method was previously equilibrated with 0.01M phosphate buffer (pH 8.0) by filtration in Sephadex G 25. 5 g (wet weight) cellulose for each ml of IgG suspension were mixed at 4ºC and the mixture stirred thoroughly every 10 min for 1 hour, then the slurry was poured onto a Buchner funnel and the supernatant containing the IgG was sucked through, the cellulose was then quickly washed with 3 vols of 20 ml of the same buffer. The combined effluents contained the IgG.

Column method

The DEAE cellulose was equilibrated as described above and packed in a column (bed vol 24 x 3.5 cm). Ig (10 ml sample), previously equilibrated with 0.01M phosphate buffer (pH 8.0) by the dialysis method, was added to the column and eluted with the same buffer. The effluent was monitored for protein content by collection of every 5 drops of the effluent into 0.5ml of TCA solution (10 % trichloroacetic acid in water). The effluent was collected as soon as a white precipitate was detected in the TCA test and until no more precipitate occured. Following purification, the IgG containing effluents were concentrated by dialysis and assayed for protein concentration.

III.2.2.2.7 Preparing F(ab)'$_2$ fractions of IgG

Pepsin hydrolysis of Ig produces a major fragment (the F(ab)'$_2$) and a small fragment (the P'fc). The former retains the ability to bind antigen.

For pepsin digestion, the IgG suspension was adjusted to 20 mg/ml and dialysed against 0.1M sodium acetate for 3 hours. Following dialysis, the pH of the suspension was adjusted to pH 4.5 with acetic acid and pre-warmed to 37°C before the application of the enzyme.

The enzyme Pepsin (2x crystallized and lyophilized powder 30-85 units/mg protei, from SIGMA CHEMICALS) was added to the sample (2 mg of pepsin for each 100 mg of IgG)

and incubated at 37°C overnight. After that, the sample was centrifuged, the precipitates discarded and the pH of the suspension adjusted to 7.4. Finally the suspension was dialysed against PBS at 4°C for 24 hours, centrifuged and the protein concentration estimated.

III.2.2.2.8 Isolation of IgG fragments

The isolation of IgG fragments was done by gel filtration through a Sephacryl S 200 column (bed volume 90 x 2.5) (PHARMACIA, UPSALA, SWEDEN) The column was equilibrated with PBS (pH 7.2) containing 0.02 % sodium azide. 2 ml (8 to 12 mg/ml) of IgG was applied to the column through a peristaltic pump and then through a flow adapter. Immediately after application of the sample, the column was run at 20 ml/hour collecting samples of 2.5 ml using a fraction collector (LKB).

Three differents peaks were recorded after gel filtration. The effluents contained in the tubes that corresponded to each peak were pooled, concentrated by negative pressure dialysis and assayed for protein concentration.

III.2.2.2.9 Immunological analysis of the fragments

Undigested IgG and fractions 1, 2, and 3 were checked for their specificity and nature by the double immunodiffusion technique. The immunoplates were prepared in Petri dishes (5 cm diameter) which were precoated by drying an agar solution (5 % in PBS) on them in order to hold the final agar in place during the washing

procedudures. The definitive agar coat of 3.5 ml of 2 % Noble agar (DIFCO) in PBS containing 0.02 % sodium azide at 60°C was poured on the precoated Petri dishes. After the agar had cooled and set, the required holes were cut. A center hole and 6 equidistant surrounding holes of 5mm in diameter and 5mm space between wells were punched out with cutters. The wells were filled with the immune reagents until the meniscus disappeared, and the petri dishes were placed in a humid chamber at 26°C for 48 hours. Thereafter, the plates were washed in PBS for 24 to 48 hours, covered with filter paper, dried overnight and stained for 5 min in 70 % of 0.5 % Amido-black in 70 % methanol, 20 % distilled water and 10 % glacial acetic acid. The plates were then differentiated in the methanol-water-acetic acid mixture until the gel was nearly colourles, but the precipitation arcs remained blue.

Two immunodiffusion systems were used. In the first one, the activity of the fractions was tested by their ability to react with goat anti-rabbit gamma globulin (whole molecule) (NORDIC LABORATORIES) (Fig.1a) as follows: the goat anti-rabbit gamma globulin was placed in the central well, fractions 1, 2, and 3 obtained after fractionation of anti-*L. mexicana* Ig were placed in wells 1, 2 and 3 respectively, the original undigested rabbit anti-*L.mexicana* and undigested anti-*L.major* IgG and PBS were placed in wells 4, 5, and 6. This resulted in a continuous precipitation line from wells 1 to 5, indicating specificity and activity of the goat anti rabbit IgG to rabbit anti-*Leishmania* antibody, before and after

pepsin hydrolysis.

The second immunodiffusion system was set up in order to identify the nature of the fragments obtained after pepsin hydrolysis of the rabbit anti- *Leishmania* IgG by their ability to react with anti-rabbit IgG (Fc) raised in goat (NORDIC LABORATORIES) (Fig. 1b). In this immuno-diffusion system, this reagent occupied the central well, while fractions 1, 2, and 3 were placed in the 1, 2, and 3 wells respectively; rabbit anti-*L. mexicana* IgG (whole molecule) served as a control and was placed in well 5, wells 4 and 6 were filled with PBS. The Fig. 1b shows the result, fraction 2 and undigested IgG (anti-*L. mexicana* IgG) reacted with goat anti rabbit IgG (Fc) while fractions 1 and 3 did not. The results indicated the presence of the Fc fragment only in wells 2 and 5. Fractions 1 and 3 did not react with anti-rabbit IgG (Fc) and therefore contain the F(ab)'$_2$ fraction. Fraction 1 contained the F(ab)'$_2$ fraction which also contained the highest protein concentration among the different fractions (see Table 1). Further experiments were performed with fraction 1, considered to be the F(ab)'$_2$ fragment of IgG *L. mexicana* antibody.

Specificity and titration of F(ab)'2 fraction of the rabbit anti-*Leishmania* IgG

The indirect immunofluorescence technique was used. The technique is explained in detail in Chapter V. Smears of *L. mexicana* promastigotes, acetone fixed, were used as

FIGURE 1.A

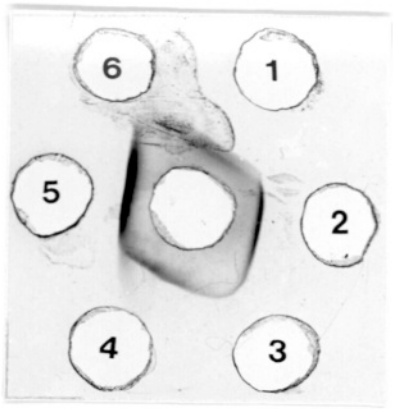


FIGURE 1.B

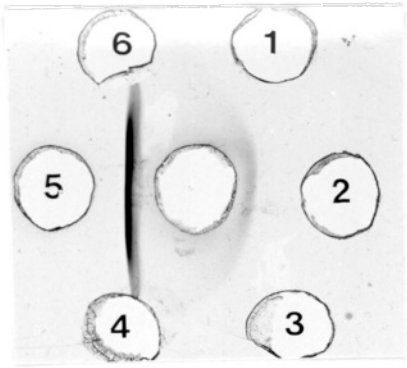


FIGURE 1 (A and B). Immunodiffusion of fractions from DEAE cellulose chromatography of pepsin digest of rabbit anti-*L.mexicana* IgG and of undigested rabbit anti-*L. mexicana* and rabbit anti-*L. major* IgG.

In (A) the activity of the digested fractions and undigested IgG is tested. Central well contained goat anti-rabbit IgG . Wells 1 to 3 contained fraction 1,2 and 3 obtained after pepsin digestion of rabbit anti-*L. mexicana* IgG, well 4 contained undigested rabbit anti-*L. mexicana* (original sample) and well 5 contained undigested rabbit anti-*L. major* IgG. Well 6 contained PBS.

Goat anti-rabbit IgG recognized the immunoreagents from wells 1 to 5.

In B, the immunological nature of the digest fragments is shown by comparing the ability of the rabbit anti-*L. mexicana* IgG (original sample, well 5) and their pepsin digested fractions 1,2 and 3 (wells 1,2 and 3 respectively) to react with goat anti-rabbit IgG (Fc) (central well). Wells 4 and 6 contained PBS.

Goat anti-rabbit IgG (*Fc*) recognized the undigested rabbit anti-L. mexicana IgG and and fraction 2, but failed to recognized fractions 1 and 3.

antigens on which F(ab)'$_2$ serial dilutions were layered (first layer), FITC conjugated goat anti-rabbit whole IgG (working dilution 1:40) represented the second layer. The immunofluorescent titer was 1:800.

Controls: PBS and normal rabbit serum substitutes for the first layer served as control.

No fluorescence was observed in these cases.

III.2.2.2.10 Immunoabsorption of the F(ab)'2 fraction of rabbit anti-*L. mexicana* IgG with mouse antigens

In order to remove any rabbit antibody cross-reacting with mouse antigen, the F(ab)'$_2$ fraction of anti-*L. mexicana* and normal rabbit serum were absorbed four times at 30 min for each period, at 37°C with 1ml of spleen cell suspension from uninfected BALB/c mice.

Table 1 summarizes the procedures used for obtaining the F(ab)'$_2$ fraction of rabbit anti- *Leishmania* IgG. It can be seen that both anti-*L. major* and anti-*L. mexicana* sera were treated in order to obtain the F(ab)'$_2$ fractions, but only the anti-*L. mexicana* IgG procedure gave sufficient protein for further analysis.

TABLE 1

PROCEDURES USED IN THE PREPARATION OF $F(ab)_2$ FRACTION OF IgG RABBIT ANTI Leishmania ANTIBODY

	ANTISERA TO		
	L. major ANTIGEN	L. mexicana I ANTIGEN	L. mexicana II ANTIGEN
AMOUNT PRECIPITATED IN $(NH_4)_2SO_4$	80 mg Ig	162.5 mg Ig	156 mg Ig
DEAE	4.5 mg IgG	40 mg IgG	30.4 mg IgG
PEPSIN DIGESTION		24 mg. FIgG	16 mg. FIgG
AMOUNT OBTAINED BY SEPHACRYL G200 FILTRATION		F1 4.7 mg. F2 2.9 mg. F3 1.3 mg.	F1 2.5 mg. F2 1.7 mg. F3 0.8 mg.

DEAE : diethyl aminoethyl cellulose.
Ig immunoglobulin IgG : immunoglobulin G.
FIgG : fractionated immunoglobulin G
DEAE fractionation for L. major and L. mexicana I antigens batch method, L. mexicana II column method.

III.2.2.3 Preparation of fluorescein isothiocinate (FITC) conjugates

Two conjugates were prepared; FITC-*L. mexicana* antigen and FITC anti-mouse IgG (Fab). The conjugation process was carried out according to GODING (1976).

L. mexicana antigen (PSA) was obtained from promastigotes using BRYCESON et al. (1970) technique (see above). Rabbit anti-mouse IgG (Fab) was a gift from C.G. SHAPLAND (Middlesex Hospital Medical School, London).

III.2.2.3.1 Evaluation of specificity and activity of the substances to be conjugated.

An estimate of the specificity and activity of the substance to be conjugated is required before carrying out the conjugation procedure. For conjugation, a good immunserum should have a minimum of 8 units/ml (reciprocal value of the precipitating titer), but best 16 to 32 units/ml.(JOHNSON et al. 1978).

The agar double immunodiffusion technique was used in order to determine the activity and specificity of the substance to be conjugated. The Figures 2a and 3a show the results. Figure 2a shows the immunoplate testing of anti-mouse IgG (Fab). The central well contained mouse IgG and the peripheral wells double serial dilutions (1:1, to 1:32) of the rabbit anti-mouse IgG (Fab). A continuous arc of precipitation is observed up to 1:32 dilution.

Figure 3a shows in the immunoplate testing the specificity and activity of the *L. mexicana* antigen.

FIGURES 2 (A and B).

Immunodiffusion for testing the specificity and potency of anti-mouse IgG (Fab) before (A) and after (B) conjugation to FITC .

In A, central well contained mouse IgG and wells 1 to 6 double serial dilutions (1:1 to 1:32) of unlabelled rabbit anti-mouse IgG (Fab).
A reaction was observed up to 1:32 dilution.

In B, central well contained mouse IgG and wells 1 to 6 double serial dilutions of anti-mouse IgG (Fab) conjugated to FITC.
A reaction was observed up to 1:8 dilution.

FIGURES 3 (A and B).

Immunodiffusion for testing the specificity and activity of *L. mexicana* antigen before (A) and after (B) conjugation to FITC.

In A, central well contained rabbit anti-*L. mexicana* serum and peripheral wells contained serial dilutions of unlabelled *L. mexicana* soluble antigen (1:1 up to 1:32).
A reaction of is observed up to 1:8 dilution.

In B, central well contained rabbit anti-*L. mexicana* serum and well 1 undiluted *L. mexicana* antigen conjugated to FITC.
A precipitation arc demonstrated the specificity of the reaction.

FIGURE 2.A

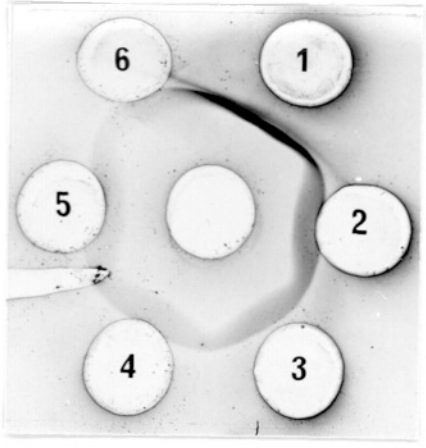


FIGURE 2.B

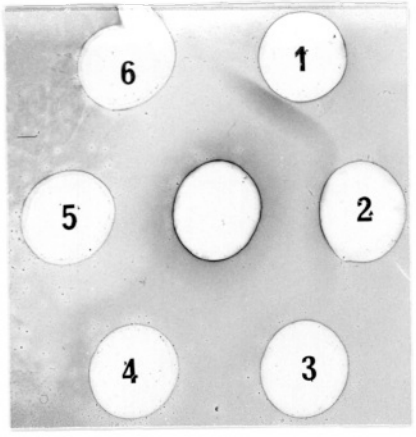


FIGURE 3.A

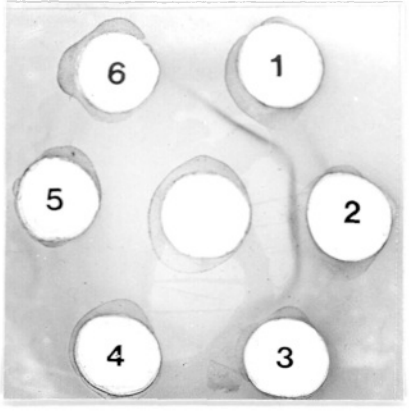
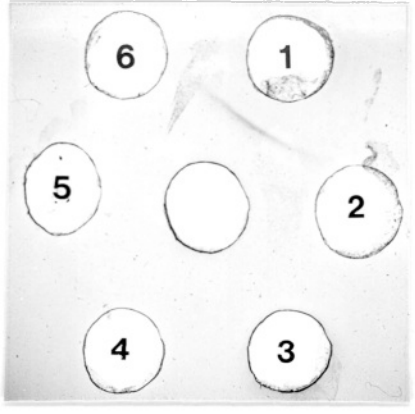


FIGURE 3.B



Anti-*L.mexicana* serum filled the center well and serial dilutions of the *L. mexicana* antigen were put in the peripheral wells. Precipitation was observed in this system up to a 1:8 dilution (Figure 3a).

III.2.2.3.2 Conjugation procedure

Prior to conjugation, protein solutions were adjusted to a pH of 9.5 by overnight dialysis at 4°C against carbonate-bicarbonate buffer 0.5 M, pH 9.5. Thereafter, 4 ml of a solution containing 5.5 mg/ml of *L. mexicana* antigen and 2 ml of a solution containing 6 mg/ml of rabbit-antimouse IgG (Fab) were conjugated with FITC (BDH isomer I 5656B0A19). The dialyzed protein was placed in a small beaker with a magnetic stirrer and allowed to warm to room temperature. FITC was dissolved in dimethylsulphoxide (DMSO, HOPKINS & WILLIAMS) at a concentration of 1 mg FITC/ml. The amount of FITC added to the protein solution was in the order of 0.6 mg/100 mg of protein for *L. mexicana* antigen and 0.8 mg/100 mg of protein for the rabbit anti-mouse IgG (Fab), which, according to BEUTNER, (1973), should give a molar FITC/Protein ratio (F/P) of 1.5 and 2 respectivelly.

The FITC solution was added dropwise to the protein solution with constant stirring, then the solution was allowed to stand for 2 hours at room temperature protected from light, after which the reaction mixture was transferred to a Sephadex G 25 column for removal of the unbound fluorescein.

III.2.2.3.3 Removal of free dye from the conjugate

Sephadex G 25 (PHARMACIA UPSALA SWEDEN) was swollen in PBS by placing the gel slurry on a boiling water bath for 1 hour. The sephadex gel was packed in a column (16 to 18 x 1.5 cm bed volume) and equilibrated with PBS pH 7.2. The upper surface of the gel bed was protected with filter paper in order to avoid disturbances by application of the sample.

The conjugate mixture was then carefully layered on top of the gel bed and elution carried out with PBS at a flow rate of approximately 0.5 ml/min. As filtration progressed, a clear separation of two yellow coloured bands by an invisible carbonate-bicarbonate band (detected by pH testing) were observed. The first coloured band to emerge contained the labelled protein, and the second coloured, slow migrating band contained unreacted free dye (KAWAMURA, 1977).

Thus, it was possible to collect by visual checking the fast migrating fraction containing the labelled protein.

III.2.2.3.4 Determination of FITC in the conjugate

Preparation of a fluorescein diacetate (FDA) reference Standard

Because of the instability of FITC in solvents, FDA is recommended as a reference standard for the preparation of the reference curve for determination of FITC in the conjugates (WICK et al. 1978).

An alcoholic solution of sodium hydroxide (3 g NaOH, 40 ml 95 % ethanol) was slowly heated to boiling point, (and the supernatant decanted from the excess of NaOH sediment) was added to 50 mg FDA (SIGMA CHEMICALS) accurately weighed, which was then dissolved on a hot plate, with stirring, allowed to cool to room temperature, transferred to a 500 ml volumetric flask and made up to the mark with distilled water (final dilution 10 mg FDA/100 ml solution).

Preparation of FDA reference standard curve.

Fig. 4 shows the FDA reference standardcurve for determinations of the FITC concentration in the conjugates.

Appropriate dilutions of the above FDA reference standard solution were made in 0.1 sodium hydroxide, to give a serie of solutions of concentrations between 1 and 4 µg of FDA/ml.

The extinction values were read in a spectrophotometer at 490 nm against NaOH as a blank and plotted (see Fig. 4).

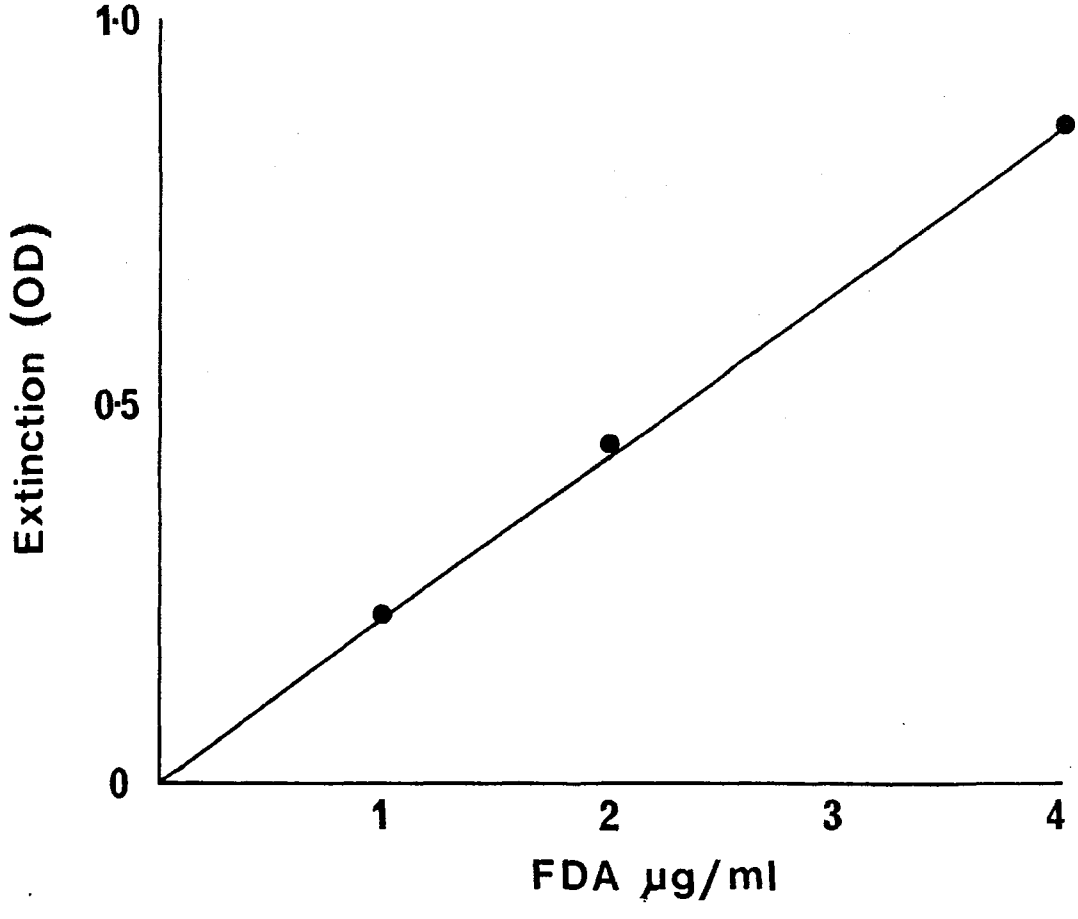
Determination of the FITC bound to protein

Once the standard curve has been determined, an appropriate dilution of the conjugate in 0.1N NaOH to give a reading between 0.05 and 0.8 (in 10mm cells) at 495 nm against 0.1 NaOH as a blank was prepared. The concentration of FITC in µg/ml was ascertained from the standard curve for FDA through multiplication by the constant 1.066. This constant results from the relationship between the coefficient of extinction of FDA, which was found to be

Figure 4

FDA Reference Curve for Determination of the FITC Concentration in Conjugates

O. D.	FDA µg./ml.
0.220	1.00
0.440	2.00
0.860	4.00



0.208 $E_{\mu g/ml}$ 490 nm and the extinction coefficient for known amounts of FITC bound to protein (E_1 µg/ml) which was found to be 0.195 (BEUTNER et al. 1973)

E (FDA)/E(FITC) bound to protein = 1.066

FDA (µg/ml)x1.06 = Protein bound FITC (µg/ml)

III.2.2.3.5 Protein determination of the conjugates by Biuret method

Preparation of standard curve

The reference curve was prepared using crystalline bovine serum albumin (BSA) 15.2 % N_2 (SIGMA CHEMICALS) as the standard.

A standard protein solution containing 10 mg N_2/ml (= 65.79 mg protein/ml) was made.

An initial dilution of 1:20 of the standard protein solution in 0.85 % NaCl was prepared. This solution was used for a dilutions in NaCl 0.85 %, containing succesive protein cocentration (see Table 2).

Biuret reagent (SIGMA CHEMICALS) was added to the protein solution mixed, and colour allowed to develop for 30 min before reading in a spectrophotometer at 560 nm and a light path of 10 mm.

For determination of the protein concentration in FITC conjugates, the extinction is measured at 560 nm since proteins undergo a change in their absorption maximum as a result of conjugation with fluorochromes (WICK et al.

TABLE 2

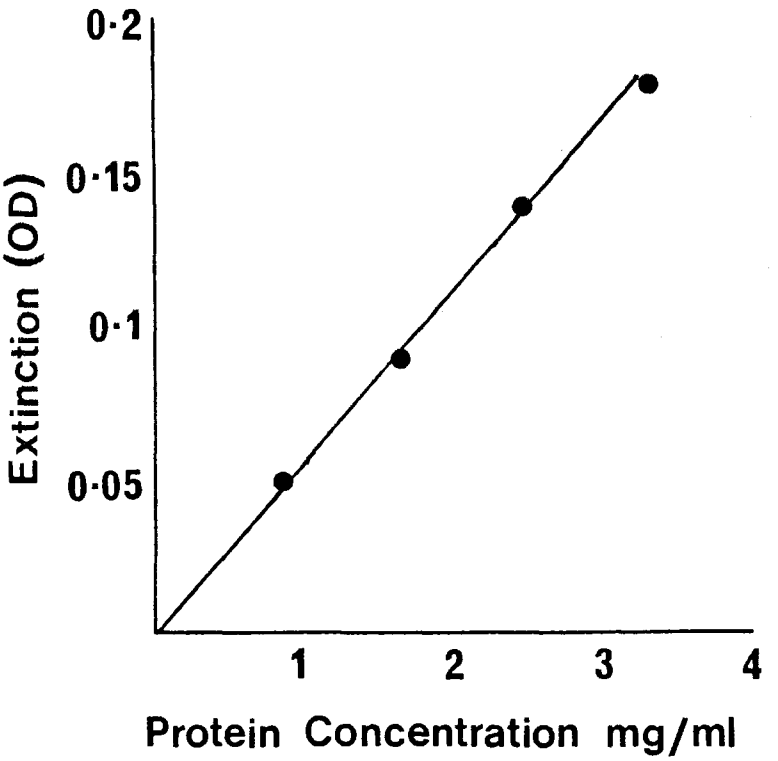
DUAL DETERMINATION OF SERIAL PROTEIN DILUTIONS OF BSA FOR A BIURET STANDARD CURVE PREPARATION

SAMPLE	NaCl 0.85% SOLUTION	1:20 BSA DILUTION	BIURET	EXTINTION (OD)	PROTEIN CONCENT.
1-2	2.0	0.0	8.0	0.000	0.00
3-4	1.5	0.5	8.0	0.050	0.88
5-6	1.0	1.0	8.0	0.090	1.64
7-8	0.5	1.5	8.0	0.140	2.46
9-10	0.0	2.0	8.0	0.180	3.29

All the reagents expressed in millilitres. Extintion expressed in units of optical density. Protein concentrations in mg./ml.

Figure 5

Reference Curve for Protein Determination by the Biuret Method



1978). The resultant OD (optical density) values obtained were plotted (see Fig. 5).

Determination of protein in the FITC-conjugates

The conjugate was dilute in NaCl 0.85 % in order to obtain readings within the OD range of the curve.

The conjugate sample and the Biuret reagent were mixed and the reaction allowed to proceed for at least 30 min. The extinction values (OD) were obtained by reading the samples in a spectrophotometer at 560 nm in 10 mm cuvettes using 0.85 % NaCl as a blank.

The protein concentration of the conjugate was ascertained from the standard curve by multiplication of that figure by the conjugate dilution used.

III.2.2.3.6 Calculation of the F/P ratio

The protein concentration was determined by the Biuret method (mg protein/ml conjugate). The FITC bound to protein in the conjugate (µg protein-bound FITC) was determined from the FDA Standar curve and application of a conversion factor of 1.066.

The F/P ratio (using weights) is expressed as

W/W F/P = µg of FITC protein bound/ mg protein

The molar F/P ratio is preferred, and may be obtained from the weight referred value by the following conversion:

molar F/P = 0.41 x (W/W) F/P

Table 3 shows the results after the characterization of the conjugates

III.2.2.3.7 Determination of conjugate specificity and titration

The specificity and potency of the conjugates were tested using the immunofluorescence and the agar double diffusion techniques. The technical procedures have been described above.

The FITC-anti-mouse IgG (Fab) was tested using the double diffusion technique. The Figure 5 shows the precipitation arcs obtained after reaction of mouse IgG (center well) and serial double dilutions (1:1 to 1:32) of FITC-anti-mouse IgG (Fab) (peripheral wells). Precipitation was observed up to 1:8 dilution.

FITC-*L. mexicana* antigen was tested using the IIFT. Acetone-fixed promastigotes served as the antigen for unlabelled anti-*L. mexicana* IgG antibody working dilution 1:40 (first Layer). Serial dilutions (1:5 to 1:40) of FITC-*L.mexicana* antigen constituted the second layer of the reaction. Positive staining was obtained up to 1:10 dilution.

In the double immunodiffusion technique, FITC labelled *L. mexicana* antigen (undiluted) gave a precipitation arc against anti-*L. mexicana* IgG (Fig.3b)

TABLE 3

CHARACTERIZATION OF FITC-*L. MEXICANA* ANTIGEN AND ANTI-MOUSE IgG (F ab) FITC

	FITC *L. mexicana* ANTIGEN	FITC ANTI-MOUSE IgG F(ab)
TOTAL PROTEIN PRIOR TO CONJUGATION (mg.)	11.3	12.0
TOTAL PROTEIN IN CONJUGATE (mg.)	5.5	5.5
MILLILITRES OF CONJUGATE	10.0	5.0
PROTEIN CONCENTRATION IN CONJUGATE (mg./ml.)	0.550	1.100
EFFICIENCY OF CONJUGATION	49.12%	45.83%
PROTEIN BOUND FITC µg./ml. X 1.066	2.63	7.03
W/W F/P RATIO	4.78	6.30
MOLAR F/P RATIO W/W FP X 0.411	1.96	2.60

III.2.2.4 Preparation of tissues for immunofluorescence technique

III.2.2.4.1 Tissues employed

The immunofluorescence technique was performed on samples of skin lesions and lymph node removed from three mice from each experimental group, every two weeks, up to the eighteenth week after infection. Pieces of the excised tissues were put on a piece of filter paper, embedded and orientated in O.C.T. embedding medium for frozen tissue specimens (RAYMOND & LAMB, England), placed in plastic vials and then frozen in liquid nitrogen where they were kept until required.

III.2.2.4.2 Sectioning frozen tissues

The tissues were transferred from the liquid nitrogen container to a cryostat chamber, then allowed to reach cryostat temperature (-20°C). After that, the tissues were attached to precooled chucks with a few drops of O.C.T. compound and placed on the cryostat microtome to be sectioned. Four to five micron sections were made and picked up on multi-spot slides (C.A. Hendley L.T.D Essex), allowed to dry at room temperature and kept overnight at -20°C in a sealed box. In each case , every other section in the series being cut for immunofluorescence was stained by haematoxylin and eosin, in order to help in the identification of the cells and structures in the fluorescent sections.

III.2.2.4.3 Immunofluorescence (IF) staining procedures.

Direct (DIFT) and indirect immunofluorescence techniques (IIFT) were performed. The slides containing the tissue sections were removed from the freezer, allowed to warm to room temperature, dried with a fan (cold current air) for half an hour, washed in PBS pH 7.2 for 10 minutes and fixed in a mixture of equal volumes of ethanol (95 %) and ether (undiluted) for 10 min. After fixation, the slides were rinsed and washed in PBS 10 min and the excess fluid blotted off before being transferred to a rack in a humid chamber.

In the case of IIFT, the unconjugated immune-serum (first layer) was layered on the section, incubated for 30 min, then the fluid blotted and the slides rinsed and washed in PBS 3 times (each wash 10 min) using a magnetic stirrer. The excess PBS was then removed and the slides were transferred back to the humid chamber, treated with the corresponding fluorescent conjugate (second layer) for 30 min and subsequently rinsed and washed in PBS 3 times for over 60 min. Finally, the sections were mounted in buffered glycerine (9/1, glycerol/PBS).

In the case of DIFT, only one layer was applied to the sections, that being the FITC conjugate intended to react directly with a component of the tissue.

III.2.2.4.4 Tissue examination

After performance of IFT, the tissues were examined using white light under dark field conditions, and then switched to incident ultra-violet illumination. The microscope used was a Leitz Orthoplan equipped with Ploemopak 2.1a2.2 fluorescence vertical illuminator, 200w/4 ultra high pressure mercury lamp and Hz filter system, consisting of heat filter Kto-1 suppressor filter LP515, exciter filter BP 339-490 and beam splitting mirror RKP510.

Ektachrome 400 (Kodak) film was used for photomicrophotographs.

III.2.2.4.5 Reagents used in the immunofluorescence technique

The FITC conjugated reagents were purchased from NORDIC IMMUNOLOGICAL LABORATORIES (Maindenhead, England) unless otherwise specified.

III.2.2.4.5.1 Reagents used for IgG detection

a. In the DIFT

Goat anti-mouse IgG (Fc) conjugated to FITC, molar F/P ratio 1-2, working dilution 1:16.

Rabbit anti-mouse IgG (Fab) conjugated to FITC (conjugated in the laboratory) protein concentration 1.1mg/ml molar F/P ratio 2.6. working dilution 1:40

b. In the IIFT

First layer: goat anti-mouse IgG (Fc), working dilution 1:40. Second layer: rabbit anti-goat Ig conjugated to FITC, working dilution 1:60.

III.2.2.4.5.2 Reagents used for IgM detection

a. In the DIFT

Goat antimouse IgM (Fc) conjugated to FITC, working dilution 1:10.

b. In IIFT

First layer goat anti-mouse IgM (Fc) working dilution 1:40. Second layer rabbit anti-goat Ig conjugated to FITC, working dilution 1:60.

III.2.2.4.5.3 Reagents used for detection of C3 complement factor

a. In the DIFT

Goat antimouse C3 conjugated to FITC, working dilution 1:20

b. In the IIFT

First layer: rabbit anti-mouse C3, (gift of Professor P. Lachman, F.R.S; M.R.C; Cambridge), working dilution 1:40. Second layer goat anti-rabbit Ig conjugated to FITC, working dilution 1:64 (gift of Prof. Lachman)

III.2.2.4.5.4 Reagents specific to *Leishmania*

L. mexicana conjugated to FITC antigen used undiluted in DIFT (see above), protein concentration 0.550 mg/ml. Molar F/P ratio 1.96.

$F(ab)'_2$ fraction of rabbit anti-*L. mexicana* IgG (see above) was used in the IIFT at a protein concentration of 2 mg/ml, working dilution 1:4. It was used in conjuction with Goat anti-rabbit Ig conjugated to FITC

as the second layer at a working dilution of 1:64.

III.2.2.4.5.5 Reagents used in the identification of the T cell surface markers. (Lyt1 and Lyt2).

Mouse anti-Lyt1.1 (CEDARLANE LABORATORIES) mouse anti-Lyt1.2 (NEW ENGLAND NUCLEAR LABORATORIES), and rat anti-Lyt2 (SALK INSTITUTE) monoclonal antibodies constituted the first layer in the IIFT. Rabbit anti-mouse IgG conjugated to FITC constituted the second layer for the identification of lymphocytes bearing the Lyt1 phenotype and FITC conjugated anti-rat IgG (Fab) for those cells bearing Lyt2 phenotypes. The characteristics of these reagents are described in detail in ChapterIV.

It is important to point out, that due to the use of anti-mouse and anti-rat immunoglobulin as the second layer in the IIFT for detection of T cells, nonspecific staining due to the reaction of such reagents with Ig contained in the tissues and cell structure was expected. In order to avoid this, the possible Ig contained in the tissues was blocked by treating the sections with unlabelled goat anti-mouse Ig prior to the application of the relevant reagents. The sections were incubated with the unlabelled Ig for 1 hour at room temperature. The efficiency of the blocking test was corroborated by the absence of staining in the pretreated sections when staining was done only with anti mouse-Ig conjugate to FITC after the performance of the blockage.

III.2.2.4.6 Purification of FITC conjugates by absorption with liver powder

In order to minimize any non-specific staining, that could interfere with the interpretation of the immunofluorescence technique, absorption of all the conjugates with acetone extracted mouse liver powder (SIGMA CHEMICALS) was performed.

The wet powder method KAWAMURA (1977) was used. 100 and 50 mg of mouse liver powder were moistened with 1 and 0.5 ml of PBS respectively. After centrifugation at 1500 rpm for 20 minutes two preparations of wet powder were obtained. The conjugates, in amounts of 1 ml, were absorbed twice, once with the 100 mg and once with the 50 mg lots of wet liver powder preparations. The conjugates were thoroughly mixed with the liver powder preparations for 4 hours at 4°C and then centrifuged at 15000 rpm for 30 min at 4°C. The supernatant was carefully removed, taking care to avoid contamination by the sediment, filtered through a millipore filter (22µ), distributed in small aliquots and stored at -20°C until required. Immediately before any staining was done, the thawed conjugates were again centrifuged at 15000 rpm for 15 minutes at 4°C.

III.2.2.4.7 Dilution of the immunoreagents used in the IFT

The working dilutions were prepared in 5 % albumin solutions (fraction V, SIGMA CHEMICALS) in PBS.

III.2.3.4 Controls used in the Immunofluorescence technique

III.2.3.4.1 Normal controls

All the immunofluorescence reactions performed on the experimental groups were also performed on skin and lymph nodes from healthy BALB/c and CBA/ca mice.

III.2.3.4.2 Positive control

Kidneys from mice with renal lesions and known to be positive for Ig and C3 were kindly provided by Dr. M. D. Steward (London School of Hygiene and Tropical Medicine).

III.2.3.4.3 PBS control

In order to estimate possible autofluorescence of the tissues, all sections studied were treated with PBS only and examined under fluorescence illumination.

III.2.3.4.4 Controls used in DIFT

Blocking test

No fluorescence should be obtained if antigenic determinants are blocked with unlabelled anti-sera of the same specificity before adding the same anti-sera conjugated to FITC. For Ig and C3 complement factor, the performance of the blocking test was done using unlabelled anti-mouse IgM or anti-mouse IgG (100-200 µg antibody/ ml, MILES YEDA LTD) or unlabelled antimouse C3 (1:10 gift of

Prof. LACHMAN) which were layered over the sections for 30 min before application of the relevant conjugate.

Absorption test

No fluorescence should be obtained if the conjugate is absorbed with the appropriate antigen or antibody before application to the preparation to be investigated.

Normal mouse serum diluted 1:10 was used as a source of immunoglobulins. This was mixed with anti-mouse IgG conjugated to FITC (final dilution 1:16 dilution), or with anti-mouse IgM conjugated to FITC (final dilution 1:10), or with anti-mouse IgG (Fab) conjugated to FITC (final dilution 1:40). After 30 minutes of incubation, the mixtures were applied to the sections.

C3 factor was obtained from fresh normal serum treated with zymosan. 1mg of zymosan (SIGMA CHEMICALS) was added per each 0.1ml of serum, mixed and incubated at 37°C for 15 minutes. The complement activated serum was mixed with goat anti-mouse C3 conjugated to FITC (final dilution 1:40) for 30 min and then applied to the sections.

Controls for FITC conjugated *L. mexicana* antigens (DIFT)

Blocking test.

Unlabelled *L. mexicana* antigen (4mg/ml) was layered onto the sections for 30 min before the application of the FITC conjugated *L.mexicana* antigen.

Absorption test

FITC conjugated *L. mexicana* antigen was mixed with equal volume of mouse serum anti-*L. mexicana* antibody (IF titer 1:320). The mixture was incubated at room temperature for 1 hour before its application to the sections.

III.2.3.4.5 Controls used in IIFT

PBS and normal serum substitution

Substitution of the unlabelled antiserum (first layer) by PBS was used in order to determine whether the heterologous Ig, that constituted the second layer, cross reacted with any determinant in the the tissue.

Normal mouse and rabbit sera were substituted for the first layer in order to know if they contained antibodies to any of the antigens under investigation.

Control for checking the F(ab)'2 fraction of the anti-*L. mexicana* IgG (IIFT)

Absorption test

The F(ab)'2 fraction of the anti-*L. mexicana* IgG was absorbed twice with living washed promastigotes (1 x 10^7) for 30 min. at 37°C. The parasites were then removed by centrifugation and the antibody applied to the sections.

III.2.3.5 Other reactions used in order to aid the interpretation of the immunoflurescence results

III.2.3.5.1 ∝ Naphthyl acetate reaction for non specific Esterase

In order to identify cells belonging to the macrophage-phagocytic system, the ∝ Naphthyl acetate staining method for esterases (PEARSE, 1972) was used. 10 to 15 μ cryostat sections were produced and picked up on clean slides. After drying, the sections were incubated for 15 min with 10 mg of ∝ Naphthyl acetate (dissolved in 0.25 ml acetone) and added with 20 ml 0.1 phosphate buffer pH 7.4 and 50 mg of Fast blue salt (BDH). After incubation, the slides were washed in running water, counterstained in Mayer's haemalum (BDH) for 5 min, washed in running water for 30 min and mounted in glycerin jelly.

III.2.3.5.2 Studies on Eosinophils

Eosinophils were abundant in *Leishmania* lesions (see ChapterII). These cells were also stained with fluorescent conjugates.

These granulocytes represent the most troublesome cells in immunofluorescence studies as they tend to stain non-specifically, therefore, control experiments were done to determine how much of their staining might be due to specific and how much to non-specific causes.

Experimental induction of tissue eosinophilia

Recruitment of eosinophils in the skin of normal BALB/c mice was done to obtain a concentration of eosinophils in skin tissue which could serve as a control.

The mice were injected weekly for two weeks, intradermally in the foot-pad with 20% bovine serum-albumin solution (SIGMA CHEMICALS) or 3% starch solution. Six hours after the second injection, the animals were sacrificed and the skin of the site of injection removed, embedded in O.C.T. compound, frozen in liquid nitrogen, sectioned in the cryostat microtome and stained with haematoxylin and eosin after fixation in formol saline. The stained sections were then examined under the microscope and the presence of eosinophils corraborated. These sections were used as a control in the performance of the immunofluorescence technique.

Blocking effects of sodium dodecyl sulphate (SDS)

SDS is an anionic detergent (BERRETY & CORMANE 1979) used to determine the specificity of eosinophils stained by FITC conjugates where non-specificity might be due to the FITC itself. The mechanism of blockage of non-specific staining seems to consist of a denaturating property of SDS which dissociates the basic proteins which bind the acid FITC.

Control and infected sections were incubated in 0.025% SDS (SIGMA CHEMICAL) in borate buffer (pH 8.4), ionic strength 0.1) before the performance of the

immunofluorescence technique.

Inhibition of Eosinophil staining by Diamino benzidine (DAB) and hydrogen peroxide

In tissues where eosinophils are abundant, there is difficulty in distinguishing between immunocytes and eosinophilic granulocytes in fluorescence preparations.

When tissue containing eosinophil, are pretreated by DAB and hydrogen peroxidase, a brown reaction product develops in presence of this substrate at the site of the endogenous peroxidase of the granulocytes, sheltering these cells from subsequent staining with FITC conjugates, but without interfering with the staining of other tissue elements (VALNES & BRANDTZAEG 1981).

The DBA method, PEARSE (1972) was used. Tissue sections were incubated for 10 min with DAB in 0.05M Tris-HCl buffer pH 7.6, containing freshly added H_2O_2.

A concentration of 0.5 g/l of DAB in combination with 0.004% H_2O_2 was used. Following DAB treatment, the slides were washed in 3 changes of distilled water, followed by fluorescence staining by the direct method. Observations of the sections was done using both bright field illumination and ultra-violet illumination.

III.2.3.5.3 Studies on muscles

Investigation of antibody in the sera of *Leishmania* infected mice reacting with muscular structures in mice.

Antigens

Whole heart and skeletal muscles from normal CBA/ca mice and mice infected with *Leishmania* 8 weeks previously (from all the expeerimental groups used in this study) served as antigen. Muscles from two mice from each group were tested.

Sera

CBA/ca mice normal sera, sera from BALB/c mice infected with *L. major* 8 weeks previously (IIFT titer 1:160) and sera from CBA/ca mice infected with *L. mexicana* 16 weeks previously (IIFT titer 1:320) were used.

Procedure

Indirect immunofluorescence technique was used. The sera were studied up to 1:320 dilution.

The fluorescent conjugate (second layer) was goat anti-mouse IgG conjugated to FITC, molar F/P ratio 1-2, working dilution 1:60.

The sections were also treated with PBS only, in order to check for the presence of any autofluorescence. PBS and normal mouse serum were substituted for the first layer in control experiments.

III.3 RESULTS

III.3.1 SKIN LESIONS

Tables 4, 5 and 6 summarize the results obtained after using the immunofluorescence technique on cryostat sections from skin lesions of *Leishmania* infected animals at biweekly intervals after inoculation of the parasite.

From the Tables it can be observed that structures in the skin, as well as the infiltrating cells, exhibited fluorescence when staining with the various immunoreagents.

In general, fluorescent staining in the infected skin infiltrate was detected from the second week of infection, and the maximum intensity was observed around the fourth week after infection in all cases. After this period, the intensity and amount of fluorescence staining declined. In CBA/ca mice infected with *L. major* this period of reduction of the fluorescence seemed to coincide with the onset of the healing process, while in CBA/ca and BALB/c infected with *L.mexicana* and BALB/c infected with *L. major* it seemed to correspond to the beginning of the evolution of the infiltrate towards a histiocytoma (mass of vacuolated, heavily parasitized macrophages), which was then consistently negative with all the immunoreagents used in all the cases.

Direct (DIFT) and indirect immunofluorescence technique (IIFT) were performed in the investigation of immunoglubulin and C3 complement factor.

TABLE 4

DISTRIBUTION OF IMMUNOFLUORESCENT STAINING IN STRUCTURES OF THE SKIN FROM CBA/ca AND BALB/c MICE INFECTED WITH Leishmania major AND Leishmania mexicana.

CBA/ca MICE

INFECTED WITH L. major — INFECTED WITH L. mexicana

Dermis-epidermis junction

	2 W	4 W	6 W	8 W	2 W	4 W	6 W	8 W
ANTI-IgM (Fc)	dl+				cl+	cl+		
ANTI-IgG (Fc)	dl+	cl++			cl++	cg++	cg++	
ANTI IgG (Fab)		dl+				cl++		
ANTI-L. mexicana	dl+				cl++	cl+++		
ANTI-C3	dl+	cl++				cg++	cg++	cg++

Blood vessels

	2 W	4 W	6 W	8 W	2 W	4 W	6 W	8 W
ANRI IgM (Fc)		cl+++	cl+++			cg+++		
ANTI-IgG (Fc)		cl++	cl++		cl++	cl++		
ANTI-IgG (Fab)		cl++				cl++		
ANTI-L. mexicana	cl++	cl++				cl++	cl++	cl++
ANTI-C3	cl++	cl+++			cl++	cl++		cl++

Muscles

	2 W	4 W	6 W	8 W	2 W	4 W	6 W	8 W
ANTI-IgM (Fc)								
ANTI-IgG (Fc)		+	+	+		+		
ANTI-IgG (Fab)								
ANTI-L. mexicana	+++	++	+++	+++	+++	++	+++	++
ANTI-C3	+++	+++	+++		+++	+++		+++

Contd. next page

BALB/C MICE

INFECTED WITH L. major | INFECTED WITH L. mexicana.

Dermis-epidermis junction

	2 W	4 W	6 W	8 W	10W	12W	2 W	4 W	6 W	8 W	10W	12W
ANTI-IgM (Fc)	cl+++	cl+++	cl+++					cl++	cl++			
ANTI-IgG (Fc)								cl++	cl++	cg++		
ANTI-IgG F(ab)												
ANTI-L. mexicana	cl++	cg+++					cg+++	cg+++		cg+++		cg+++
ANTI-C3	cl++	cg+++		cg+++		cg+++	cg+++	cg+++		cg+++		

Blood vessels

	2 W	4 W	6 W	8 W	10W	12W	2 W	4 W	6 W	8 W	10W	12W
ANTI-IgM (Fc)	cl+++	cl+++	cl+++									
ANTI-IgG (Fc)	cl+++	cl+++	cl+++	cl+++			cg++	cg++				
ANTI-IgG(Fab)	cl++	cl++					cl++					
ANTI-L. mexicana	cl++	cl+++	cl++			cl++						
ANTI-C3	cg+++	cg+++	cg+++	cg+++			cl++	cg++	cg+++			

Muscles

	2 W	4 W	6 W	8 W	10W	12W	2 W	4 W	6 W	8 W	10W	12W
ANTI-IgM (Fc)												
ANTI-IgG (Fc)		cl++	cl++	cl+++	cl++	cl++		cl+++*				
ANTI-IgG (Fab)												
ANTI-L. mexicana	cl+++*	cl+++*					cl++	cl+++	cl+++*	cl+++*		
ANTI-C3	cl++	cl+++*	cl+++*	cl+++*				cl+++	cl+++	cl+++		

Fluorescence patterns = cg: continuous granular, cl: continuous linear, dl: discontinuous linear

Intensity of fluorescence = + weak, ++ moderate, +++ intense. * hyaline collections also stained.

W = WEEKS AFTER INFECTION

TABLE 5

TITRATION OF ANTI-MUSCULAR ANTIBODY IN SERA FROM MICE INFECTED WITH L. major AND L. mexicana.

ANTIGEN PREPARATION		SERUM FROM : NORMAL MOUSE	MOUSE INFECTED WITH L. major	MOUSE INFECTED WITH L. mexicana
		NORMAL CBA/ca MICE		
SKELETAL MUSCLE	N		80	20
HEART	N		80	20
		CBA/ca MICE INFECTED WITH L. major		
SKELETAL MUSCLE	N		N	N
HEART	N		N	N
		CBA/ca MICE INFECTED WITH L. mexicana		
SKELETAL MUSCLE	N		40	20
HEART	N		20	20
		BALB/c MICE INFECTED WITH L. major		
SKELETAL MUSCLE	N		320	20
HEART	N		320	320
		BALB/c MICE INFECTED WITH L. mexicana		
SKELETAL MUSCLE	N		20	20
HEART	N		320	320

N : negative. Results expressed as the reciprocals of the highest serum dilution giving ++ immunofluorescence.
L. major antisera from a pool of 3 BALB/c mice, immunofluorescence titre 1 : 160.
L. mexicana antisera from a pool of 3 CBA/ca mice, immunofluorescence titre 1 : 320.
Muscles taken from infected mice 8 weeks after infection.

TABLE 6

DISTRIBUTION OF IMMUNOFLUORESCENT STAINING IN CELLS OF THE INFILTRATE OF SKIN LESIONS OF CBA/ca AND BALB/c MICE INFECTED WITH L. major AND L. mexicana.

INFECTION MODELS

	CBA/ca MICE INFECTED WITH													BALB/c MICE INFECTED WITH												
	L. maj.		L. mexicana							L. major							L. mexicana								
RETICULAR SHAPED CELLS																									
	2W	4W	2W	4W	6W	8W	10W	12W		2W	4W	6W	8W	10W	12W	14W		2W	4W	6W	8W	10W	12W	14W	
ANTI-IgM (Fc)		+±																							
ANTI-IgG (Fc)	+		+±	+±	+					+±															
ANTI-IgG (Fab)	+	+		+						+	+	+	+												
ANTI-L. mexicana	+	+±		+	+					+	+	+													
FITC-L. mexicana Ag.	+		+	+						+	+	+	+												
ANTI-C3	+	+		+	+					+	+	+													
MACROPHAGES																									
	2W	4W	2W	4W	6W	8W	10W	12W		2W	4W	6W	8W	10W	12W	14W		2W	4W	6W	8W	10W	12W	14W	
ANTI-IgM (Fc)		+±								+	+	+						+	+±	+	+				
ANTI-IgG (Fc)	+	+±	+	+	+	+	+			+	+	+	+	+	+	+			+		+				
ANTI-IgG (Fab)		+	+	+		+				+	+	+	+												
ANTI-L. mexicana	+±	+±	+	+	+	+	+	+		+	+±	+	+												
FITC-L. mexicana Ag.										+	+	+						+	+	+	+				
ANTI-C3		+		+±		+		+		+	+±	+						+	+	+					
LYMPHOCYTES																									
	2W	4W	2W	4W	6W	8W	10W	12W		2W	4W	6W	8W	10W	12W	14W		2W	4W	6W	8W	10W	12W	14W	
ANTI-IgM (Fc)	+																								
ANTI-Igg (Fc)										+	+	+	+	+	+										
ANTI-IgG (Fab)	+			Pc							+														
ANTI-L. mexicana																									
FITC-L. mexicana Ag.				Pc											+										
ANTI-C3																									
ANTI-Lyt1	+	+	+	+		+					+	+							+	+	+	+			
ANTI-Lyt2			+	+						+	+	+	+						+	+	+	+			

Contd. next page.

INFECTION MODELS

	CBA/ca MICE INFECTED WITH													BALB/c MICE INFECTED WITH												
	L. maj.		L. mexicana						L. major							L. mexicana									
EOSINOPHILS																									
	2W	4W	2W	4W	6W	8W	10W	12W	2W	4W	6W	8W	10W	12W	14W	2W	4W	6W	8W	10W	12W	14W
ANTI-IgM (Fc)																						
ANTI-IgG (Fc)				+	+	+	+		+	+	+	+	+	+	+		+	+			+	+
ANTI-IgG (Fab)		+		+	+		+		+	+	+	+	+				+	+	+			
ANTI-L. mexicana										+	+	+										
FITC-L. mexicana Ag.	+			+	+	+				+	+	+					+	+				
ANTI-C3																	+	+	+	+	+	+
AMASTIGOTES																						
	2W	4W	2W	4W	6W	8W	10W	12W	2W	4W	6W	8W	10W	12W	14W	2W	4W	6W	8W	10W	12W	14W
ANTI-IgM (Fc)																						
ANTI-IgG (Fc)		+																				
ANTI-IgG (Fab)				+	+	+						+										
ANTI-L. mexicana	+	+		+	+	+				+		+					+					
FITC-L. mexicana Ag.		+		+	+			+	+	+	+	+					+	+	+	+		
ANTI-C3				+	+			+	+	+	+						+		+	+		

Ag. : antigen. Pc : plasma cells. + cells stained in dermis. * Cells stained in epidermis as well

The sensitivity of the reaction was similar for both Ig and C3. The skin structures which stained were: the dermal-epidermal junction, the blood vessels and the muscles.

Fluorescent deposits on these structures reacted as immunoglobulins (G and M), C3 factor of complement and *Leishmania* antigen. Most of the deposits observed were associated with each other and in the same localities (antigen-antibody, or antigen-antibody-complement complexes).

Dermal-epidermal junction

The pattern of deposition of staining elements at this level was variable and seemed to be related to the clinical stage of the disease. Thus, in skin sections from CBA/ca mice infected with *L. major*, in which self-healing lesions occured, the pattern of staining detected by the second week after infection at this level, was discontinuous and consisted of deposits of antigen (Ag), immunoglobulins (M and G) and C3. By the fourth week, the pattern of staining became continuous and linear, produced by deposits of IgG and C3 complement factor. The intensity of fluorescence in these deposits was weak at the second week and slightly greater by the fourth week of infection. Thereafter fluorescence was no longer detected.

In mice exhibiting non-healing lesions (CBA/ca, BALB/c infected with *L. mexicana* and BALB/c infected with *L. major*), on the other hand, the deposits were continuous and linear by the second week of infection, and

as long as the infection progressed, they became thicker and frequently granular in appearance (Fig. 6). Ag and C3 deposits in CBA/ca and BALB/c mice infected with *L. mexicana* were associated with IgG and IgM immunoglobulins, while in BALb/c infected with *L. major* only IgM deposits were observed. The staining at this level was observed up to the twelfth week in Balb/c mice infected either with *L. major* or *L mexicana* and up to the eighth week in CBA/ca mice infected with *L. mexicana*.

Blood vessels

Small blood vessels (capillaries and venules) and rarely arterioles were found to be stained.

The stained deposits were located at the intima levels in small vessels (Fig. 7) and in all the three layers (intima, media and adventitia) in arterioles. In most of the cases, the pattern of deposition was linear, although granular deposits were observed in blood vessels from mice exhibiting non-healing lesions especially when stained with anti-C3 reagents.

Association between deposits of Ag, Ig(G and M) and C3 were found by the fourth week of infection in CBA/ca infected with *L.major* and *L. mexicana*, by 2, 4 and 6 weeks after infection in BALB/c infected with *L. major* and by the second week in BALB/c infected with *L. mexicana*.

The intensity of fluorescence was moderate to intense in all cases.

FIGURE 6

Direct immunofluorescence on skin lesion section of CBA/ca mouse, 4 weeks infected with *L. mexicana*. Skin section treated with rabbit anti-mouse C3 conjugated to FITC (dilution 1:20) demonstrating deposition of staining at the dermal epidermal junction. X 125.

FIGURE 7

Indirect immunofluorescence on skin lesion section from BALB/c mouse 4 weeks infected with *L. major*. Skin section treated with $F(ab)'_2$ fraction of rabbit anti-*L. mexicana* IgG (dilution 1:4) demonstrating fluorescence in blood vessel. X 128.

FIGURE 6

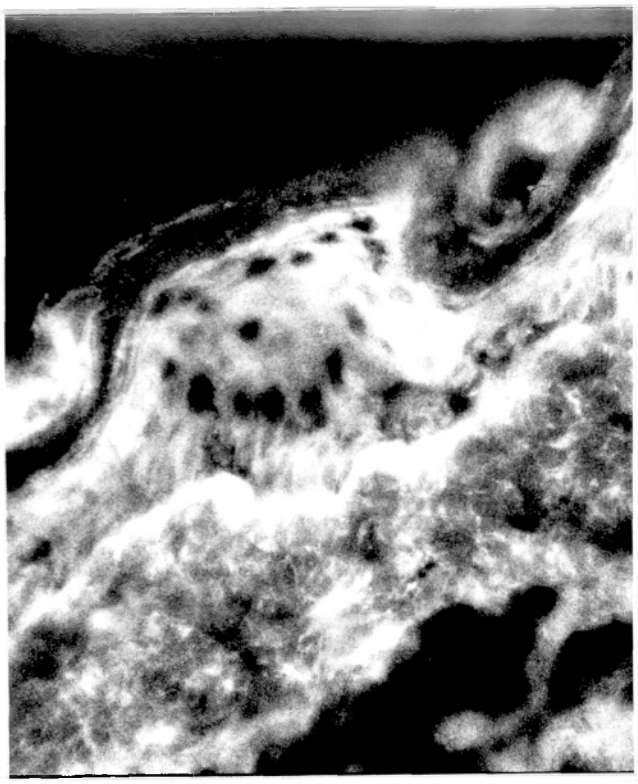
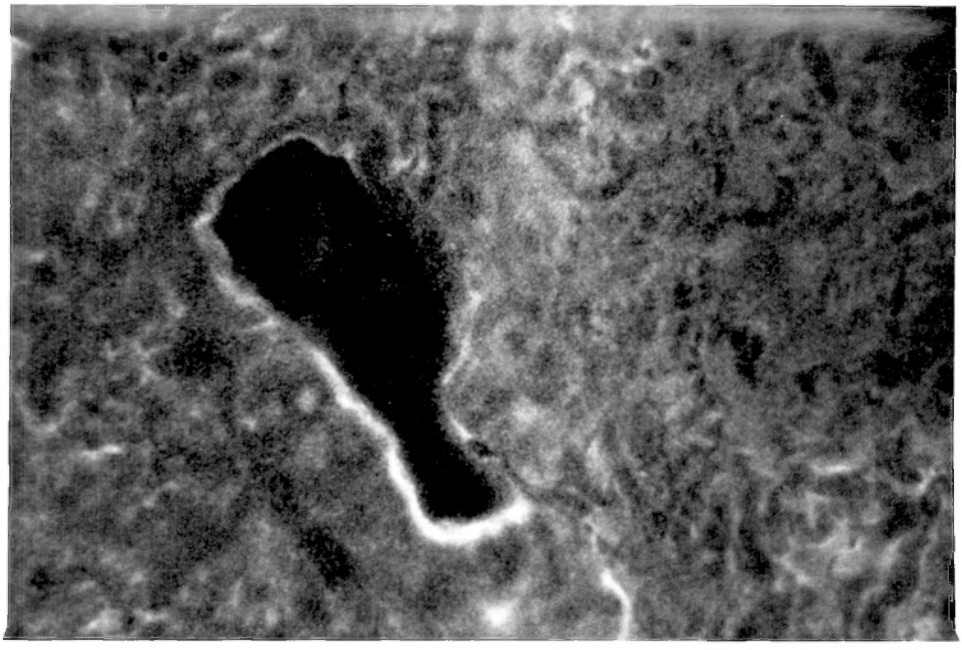


FIGURE 7



Muscles

Muscles were found to be intensely stained in a thick linear fashion surrounding the sarcolemma (Fig. 8). Fluorescent deposits at this level were mainly detected by anti-C3 and anti-*L. mexicana* antibody ($F(ab)'_2$ fraction of IgG). IgG deposits were also detected at this level when using anti-IgG (Fc) preparations, but not with the Fab fraction of anti-IgG.

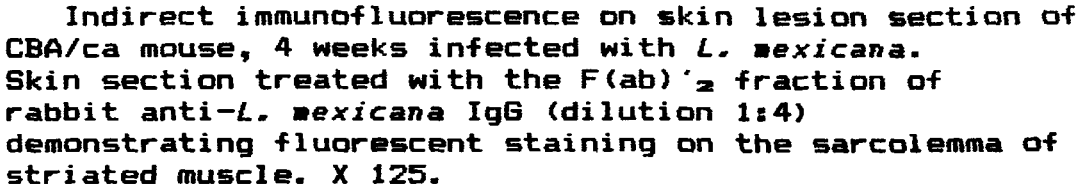
Ag, IgG and C3 were found in muscular structures by the fourth week in all the non-healing infection models and in CBA/ca mice infected with *L. major* by the fourth and sixth weeks after infection.

Hyaline collections, thought to originate from muscular degeneration (see Results, Chapter II) were observed to fluoresce with the same reagents as the muscles (see Table 4).

It was found that normal muscular structures from non-infected mice were also stained by anti-*L. mexicana* antibody. This led to an investigation of the possible presence of an antibody reacting with muscles in the serum of mice infected with *Leishmania*. Skeletal muscle and heart from normal mice and from mice from the various host parasite combinations studied served as antigen. Sera from BALB/c mice infected with *L. mexicana* , CBA/ca mice infected with *L. major* and normal serum were investigated for antibody reacting with muscles using the IIFT.

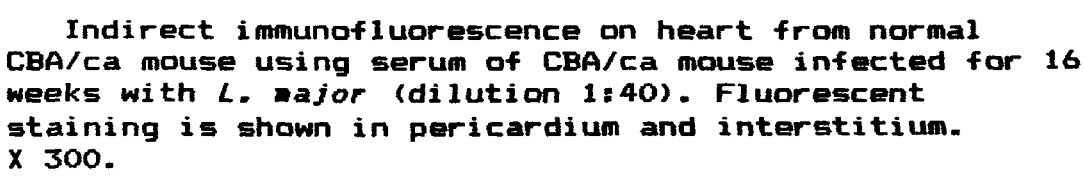
The results are recorded in Table 5, where it can be

FIGURE 8



Indirect immunofluorescence on skin lesion section of CBA/ca mouse, 4 weeks infected with *L. mexicana*. Skin section treated with the F(ab)'$_2$ fraction of rabbit anti-*L. mexicana* IgG (dilution 1:4) demonstrating fluorescent staining on the sarcolemma of striated muscle. X 125.

FIGURE 9



Indirect immunofluorescence on heart from normal CBA/ca mouse using serum of CBA/ca mouse infected for 16 weeks with *L. major* (dilution 1:40). Fluorescent staining is shown in pericardium and interstitium. X 300.

FIGURE 8

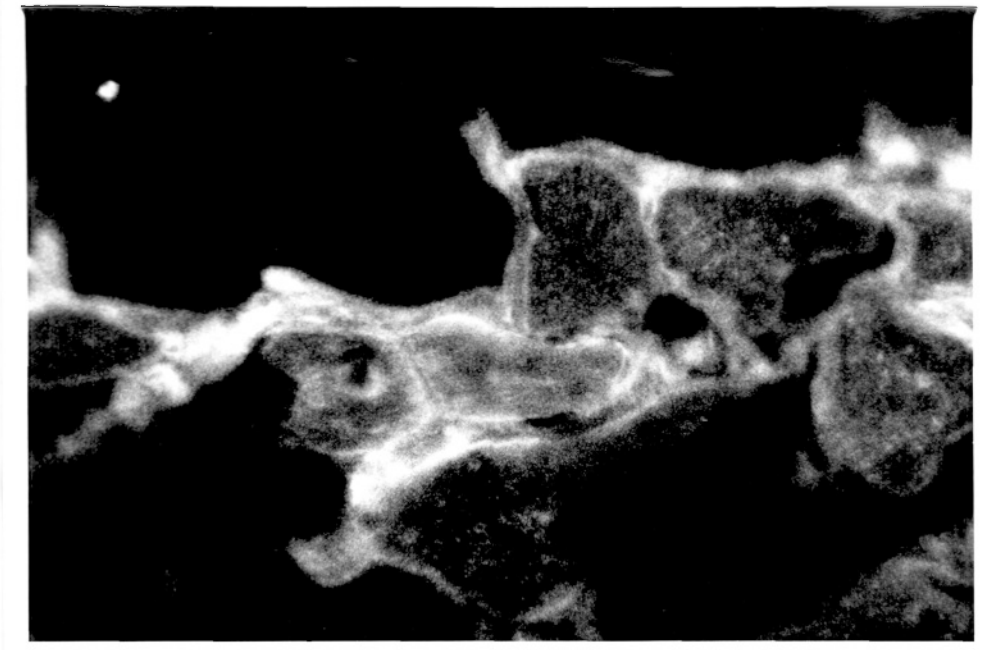
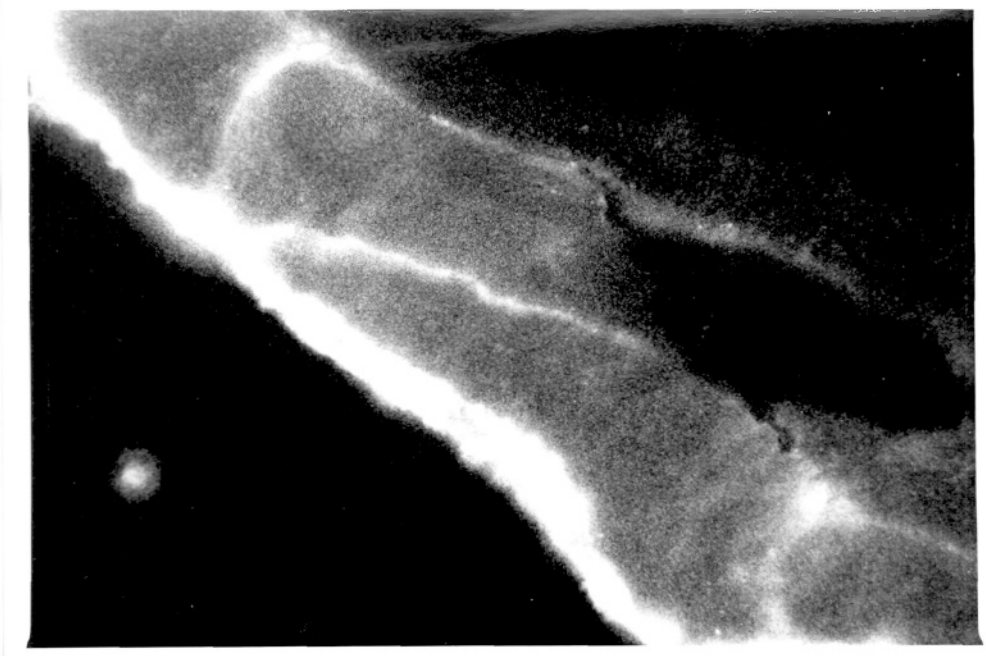


FIGURE 9



seen that all the sera from the infected animals, but not the control serum, stained the muscular structures of non-infected mice and of CBA/ca mice infected with *L. mexicana* and BALB/c infected with *L. major* or *L. mexicana*. Muscular structures in CBA/ca mice infected with *L. major* were not stained by any of the sera used.

The titers varied with the antisera and the muscle preparation under investigation, but in general, higher antibody titers were observed with *L. major* serum when skeletal muscle was used as the antigen. Similar titers with all antisera were observed when using cardiac muscle.

The staining of skeletal muscles exhibited a similar pattern to that described in muscles in skin lesions. With cardiac muscles, on the other hand, the staining was localized to the interstitium of the myocardium between muscular fibers (Fig.9), in pericardium, endocardium and blood vessels; the deposits were linear. In addition, a granular apparently intracytoplasmic pattern was seen in cardiac muscle from CBA/ca mice infected with *L mexicana*; this pattern of staining was observed with both antisera used.

Cells of the infiltrate

Regarding the fluorescence observed in the cells constituting the skin infiltrate, non-infected macrophages, or macrophages exhibiting low parasitic load showed membrane staining, which was seen as a ring partially or completely surrounded the cell (Fig.10). These cells were detected staining in the skin infiltrate of all the

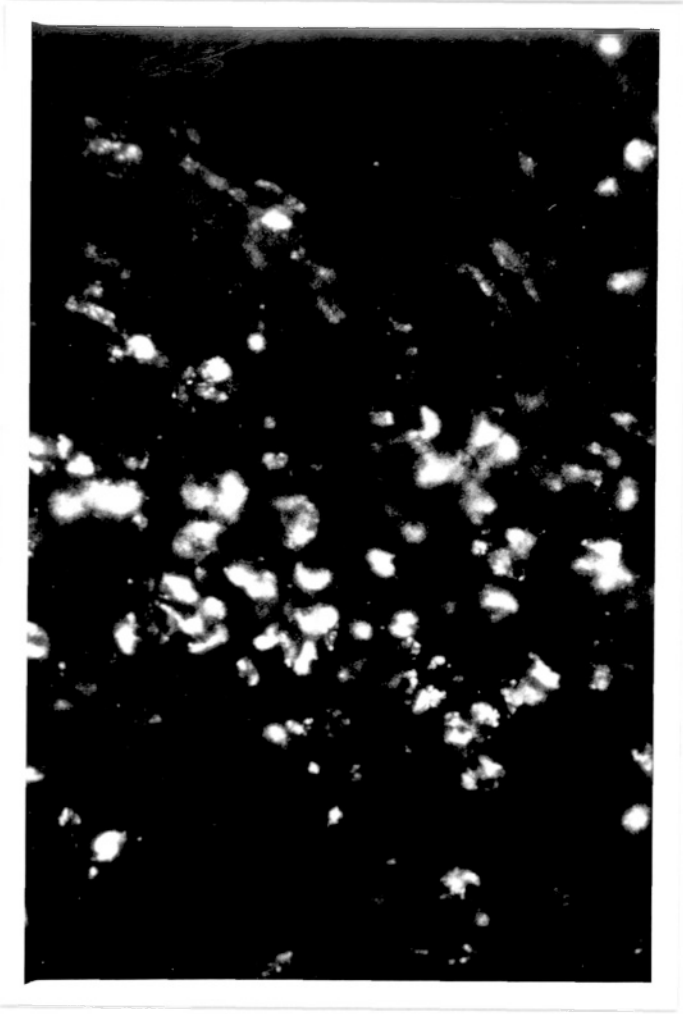


FIGURE 10

Indirect immunofluorescence on skin lesion section from BALB/c mouse, 4 weeks infected with *L.major* using the $F(ab)'_2$ fraction of rabbit anti-*L. mexicana* IgG. Macrophages exhibited fluorescent staining on their surfaces. X 125.

the infection models used and with all the immunoreactants used for the identification of immunoglobulins, C3 factor and reactants specific for *Leishmania* (see Table 6). However, as mentioned above, only non-infected macrophages or those macrophages exhibiting low parasitic load were stained. Heavily parasitized and vacuolated macrophage, observed as part of the histiocytomas in mice exhibiting non-healing lesions were always negative with all the immunoreactants used. As a consequence of the selective staining in the macrophage population, the distribution of stained macrophages in the infiltrate varied with the development of the infection. Thus, in mice in which self-healing lesions occurred (CBA/ca infected with *L. major*) and in early infections (up to the four week) in groups of mice whose lesions evolved to chronicity (CBA/ca, BALB/c infected with *L. mexicana* and BALB/c infected with *L. major*.) these stained cells were seen throughout the infiltrate, but as the infection progressed in CBA/ca infected with *L. major*, stained macrophages disappeared as a consequence of the healing process and in the rest of the groups (non-healing lesions) fluorescent macrophages appeared towards the periphery of the infiltrate leaving unstained the cells of the central histiocytoma.

Irregularly shaped cells (reticular or spindle cells) were stained with the same reagents as macrophages. These cells were localized immediately under the epidermis, among bundles of collagen and occasionally in the epidermis

itself (see Table 6). The staining in these reticular shaped cells was observed outlining the body and its prolongations (Fig. 11). Some of these cells were laden with pigment in homologous sections stained by haematoxylin and eosin. The performance of the α napthyl-acetate method for non-specific esterase showed the presence of this enzyme in the cytoplasm of the reticular shaped cells (Fig. 12) and in the macrophages of the infiltrate.

Macrophages and reticular shaped cells were stained from the second week after infection in infiltrates from all the groups of mice in the different host-parasite combinations, the exception being the BALB/c *L. mexicana* group of mice, in which reticular shaped cells were not stained at all.

On the periods up to which the staining occured in these cells, in CBA/ca *L.major*, macrophages and reticular shaped cells were detected up to the fourth week of infection in CBA/ca infected with *L. mexicana* and in BALB/c infected with *L. major*, reticular shaped cells were seen stained up to the sixth and eight week, while macrophages were stained up to the twelfth and fourteenth week respectively. In BALB/c infected with *L. mexicana*, only macrophages were surrounded with fluorescence and these lasted up to the tenth week. In contrast to the other experimental groups, in BALB/c -*L.mexicana* infected mice, anti-*L. mexicana* Ig (F(ab)'$_2$). did not produce any staining in the macrophages.

Lymphocytes were hard to identify and differentiate from monocytes and sometimes histiocytes in the

FIGURE 11

Indirect immunofluorescence on skin lesion section from CBA/ca mouse, 4 weeks infected with *L. mexicana* using the $F(ab)'_2$ fraction of rabbit anti- *L. mexicana* IgG (dilution 1:4). fluorescence is shown on the membrane surface of dendritic shaped cell of the subepidermal area. X 400.

FIGURE 12

Non-specific esterase test on frozen skin lesion section from CBA/ca mouse, 4 weeks infected with *L. mexicana*. Note reticular or dendritic shaped cells of subepidermis giving positive staining. X 400.

FIGURE 11

FIGURE 12

immunofluorescence technique performed on cryostat sections, however, with the aid of homologous haematoxylin and eosin staining of alternate sections they could sometimes be identified (see Table 6). These cells were stained on their surface with anti-sera to immunoglobulin and FITC-*L. mexicana* only. C3 was not detected.

Plasma cells were detected and found to stain in their cytoplasm with anti-IgG (Fab) and FITC- *L.mexicana* antigen in the infiltrate from skin lesions of CBA/ca mice infected with *L. mexicana* by the fourth week of infection.

Monoclonal antibodies (anti-Lyt1 and anti-Lyt2), used for identification of T cells subpopulations, produced a very weak fluorescence on the surface of lymphoid cells in all the experimental groups. The stained cells were observed as a palisade around the lesions and occasionaly surrounding the blood vessels.

Due to the difficulties in the identification of these cells in tissue sections, experiments with tissue disaggregation for isolation and further characterization of lymphocytes were performed (see next Chapter).

Polymorphonuclear eosinophils were seen to stain in their cytoplasm using anti-IgG and FITC-*L. mexicana* antigen in the infiltrates of all skin lesions (Fig 13). In addition, they were stained by *L. mexicana* antibody in BALB/c mice infected with *L. major* and by anti-C3 in BALB/c infected with *L. mexicana*. But, while they were rarely stained in CBA/ca mice infected with *L. major*, they were stained and abundant in BALB/c and CBA/ca mice

FIGURE 13

Direct immunofluorescence on skin lesion section from CBA/ca mouse, 4 weeks infected with *L. mexicana* using *L. mexicana* soluble antigen conjugated to FITC. Observe polymorphonuclear eosinophils exhibiting cytoplasm fluorescence. X 125.

FIGURE 14

Indirect immunofluorescence on skin lesion section from CBA/ca mouse, 4 weeks infected with *L. mexicana* using the $F(ab)'_2$ fraction of rabbit anti-*L. mexicana* IgG (dilution 1:4). Observe fluorescent amastigotes inside a macrophage. X 400.

FIGURE 13

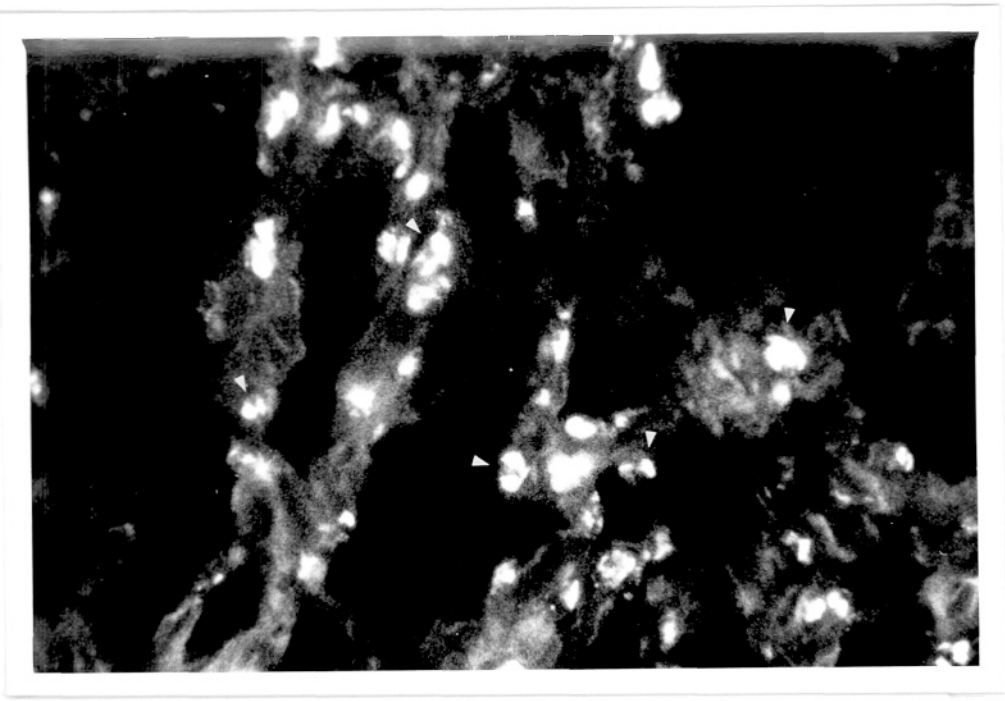
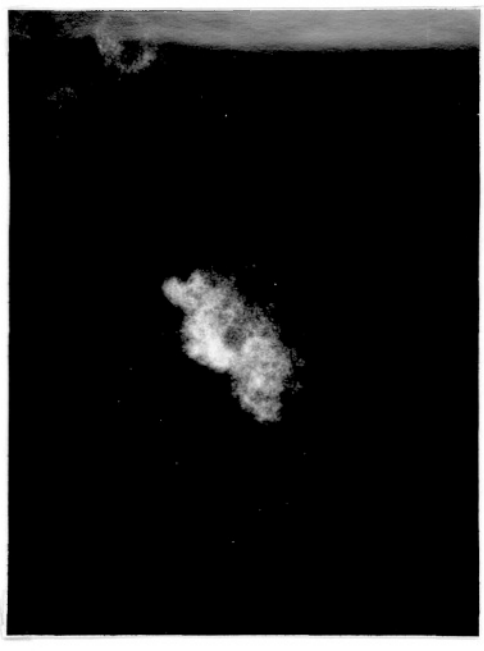


FIGURE 14



infected with *L. mexicana* and in BALB/c infected with *L. major* and were observed in these lesions for longer periods of time (see Table 6).

Control experiments for the specificity of the staining observed in these cells showed that eosinophils recruited in the foot-pad of healthy animals after two weekly injections with bovine serum albumin or starch, were stained by the same immunoconjugates as the eosinophils in *Leishmania* infected tissues. However, blocking of the non-specific staining using Sodium Dodecyl sulphate (SDS), showed ablation of the staining in the tissue eosinophils induced by albumin or starch injections and not in eosinophils from *Leishmania* infected animals.

On the other hand, control experiments designed to corroborate the specificity of the staining of *Leishmania* parasites showed complete ablation of the fluorescence with the blocking test by unconjugated antigen, but not with the absorption test (absorbtion of the conjugated antigen with *Leismania* antisera).

Due to the abundance and the intensity of fluorescence of the eosinophils, other cells as for instance antibody forming cells, which should also stain in their cytoplasm, could be masked by the fluorescence of eosinophils. In order to investigate this possibility, the tissue sections were treated with DBA/H_2O_2 before staining with the conjugate. Untreated sections served as control.

The examination of the DBA/H_2/O_2 pretreated sections under bright field conditions demonstrated the presence of a brown intra-cytoplasmic

deposit in eosinophil granulocytes, reticular shaped cells, monocytes and immature macrophages. The examination of the same section after switching to ultra-violet illumination revealed that polymorphonuclear eosinophils were completely sheltered from fluorescence, but reticular shaped cells, macrophages and monocytes still exhibited surface staining.

Antibody forming cells were observed only in small amounts and only in sections from CBA/ca mice infected with *L.mexicana* corroborating the previous immunofluorescence findings (see Table 6)

Amastigotes

Amastigotes free or inside macrophages were detected by anti-*L. mexicana* IgG ($F(ab)'_2$) anti-IgG, anti-C3 and FITC-*L. mexicana* antigen in sections from all the mice of the different experimental groups. The parasites were not stained by anti-IgM preparations. Amastigotes inside macrophages of the fully developed histiocytoma were never stained.

III.3.2 LYMPH NODE

Table 7(a,b,c,d) summarizes the distribution of fluorescence staining in cryostat sections of lymph node tissue from the different host-parasite combinationsat different periods of time using anti-IgG, anti-IgM, anti-C3, anti-*L.mexicana*, anti-Lyt1 anti-Lyt2 sera and FITC-*L. mexicana* antigen.

The anti-IgM and anti-IgG sera resulted in a peripheral

TABLE 7 A

DISTRIBUTION OF FLUORESCENT STAINING IN THE DRAINING LYMPH NODES OF CBA/ca MICE INFECTED WITH Leishmania major.

REAGENTS	LYMPHOCYTES	Ab. FORMING CELLS	EOSINOPHILS	MACROPHAGES	IN.CELL. DEPOSITS	SYNUS SYSTEM
		DISTRIBUTION OF FLUORESCENT STAINING IN THE CORTEX				
ANTI IgM	2 + 4-10 +++	4-10 ++	N. S.	N. S.	N. S.	2-5
ANTI IgG	2 + 4-6 +++	N. S.	N. S.	N. S.	N. S.	4
ANTI IgG Fab	2-10 +++	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI L. mex.	N. S.	N. S.	N. S.	N. S.	2-8	2-12
L. mex. Ag.	N. S.	N. S.	N. S.	N. S.	2-8	2 4 12
ANTI C3	N. S.	N. S.	N. S.	N. S.	2-12	2-12
ANTI LY T1	2-6 ++	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
		DISTRIBUTION OF FLUORESCENT STAINING IN THE PARACORTEX				
ANTI IgM	4 +	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI IgG	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI IgG Fab	N. S.	N. S.	N. S.	4 +	N. S.	N. S.
ANTI L. mex.	N. S.	N. S.	N. S.	N. S.	2-8	N. S.
L. mex. Ag.	N. S.	N. S.	4-8 +	N. S.	6	N. S.
ANTI C3	N. S.	N. S.	N. S.	N. S.	2-12	N. S.
ANTI LY T1	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S.	2	N. S.
		DISTRIBUTION OF FLUORESCENT STAINING IN THE MEDULLA				
ANTI IgM	2 + 4-10 ++	4-10 ++	N. S.	N. S.	N. S.	N. S.
ANTI IgG	2-6 ++	2-6 ++	N. S.	N. S.	N. S.	2-6
ANTI IgG Fab	4-8 ++	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI L. mex.	N. S.	N. S.	N. S.	N. S.	N. S.	2-8
L. mex. Ag.	N. S.	N. S.	4 +	N. S.	N. S.	4
ANTI C3	N. S.	N. S.	N. S.	N. S.	N. S.	2-12
ANTI LYT1	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.

Figures are weeks after the infection. +, ++, +++ indicate few, moderate or abundant respectively. N. S. indicates no fluorescence detected in the structure. IN. CELL. stands for intracellular. In macrophages fluorescent staining was circumscribed to intracellular amasti gotes.

TABLE 7 B

DISTRIBUTION OF FLUORESCENT STAINING IN THE DRAINING LYMPH NODES OF CBA/ca MICE INFECTED WITH Leishmania mexicana

REAGENTS	LYMPHOCYTES	Ab. FORMING CELLS	EOSINOPHILS	MACROPHAGES	IN.CELL. DEPOSITS	SYNUS SYSTEM
DISTRIBUTION OF FLUORESCENT STAINING IN THE CORTEX						
ANTI IgM	2-6 +++ 8-12 +	N. S.	N. S.	N. S.	N. S.	2-6
ANTI Ig6	2-4 +++ 6-10 +	2-4 ++	N. S.	N. S.	N. S.	12
ANTI Ig6 Fab	4 ++	N. S.	4 ++	N. S.	2-4	4
ANTI L. mex.	N. S.	N. S.	N. S.	N. S.	2-12	2-4
L. mex. Ag.	N. S.	N. S.	2 +	4 +	N. S	N. S.
ANTI C3	N. S.	N. S.	N. S.	N. S.	2-12	2
ANTI LY T1	2-12 ++	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S	N. S.	N. S.
DISTRIBUTION OF FLUORESCENT STAINING IN THE PARACORTEX						
ANTI IgM	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI Ig6	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI Ig6 Fab	N. S.	N. S.	4 +	2-4 +	N. S.	N. S.
ANTI L. mex.	N. S.	N. S.	N. S.	4 +	2-12	N. S.
L. mex. Ag.	N. S.	N. S.	2 +	2 +	4	N. S.
ANTI C3	N. S.	N. S.	8 +	8-12 +	4-6	N. S.
ANTI LY T1	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S.	2-12	N. S.
DISTRIBUTION OF FLUORESCENT STAINING IN THE MEDULLA						
ANTI IgM	2-12 +	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI Ig6	N. S.	4-10 ++	N. S.	N. S.	N. S.	2-6
ANTI Ig6 Fab	8 +	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI L. mex.	N. S.	N. S.	N. S.	N. S.	N. S.	2-12
L. mex. Ag.	N. S.	N. S.	N. S.	N. S.	N. S.	4
ANTI C3	N. S.	N. S.	N. S.	12 +	N. S.	2-16
ANTI LYT1	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.

Figures are weeks after the infection. +, ++, +++ indicate few, moderate or abundant respectively. N. S. indicates no fluorescence detected in the structure. IN. CELL. stands for intracellular. In macrophages fluorescent staining was circumscribed to intracellular amastiogotes.

TABLE 7 C

DISTRIBUTION OF FLUORESCENT STAINING IN THE DRAINING LYMPH NODES OF BALB/c MICE INFECTED WITH Leishmania major

REAGENTS	LYMPHOCYTES	Ab. FORMING CELLS	EOSINOPHILS	MACROPHAGES	IN.CELL. DEPOSITS	SYNUS SYSTEM
			DISTRIBUTION OF FLUORESCENT STAINING IN THE CORTEX			
ANTI IgM	2 6 + 4 ++	N. S.	N. S.	N. S.	N. S.	4
ANTI IgG	2-8 +++ 10-12 +	N. S.	N. S.	N. S.	N. S.	2-6
ANTI IgG Fab	2-4 +++ 8 +	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI L. mex.	N. S.	N. S.	12 +	N. S.	2-8	2-12
L. mex. Ag.	N. S.	N. S.	4-12 +	N. S.	2-8	N. S.
ANTI C3	N. S.	N. S.	N. S.	N. S.	2-8	2-6
ANTI LY T1	2-8 ++	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S	N. S.	N. S.
			DISTRIBUTION OF FLUORESCENT STAINING IN THE PARACORTEX			
ANTI IgM	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI IgG	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI IgG Fab	N. S.	N. S.	2-4 ++	2-4++	N. S.	N. S.
ANTI L. mex.	N. S.	N. S.	12 +	12 +	N. S.	N. S.
L. mex. Ag.	N. S.	N. S.	4-12 +	4-12 +	N. S.	N. S.
ANTI C3	N. S.	N. S.	N. S.	N. S.	4-8	N. S.
ANTI LY T1	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S.	2-12	N. S.
			DISTRIBUTION OF FLUORESCENT STAINING IN THE MEDULLA			
ANTI IgM	2-6 +	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI IgG	6-12 ++	N. S.	N. S.	N. S.	N. S.	N.S.
ANTI IgG Fab	2-4 + 8 ++	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI L. mex.	N. S.	N. S.	12 +	N. S.	N. S.	8
L. mex. Ag.	N. S.	N. S.	4-12 +	4-12 +	N. S.	2-8
ANTI C3	N. S.	N. S.	N. S.	6 +	N. S.	4-8
ANTI LYT1	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.

Figures are weeks after the infection. +, ++, +++ indicate few, moderate or abundant respectively.
N. S. indicates no fluorescence detected in the structure. IN. CELL. stands for intracellular. In macrophages fluorescent staining was circumscribed to intracellular amastiogotes.

TABLE 7 D

DISTRIBUTION OF FLUORESCENT STAINING IN THE DRAINING LYMPH NODES OF BALB/c MICE INFECTED WITH Leishmania mexicana

REAGENTS	LYMPHOCYTES	Ab. FORMING CELLS	EOSINOPHILS	MACROPHAGES	IN.CELL. DEPOSITS	SYNUS SYSTEM
DISTRIBUTION OF FLUORESCENT STAINING IN THE CORTEX						
ANTI IgM	4-6 +++ 8-12 +	N. S.	N. S.	N. S.	N. S.	4-6
ANTI IgG	2-8 +++	N. S.	N. S.	N. S.	N. S.	4
ANTI IgG Fab	2-8 +++	N. S.	N. S.	N. S.	N. S.	4
ANTI L. mex.	N. S.	N. S.	N. S.	N. S.	2-4	2-6
L. mex. Ag.	N. S.	N. S.	6 +	N. S.	6	2-6
ANTI C3	N. S.	N. S.	8 +	8 +	2-8	N. S.
ANTI LY T1	2-8 ++	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S	N. S.	N. S.
DISTRIBUTION OF FLUORESCENT STAINING IN THE PARACORTEX						
ANTI IgM	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI IgG	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI IgG Fab	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI L. mex.	N. S.	N. S.	N. S.	4 +	N. S.	N. S.
L. mex. Ag.	N. S.	N. S.	6 +	6 +	6	N. S.
ANTI C3	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T1	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S.	2-8	N. S.
DISTRIBUTION OF FLUORESCENT STAINING IN THE MEDULLA						
ANTI IgM	2 + 4-6 ++.	N. S.	N. S.	N. S.	N. S.	N. S
ANTI IgG	2-6 ++	N. S.	N. S.	N. S.	N. S.	8
ANTI IgG Fab	2-6 +	N. S.	N. S.	N. S.	N. S.	8
ANTI L. mex.	N. S.	N. S.	N. S.	N. S.	N. S.	2-6
L. mex. Ag.	N. S.	N. S.	N. S.	6 +	N. S.	N.S
ANTI C3	N. S.	N. S.	N. S.	N. S.	N. S.	2-8
ANTI LYT1	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
ANTI LY T2	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.

Figures are weeks after the infection. +, ++, +++ indicate few, moderate or abundant respectively. N. S. indicates no fluorescence detected in the structure. IN. CELL. stands for intracellular. In macrophages fluorescent staining was circumscribed to intracellular amastiogotes.

staining of small lymphocytes, intracytoplasmic staining of large lymphoid cells and linear staining of the lymph node sinus system.

In general, by the second week after infection, scanty small lymphocytes were detected in the follicular areas. By the fourth week of infection, the majority of these cells displayed peripheral fluorescence, remaining like this up to the tenth week of infection in CBA/ca mice infected with *L. major* and up to the sixth to the eighth week of infection in CBA/ca infected with *L. mexicana* , and BALB/c infected with *L. major* or *L. mexicana*. After that the number of stained small lymphocytes decreased. Small stained lymphocytes were also detected in the medulla, but only in low numbers. In the paracortical areas, they were rarely seen. Lymphoid cells exhibiting fluorescence staining were only observed in the follicular and medullary areas of the lymph nodes from CBA/ca mice infected with *L. mexicana* or *L. major*. These cells, which were apparently forming antibody, did not stain with specific antigen as FITC conjugated to *L. mexicana*.

Anti-*L. mexicana* $(F(ab)'_2)$, anti-C3 antisera and FITC-*L. mexicana* resulted in associated intercellular staining in the cortical and paracortical areas of the lymphoid structure (Figs. 15, 16, 17).

This intercellular deposition of staining adopted different patterns. It was seen as:

a- reticular pattern,

b- as a fine or thick intercellular line and

c- outlining groups of lymphocytes. In some areas, these

FIGURE 15

Indirect immunofluorescence on lymph node section from from CBA/ca mouse, 4 weeks infected with *L. major* using the $F(ab)'_2$ fraction of rabbit anti-*L. mexicana* IgG (dilution 1:4). Thick dendritic intercellular pattern is observed in the germinal centre. X 125.

FIGURE 16

Indirect immunofluorescence on lymph node section from BALB/c mouse, 4 weeks infected with *L. mexicana* using the $F(ab)'_2$ fraction of rabbit anti-*L. mexicana* IgG. Note fluorescent staining in the marginal sinus and long intercellular deposits in the follicular area. X 160.

FIGURE 15

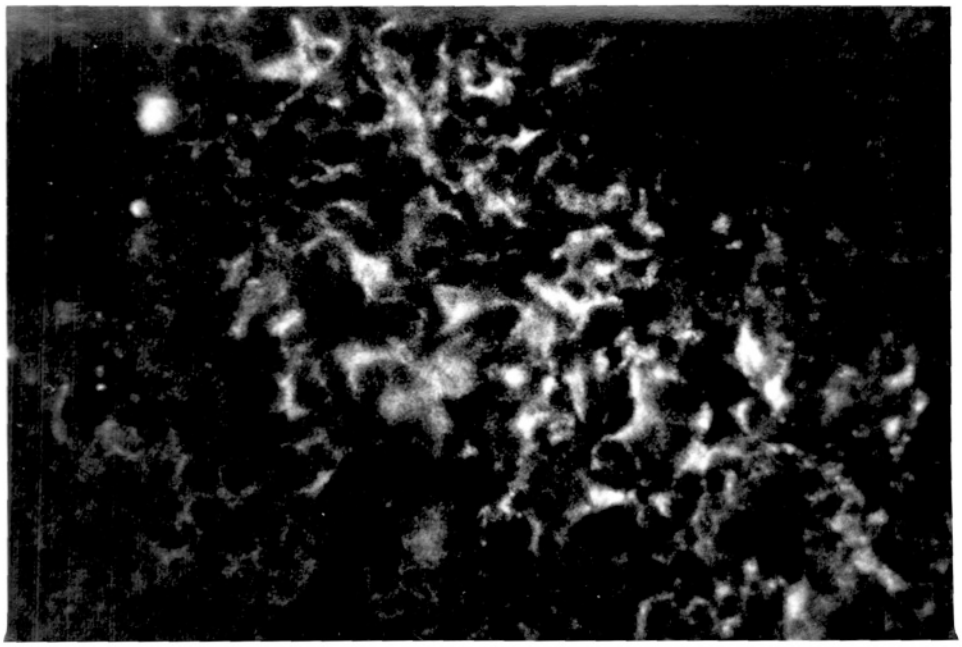
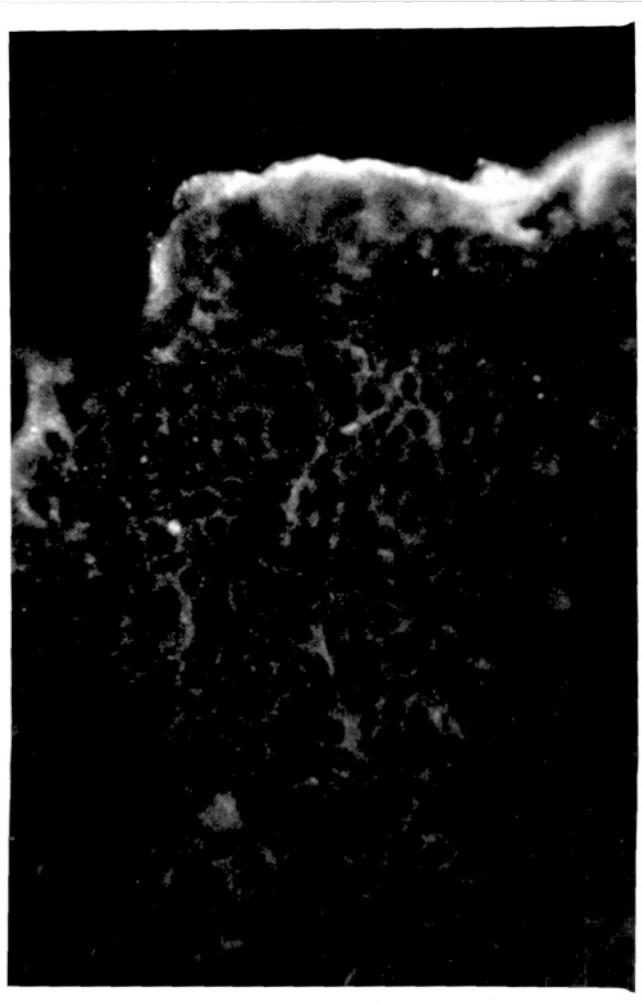


FIGURE 16



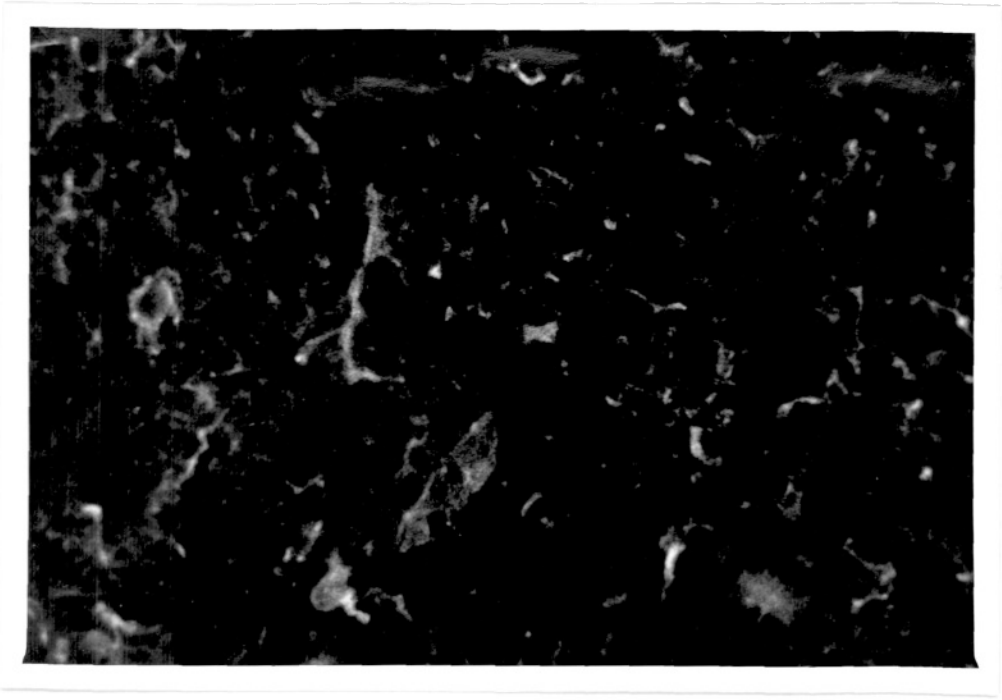


FIGURE. 17

Direct immunofluorescence on lymph node section from CBA/ca mouse, 2 weeks infected with *L. major* using rabbit anti-mouse C3 (dilution 1:40). Note short and large reticular and linear fluorescent patterns of deposition in the paracortical area. X 100.

intercellular deposits were so closely spaced that the lack of staining of the intervening cells could not be ascertained with certainty.

The appearance and duration of these deposits varied little in the different groups studied. In general, they were detected for first time by the second week of infection and lasted up to the twelfth week after infection in CBA/ca mice infected with *L. major* or *L. mexicana* and up to the eighth week in BALB/c mice infected with *L. major* or *L. mexicana*

The sinus system was lined with fluorescence with all the immunoreagents in all the groups (Fig. 16). Frequently the macrophages inside the sinus system were seen to be stained.

Polymorphonuclear eosinophils were stained within their cytoplasm, and amastigotes inside macrophages were observed in paracortical, interfollicular and medullary areas. The eosinophils were usually stained by FITC-*L. mexicana* antigen, anti-IgG and sometimes anti-C3 antisera and the amastigotes by anti-*L. mexicana* antibody, by anti-IgG (Fab) and sometimes by anti-C3 antisera.

Anti-Lyt1 monoclonal antibody resulted in a peripheral staining of lymphocytes situated towards the subcapsular area, in which they produced a band of fluorescence. Anti-Lyt2, on the other hand, produced intercellular staining in the paracortical areas. Staining with these two antibodies was observed to occur from the second week after infection in all the cases and it lasted up to the sixth week of infection in the CBA/ca-*L. major* group and to

the eighth week in BALB/c mice infected with *L. major* or *L. mexicana* and up to the twelfth week after infection in CBA/ca mice infected with *L. mexicana*.

III.3.3 CONTROLS

III.3.3.1 PBS

No autofluorescence was seen when normal or infected sections were treated with PBS only.

III.3.3.2 Blocking and absorption test

After blocking, fluorescence became negative in all the cases.

Absorption experiments removed fluorescence in all cases, except in the case of absorption of FITC-*L. mexicana* antigen by *L. mexicana* antibody, which failed to removed fluorescence from eosinophils in the tissues.

III.3.3.3 Substitution test

In the IIFT,the substitution of unlabeled anti-sera by PBS or normal serum did not produce fluorescent staining.

III.3.3.4 Normal tissues

When normal tissue, skin or lymph node were used as antigen for immunofluorescence reactions, non specific fluorescence was observed in the corneum stratum and hair follicles in the skin and in mast-cells in skin lesions and lymph nodes.

In addition, and as referred to before, anti-*L. mexicana* antibody IgG ($F(ab')_2$) produced staining in the sarcolemma of muscles in normal tissues.

III.4 DISCUSSION

III.4.1 SKIN LESIONS

The results presented in this section demonstrate a correlated presence of antigen, immunoglobulins and C3 factor of complement in the skin structures and cells of the infiltrate in the lesions from mice of the different experimental groups.

III.4.1.1 Skin structures

The fluorescent deposits observed in skin structures were localized in the dermis-epidermis junction, blood vessels and muscles.

Deposits similar to this have been reported in a wide range of dermal pathologies, such as Lupus erythematosus, Bullous dermatoses, Bullus pemphigoid, Dermatitis herpetiforme (BEUTNER et al. 1973,1978); cutaneous candidiasis (SOHNLE & KIRPATRICK, 1976) and Leprosy (QUISMORIO et al. 1975; WALLACH et al. 1979), among other diseases.

The mechanisms and immunological significance of these deposits is unknown, the following possibilities have been proposed:

1.- They may represent immune-complexes depositing on antigen.

2.-They may consist of auto-antibody immune-complexes involving tissue antigen.

3.- They may be the result of a passive deposition of immune-complexes at the various sites.

Generally the nature of these deposits has been characterized by their morphological appearance, thus, granular or lumpy deposits have been attributed to immune-complexes and characteristic of diseases such as Lupus erythematosus, while linear ones have been related to the presence of auto-antibodies and characteristic of such diseases as Bullous phemphigoid, or have been related to the passive deposition of immune components. However, there are many contradictory reports in the literature that suggest that merely the appearance of these deposits is insufficient to characterise them. For instance:

1.- In skin biopsies, obtained from Lupus erythematosus, and studied by immunofluorescence, not only granular (TUFANELLY, 1969; SCHREINER & WOLFF, 1970; BEUTNER et al. 1978), but also linear (JABLONSKA & CHORZELSKY 1974; GROSSMAN et al. 1974) depositions have been described at the dermal-epidermal junction.

2.- Acid eluates from Lupus erythematosus skin biopsies, which by DIFT have shown granular deposits of Ig at the dermal epidermal junction, were employed in the IIFT using frozen sections of various tissues, and it was found that the skin eluates contained Ig fixed in a distinctly linear fashion to the basal membrane of skin and oesophagus (LANDRY & SAMS, 1972).

3.-In spontaneous auto-immune conditions, such as these found in (NZB X NZW)F_1 mice, in some cases the pattern of Ig deposition at the dermal-epidermal junction was granular, in other cases a homogeneous and more linear pattern was observed in other animals from the same group (GILLIAM et al. 1975).

4. UEKI (1977) has found, in several cases of typical Bullous phemphigoid a granular pattern of fluorescence instead of the typical linear one.

Several factors could be responsible for differences in the immunofluorescence patterns. In this study, it was found that the appearance of fluorescent deposits at the dermal-epidermal-junction seemed to vary with the clinical form of the disease, the stage of development of the the infection and the antisera used. Thus, while in mice exhibiting self-healing lesions (CBA/ca mice infected with *L. major*), the pattern of fluorescence at the dermal epidermal-junction was discontinuous, or continuous and linear, in mice exhibiting non-healing lesions (CBA/ca and BALB/c mice infected with *L. mexicana* and BALB/c mice infected with *L. major*), the pattern changed from homogeneous linear to granular with the progress of the infection. Similar observations have been reported by TUFANELLY et al. (1969) and JABLONSKA & CHORZELSKI (1974) in Lupus erythematosus. In these cases, the authors reported changes in the fluorescent pattern in the dermal-epidermal junction in relation to structural changes and the chronicity of the disease.

The pattern of staining also varied with the antisera

used. Different anti-sera resulted in a different pattern of staining in the same tissue at the same time of infection, which could be related to the different composition and concentration of antibodies and antigens in the different immune reagents or in the lesions (JABLONSKA & CHORZELSKI, 1974). In a comparative study of immunofluorescent deposits in various dermatoses, CORMANE et al. (1970) observed that the pattern of deposition varied, not only with the characteristics of the anti-sera and antigen used, but also with the illumination conditions. While with transmitted illumination, relative coarse grained deposits were detected in the dermal epidermal-junction and blood vessels, incident illumination showed more homogeneous deposits in the same areas.

The specific staining observed whith anti- *L. mexicana* serum at the dermis epidermis-junction, and other structures in which similar depositions were observed (blood vessels and muscles), could be explained by the presence of parasite derivatives in these structures, or by a cross-reaction between *Leishmania* and tissue antigens. The latter possibility seems unlikely in the case of the dermal-epidermal junction and blood vessel staining because the specific *L. mexicana* anti-sera did not stain these structures in normal skin. The exception is the muscular structures, where the specific antiserum did stain normal tissue. Muscle will be considered later in this discussion.

Discussing the possibility of a passive deposition of immune components in the skin structures, UEKI (1977)

stated that an homogeneous localization of immunoglobulins at the dermis-epidermis junction and also in the perivascular spaces suggested a non-specific deposit of serum-globulins, which could precipitate non-specifically in the vascular walls or the perivascular spaces through damage to the blood vessels with consequent leakage and at dermis-epidermis junction as a result of a "backwater" phenomenon. However, the fact that the the basal lamina is permeable to a variety of proteins and non-protein tracers (SCHREINER & WOLFF, 1970) which can therefore extend beyond the basal lamina, makes this possibility unlikely and suggests that selective binding must occur in this area.

Regarding the deposition of immune components in blood vessels, in this study I have reported the presence of deposits of Ag, Ab and C3 complement factor in the intima of small blood vessels and also in the intima, media and adventitia of arterioles. The pattern of fluorescence was homogeneous and linear most of the time. Such deposits in blood vessels have been found in most cases to be coincident with deposits in other skin structures such as the dermal-epidermal junction in Lupus erythematosus (TUFANELLY et al.1969; Leprosy (WALLACH et al. 1979) and in Pemphigus vulgaris (CORMANE et al. 1970). Obviously, immunological reactants leave the blood stream through the walls of the blood vessels of the dermis, but before they pass into the tissue, damage to the blood vessels walls can cause them to become trapped and subsequently detectable by immunofluorescence.

On the other hand, detailed immunofluorescent studies on

blood vessel changes associated with the presence of immunoglobulin and complement in Purpura pigmentosa chronica, (IWATSUKI et al. 1980) and in Lupus erythematosus (IGARASHI et al. 1981) has suggested the possibility that immunological processes were involved in producing the histopathological alterations observed in blood vessels. SCOTT & ROWELL (1965) pointed to a relationship between the accumulation of immunoglobulins in cutaneous blood vessel walls and cellular infiltration of the perivascular area. The authors found that deposition of Ig was frequent in the vascular lesions in various skin conditions where marked changes in tissues occured, though less frequently when tissue changes were of a mild intensity. In the histopathological study reported in this thesis (Chapter II), damage to blood vessels increased with the progress of the infection. Furthermore, the cellular infiltrate adopted a perivascular distribution, and polymorphonuclear eosinophils were abundant in it. This finding suggests an association between the immune deposits, infiltrating cells and damage of the blood vessels. There is experimental evidence that a major role is played by the leukocyte as well as by antigen antibody complexes and complement (WARD & COCHRANE, 1965) in the development of vasculitis. The mechanisms proposed are as follows: leukocytes are induced to phagocytose immune complexes in the intima of the blood vessels, during that process they release enzymes causing tissue damage including the damage to vessels' walls.

In conclusion, the nature of the immune deposits observed in the dermal-epidermal junction and in the blood

vessels is not completely apparent. The evidence presented above indicates that the pattern of deposition of fluorescing material is not an adequate guide for determining the nature of these deposits. However, the association of *Leishmania* antigen, antibodies and C3 factor of the complement at the same time span and in the same structure strongly suggests the presence of immune-complexes at this level. More work needs to be done in order to elucidate the nature and the cause of these deposits. Experiments on elution of the immunological components from the skin structures and subsequent analysis of them should be done.

The origin of the possible immune-complexes cannot be deduced from the results reported in this study. An investigation of circulating immune-complexes and searching for them in other tissues would be of interest not only to establish their origin (from the circulation or locally formed), but also in evaluating their participation in the various mechanisms of suppression of the immune response.

Immune-complexes have been reported in visceral leishmaniasis (DE BRITO et al. 1975 and KHARAZMI et al. 1982) but not in cutaneous leishmaniasis even though it has been suggested that immune-complexes could block the expression of anti-parasite effector mechanisms in murine cutaneous leishmaniasis (ALEXANDER & PHILLIPS, 1978).

In muscles, fluorescent deposits corresponding to antigen, IgG and C3 factor were detected. The pattern of deposition of the fluorescent staining was homogeneous, linear and surrounded the sarcolemma.

Surprisingly, while immunoglobulin deposits were detected by anti- IgG (Fc) FITC, the Fab fraction of anti-IgG FITC failed consistently to produce staining at this level. Experiments of AARLI & TONDER (1974) suggest an explanation for this fact. These authors, in an *in vitro* system, demonstrated that IgG binds to muscular structures only by the Fc fragment.

Muscles from infected and uninfected skin were stained by the rabbit F(ab)'$_2$ fraction of IgG *L. mexicana* in the indirect immunofluorescence technique. The fact that normal muscular structures were also stained by this anti-serum suggests a cross-reaction between *Leishmania* antigen and tissues structures rather, than the presence of *Leishmania* antigen itself on these structure.

The specificity of the staining was corroborated by the ablation of the staining by prior absorption of the specific antisera (rabbit F(ab)'$_2$ fraction of IgG *L. mexicana* antibody) with *L. mexicana* promastigotes. The possible interference of the heterologous antibody used as a second layer in the IIFT or the presence of natural antibody to muscles in normal rabbit serum was ruled out by the absence of staining when PBS or normal serum was substituted for the specific antiserum in the middle layer.

In this study, also, an antibody reacting with skeletal and cardiac muscle of normal and *Leishmania* infected mice was found in the sera of *L.major* and *L. mexicana*

infected mice. While the pattern of the fluorescent staining in skeletal muscles was similar to that previously described in muscles of skin lesions, the heart exhibited staining in the interstitium of the muscle, endocardium, pericardium and vessels. A similar interaction between micro-organisms and tissue structures has been suggested in Rheumatic fever, between *Streptococcus* and cardiac mucle (KAPLAN & MEYESERIAN, 1962; ZABRISKIE et al. 1966) and between *Trypanosoma cruzi* and heart in Chagas disease (COSSIO et al. 1974).

COSSIO and coworkers (1974), using immunofluorescence techniques demonstrated in human Chagas' disease a serum gamma globulin element which reacted with endocardium, vascular structures and the interstitium of striated muscle in the heart (EVI antibody). This antibody was present in 95 % of patients with Chagas' cardiopathy and only in 45 % of asymptomatic patients; it has not been detected in normal controls or in a number of sera from patients with different cardiopathies or auto-immune diseases. Ultrastructural immunochemical methods demonstrated that reactions were occurring in the plasma membrane of heart and skeletal muscle fibres as well as of endothelial cells of blood vessels. Absorption of the positive sera with *Trypanosoma cruzi* epimastigotes abolished the reaction. The EVI antibody is probably the expression of antigens cross-reacting between *T. cruzi* and some tissues of the vertebrate (COSSIO et al. 1974).

In leishmaniasis, SZARFMAN et al.(1975) demonstrated fluorescent anti-skeletal muscular antibody in three sera

out of eight from patients suffering from Kala-azar and in one other serum which was only positive with cardiac structures. The sera reacting with skeletal muscle produced a linear pattern on the sarcolemma while the serum reacting with cardiac structures stained endocardium, vessels and interstitium (EVI factor).

The same authors, after the performance of an antisera tissue absorption test, demonstrated that EVI factor and anti-skeletal muscle antibody were different. As a possible explanation for the reaction of *Leishmania* antibody with muscular structures, SZARFMAN et al. (1975) proposed: a.- cross-reaction of *Leishmania* antibodies with host structures, or b.- the presence of anti-muscular antibodies which are formed in response to antigen liberation from the damaged tissues. However, it is of note that anti-*Leishmania mexicana* antibody, raised by immunization of rabbit with *Leishmania* antigen, rather than infection, produced similar deposits in the muscles of *Leishmania* lesions in mice, suggests the presence of a cross reacting antibody rather than auto-antibodies reacting with muscles in the mice sera.

Hyaline collections, thought to be the product of degeneration of muscular structures (see Chapter II), were sometimes found to be fluorescent with the same antisera as reacted with muscles.

III.4.1.2 Cells of the infiltrate

Regarding the fluorescent staining of cells of the infiltrate, I shall first discuss the staining of macrophages and reticular shaped cells and then the staining of polymorphonuclear eosinophils. For the reasons explained in the results, lymphocytes will be the subject of a separate section.

Macrophages and reticular shaped cells stained with anti-Ig (G and M), anti C3 antisera and by the specific reagents to *Leishmania* (anti-*L.mexicana* IgG $(F(ab)'_2)$ and FITC-*L.mexicana* antigen) in the lesions resulting from the infection of BALB/c mice with *L. major* and from the infection of CBA/ca mice with either species of parasite. In BALB/C mice infected with *L. mexicana*, macrophages were stained by all the immunoreagents except by *L. mexicana* antibody, while reticular shaped cells were not stained by any of the immunoreagents.

The staining of reticular shaped cells and macrophages with the $F(ab)'_2$ fraction of the anti- *L. mexicana* IgG indicated the presence of *Leishmania* antigen on the surface of these cells. The concomitant staining with anti-Ig (M and G) and anti-C3 suggests the possibility that the antigen is presented already complexed as an antigen-antibody-complement complex. The alternative possibility that immunoglobulins attached to Fc receptors can be ruled out in the case of anti-*L. mexicana* antibody and anti-IgG antiserum, because of the use of

immunoglobulins devoid of the Fc fraction. In the case of anti-C3 and anti-IgM antisera, however, such a possibility could not be discarded as whole immunoglobulin molecules were used.

The nature of what I have called reticular shaped cells cannot be established with certainty. However, they resemble Langerhans cells in:

1.- their morphology and distribution within the skin.

2 - their positive reaction to the non-specific ester ase test.

3 - Their ability to present antigens.

During the initial stages of infection, non-parasitized or slightly parasitized macrophages were detected presenting antigens. As the infection progressed, in the infection models whose lesions healed macrophages eventually disappeared from the lesions. In all the cases where lesions did not heal, a predominance of heavily parasitized monovacuolated macrophages, consistently negative to the staining with all the immune-reactants, was observed. Only in the periphery of the lesion a few non-parasitized or lightly parasitized macrophages were detected fluorescing.

This would seem to indicate that the large heavily parasitized macrophages have lost their ability to present antigen and were probably deprived of their Fc and C3 receptors. A comparable relationship between parasitic load and detection of macrophages' Fc and C3 receptors has been reported by RIDLEY et al. (1978) in leprosy. These authors found that the macrophages constituting the granuloma of

active border-line lepromatous Leprosy (BL-LL), which had not phagocytosed *Mycobacterium*, had Fc and C3 receptors and were, at this stage, indistinguishable from those of border-line tuberculoid Leprosy (BT). By contrast, the macrophages of the same granuloma which had phagocytosed *Mycobacterium leprae*, had lost their C3 receptors and experienced a reduction of Fc sites. Finally, in the heavily parasitized macrophages in the foamy cell stage, no receptor sites were found.

The association of the loss of the ability to present antigen, or the loss of receptors sites on macrophage surfaces (considering that antigens may be presented in a complexed Ag-Ab-C form) with the increase of the parasitic load in macrophages, could be the result of the evolution of the infection in an homogeneous population of macrophages, or as recent evidence would suggest, the consequence of the existence of different populations of these cells with different surface markers and susceptibility to infection.

GORCZYNSKI & MACRAE (1982) demonstrated that macrophages from the skin of BALB/c and CBA mice could be separated into two populations according to their sedimentation velocity. Furthermore, at least in one mice strain, the two populations differed in their ability to support *L. major* growth. Large cells (peak sedimentation velocity 10 to 14 mm/hour) from both strains of mice allowed an early proliferation of parasites, followed by a lessening of their replication.

Smaller cells (peak sedimentation velocity 5 to 8

mm/hour) of the BALB/c strain supported a prolonged and increasing parasitic load as the infection progressed. The smaller cells from CBA mice, however, behaved in the same way as the large cells.

It is interesting to note that macrophages from resistant (CBA) and susceptible (BALB/c) mice differed not only in their ability to support parasite growth, but also in their ability to present antigen, as revealed by the fact that only *L. major* infected cells from CBA mice were able to act as accesory cells in stimulating proliferation of sensitized lymphocytes from BALB/c, CBA and (BALB/c x CBA)F1 origin. In conclusion, the observations of GORCZYNSKI & MACRAE (1982) would seem to support the possibility that the differences observed in this study, with reference to the distribution of immunofluorescent staining between macrophages of the same histiocytoma observed in mice exhibiting non-healing lesions, could reflect the existence of different populations of macrophages.

As mentioned before, in BALB/c mice infected with *L. mexicana*, cells bearing antigen were not detected. However, macrophages in this experimental group did stain with anti-IgG, anti-C3 and FITC-*L. mexicana* antigen. The distribution of fluorescent macrophages was similar to that observed for macrophages in the rest of the experimental groups exhibiting non-healing lesions.

The failure to detect antigen on cells of the BALB/c mice infected with *L. mexicana* could be due to the presence of only small amounts of antigen that could not be

detected by the system used, or to a real lack of the antigen due to a defect in presentation.

The results reported in this study confirm *in vivo* those obtained *in vitro* by HANDMAN et al. (1979) and BERMAN & DWYER (1981) showing, by means of the immunofluorescence technique, that *Leishmania* antigen can be expressed on macrophage surfaces. Furthemore, the results also agree with those of HANDMAN and coworkers in that they showed that *L. major* antigen could be found on the surface of macrophages from strains of mice exhibiting different susceptibilities to the parasite (BALB/c susceptible, CBA resistant). HANDMAN and coworkers (1979) also reported that BALB/c infected macrophages, and not CBA infected macrophages, were unable to sensitize syngeneic recipients for a delayed type hypersensitivity to *L. major* antigen, which was in turn associated with a diminished expression of H-2 exo-antigens on BALB/c macrophages. This, however, has been recently challenged by MAUEL's co-workers (personal communication).

It is of note that, in this study the antigen was detected on the surface of uninfected macrophages as well as on macrophages exhibiting a low parasitic load. The mechanisms by which the antigen (s) becomes incorporated on the macrophage surface are unknown. However, it has been proposed that the antigen could be incorporated into the phagolysosomal membrane with subsequent fusion of that membrane with the macrophage surface membrane (DWYER, 1978), or alternatively the antigen could be excreted into the extracellular fluid and then absorbed alone, or as part

of immune-complexes onto the macrophage surface (HANDMAN et al. 1979).

Amastigotes, free or inside macrophages, were detected by staining with anti-*L. mexicana* IgG $(F(ab)'_2$, anti-IgG and anti-C3 antisera. The fluorescent parasites showed a similar distribution in the lesion as stained macrophages and areas of necrosis. Sometimes, the stained amastigotes were observed inside macrophages presenting surface staining. Amastigotes inside monovacuolated heavily parasitized macrophages were never stained. I cannot offer a suitable explanation for the differential staining of intracellular amastigotes observed in these study. RADWANSKY et al. (1974) reported no staining of intracellular amastigotes by specific anti-serum when using the immunofluorescent technique on frozen sections from lesions produce by *L. enriettii* in guinea pigs. These authors offered, as a possible explanation, the inability of the immunoreactant to penetrate inside the cells. However, in cut cryostat section at least some of the amastigotes should be cut and therefore exposed to the immune-reagents in question.

Polymorphonuclear eosinophils were particularly abundant in skin lesions from mice exhibiting non-healing lesions. Their cytoplasm was stained by FITC- *L.mexicana* and anti-IgG in all the experimental groups and also by anti-*L. mexicana* antibody in BALB/C mice infected with *L. major* and by anti-C3 antisera in BALB/c mice infected with *L. mexicana*.

As is well known, non-specific staining of eosinophil

granulocytes by FITC conjugates has represented a problem in the interpretation of immunofluorescence since LACHMANN (1964) reported it. The non-specific staining of these cells by FITC conjugates could be explained by antigen cross-reactivity between the conjugate and cell components, or by physico-chemical adsorption of the conjugates into cellular components, not based on an immune reaction (BERRETY & CORMANE, 1979).

In this study, the staining of tissue eosinophils not related to *Leishmania* infections (produced by inoculating uninfected mice with bovine serum albumin or starch) suggests the non-specificity of the staining by the conjugates. However, the pretreatment of sections containing eosinophils from *Leishmania* infected mice and from bovine serum albumin or starch inoculated mice with SDS before the application of the conjugate, resulted in the ablation of the staining in eosinophils produced by inoculation with bovine sera albumin or starch, but not in those elicited as a consequence of *Leishmania* infections. This result suggests that, despite the fact that the conjugates stained non-specifically elicited eosinophils, in *Leishmania* infections at least part of the fluorescence exhibited by eosinophils is specific.

The specificity of the staining by FITC *L. mexicana* antigen was shown by the blockage of the staining when the sections were treated with unlabelled antigen prior to treatment with the conjugate. Paradoxically, absorption of FITC- *L. mexicana* antigen with anti-*Leishmania* antisera did not eliminate the

staining capacity of the conjugate. This might be explained by the presence of low affinity antibodies in the serum, or by different concentrations of antigen and antibodies in the mixture that still allowed an excess of the conjugate.

Finally, in order to help in the identification of any other cytoplasm staining cells (antibody forming cells) that could be masked by the intensely stained eosinophils, inhibition of the staining of these cells was carried out by treatment of the sections with DAB/H_2O_2 before the application of the conjugate. As a result,

a.- eosinophils were completely sheltered from fluorescence,

b.- scanty plasma cells were only detected in CBA/ca mice infected with *L. mexicana* and

c.- Monocytes, immature macrophages and reticular shaped cells were stained on their surfaces.

It is important to note that the blocking of staining by this method is incompatible with attempts to demostrate immune components inside cells containing endogenous peroxidase because the deposits of DAB interfere with specific immunofluorescence, even when the antigenic sites are located in molecules other than those initiating the DBA reaction in the same cell (VALNES & BRANDZAEG, 1981). Thus, by this method, the possibility of specific staining in the eosinophils cannot be ruled out. The presence of fluorescence in cells other than eosinophils, but containing peroxidase in their cytoplasm (monocytes,

immature macrophages and reticular shaped cells), is explained by the fact that fluorescence was observed on cell membranes and not in the cytoplasm of these cells.

In conclusion, even when it is obvious that FITC conjugates attached non-specifically to eosinophils, the SDS and blocking test controls suggest the presence of antibody specific to *Leishmania* inside eosinophils. This antibody could be a component of immune-complexes, as C3 was also identified in eosinophils from BALB/c mice infected with *L. mexicana* and specific antigen was found in eosinophils from BALB/c mice infected with *L. major*. However, the alternative possibility of a cross-reaction between the conjugates and eosinophils cellular antigen was not ruled out by these experiments.

III.4.2 LYMPH NODES

The immunofluorescence studies of draining lymph nodes from the different experimental groups at different periods of time after infection showed that in general, after the treatment of the tissue sections with FITC *L. mexicana* antigen, $F(ab)'_2$ fraction of anti-*L. mexicana* IgG and anti-C3 serum, an intercellular deposition of staining in the cortical and paracortical areas could be seen . Anti-IgG and anti-IgM sera resulted in staining of the lymphocyte surfaces and the antibody forming cells in the cortical and medullar areas. The sinus system was also stained with all the immunoreagents.

The distribution of antigen (Ag) and immunoglobulins (Ig) in lymphoid structures has been demonstrated using

immunofluorescence. It was noted as early as 1950, by KAPLAN and coworkers, that Ag could localize in lymphoid follicles. In 1962, MELLORS & BRZORSKO reported that injected immune-complexes were trapped in germinal centers of lymphoid organs of immune animals. Thereafter, WHITE (1963) and WHITE et al. (1967) described a characteristic reticular or dendritic pattern of fluorescence staining of antigen (Ag) in lymphoid follicles following injection of the Ag.

An improvement in the detection of this sort of deposit was introduced by NOSSAL & ADA (reviewed by NOSSAL & ADA, 1971) when they used autoradiography techniques which showed a higher sensitivity than immunofluorescence.

Autoradiography studies of Ag draining lymph nodes in the mouse (BALFOUR & HUMPHREY, 1967; TEW & MANDEL, 1978,1979,) and the rat (NOSSAL & ADA, 1971) showed that, in immunized animals, the radioactive Ag was localized in the follicles a short time after challenge. By contrast, in non-immunized mice, much of the radioactivity was in the non-follicullar regions (subcapsular and medullary sinuses) (BALFOUR & HUMPHREY, 1967; TEW & MANDEL, 1979; TEW et al. 1980). However, seven days after the injection of the antigen, when antibody (Ab) was first detected, the antigen began to localize in the germinal centers as well (BALFOUR & HUMPHREY, 1967).

The combination of autoradiography and immunofluorescence technique for the sudy of Ig distribution used by BALFOUR & HUMPHREY (1967) showed that,

in primed animals, an association in the localization of antigen and immunoglobulins in the cortex occured, suggesting that the cortical localization of the antigen during the secondary response is associated with the presence of preformed antibody. However, the authors did not find specific antibody in the same situation, and this was interpreted as being due to low levels of antibody, or to the relative insensitivity of the method.

The electron microscopic studies of PERNIS (1967) and HOEFSMIT (1975) in germinal centers, revealed the presence of non-lymphoid cells with long interlacing cell processes separating the lymphoid cells. The cells concerned were also differentiated from macrophages at the fine structural level (HOEFSMIT, 1975). The association of autoradiography and electron microscopy (NOSSAL et al. 1968; CARR, 1975; MANDEL et al. 1980) demonstrated an association of the labelled antigen with the cytoplasmic processess of dendritic reticular cells.

NOSSAL & ADA (1971) proposed two possible explanations for the presence of Ig in relation with Ag localized in dendritic reticular cells.

1- The globulins localized in these cells because they contain antibody which could react with Ag which was already present in the follicle, or

2.-because there was a reaction between the reticular cell membrane and the globulin molecule. The evidence seems to support the second possibility. In fact, the presence of antibody either natural or specific is a requirement for the localization of the Ag (NOSSAL & ADA, 1971; TEW et al.

1980) but it is also essential that intact antibody be present at that site. Removal of the Fc fragment from immunoglobulins (NOSSAL & ADA, 1971; KLAUS, 1978) diminished or abrogates Ag retention. On the other hand, Fc, but not Fab, fragments isolated from syngeneic or xenogeneic IgG preparation, became attached to dendritic cells, indicating that an IgG molecule could attach to the cell wall by the Fc portion of the molecule, leaving the Fab portion free to bind Ag. These experiments suggest the presence of Fc receptors on these cells.

It has been shown by PHIPPS et al. (1980), and EMBLING et al.(1978) that Ig classes and sub-classes differ in their ability to mediate Ag retention, which may be related to their different complement binding capacity (MANDEL et al. 1980). Complement and antibody-dependent Ag retention is consistent with the results of PAPAMICHAEL et al. (1975) who showed that localization of aggregated IgG in spleen lymphoid follicles was C3 dependent. KLAUS & HUMPHREY (1977) reported that levan and DNP-levan localized in germinal centers within hours of inoculation, and before antibody production had occured. This may also be the case for polymerized bacterial flagellin (NOSSAL & ADA, 1971). As these substances themselves activate complement, it is possible that C3 fixation is the only requirement for germinal center localization.

EMBLING et al. (1978) studied the capacity of non-heat-aggregated monoclonal human Ig of different classes to localize in murine splenic germinal centers within 24 hours after intravenous injection. The results

showed that at least trimerization of IgG must occur before any germinal centre trapping is manifest. Studies of complement fixation by these IgG preparation *in vitro*, together with studies of the germinal center trapping of various monoclonal Igs, have indicated that the sole structural requirement for germinal center localization of Ig aggregates is the ability to fix complement. However, the authors failed to demonstrate fixed C3 concomitant with heated aggregated IgG in a dendritic pattern in mouse spleen germinal centers by immunofluorescence. GAJI-PECKZALSKA et al. (1969), however, did find C3 in the same distribution as immunoglobulin in human and rabbit lymph nodes.

It is of note that, in this thesis, C3 was localized in a dendritic pattern concomitant with *Leismania* antigen and antibody.

In relation to the mode of entry of the antigen into the lymphoid tissue, and its contact with the follicular dendritic cells (FDC), various mechanisms have been postulated:

1. soluble circulating antigen, either alone or already complexed with antibody, is trapped as it percolates through the lymph node and splenic follicles (TEW & MANDEL, 1978).

2. Antigen may be carried to the FDC on other cells and is then deposited on the FDC. It has been suggested that Ag is carried into the spleen attached to appropriate Ig receptors on the surface of B cells (VAN ROOIJEN, 1973). It is also possible that antigen attaches to the surface of

macrophages or macrophage like cells. It is believed that macrophages can retain a small portion of Ag on their surface while at the same time ingesting and degrading the majority of Ag they encounter (UNANUE et al. 1969).
3. A third possibility is that Ag is carried into follicles by cells, which are not classical macrophages, such as Langerhans cells, which have been shown to possess Fc and C3 receptors (STINGL et al. 1977 and SOUTHEIMER & GILLIAM, 1981) by which means immune-complexes could be attached to their surfaces. These cells were seen in the afferent lymph (veil cells) and in the subcapsular sinuses of nodes draining the dermal challenge site in the guinea pig (SILBERBERG-SINAKING et al. 1976). However, once they enter the node, these cells apparently migrate to the paracortex (interdigitating cells), (SILBERBEG-SINAKIN et al. 1976), a site were Ag binding FDC is not found. However, in this thesis, I have described similar intercellular deposits in paracortical as in cortical areas. The possibility that these deposits are associated with Langerhans-like cells or interdigitating cells as they are called at this level (VAN FURTTH, 1980),is not unlikely and is supported by the detection in skin lesions of reticular shaped cells which were found to be fluorescent when stained by the same immune reagents as were the deposits in lymph nodes. The presence of these cells in the paracortical area could be involved in an interaction between presented Ag and T cells at this level.

The nature and origin of the FDC has not been completely elucidated. These cells are present in nodes from passively

immunized mice (NOSSAL & ADA 1971; MANDEL et al.1980) but, in contrast with actively immunized mice they show only poorly developed processes (MANDEL et al. 1980). In athymic nude mice these cells have also shown to have the capacity to bind Ag (TEW et al. 1979) showing that their presence is independent of T cells.

MANDEL et al. (1980) suggested that it is possible that FDC shows hypertrophy when the nodes are stimulated and develop their processess *in situ*, but it is possible that, while FDC may proliferate locally, they may instead develop from precursors which enter the node from the peripheral tissues for instance as Langerhans cell (SILBERBERG-SINAKIN et al. 1976). Langerhans cells, however, differ from FDC as they tend to localize in the paracortex only and in the presence of Birbeck granules and intracytoplasmic fibrills not found in FDC (MANDEL et al. 1980).

Another cell candidate for the role of antigen retention is the dendritic cell described in the spleen by STEINMAN and colleagues (1973, 1979). These dendritic cells share some of the features of the Ag binding cells but, lack Fc receptors and therefore are unable to bind immune-complexes. FDCs also differ from macrophages in their inability to phagocytose (MANDEL et al. 1980) and in their morphology at the fine structural level (HOEFSMIT, 1975).

Regarding the persistence of Ag in the spleen and the draining lymph node of immunized animals TEW et al. (1980), found that Ag can persist for months or years on the

surface of dendritic cells. These data, and a variety of studies on *in vitro* model systems by the same authors have made it possible to propose a feed-back hypothesis concerning the participation of Ag attached to dendritic cells in the mantainance of the humoral response over time. A multiple dynamic equilibrium was postulated to exist between persisting Ag, free specific antibody and Ag-Ab complexes at various ratios in the lymphoid organs. The immunogenicity of the complexes is directly related to the Ag-Ab ratio, and alterations in serum antibody levels result in the formation or dissociation of these complexes. When Ab levels in the circulation decline, Ag determinants are exposed, memory B cells are stimulated and a new cycle of Ab synthesis is induced. Then, the newly produced Ab feeds-back into the system, it inactivates the exposed determinants and terminates the immunogenic stimulus.

In relation to the fluorescent staining observed lining marginal and medullar sinuses, in this thesis, it could be associated with staining of macrophage lining cells of the medullar and marginal cords. MANDEL et al. (1980), made autoradiography studies on lymph node thin sections (1 μm) and demonstrated that most of the labelled antigen was associated with sinus lining cells presumably macrophages. The follicle associated-label could not be localized to any particular cell type by light microscopy. The participation of stained antigenic determinants with clusters of histiocytes invading lymphoid structures seen in conventionally stained sections (ChapterII) could not be ascertained from the fluorescent sections.

In this study, it was also found that small lymphocytes were surface stained by anti-IgG and anti-IgM sera in follicular areas. Large lymphocytes were also stained in their cytoplasm by these antisera in follicular and medullary areas. However, there was no correspondence between the number of pyroninophilic cells detected by routine histology and these cells seen to be stained by immunofluorescence. Furthermore, in BALB/c mice infected with *L. major* or *L. mexicana*, Ab forming cells were not detected at all by the immunofluorescence technique. This fact may be explained by immaturity of these cells which therefore did not contain detectable Ab (WHITE, 1963). This in turn suggests the possibility that the Ab involved in the intercellular staining came from somewhere else. This suggestion may find support in the experiments of PERNIS (1967), which adduce evidence to indicate that at least part of the immunoglobulin found in the germinal centers is not synthesized locally but is bound there secondarily. Interestingly, Ab specific to *Leishmania* as demonstrated by FITC-*L. mexicana* Ag was found in intercellular deposits, but not in correspondence with IgG or IgM detected in the cytoplasm of large cells. Only in one case, intercellular deposits were demonstrated by anti-sera to Ig, and that was in sections from CBA/ca mice infected with *L. mexicana* treated with anti-IgG (Fab) antisera. After reviewing the literature, it seems clear that the intercellular deposits observed in paracortical and cortical areas are associated

with cells of dendritic appearance (follicular dendritic cells and interdigitating cells), which are known to exist at these levels and have been associated with complexes of antigen, antibody and complement. The staining in the sinus system on the other hand, seems to be associated with the macrophages found in these areas.

Anti-Lyt1 and anti-Lyt2 monoclonal antibodies stained lymph node sections in unexpected but consistent patterns. Anti-Lyt1 was localized as a fluorescent band in the subcapsular area, and apparently the staining was surrounding lymphocytes. Anti-Lyt2, on the other hand, produced an intercellular fine dendritic pattern in the paracortical area.

III.4.3 Remarks

In general, in the immunofluorescence studies carried out on skin lesions and lymph nodes, it was found that, most of the time, antigen, antibody and C3 factor coincided in the same structures at the same time spans of infection. However, on occasion, only one or two of the immunecomponents were detected, in absence of the others. This could be explained by:

1.- A real deficiency of the particular immune-reagent content at the site of investigation.

2.- Different equivalent concentrations of the immune components in the tissues.

3.- Low antisera strength.

4.- Because the threshold of sensitivity of the system was too high to reveal these components in every situation. For instance, complement seems to be more readily detected

by immunofluorescence than antigen or antibody (SOHNLE & KIRPATRICK 1976).

It is noteworthy that FITC *L. mexicana* antigen failed to recognise specific antibody on certain skin structures when it did on many other cell surfaces. It is possible that the antibodies found at these levels are different, and are produced against antigenic determinants which may not be present in the FITC *L.mexicana* antigen used in this study, which in turn could be due to: a.- the conjugate antigen was prepared using only the soluble portion of the antigen, b.-the antigen was prepared from a different stage of the parasite (promastigote) to that (amastigote) causing the intracellular infection and even though both forms of the parasite apparently share most antigenic determinants, there is evidence that some antigens are unique to each form. Absorption of serum from *L. donovani* infected hamster with *L. donovani* promastigotes reduced, but did not eliminate, binding of the serum to homologous amastigotes (DWYER, 1976). Differences in immunocytochemical staining, between amastigotes and promastigotes, have been reported by DWYER et al. (1974). HANDMAN & CURTIS (1981) reported in *L.major* the existence of two polypeptides; one of them (Mw of 69000 d), is common to amastigotes and promastigotes of the parasite and is recognised by antibodies raised against amastigotes and promastigotes. The other polypeptide (Mw 94000 d), was detected in amastigotes, but not in promastigotes and was recognised by antibodies

against amastigotes only.

HUNTER & COOK (1983) reported antigenic similarities and differences between promastigotes and amastigotes of *L. mexicana*, demonstrated by crossed immuno-electrophoresis analysis using hyperimmune rabbit antisera to both forms of the parasite.

III.4.4 Conclusions:

By the use of the immunofluorescence system outlined above, regular deposits of antigen, antibody and complement were detected along skin structures (dermal-epidermal junction, blood vessels and muscles), cells of the infiltrate in cutaneous lesions and in cells of the lymph nodes draining the skin lesion in all the experimental groups studied.

The nature and immunological significance of these deposits in skin structures, such as the dermis-epidermis junction and the blood vessels is not clear, but the association of antigen, antibody and C3 factor in these areas suggests they are immune complexes. Similar deposits along muscular structures and the demonstration of antimuscular antibodies in *Leishmania* infected mice reacting with muscular structures from both normal and *Leishmania* infected mice suggests a cross-reaction between *Leishmania* antigens and host tissues, and/or the presence of an auto -antibody to muscle.

Macrophages and reticular shaped cells in skin lesions were detected presenting antigen (except in BALB/c mice infected with *L. mexicana*) which was indicated by the positive staining with anti-*L. mexicana* antibody

$(F(ab)'_2$ fragment of IgG). The presence of antibody and C3 factor in the same areas suggests that the antigen is perhaps even presented in a complexed form. The nature of the so called reticular cells is not clear, but these cells share some characteristics with Langerhans cells.

Polymorphonuclear eosinophils exhibited cytoplasmic staining which may be related to immunoglobulin or immune complexes, or could be due to cross reaction between conjugates and cellular antigens of the eosinophils.

In lymph nodes, immunofluorescent intercellular deposits seem to be associated with reticular cells known to exist in the cortex and paracortex (follicular dendritic cells and interdigitating cells) apparently found in these areas having the role of presenting antigen. Staining of marginal sinus may correspond to macrophages lining the medullary and marginal cords. Intercellular and sinus staining was observed in lymph nodes draining the lesions in all the experimental groups. Cells presenting Ag were not observed in skin lesions of BALB/C mice infected with *L. mexicana*, but they were found in lymph nodes.

Fine structure studies with the electron microscope on all of these tissues, will be the subject of future studies and may resolve some of the question raised.

CHAPTER IV

ISOLATION AND CHARACTERIZATION OF THE LYMPHOCYTE SUBPOPULATIONS FROM SKIN LESIONS AND DRAINING LYMPH NODES IN CBA/ca AND BALB/c INFECTED WITH *L. major* AND *L. mexicana*

IV.1 INTRODUCTION

In this study, cellular extractions from skin lesions and lymph nodes was performed on mice with the four host-parasite combinations previously described.

The objective of this study was the characterization of the B and T lymphocyte subpopulations in dispersed cellular suspensions prepared from these tissues, as the immunofluorescence technique performed on cryostat sections for the same purpose (Chapter III) did not always allow a clear differentiation of the lymphocytes from monocytes and sometimes histiocytes.

Characterization of the cells from tissue suspensions allowed counting and more accurate and easier identification of the cells. However, when the pattern of localization of the cells in tissues is required, studies on tissue sections is unavoidable.

IV.2 MATERIALS AND METHODS

Two experiments were carried out at the sixth and twelfth weeks after infection. Two replicates were done in each case. Three mice from each group (CBA/CA-*L. major*, CBA/ca-*L. mexicana*, BALB/c-*L. major* and BALB/c-*L. mexicana*) were killed by cervical dislocation, and the skin from the infection site and the draining lymph node were removed.

IV.2.1 Preparation of cell suspensions

The different proportion of lymphoid cells in lymph nodes and skin lesions forced the use of different methods for the isolation of these cells from these tissues. Thus, in lymph nodes, lymphocytes were very abundant despite the infiltration of the organ by cells of the macrophage system (see Chapter II), while in skin lesions the predominant cells in the infiltrate were macrophages and lymphocytes were rather scanty.

In lymph node disaggregates, filtration of the cell suspensions through a glass wool column resulted in a population of lymphocytes reasonably clear of macrophages. Separation of T lymphocytes from B lymphocytes was then done by loading the glass wool filtered cell suspension onto a nylon wool column at 37°C.

In skin cell disaggregates the same method was attempted, however, even when the cell suspensions were

repeatedly passed (3 times) through the glass wool column, a high contamination with macrophages occured. Separation using Percoll density gradients was attempted resulting in an effective separation of macrophages from other mononuclear cells. However, T and B lymphocytes can not be separated from each other by this method, and further filtration through nylon wool columns could not be done due to the small amount of lymphoid cells present in the skin infiltrates. Determination of both T and B lymphocytes from skin infiltrates was then carried out using the whole population of macrophage depleted lymphocytes. The methods used for such a separation are described in detail below.

IV.2.1.1 Preparation of cell suspensions from skin infiltrates

The skin was disrupted using the TAN et al.(1975) technique. The skin tissues were finely chopped with scissors in RPMI 1640 medium (GIBCO), buffered with sodium bicarbonate 7.5 % W/V containing 2 mM L glutamine, 12 mM Hepes, Penicillin (100 unit/ml), Streptomycin (100 µg/ml) and Collagenase (type 2 Nº C6885 SIGMA CHEMICALS) 40 units/ml.

The cellular suspension was left at 4ºC overnight. The following morning it was disaggregated using a magnetic stirrer for 1-1/2 hours at 37ºC and further forced through a stainless steel sieve with the plunger of a syringe. Cell debris and clumps were removed by gravity sedimentation for 10 min and the supernatants filtered through glass wool (room temperature) to remove dead cells,

cell debris and most of the macrophages (JULIUS et al. 1973).

IV.2.1.1.1 Glass wool filtration

A small amount of previously washed glass wool was loosely packed into a Pasteur pipette with the aid of an applicator stick. The column was thoroughly rinsed with medium (RPMI plus additives) containing 5 % fetal calf serum (FCS) before and after passing the cell suspension through it. Finally, the filtered cells were pellet ed at 200g for 10 min, and then resuspended in 1 ml of RPMI/5 % FCS for further loading onto Percoll density gradients.

IV.2.1.1.2 Percoll density gradients

Percoll stock working solution of isotonic Percoll (PHARMACIA UPSALA SWEDEN) was made up according to ULMER & FLAD (1979). Solutions of density 1055 g/ml and 1085 g/ml were made up from the 1120 g/ml stock by dilution with RPMI only.

A stock solution of Percoll was prepared by mixing 90 ml of Percoll with 8.965 ml of Hank's solution (10x), 1 ml 12 mM Hepes and 455 µl HCl (1N). The mixing ratio at the desired densities were calculated according to the equation:

Density (g/ml) = (% Percoll stock solution 0.001186) + 1.041

Densities were checked using a density marker beads kit

(PHARMACIA).

Density steps were made up according to DAVIES & PARROT (1981) at room temperature in conical 10 ml plastic tubes. 1 ml 1,085 g/ml Percoll was overlaid with 2 ml 1.055 g/ml and the latter in turn overlaid with 2ml of RPMI 5 % FCS.

The cell suspension contained in 1ml of RPMI/5 % FCS was overlaid on the uppermost layer.

Density gradient centrifugation was carried out at 600g for 20 min at room temperature in a swing-out rotor using a MSE centrifuge. To prevent disturbance of the gradients, a careful acceleration of the rotor was done by hand, and the run allowed to stop without braking.

Mononuclear cells were recovered from the 1.055-1.085 g/ml interface with a Pasteur pipette. Dead cells, debris, macrophages and amastigotes were found in the top interface and red blod cells in the pellet. The recovered mononuclear cells were washed two or three times with RPMI/5 % FCS before further processing.

IV.2.1.2 Preparation of cell suspensions from lymph nodes

Lymph nodes were chopped in Hank's balanced solution contained 10µ/ml of gentamicin and buffered with sodium bicarbonate. The resulting suspension was forced through a stainless steel sieve, clumps removed by gravity sedimentation for 10 min and the supernatant filtered through glass wool columns following the technique described above. Finally, the cell suspension was pelleted at 200 g for 10 min and resuspended in fresh RPMI/5 % FCS.

Aliquots of these suspensions containing about 1 x 10^7 cells were taken for cell counting and the immunofluorescence technique for identification of B lymphocytes.

IV.2.1.2.1 Isolation of T lymphocytes. Nylon wool filtration tehcnique.

After glass wool filtration, the cellular suspension contained in a volume of 2 ml was loaded onto a nylon wool column using the technique described by JULIUS et al. (1973). A 10 ml syringe barrel was loosely packed with 0.6 g of prewashed nylon wool (FT-242 scrubbed nylon fiber FENWALL LABORATORIES) up to the 6 ml mark and subsequently rinsed with about 20 ml of warm RPMI/5 % FCS.

The column was drained of excess medium, covered with parafilm to avoid evaporation and placed in an incubator at 37°C for one hour. After incubation, the column was flushed with 5 to 10 ml of warm RPMI and loaded with the cell suspension, which was added dropwise to the top of the column and subsequently washed into the nylon wool with 0.5 to 1 ml of warm medium (37°C). The column was covered with parafilm and placed back in the incubator at 37°C for 45 min, after which the cells were eluted slowly (1 drop/sec) by warm RPMI/5 % FCS and the free cells collected in the first 25 ml of effluent, pelleted at 290 g at 4°C and resuspended in EDTA/ovalbumin buffer (NAIRN, 1970).

IV.2.1.3 Cell counting and estimation of viability in the cell suspension.

Mononuclear cells from skin infiltrates recovered from the Percoll gradient interface and lymph node cells obtained after glass-wool and nylon-wool filtration were counted in an haemocytometer and their viability estimated by their ability to exclude Trypan-blue (see Chapter II).

IV.2.1.4 Cytology

Smears from cells recovered from Percoll interface and from glass-wool effluents were fixed in methanol and stained with Giemsa 5 % in phosphate buffered water pH 7.2.

IV.2.1.5 Glassware

All glassware was thoroughly washed with detergent, rinsed in tap-water and distilled water, oven dried and further rinsed with Repelcote (HOPKINS & WILLIAMNS) and dried at room temperature.

IV.2.1.6 Characterization of B cells

The direct immunofluorescence technique (DIFT) using anti-IgM and anti-IgG FITC conjugated antisera, and FITC conjugated *L. mexicana* antigen was performed on lymph node cell suspensions obtained after glass-wool filtration and on skin cell suspensions from the 1.055-1.085 g/ml Percoll interface. DIFT when performed on lymph node suspensions was applied to the living cell suspensions and on fixed smears, while with skin cell suspensions, it was

done on fixed smears only.

IV.2.1.6.1 Reagents

The reagents used were purchased from NORDIC LABORATORIES.

1.- Goat-antimouse IgM (Fc) FITC F/P 1-2; working dilution 1:10

2.- Goat-antimouse IgG (Fc) FITC, F/P ratio 1-2, working dilution 1:16

3.- *L. mexicana*-antigen FITC, F/P ratio 1.96, undiluted see above.

IV.2.1.6.2 Procedure

Smears were made by placing a drop of the cell suspension on a grease free multi-spot slide (C.A. HENDLEY (ESSEX) LTD), allowed to dry at room temperature and fixed in a mixture of equal volumes of Ethanol (95 %) and Ether (undiluted) for 10 min, rinsed and washed in PBS.

The conjugates were then put onto the fixed smears for 30 min, rinsed and washed in PBS (three changes of ten min each) and mounted in buffered glycerol.

When the staining was performed on living cell suspensions, cells were adjusted to 0.5 x 10^7/ml in EDTA/ovalbumin buffer. 50 µl of the suspension containing lymphocytes were mixed with 50 µl of the conjugate and placed into a U shaped microtritation plate (FLOW LABORATORIES), incubated for 30 min at room temperature, washed three times by centrifugation at 150 g for 10 min at 4°C with the same buffer. Smears were

then prepared on multi-spot slides, dried, fixed as above and mounted in buffered glycerol.

IV.2.1.7 Characterization of T cells

Indirect immunofluorescence technique (IIFT) (see Chapter III) was performed in order to identify lymphocytes bearing the Lyt1 and Lyt2 surface markers in cells obtained after nylon wool filtration of lymph node suspensions and skin lesion cells obtained from the 1.055-1.085 Percoll interface. As T cells were mixed with B cells in suspensions from Percoll gradients, blockage of B cells was carried out by pre-treatment of the cell suspension with unlabelled anti-mouse IgG as described in Chapter III.

Living cell suspensions from lymph nodes and Ethanol-ether fixed smears from lymph nodes and skin cell suspensions constituted the antigen for the reaction. The preparation of smears and staining of cells in suspension was described above.

IV.2.1.7.1 Reagents

The reagents used were:

1.- Mouse anti-mouse Lyt1.1 monoclonal antibody (CEDARLANE LABORATORIES), working dilution 1:100. This antibody reacts with cells expressing the Lyt1.1. phenotype (CBA/ca).

2.- Mouse anti-mouse Lyt1.2 monoclonal antibody (NEW ENGLAND NUCLEAR LABORATORIES), working dilution 1:20. This antibody reacts with cells expressing the Lyt1.2 phenotype (BALB/c).

3.- Rat anti-mouse Lyt2 monoclonal antibody was obtained from a cell culture secreting this antibody. This antibody reacts with cells expressing all Lyt2 alleles (BALB/c and CBA/ca) and was used undiluted.

4.- Rabbit anti-mouse IgG conjugated to FITC (MILES YEDA Laboratories), F/P ratio 4.6, working dilution 1:60 constituted the second layer in the IIFT for anti-Lyt1.1 and anti-Lyt1.2 antibodies.

5.- Sheep anti-rat IgG (Fab) conjugated to FITC (NORDIC LABORATORIES), F/P ratio 1-4, working dilution 1:20 constituted the second layer in the IIFT for anti-Lyt2.

All the FITC conjugates were absorbed with mouse liver powder acetone (see chapter III), filtered through a Millipore filter (22mμ) and centrifuged immediately before use.

IV.2.1.7.2 Cell culture secreting anti-Lyt2 monoclonal antibody.

IV.2.1.7.2.1 Maintenance

The cell line was obtained from the SALK Institute. Upon receipt of the cells, they were handled according to the attached instructions and seeded in a culture flask (FALCON 25 Sqcm) containing 5 ml of RPMI 1640 medium plus additives as described above , and 10 % FCS.

The culture cells were then incubated at 37°C, and medium was added every 2 to 3 days depending on the cell density. The cell line was allowed to grow

under these conditions and, when a high density was reached, the culture was either split or transferred to a bigger flask. Aliquots of the cultured cells were also frozen and kept in a liquid nitrogen container.

IV.2.1.7.2.2 Cryopreservation of cells

The cells were harvested by centrifugation at 150 g for 10 min at 4°C and resuspended (1 x 10^6) in 0.5 mls of a solution of 90% FCS and 10 % Dimethyl sulphoxide (DMSO), placed in plastic vials and frozen slowly (1°C/min to 40°C) by placing the vials into the gas phase of liquid nitrogen. Thereafter, the vials containing the cells were immersed in a liquid nitrogen container.

The cells recovered from liquid nitrogen storage were thawed by immersing the vials in a water bath at 37°C, the cells were then washed two to three times in tissue culture medium by centrifugation and their viability estimated.

IV.2.1.7.2.3 Viability estimation of cell cultures

Viability was estimated by the Acridine orange/ Ethidium bromide method. A one part per million solution of Acridine orange/Ethidium bromide was mixed with one part of the cell suspension v/v, placed in an haemocytometer and the counting of green (viables) and orange (non-viable) cells performed under UV illumination.

IV.2.1.7.2.4 Antibody collection

Supernatant from the cells in culture was collected and the antibody precipitated using ammonium sulphate (see Chapter III). Thereafter, the sediment containing the precipitated Ig was dialysed against PBS and the protein concentration estimated (7 to 10 mg/ml).

Fresh RPMI/10 % FCS medium was processed in the same way as the supernatant from the tissues and used as a control.

IV.2.1.7.3 Controls used in IIFT

PBS

Substitution of the unlabelled antibodies(first layer) by PBS was carried out in all the cases in order to evaluate the possible participation of the FITC conjugates alone (second layer) in the staining.

RPMI/10 % FCS

In order to evaluate the possible participation of Ig contained in FCS in the medium from which anti-Lyt2 was harvested, controls were performed by substitution of the relevant antibody by ammonium sulphate precipitates of RPMI/10 % FCS.

Cross substitution

The specificity of anti-Lyt1.1 and anti-Lyt1.2 was tested by cross-substitution in the first layer of these antibodies.

IV.2.1.7.4 Cell counting of stained cells

The smears were examined with a Leitz Orthoplan microscope equipped for incident fluorescence (see Chapter III.

The cells were first counted under white light dark field conditions, then switched to fluorescence illumination. Cells exhibiting speckled, ringed or crescent fluorescence were considered positive. Two to six hundred cells were counted on smears from lymph node suspensions and over one hundred cells on smears from skin cell suspensions.

IV.3 RESULTS

IV.3.1 CYtology from giemsa stained smears

IV.3.1.1 Lymph node suspensions

Smears made from the cell suspensions obtained after glass wool filtration contained mainly small and medium sized lymphocytes, immunoblasts, some plasma cells, macrophages, amastigotes either free or inside macrophages scanty eosinophils and cellular debris. Smears made after nylon wool filtration on the other hand, were cleaner, and consisted mainly of small lymphocytes, however, occasionally macrophages and amastigotes were observed contaminating the preparation, especially with the suspensions from BALB/c mice infected with *L.major* or *L.mexicana*.

IV.3.1.2 Skin cell suspensions

Smears made from skin cell suspensions obtained from the Percoll gradient interface (1058-1085 g/ml) showed mononuclear cells predominating (lymphocytes and monocytes) with scanty macrophages, plasma cells, eosinophils and amastigotes either free or contained inside macrophages.

IV.3.2 Estimation of cell numbers and percentage of viability

Table 1 and 3 record the number of cells and percentage of viability in lymph node and skin cell suspensions from mice conforming the different experimental groups studied. In these two Tables, it can be observed that, after processing lymph nodes and filtrating the resulting cell suspension through glass wool, yields ranging from 10^7 to 10^8 cells with a viability of over 97 % were obtained. When the effluent cells were then filtered through a nylon wool columm, the percentage of cells recovered in most of the cases was over 34 %, which is consistent with the results of JULIUS et al. (1973). Viability in these suspensions was always over 92 %.

In skin cell suspensions, the total yield of the cells obtained from the percoll gradient interface (1050-1085 g/ml) was about 10^8 cells and viability was over 80 % with the exception of cell suspensions obtained from BALB/c mice infected with *L. mexicana* where, the percentage of live cells was only 53 %.

IV.3.3 Characterization of fluorescent cells

Fluorescent staining was associated with cell membrane surfaces, as dispersed spots, crescent shapes, or forming a continuous circle around the edge of the cell. Only those cells exhibiting surface fluorescence were taken into account when counts were performed. In general, counts recorded the number of lymphoid cells, but sometimes may also have included other mononuclear cells (monocytes), since lymphocytes are often indistinguishable from monocytes at the light microscope level (SYMMERS, 1978).

Cytoplasmic fluorescence was observed in cells resembling plasma cells and in eosinophils with anti-IgG and FITC-*L. mexicana* antigen. These cells, even when scanty, were detected in cell suspensions from all the experimental groups.

IV.3.4 Controls

In lymph nodes cell suspension substitution of monoclonal anti-Lyt1.1 and anti-Lyt1.2 antibodies (first layer in the IIFT) by PBS, resulted in weak staining of some cells of the suspension. This result can be interpreted as: a.- reaction of the FITC anti-IgG conjugate with contaminant B cells, or b.- binding of IgG to Fc receptors on the lymphocyte surfaces. This last suggestion was confirmed by the removal of all staining in the control suspension of cells when previous blockage with unlabelled goat-antimouse Ig was performed. Therefore, the results reported for anti-Lyt1$^+$ in Table 2 are the result of substracting the number of staining cells in

control experiments from the total cells stained by the reagents in question in the suspensions.

Substitution of anti-Lyt2 monoclonal antibody by PBS or RPMI/ 10 % FCS gave no fluorescence in the cells from skin lesions and lymph node suspensions. Finally, cross-substitution of monoclonal antibodies to specific phenotypes gave negative results.

IV.3.5 Characterization of B and T cell sub-population of lymphocytes

Tables 2 and 4 record the results of the characterization of B and T cell populations of lymphocytes from skin and lymph node suspensions, respectively, performed 6 and 12 weeks after infection in each of the infection models.

IV.3.5.1 B cell characterization

IV.3.5.1.1 Lymph nodes

Lymphocytes bearing IgG (Fig. 1) and IgM were detected in lymph node cell suspensions prepared from all four infection models at six weeks of infection. At the twelfth week of infection, no cell suspension was prepared from recovered CBA/ca previously infected with *L. major*. All other infection models showed a marked decrease of B cells, especially of those bearing IgG.

The percentage of B cells in BALB/c mice infected with *L. mexicana* 6 weeks after the infection was one

order of magnitude smaller than in any of the other groups. By the 12th week after infection, only IgG bearing lymphocytes were detected in this group and then in a considerably reduced percentage.

IV.3.5.1.2 Skin lesions

In skin lesions, the only type of B cell detected consistently in cell suspensions from all infection models, six week after infection, were IgG bearing cells. IgM bearing cells were detected solely in CBA/ca mice infected with *L. major* 6 weeks after infection. 12 weeks after the infection, the lesions of CBA/ca mice infected with *L. major* were cured, while in the mice of the same strain infected with *L. mexicana* the percentage of IgG bearing cells remained unchanged. Regarding BALB/c mice, IgG bearing B cells were present only in considerably reduced numbers in the group infected with *L. major* and altogether absent from those infected with *L. mexicana.*

Lymphocytes bearing specific anti-*Leishmania* antibodies were detected stained by FITC-*L. mexicana* in both skin and lymph nodes from all experimental groups six weeks after infection. Like IgG and IgM bearing cells, their numbers were conspicuously reduced by the the twelfth week of the infection in the animals still showing lesions.

IV.3.5.2 T cell characterization

As Tables 2 and 4 show, T cells were detected in cell suspensions made from skin lesions and lymph nodes taken from all infection models, with the exception of the skin suspensions from BALB/C mice infected with *L. mexicana*. In all cases, cells exhibiting the Lyt 1+ marker predominated overwhemlingly at 6 and 12 weeks of infection (Fig.12) over the exiguous percentage of cells exhibiting the Lyt 2 + phenotype.

The percentages of T and B cells in lymph node suspensions (Table 2) are not additive, as they were calculated from different samples. T cell subpopulations were estimated from an enriched population of these cells obtained after nylon wool filration, while the percentages of B cell were estimated from a mixed population of B and T cells.

In skin cell suspensions, on the other hand, the percentages of B and T cells (Table 4) should add to 100 % if there were no other types of cells in the suspension. The fact that they do not could be due to

a - The presence of cells bearing other Ig surface markers than those searched for in this study.

b - The inclusion of cells which resembled lymphocytes but, were not lymphhocytes in the total count.

c - The presence of lymphocytes lacking surface markers (null cells).

TABLE 1

COUNTS AND VIABILITY OF LYMPHOCYTES IN CELL SUSPENSIONS OBTAINED FROM CBA/ca AND BALB/c MICE INFECTED WITH Leishmania major AND L. mexicana, AT 6 AND 12 WEEKS AFTER INFECTION.

RESULTS 6 WEEKS AFTER INFECTION

CELL COUNTS AFTER GLASS WOOL FILTRATION

	CBA/ca MICE			BALB/c MICE		
	L. major	L. mexicana	CONTROL	L. major	L. mexicana	CONTROL
LIVE	4.50 (0.92)	4.69 (3.40)	5.00	11.80 (1.41)	10.45 (0.49)	3.00
DEAD	0.09 (0.01)	0.02 (0.01)	0.60	0.22 (0.03)	0.14 (0.03)	0.2
TOTAL	4.64 (0.93)	4.71 (3.40)	5.60	12.02 (1.39)	10.59 (0.52)	3.2
% VIAB.	98.10 (0.28)	99.48 (0.25)	89.29	97.92 (0.76)	98.44 (0.15)	93.7

CELL COUNTS AFTER NYLON WOOL FILTRATION

LIVE	1.23 (0.47)	0.97 (0.62)	2.90	2.65 (0.92)	2.32 (0.74)	1.00
DEAD	0.08 (0.05)	0.07 (0.04)	0.10	0.22 (0.26)	0.16 (0.19)	0.09
TOTAL	1.31 (0.51)	1.04 (0.66)	3.00	2.87 (1.17)	2.52 (0.54)	1.09
% VIAB.	94.40 (1.41)	93.16 (0.25)	96.67	93.64 (6.29)	92.64 (9.42)	91.74
CELL RECOV.	35.29 (5.29)	34.65 (14.47)	63.04	26.89 (14.09)	26.21 (4.24)	41.22

Counts given as 10^7 cells. Each experimental group consisted of 3 mice. 10^7 cells were taken after glass wool filtration for counting and B cell characterization

% VIAB. : Percentage of viable cells. CELL RECOV. : % Cell recovery.

Results obtained 12 weeks after infection in next page.

RESULTS 12 WEEKS AFTER INFECTION

CELL COUNTS AFTER GLASS WOOL FILTRATION

	CBA/ca MICE		BALB/c MICE	
	L. major	L. mexicana	L. major	L. mexicana
LIVE	ND	3.05 (0.21)	7.90 (1.27)	7.77 (1.12)
DEAD	ND	0.04 (0.03)	0.19 (0.04)	0.03 (0.01)
TOTAL	ND	3.09 (0.24)	7.94 (1.03)	7.79 (1.13)
% VIAB.	ND	98.68 (0.91)	97.62 (0.75)	99.68 (0.07)

COUNTS AFTER NYLON WOOL FILTRATION

LIVE	ND	1.07 (0.19)	3.68 (0.34)	2.22 (0.68)
DEAD	ND	0.06 (0.01)	0.11 (0.12)	0.02 (0.01)
TOTAL	ND	1.13 (0.25)	3.79 (0.46)	2.24 (0.68)
% VIAB.	ND	95.05 (4.02)	97.16 (2.84)	99.09 (0.24)
CELL RECOV.	ND	52.23 (14.50)	50.89 (4.10)	34.30 (15.73)

TABLE 2

CHARACTERIZATION OF B AND T LYMPHOCYTE POPULATIONS IN LYMPH NODES CELL SUSPENSIONS FROM CBA/ca AND BALB/c MICE INFECTED WITH L. major AND L. mexicana, 6 AND 12 WEEKS AFTER INFECTION.

RESULTS 6 WEEKS AFTER INFECTION

	B CELL CHARACTERIZATION			T CELL CHARACTERIZATION	
INFECTION MODELS	IgM	IgG	Leish. Ig	Lyt 1	Lyt 2
CBA/ca L. major	1113 7.08% (1.53)	1021 36.97% (6.20)	1127 2.84	860 70.25 (16.50)	729 1.09% (0.33)
CBA/ca L. mexicana	898 8.29% (5.82)	1205 28.46% (2.75)	1293 2.84%	1530 76.87% (15.30)	1257 1.47%. (0.32)
NON INFECTED CBA/ca	1104 0.52%	553 1.09%	N.D.	541 0.75%	797 3.59%
BALB/c L. major	614 1.23% (1.31)	1109 16.43% (7.21)	1203 5.96%	944 58.78% (2.59)	1011 3.31%. (1.24)
BALB/c L. mexicana	451 0.19% (0.13)	853 2.73% (0.25)	600 2.67%	1226 33.63% (9.02)	938 1.62% (0.81)
NON INFECTED BALB/c	585 0.77%	593 0.36%	N.D.	NEG.	302 1.51%

RESULTS 12 WEEKS AFTER INFECTION

CBA/ca L. major	N.D.	N.D.	N.D.	N.D.	N.D.
CBA/ca L. mexicana	500 0.20%	896 3.55% (1.34)	840 2.23%	945 50.08% (18.49)	885 0.89% (0.16)
BALB/c L. major	1100 0.29% (0.16)	1100 0.75% (0.35)	1120 0.46%	1101 47.13% (10.70)	1285 0.43%. (0.24)
BALB/c L. mexicana	NEG.	1150 0.94% (0.23)	NEG.	1108 31.90% (4.60)	928 1.65%. (0.85)

IgM : cells bearing immunoglobulin M. IgG : cells bearing immunoglobulin G. Leish. Ig.: cells bearing Leishmania antibody. NEG. : negative, N.D. : not done, Observations: number of cells counted and percentage of total cells. Figures in brackets are standard deviations of the percentages. All figures average of two experiments except for the identification of specific leishmania antibody which was done only once.

TABLE 3

COUNTS AND VIABILITY OF LYMPHOCYTES IN SKIN CELL SUSPENSIONS PREPARED FROM CBA/ca AND BALB/c MICE INFECTED WITH L. major OR L. mexicana, 6 AND 12 WEEKS AFTER INFECTION

CELL COUNTS 6 WEEKS AFTER INFECTION

	CBA/ca MICE		BALB/c MICE	
	L. major	L. mexicana	L. major	L. mexicana
LIVE	0.98 (1.10)	13.45 (5.02)	4.50 (2.90)	7.96 (0.07)
DEAD	0.12 (0.11)	2.10 (1.56)	0.10 (0.14)	0.18 (0.11)
TOTAL	1.00 (1.27)	15.55 (6.50)	4.54 (3.00)	8.13 (0.18)
% VIAB.	85.00 (7.00)	87.50 (4.72)	99.40 (0.85)	97.86 (1.26)

CELL COUNTS 12 WEEKS AFTER INFECTION

	CBA/ca MICE		BALB/c MICE	
	L. major	L. mexicana	L. major	L. mexicana
LIVE	ND	32.00 (4.24)	5.65 (1.34)	0.80
DEAD	ND	0.30 (0.14)	0.19 (0.02)	0.70
TOTAL.	ND	32.30 (4.38)	5.84 (1.36)	1.50
% VIAB.	ND	99.10 (0.32)	97.23 (1.01)	53.30

Cell counts x 10^5, each value corresponds to the mean of two experiments using 3 mice each. Cells were separated in Percoll density gradients.

TABLE 4

CHARACTERIZATION OF B AND T LYMPHOCYTE POPULATIONS IN SKIN CELL SUSPENSIONS FROM CBA/ca AND BALB/c MICE INFECTED WITH L. major AND L. mexicana, 6 AND 12 WEEKS AFTER INFECTION.

RESULTS 6 WEEKS AFTER INFECTION

	B CELL CHARACTERIZATION			T CELL CHARACTERIZATION	
INFECTION MODELS	IgM	IgG	Leish. Ig	Lyt 1 $^{+}$	Lyt 2 $^{+}$
CBA/ca L. major	407 1.27% (0.85)	293 9.84% (1.30)	440 5.00	315 1.63 (0.20)	NEG.
CBA/ca L. mexicana	NEG.	490 9.26% (1.94)	470 4.25%	190 10.84% (5.89)	NEG.
BALB/c L. major	NEG.	472 4.75% (0.30)	461 3.25%	185 28.93% (4.30)	NEG.
BALB/c L. mexicana	NEG.	898 5.75% (1.56)	323 4.64%	NEG	NEG.

RESULTS 12 WEEKS AFTER INFECTION

CBA/ca L. major	N.D.	N.D.	N.D.	N.D.	N.D.
CBA/ca L. mexicana	*	1012 8.43% (1.43)	1000 1.05%	379 2.43% (0.30)	NEG
BALB/c L. major	*	880 0.57% (0.05)	1000 0.40%	302 22.4% (12.10)	NEG.
BALB/c L. mexicana	NEG.	NEG.	NEG.	NEG.	NEG.

IgM : cells bearing immunoglobulin M. IgG : cells bearing immunoglobulin G. Leish. Ig.: cells bearing Leishmania antibody. NEG. : negative, N.D. : not done, Observations: number of cells counted and percentage of total cells. Figures in brackets are standard deviation of the percentages. All figures average of two experiments except for the identification of specific leishmania antibody which was done only once. * indicates less than one cell bearing Ig in 500 cells counted.

FIGURE 1

Direct immunofluorescence on lymph node cell suspension (after glass wool filtration) from CBA/ca mice, 6 weeks infected with *L. major* using anti-mouse IgG conjugated to FITC. Lymphocytes exhibit membrane staining and immunoblasts cytoplasm staining.
X 400.

FIGURE 2

Indirect immunofluorescence on lymph node cell suspension (after nylon wool filtration) from CBA/ca mice, 6 weeks infected with *L. major* using monoclonal mouse anti-mouse Lyt1. Lymphocytes exhibit membrane staining.
X 125.

FIGURE 1

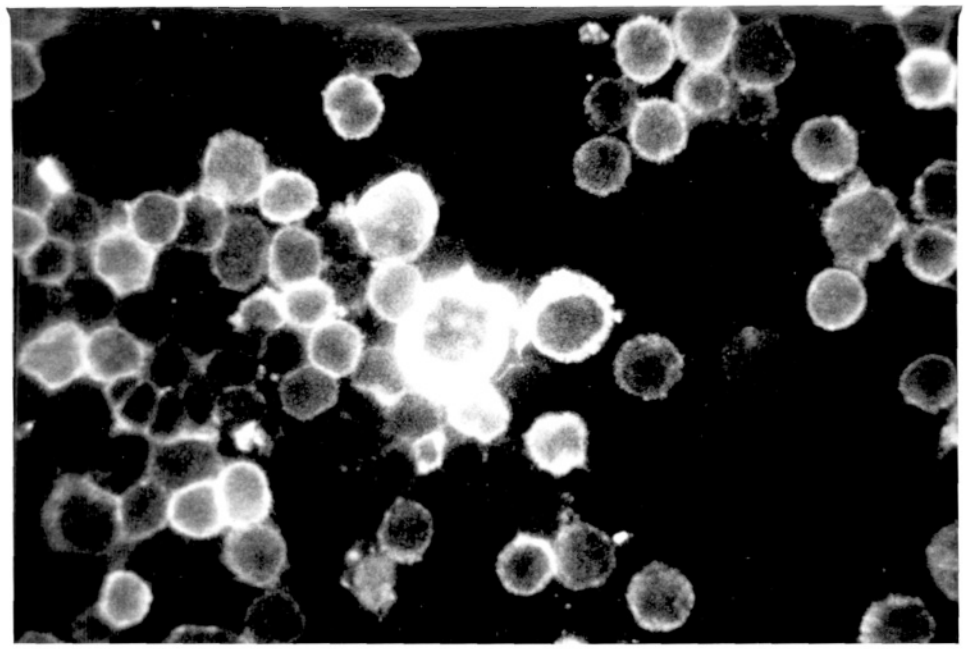
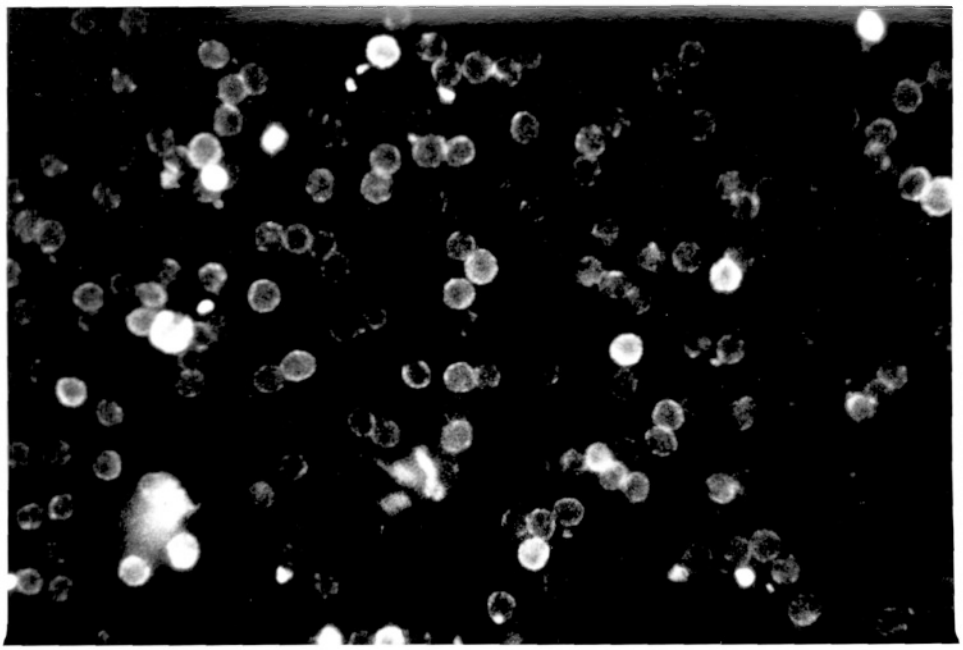


FIGURE 2



IV.4 DISCUSSION

In general, Ig bearing lymphocytes bore IgG for the most part in both skin lesions and their draining lymph nodes. Although it was not possible to establish by direct means which Ig class the anti-*Leishmania* antibodies detected in lymphocyte surfaces belonged to, the following evidence suggest that they were IgG:

a.- Anti-*Leishmania* antibody-bearing lymphocytes were detected in the skin lesions of CBA/ca mice infected with *L. mexicana* and of BALB/c mice infected with either species of parasites in the absence of any IgM bearing lymphocytes.

b.- In the lymph nodes, although the relationship was less clear-cut due to the detection of IgM bearing lymphocytes in all the infection models, the percentages of lymphocytes bearing specific antibodies were larger than those bearing IgM in all BALB/c mice, regardless of the species of parasite with which they were infected.

c - Antibody forming cells in lymph node suspensions were detected with their cytoplasm stained by anti-IgG antiserum and by FITC *Leishmania* antigen, but not by anti-IgM antiserum.

d.- In the experiments described in Chapter III, amastigotes were stained by anti-IgG but not anti-IgM antisera.

In relation to plasma cells it is worthwhile pointing out that, contrary to the observations in cryostat

sections, where these cells were observed to be stained by anti-IgG and anti-IgM antisera only in lymph nodes sections from CBA/ca mice infected with *L. major* or *L. mexicana*. In lymph node cell suspensions, plasma cells were readily detected stained by anti-IgG antisera and FITC *Leishmania* antigen in all the experimental groups. This apparently contradictory result could be due to the fact that these cells are more easily stained when isolated than in sections; in fact, HUDSON & HAY (1980) pointed out the difficulties encountered in the detection of plasma cells by IFT in sections as compared to smears from spleen. The authors suggested that, in sections, the antibody may be trapped and thereby difficult to stain.

With reference to the specific Ig involved in *Leishmania* infections, the literature reports fluorescent antibodies presumably G2 in sera from guinea pigs infected with *L. enriettii* (TURK & BRYCESON (1971) and G1 and G2 in CBA mice infected with *L. major* (PRESTON & DUMONDE, 1976).

RADWANSKY et al. (1974), on the other hand, using the immunofluorescent technique, reported amastigotes coated with IgG in lymph node sections from guinea pigs infected with *Leishmania enriettii*. In the studies of MORIEARTY et al. (1982), on Brazilian cutaneous leishmaniasis using the immunofluorescence technique, it was shown that most of the plasma cells isolated from the lesions, contained IgG, suggesting that the humoral mechanism most probably involved IgG production and this may have been an anti-leishmanial antibody, at least in part.

The comparison of the percentages of lymphocytes bearing IgG with that of lymphocytes bearing specific anti-leishmanial Ig in lymph node suspensions prepared 6 weeks after infection, showed that, with the exception of BALB/c mice infected with *L. mexicana*, all infection models showed a far greater percentage of IgG bearing lymphocytes than of anti-leishmanial Ig bearing lymphocytes. This disparity suggests the presence of non-specific antibodies which, could in turn be the consequence of polyclonal stimulation. In fact, very recent reports (COLLE et al. 1983) suggest that *L. major* infections induce polyclonal activation in susceptible (BALB/c) and resistant (C57BL/6) strains of mice as measured by the response of both strains of infected mice to thymus independent antigens (trinitrophenylated lipopolysacharide and dinitrophenylated Ficoll), which was statistically above the response of uninfected mice. In addition, it has recently been shown that *L. major* promastigotes contain a B cell mitogenic factor (WEINTRAUB et al. 1982) and it is well known that mitogenic stimulation may lead to autoimmune responses (HAMMERSTROM et al. 1976). Polyclonal activation, leading to autoantibodies, has been reported in parasitic diseases (ORTIZ-ORTIZ et al. 1980; HOUBA & ALLISON, 1966). This evidence may support the speculation made in relation to immunofluorescence deposits in skin lesions, suggesting that they might represent autoantibody to the related host structures.

In relation to the characterization of T lymphocyte

subpopulations according to the phenotype exhibited by these cells, it was found that, in all the host-parasite models used in this study, T cells exhibiting Lyt1+ phenotype predominated, and almost no lymphocytes exhibiting the Lyt2+ phenotype were found. These results are in agreement with those obtained by LOUIS et al. (1981) and LIEW et al. (1982) in the identification of the phenotype of T cells induced by *L. major* infections both as a helper and suppressor in mice. It has also been shown that Lyt1+ 2− lymphocytes from CBA (resistant) mice recovering from cutaneous lesions induced by *L. major*, are capable of conferring protective immunity upon transfer into normal mice (LIEW et al. 1982). In contrast, Lyt1+2− splenic lymphocytes from infected susceptible BALB/c mice were able to inhibit the induction of *Leishmania* specific DTH reaction in syngeneic recipients (HOWARD et al. 1981; LIEW et al. 1982).

Because T suppressor and effector cells exhibit the same phenotype, the question arises as to whether or not they are the same cell, in which case the observed differences in function would merely reflect different thresholds for immunity and suppression. MITCHELL et al. (1981), in experiments with nu/nu BALB/c athymic mice indicated that, depending upon the number of bone marrow cells from a normal donor injected, Lyt1+2− from normal mice either conferred some resistance (10^6 cells injected) or had a suppressive effect (10^8 cells injected).

Studies with cloned T cell lines have suggested that T cells may be multifunctional with functions other than the activities ascribed to their Lyt phenotype, e.g.,
1.- Cloned Lyt1^{+}2^{-} helper T cells for antibody response to SRBC could transfer a specific delayed-type hypersensitivity (DTH) response (BIANCHI et al. 1981),
2.- An Lyt1^{-}2^{+} T cell line specific for influenza virus mediated both killer cell and local DTH reactivities (LIN & ASKONAS, 1981). Recent experiments by LOUIS et al. (1982, 1983) with T cell clones specific to *L. major* antigen, demonstrated that these parasite-specific T cells exhibited the Lyt1^{+}2^{-} phenotype and were capable of mediating the following immunological functions :
a.- helper activity for antibody response *in vitro* in a hapten-carrier system b.- transfer of antigen-specific DTH responses to normal mice and c.- specific activation of parasitized macrophages resulting in the destruction of intracellular parasites. These results confirm in a parasite antigen system, the observations of BIANCHI et al. (1981) and LIN & ASKONAS (1981) which demonstrate that a given T cell clone can perform a variety of immunological functions.

These heterodox results could have among others the following explanations;

1- T cell subsets may be potentially capable of expressing multiple activities, and that the apparent monofunction of subsets is attributable to a specific *in vivo* regulation of these activities at any one time

(DENNER et al. 1981).

2- These activities are due to different cell subsets that are descendants of a common precursor of the clone (LIEW et al. 1982).

Very recently, LIEW (1983) confirmed at the clonal level, the existence of $Lyt1^{+}2^{-}$, $I\text{-}J^{-}$ T cells with suppressor, but not cytotoxic or helperactivities, generated during *L. major* infection of BALB/c mice. This clone could intensify the infection of *L. major* in BALB/c mice *in vivo*, suppress T cell induced macrophage killing of *L. major in vitro* and abrogate specific transfered delayed-type hypersensitivity responses.

The evidence seems to indicate that both curative cell-mediated immunity to *L. major* and its suppression in mice are attributable to $Lyt1^{+}2^{-}$ T cells. Studies with cloned lines on the other hand, support the cells' ability to dissociate into separate functions on separate occasions.

IV.5 Conclusions

In this thesis, the characterization of B and T cell populations in lymph node and skin lesion cell suspensions by IFT in the different host parasite models showed:

1- B lymphocytes bearing IgG predominated over lymphocytes bearing IgM in all the cases.

2- In lymph node cell suspensions, it was clear that in

CBA/ca mice infected with *L. major* or *L.mexicana* and in BALB/C infected with *L. major*, the percentage of B lymphocytes bearing IgG exceeded substantially the percentage of lymphocytes bearing specific antibody which led to the speculation about the occurrence of polyclonal stimulation.

3- An analysis of the percentage of B lymphocytes bearing IgG, IgM and *Leismania*-specific antibody in lymph node and skin cell suspensions suggests an association of IgG and anti-*Leishmania* Ig.

4- In relation to T cells, it is shown that, in both skin lesions and draining lymph nodes of mice exhibiting different susceptibilities to *L. major*, (CBA (resistant) BALB/c (susceptible)) and in mice susceptible to *L. mexicana* (CBA/ca and BALB/c), T cells exhibited the $Lyt1^{+}2^{-}$ phenotype.

CHAPTER V

IMMUNE RESPONSE IN CBA/ca AND BALB/C MICE INFECTED WITH L. major AND L. mexicana

V.1 INTRODUCTION

The acquisition of a protective immune response against leishmaniasis has been associated mainly with cell mediated responses (BRYCESON 1970; ZUCKERMAN 1975; PRESTON & DUMONDE 1976).

Classically, cell mediated immunity has been measured by the ability to produce a delayed hypersensitivity response (DHR) against the specific antigen. The Montenegro test has been used for several decades to measure DHR in relation to *Leishmania* infections by the response to the intradermal inoculation of leishmanin.

Leishmanin consists of promastigotes grown *in vitro*, killed and preserved by the addition of phenol. A positive leishmanin test reaction consists of an area of induration and erythema surrounding the site of inoculation of leishmanin, reaching a peak after 24-48 hours and gradually fading thereafter. The test is not species-specific and is evoked by antigens from different species of *Leihmania* (reviewed by MAEKELT, 1972). In self-healing cutaneous leishmaniasis, the leishmanin test becomes positive before any protective immunity develops

and while viable parasites are still abundant in the lesion. Once positive, the test remains so for life (ZUCKERMAN, 1975).

In the allergic non-healing forms of cutaneous leishmaniasis (Leishmaniasis lupoid or recidivans), and in mucocutaneous forms of the disease (espundia), leishmanin positivity is very marked while in diffuse cutaneous leishmaniasis (DCL) and visceral leishmaniasis (Kala-azar), (anergic forms of the disease), the leishmanin test is negative.

The leishmanin test is widely used to assess past and present *Leishmania* morbidity in a given area. In an endemic area, the percentage of positive tests rises with age and with duration of residence in the area (reviewed by ZUCKERMAN, 1975).

The numerous laboratory studies on cutaneous leishmaniasis have also indicated a close correlation between a positive DHR and the acquisition of a protective cell mediated response(BRYCESON et al. 1970; PRESTON et al. 1978; HANDMAN et al. 1979; PEREZ et al. 1979; HOWARD et al. 1980 a).

Thus, BRYCESON et al. (1970) demonstrated delayed hypersensitivity response in guinea-pigs infected with *L. enriettii* four weeks after infection. The delayed sensitivity could be transferred passively by lymphoid cells to normal animals. Various *in vitro* correlates of cell mediated immunity have also been demonstrated. When lymphoid cells from infected, convalescent or immunized animals are exposed to *L. enriettii* antigens, cell

mediated immunity can be measured by lymphocyte transformation. The effects of the products of lymphocytes transformation (lymphokines), such as macrophage migration inhibition factor (MIF) and a mitogenic factor (MF), have also been demonstrated (BRYCESON et al. 1970).

It has been observed that different inbred strains of mice show varying degrees of resistance to infection with *Leishmania* parasites (PRESTON et al. 1978; NASSER & MODABBER ,1979; HANDMAN et al. 1979; HOWARD et al. 1980 a) (see Chapter I)

Association between resistance to infection and DHR in the mouse model has been reported by PRESTON et al. (1978), HANDMAN et al. (1979), HOWARD et al. (1980 a), among other authors. Thus, in *Leishmania* infections which lead to healing of the lesions, a protracted DHR develops, whereas non-healing infections either do not develop DHR, or the desensitization occurs as the infection progresses (PRESTON et al. 1978, HOWARD et al. 1980 a).

Antibody specific to *Leismania* has been demonstrated during the course of infection in human and experimental leishmaniasis (see Chapter I). However, relatively little attention has been paid to the possible role of specific anti-*Leishmania* antibody as an agent in the resolution of the infection.

The high titers of antibodies to *Leishmania* antigens observed in the serum of patients suffering from DCL and Kala-azar have lead to the suggestion that these antibodies are not protective (GARNHAM & HUMPHREY, 1969). On the other hand, MAUEL et al. (1974); BRAY et al. (1974); BELEHU et

al. (1976); PRESTON & DUMONDE (1976); PRESTON et al. (1978); HERMAN (1980); and POULTER (1980) among other workers, have suggested that the humoral response against *Leishmania* parasites may play a role in effective immunity.

In this study, the immunological response in *L.major*-CBA/ca, *L. mexicana*-CBA/ca, *L. major*-BALB/c and *L.mexicana*-BALB/c models was investigated. The cellular response was estimated by the ability of the mice to produce DHR to homologous and heterologous antigens at 2, 4, 8, 12 and 16 weeks after infection. The humoral response was estimated concurrently, measuring antibody levels by agglutination and indirect immunofluorescence techniques.

V.2 MATERIALS AND METHODS

Mice, strains of parasite, preparation of the infecting inoculum and infection procedure are described in Chapter II.

The growth of promastigotes in culture and preparation of parasite antigens are described in Chapter III.

V.2.1 Skin testing

Skin testing was performed by the intradermal injection of 80 µg of PSA in 0.02 ml. PBS into the right hind foot pad of three to six mice in each experimental group at the intervals of time specified above. DHR was recorded as the increase in thickness of the injected foot pad, which was

measured with a POCOTEST A dial gauge micrometer, before injection of the antigen and 24, 48 and 72 hours after. Uninfected animals which were inoculated with PBS into one foot pad and with antigen into the other, served as controls.

V.2.2 Preparation of tissues for histology

The skin test site and the draining lymph nodes were prepared for histological examination 48 hours after the last reading.

The tissues were removed after killing the mouse, fixed in Carnoy's solution, embedded in paraffin wax, sectioned at 5 mμ and stained with haematoxylin-eosin or methyl-green pyronin, as described in Chapter II.

V.2.3 Antibody assays

Blood was taken from 3 mice of each experimental group by sectioning the axillary plexus of the animals under anaesthesia. After collection, the blood was pooled and allowed to clot at room temperature, transferred to 37° C for 20 min and then to 5° C for one hour, after which the serum was separated by centrifugation at 1000 rpm at 5° C. After separation the serum was stored in a liquid nitrogen container. The serum was not inactivated. The sera were then assayed for antibody content by using the agglutination and immunofluorescence techniques.

V.2.3.1 Agglutination technique

Agglutinating antibodies were detected using the technique of NUSSENZWEIG et al. (1963). All serum and antigen dilutions were made in PBS pH 7.2. Parasites were harvested after 8 to 10 days growth in a protein-free dialysed medium, washed once in PBS and adjusted to 1 x 10^6 per ml in the same solution.

To perform the test, 0.1 ml of the promastigote suspension was mixed with 0.1 ml of two-fold serial dilution of unheated test serum, starting from 1:10 to 1:20480, in small glass vials, incubated at 37° C for 30 min and subsequently kept in the refrigerator for 18 hours.

The tubes were then gently tapped and drops of the mixture were placed on slides, covered with cover-slips and examined by phase-contrast microscopy.

Results were graded from - to ++++ as follows:

- No agglutination

+ Small clumps; about 1/4 of the organisms agglutinated

++ Small and large clumps; about half of the organisms agglutinated

+++ Large clumps; 3/4 of the organisms agglutinated

++++ All the organisms were agglutinated

Normal mouse sera controls and a control of the promastigote suspension were included in all tests in order to exclude non-specific and spontaneous agglutination.

Agglutinating titers were expressed as the reciprocal of

the highest final serum dilution giving a ++ agglutination.

V.2.3.2 Immunofluorescence technique

The indirect immunofluorescent technique was used for determining IgM and IgG antibodies.

Antigens

Promastigotes of *L. major* and *L. mexicana* were used as antigens, suspended in EDTA/ovalbumin buffer (NAIRN, 1969) at a concentration of about 2 to 4 x 10^6 parasites per ml using a Pasteur pipette, a small drop of the parasite suspension was placed on a grease-free multispot slide and the excess fluid immediately sucked back. After fan drying, a second drop was similarly applied on top of the first and dried again. The slides were then wrapped in absorbent paper and stored (usually no longer than a week) at -20° C.

Sera

Two-fold dilution of the test sera from 1:10 to 1:20480 were made in PBS.

Conjugate antisera

The conjugate antisera used were fluorescein-labelled goat anti-mouse FC IgG (NORDIC LABORATORIES), F/P ratio between 1 and 4, working dilution 1:60 and fluorescein-labelled goat anti-mouse Fc IgM (same source

and F/P ratio), working dilution 1:40. The conjugates were diluted in PBS containing 4% bovine serum albumin fraction V (SIGMA CHEMICAL).

Indirect immunofluorescence staining

Microscope slides containing dried promastigotes were removed from -20 ° C and dried with a fan (cold air current) for 20 min fixed in acetone for 5 min, washed in PBS and set horizontally on a rack in a humid chamber.

Sera dilutions were overlaid on the antigen spot on the prepared slides and allowed to react with the antigens for 30 min, this was followed by 3 x 10 min washes with PBS. Then, the PBS was poured off and the excess fluid dried. The slides were then returned to the humid chamber and the fluorescent conjugate applied for 30 min. The slides were then washed three further times in PBS, mounted in buffered glycerin and examined under fluorescent illumination using a Leitz Orthoplan microscope, as described in Chapter III. Immunofluorescence was graded visually on a scale from - to ++++ by comparison with control smears exposed to serum dilutions from uninfected CBA/ca and BALB/c mice. The titers were expressed as the reciprocal of the highest serum dilution giving ++ cytoplasmic fluorescence.

V.3 RESULTS

V.3.1 DELAYED HYPERSENSITIVITY

The results of measuring the DHR in the four experimental groups are recorded in Tables 1 to 5 and Figures 1 to 4. Two weeks after the infection with *L. major*, CBA/ca mice demonstrated a feeble response to the homologous and heterologous antigen (Figure 1 a, b). The response to the antigen derived from *L. mexicana* increased rapidly thereafter and reached it's ~~greater magnitude~~ maximum by the fourth week. When the test antigen was derived from *L. major*, the intensity of the reaction did increase steadily, but a response of similar magnitude to that induced by *L. mexicana* derived antigen did not develop until 8 to 12 weeks after infection. In both cases, this response was mantained until the end of observations.

The kinetic study of the response in this model showed that it was of the tuberculin-type, peaking at 24 hours and persisting up to, and beyond 48 hours.

CBA/ca and BALB/c mice infected with *L. mexicana* (Tables 2 and 4; Figs 2a, 2b, 4a, 4b,) produced a DHR to the homologous antigen, but not to the heterologous antigen. The response against *L. mexicana* antigen was detected by the second week and from then on the intensity of the response increased, reaching a peak by the fourth week, persisting up to 8 weeks in BALB/c mice and up to 12 weeks in CBA/ca mice and declining thereafter.

TABLE 1

DELAYED HYPERSENSITIVITY RESPONSE TO HOMOLOGOUS AND HETEROLOGOUS ANTIGENS IN CBA/ca MICE INFECTED WITH L. major.

TESTED WITH L. major ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	4	0.25	0.03	0.23	0.07	0.15	0.03
4	4	0.30	0.04	0.43	0.07	0.43	0.05
8	4	0.55	0.09	0.65	0.03	0.65	0.05
12	4	0.70	0.12	0.73	0.11	0.45	0.05
16	4	0.63	0.09	0.60	0.07	0.45	0.05

TESTED WITH L. mexicana ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	3	0.30	0.10	0.20	0.12	0.05	0.04
4	6	0.72	0.05	0.62	0.11	0.50	0.12
8	4	0.78	0.25	0.70	0.18	0.23	0.07
12	3	0.63	0.18	0.60	0.12	0.25	0.15
16	6	0.75	0.13	0.57	0.13	0.32	0.08

S.E. stands for standard error, column "mice" records the number of mice used in each experiments. Weeks are weeks after the infection.

TABLE 2

DELAYED HYPERSENSITIVITY RESPONSE TO HOMOLOGOUS AND HETEROLOGOUS ANTIGENS IN CBA/ca MICE INFECTED WITH L. mexicana.

TESTED WITH L. major ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	4	0.10	0.04	0.00	0.00	0.07	0.03
4	4	0.15	0.03	0.13	0.03	0.00	0.00
8	4	0.03	0.03	0.10	0.06	0.00	0.00
12	4	0.05	0.05	0.00	0.00	0.05	0.05
16	4	0.05	0.05	0.05	0.05	0.05	0.05

TESTED WITH L. mexicana ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	4	0.33	0.15	0.25	0.05	0.05	0.05
4	4	0.73	0.23	0.38	0.13	0.25	0.15
8	4	0.73	0.13	0.48	0.11	0.33	0.09
12	5	0.52	0.08	0.16	0.07	0.18	0.08
16	6	0.28	0.05	0.17	0.03	0.10	0.03

S.E. stands for standard error, column "mice" records the number of mice used in each experiments. Weeks are weeks after the infection.

TABLE 3

DELAYED HYPERSENSITIVITY RESPONSE TO HOMOLOGOUS AND HETEROLOGOUS ANTIGENS IN BALB/c MICE INFECTED WITH L. major.

TESTED WITH L. major ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	4	0.08	0.03	0.03	0.03	0.03	0.02
4	4	0.15	0.09	0.05	0.03	0.13	0.05
8	4	0.03	0.03	0.05	0.03	0.05	0.05
12	4	0.05	0.05	0.10	0.04	0.10	0.00
16	4	0.03	0.03	0.03	0.03	0.03	0.03

TESTED WITH L. mexicana ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	4	0.35	0.16	0.15	0.06	0.00	0.00
4	4	0.35	0.13	0.23	0.05	0.13	0.03
8	4	0.25	0.05	0.18	0.08	0.17	0.03
12	5	0.16	0.05	0.10	0.04	0.05	0.05
16	4	0.20	0.04	0.10	0.00	0.10	0.00

S.E. stands for standard error, column "mice" records the number of mice used in each experiments. Weeks are weeks after the infection.

TABLE 4

DELAYED HYPERSENSITIVITY RESPONSE TO HOMOLOGOUS AND HETEROLOGOUS ANTIGENS IN BALB/c MICE INFECTED WITH L. mexicana.

TESTED WITH L. major ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	3	0.10	0.10	0.03	0.03	0.03	0.03
4	3	0.00	0.00	0.03	0.03	0.07	0.07
8	3	0.03	0.03	0.07	0.07	0.10	0.00
12	4	0.03	0.03	0.03	0.03	0.00	0.00
16	4	0.00	0.00	0.00	0.00	0.00	0.00

TESTED WITH L. mexicana ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	4	0.50	0.09	0.15	0.03	0.15	0.05
4	5	1.06	0.10	0.34	0.08	0.43	0.06
8	4	0.95	0.10	0.35	0.05	0.07	0.07
12	5	0.20	0.05	0.08	0.04	0.00	0.00
16	6	0.25	0.04	0.22	0.05	0.11	0.03

S.E. stands for standard error, column "mice" records the number of mice used in each experiments. Weeks are weeks after the infection.

TABLE 5 A

DELAYED HYPERSENSITIVITY RESPONSE CONTROL TESTS.
MEASUREMENTS OF FOOT PADS OF UNINFECTED CBA/ca
MICE INJECTED WITH L. major ANTIGEN,
L. mexicana ANTIGEN AND PBS SOLUTION

TESTED WITH L. major ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	3	0.03	0.03	0.03	0.03	0.00	0.00
4	3	0.03	0.03	0.03	0.03	0.00	0.00
8	3	0.07	0.03	0.06	0.03	0.00	0.00
12	3	0.07	0.03	0.03	0.00	0.05	0.05

TESTED WITH L. mexicana ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	3	0.03	0.03	0.00	0.00	0.00	0.00
4	3	0.07	0.03	0.07	0.03	0.00	0.00
8	3	0.03	0.03	0.00	0.00	0.00	0.00
12	3	0.03	0.07	0.03	0.03	0.00	0.00

TESTED WITH PBS

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E	MEAN	S.E.	MEAN	S.E.
2	3	0.00	0.00	0.03	0.03	0.00	0.00
4	3	0.05	0.02	0.10	0.00	0.00	0.00
8	3	0.00	0.00	0.00	0.00	0.00	0.00
12	3	0.10	0.00	0.05	0.05	0.00	0.00

S.E. stands for standard error, column "mice" records the number of mice used in each experiments. Weeks are weeks after the infection.

TABLE 5 B

DELAYED HYPERSENSITIVITY RESPONSE CONTROL TESTS.
MEASUREMENTS OF FOOT PADS OF UNINFECTED BALB/c
MICE INJECTED WITH L. major ANTIGEN,
L. mexicana ANTIGEN AND PBS SOLUTION

TESTED WITH L. major ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	3	0.03	0.03	0.03	0.03	0.00	0.00
4	3	0.00	0.00	0.03	0.03	0.00	0.00
8	3	0.07	0.03	0.03	0.03	0.00	0.00
12	3	0.00	0.00	0.07	0.03	0.00	0.00

TESTED WITH L. mexicana ANTIGEN

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.
2	3	0.03	0.03	0.00	0.00	0.00	0.00
4	3	0.07	0.03	0.03	0.03	0.00	0.00
8	3	0.00	0.00	0.03	0.03	0.00	0.00
12	3	0.03	0.03	0.03	0.03	0.00	0.00

TESTED WITH PBS

		24 H		48 H		72 H	
WEEKS	MICE	MEAN	S.E	MEAN	S.E.	MEAN	S.E.
2	3	0.05	0.05	0.00	0.00	0.00	0.00
4	3	0.00	0.00	0.10	0.00	0.00	0.00
8	3	0.00	0.00	0.05	0.05	0.00	0.00
12	3	0.00	0.00	0.05	0.05	0.00	0.00

S.E. stands for standard error, column "mice" records the number of mice used in each experiments. Weeks are weeks after the infection.

FIGURE 1

Foot pad DHR of CBA/ca mice infected with 10^5 amastigotes *L. major*. Each point represents the mean (+/- standard error) of 3 to 6 mice intradermally tested with 80 µg of *L. mexicana* (A) or *L. major* (B) antigen at 24 ●- - - -● and 48 ○----○ hours after the injection. ▲——▲ represents the average of measurements at 24 and 48 hour of non-infected mice (controls) injected with the respective antigen.

FIGURE 2

Foot pad DHR of CBA/ca mice infected with 10^5 amastigotes of *L. mexicana*. Each point represents the mean (+/- standard error) of 4 to 6 mice tested at 24 ●- - -● and 48 ○----○ hours with *L. mexicana* (A) or *L. major* (B) antigen. ▲——▲ represents the average of the measurements at 24 and 48 hour of non-infected mice (controls) tested with the respective antigen.

FIGURE 1

CBA/ca L. major

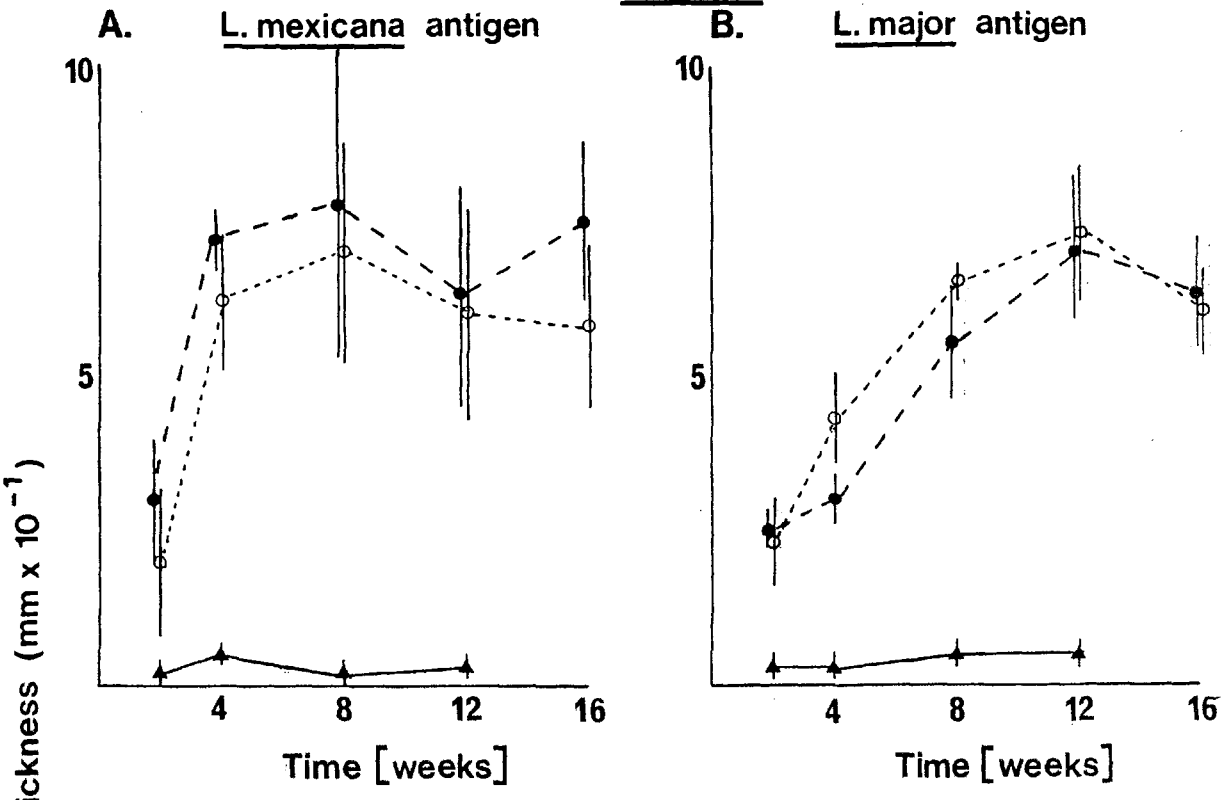


FIGURE 2

CBA/ca L. mexicana

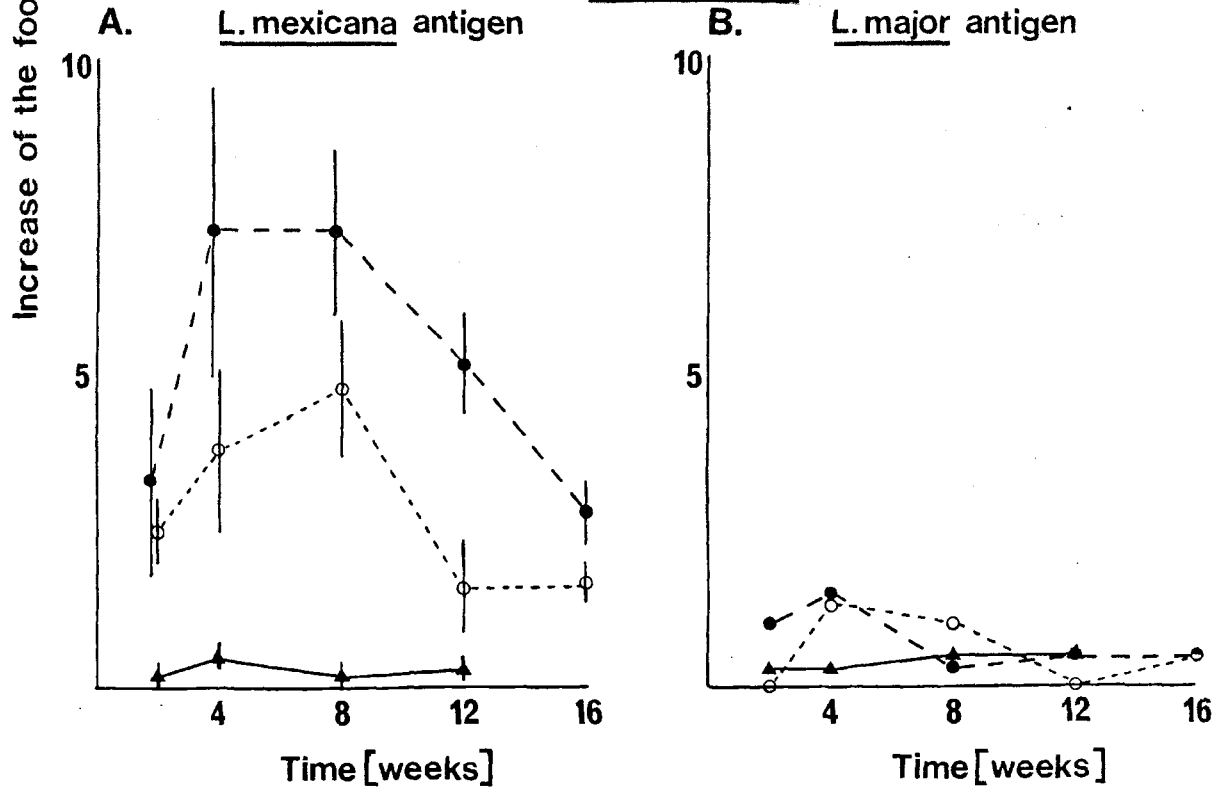


FIGURE 3

Foot pad DHR of BALB/c mice infected with 10^5 amastigotes *L. major*. Each point represents the mean (+/- standard error) of 4 mice intradermally tested with 80 µg of *L. mexicana* (A) or *L. major* (B) antigen at 24 ●---● and 48 hours O---O after the injection. ▲——▲ represents the average of measurements at 24 and 48 hours of non-infected mice (controls) injected with the respective antigen.

FIGURE 4

Foot pad DHR of BALB/c mice infected with 10^5 amastigotes of*L. mexicana*. Each point represents the mean (+/- standard error) of 3 to 6 mice tested at 24 ●---● and 48 hours O----O with *L. mexicana* (A) or *L. major* (B) antigen. ▲——▲ represent the average of the measurement at 24 and 48 hour of non-infected mice (controls) tested with the respective antigen.

FIGURE 3

BALB/C L.major

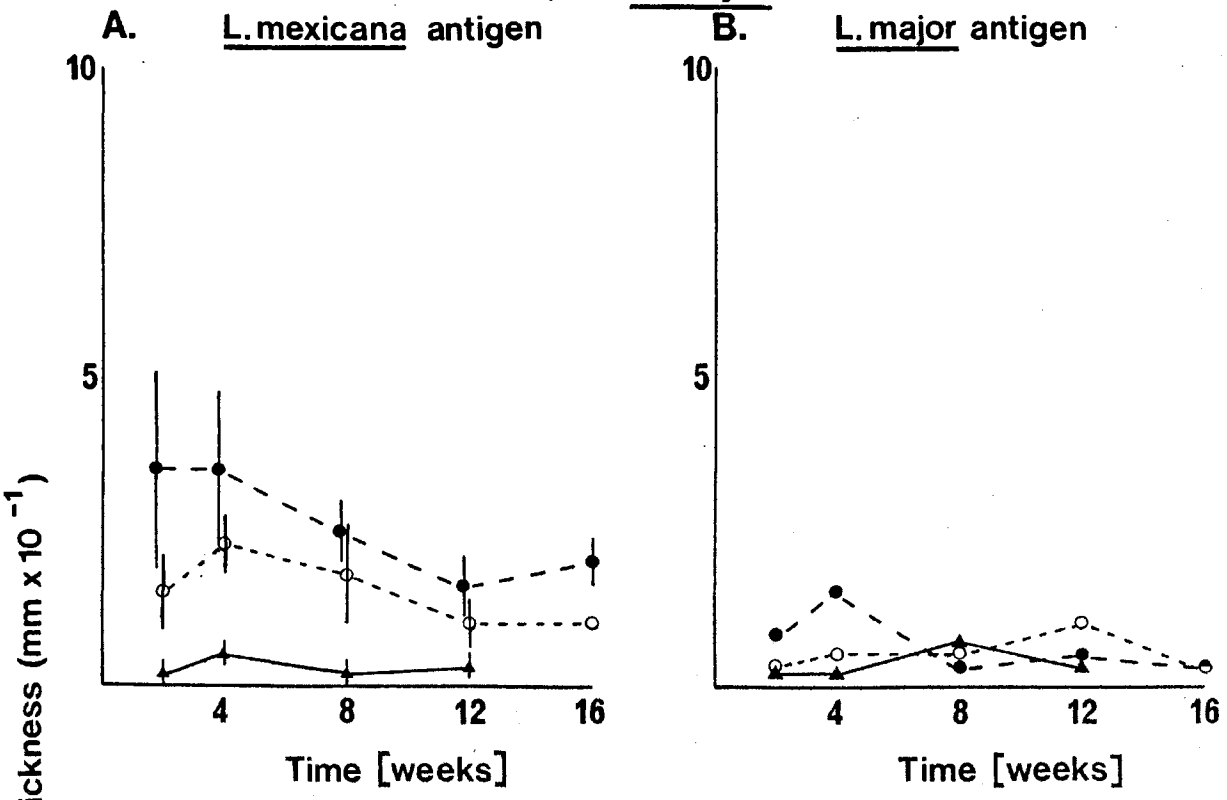
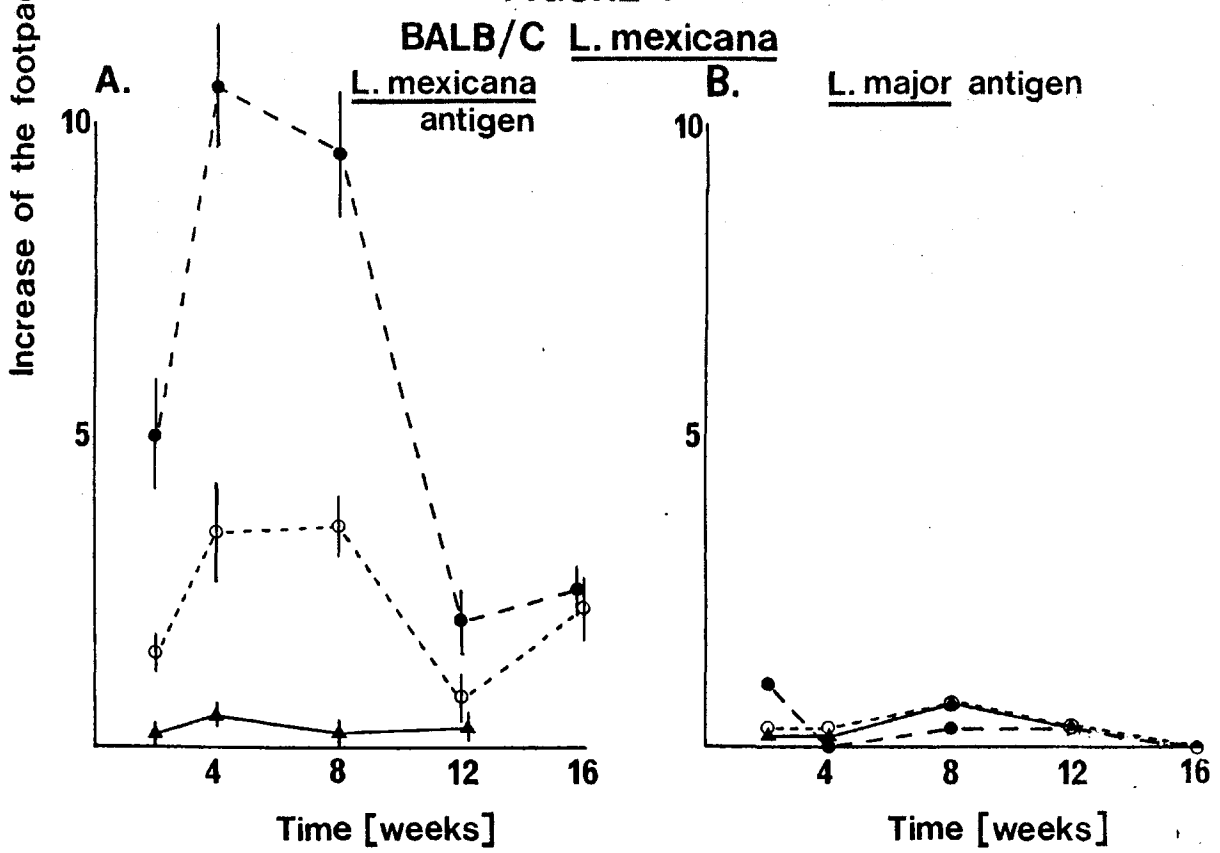


FIGURE 4

BALB/C L. mexicana



The kinetics of the response, however, differed markedly from that of CBA/ca *L. major* infected mice, in that the swelling reached its peak at 24 hours, but had dropped significantly by 48 hours and thus resembled a Jones-Mote-type response.

BALB/c mice which are susceptible to infection with *L. major*, unlike CBA/ca mice, did not develop a DHR to the homologous antigen (Table 3; Figs 3a, 3b.). They did, however, develop a response to *L. mexicana* antigen, lasting 8 weeks.

No difference was observed between the response to PBS and to antigen in uninfected animals (Table 5 a, b)

V.3.1.1 Histology

V.3.1.1.1 Inoculation site

Healing infection

(CBA/ca infected with *L. major*)

In these mice, the histological changes at the skin test site varied in intensity during the evolution of the infection and with the antigen used. Thus, by the second week, in the animals tested for DHR with *L. major* derived antigen, the changes were restricted to the vascular system. The small vessels situated between papillar and reticular dermis and the large vessels in the deep dermis were dilated, congested and exhibited tight intravascular packing of red blood cells. After four weeks

of the infection, abundant white cells were detected in the lumina of the vessels, and the alterations of the vascular structures increased; blurring of the vascular margins, hyperplasia and hypertrophy of endothelial cells and sometimes obliteration of the vessel's lumina were observed. From the twelfth week onwards the large blood vessels of the deep dermis showed scanty cells in their lumina and exhibited vacuolization of their walls.

The inflammatory cells at the site of antigen testing were lymphocytes, monocytes, macrophages, mast cells and eosinophils. These cells were apparent by the fourth week of infection, and were observed adopting intervascular distribution, and, very rarely, a nodular arrangement. As the infection evolved, the infiltrate grew bigger and extended from the central to the deep dermis, involving the muscular layer, and although it continued to be predominantly intervascular, a perivascular distribution of cells was also seen.

The relative proportions of the infiltrating cells varied during the course of the delayed hypersensitivity response, thus, by the eight week, eosinophils were as abundant as mononuclear monocytes; after the twelfth week, mononuclear cells predominated (Figs. 5,6).

Muscular structures exhibited changes that consisted of hyaline degeneration (Fig. 5) and disruption of muscle bundles which sometimes were invaded by polymorphonuclear leucocytes.

Nerves showed a vacuolar appearance due to retraction of the nerve bundles and monocytes and mast-cells were

FIGURE 5

CBA/ca mouse, 8 weeks infected with *L. major*. Dermal infiltrate at the DHR testing site as a result of the intradermal injection of 80 µg of *L. major* soluble antigen. The infiltrate consisted of mononuclear cells and eosinophils. Note in the area adjacent to the infiltrate a zone of muscular degeneration (m). 48 hour reaction. Section, H & E. X 50.

FIGURE 6

CBA/ca mouse, 12 weeks *L. major* infected. Dermal infiltrate at the DHR testing site after injection of 80 µg of soluble *L. major* antigen. The infiltrate consists mainly of mononuclear cells. 48 hour reaction. Section, H & E. X 320.

FIGURE 5

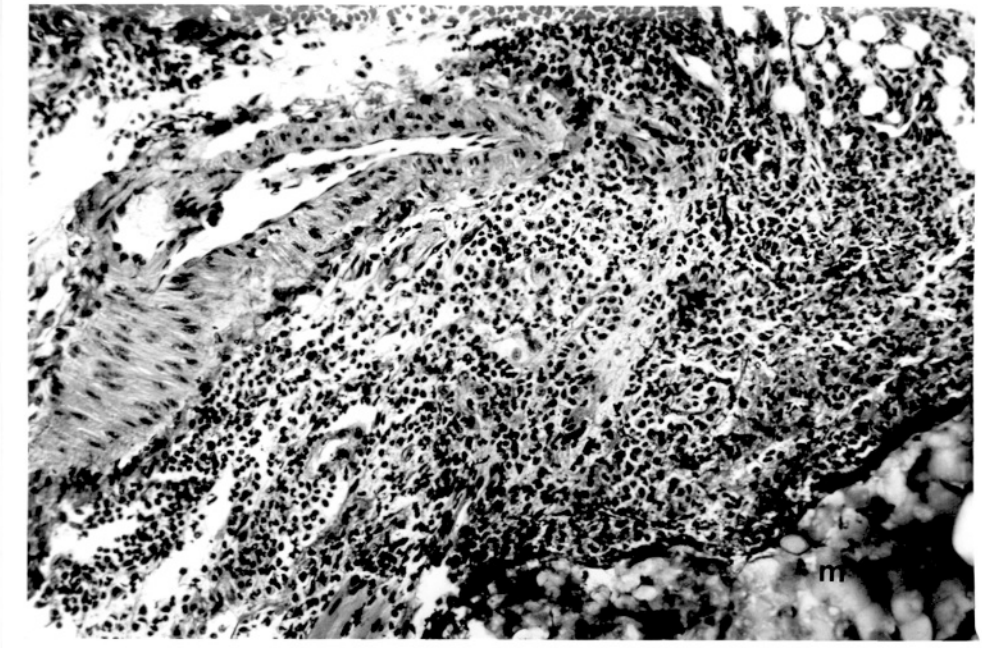
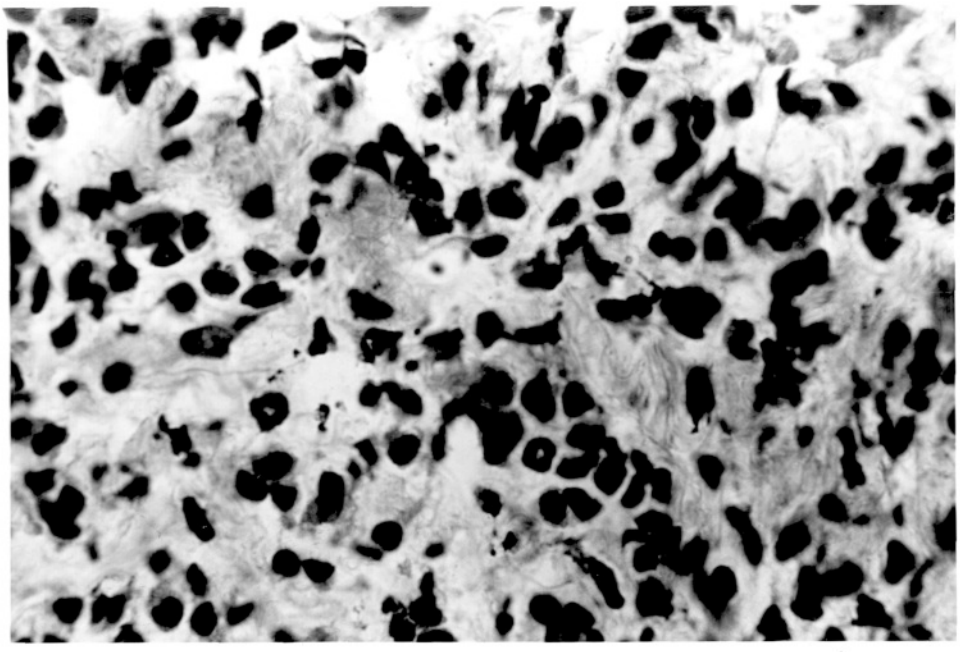


FIGURE 6



observed infiltrating the structure.

The epidermis alterations consisted of focal zones of large heavily vacuolated areas in the prickle cell and basal layer. These alterations were first observed by the eighth week. By the twelfth week, the changes described extended to most of the epidermis, and by the sixteenth week, acanthosis was also observed.

When the DHR was tested in the same experimental model (*L. major-CBA/ca*) using *L. mexicana* antigen, the changes in the skin were similar to those described for *L. major* antigen, but the alterations in the dermis and epidermis were more intense and detected earlier. Thus, two weeks after infection *L. mexicana* antigen elicited at the testing site, an inflammatory response similar to that reached by the fourth to eighth week when the testing antigen was *L. major*. Concentric aggregates of cells (Fig. 7) were more frequent when the challenging antigen was *L. mexicana* than when it was *L. major*.

Non-healing infections (CBA/ca and BALB/c infected with *L. mexicana* and BALB/c infected with *L. major*)

In these mice, the inflammatory infiltrate elicited after testing with *L. mexicana* derived antigen, had the same cellular composition as described above, although eosinophils and mast-cells were particularly abundant, especially by the fourth and eighth week (Figs. 8,9). The distribution pattern of the infiltrate appeared predominantly intervascular; nodular granuloma were seldom if ever seen. After 8 weeks of infection, the DHR started

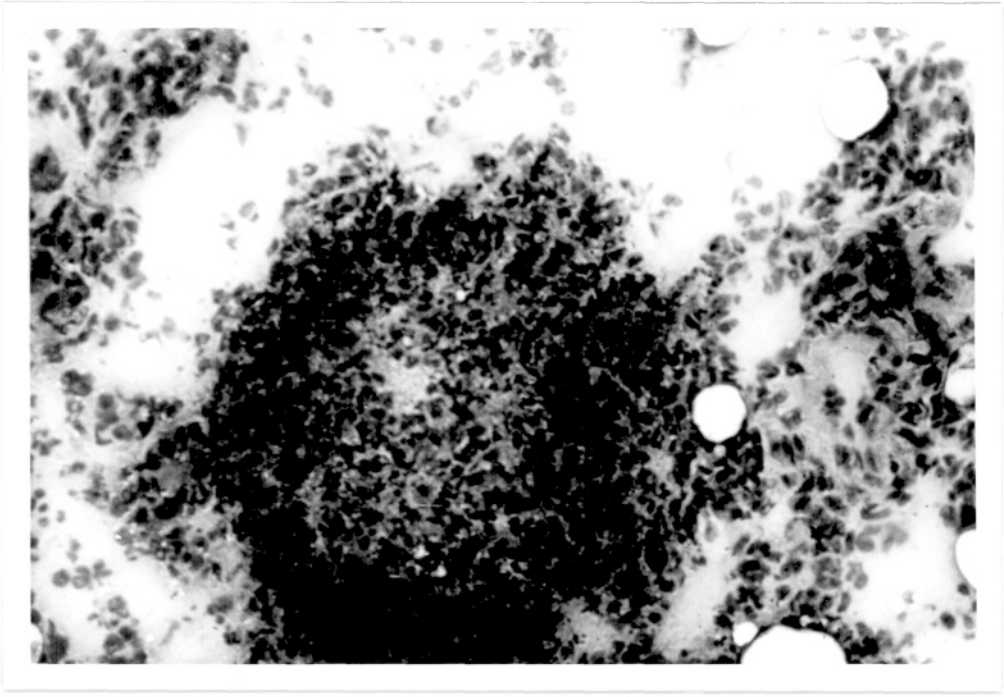


FIGURE 7

CBA/ca mouse 8 weeks infected with *L. major*. Dermal infiltrate showing a nodular granuloma at the DHR testing site after intradermal injection of 80 ug of soluble *L. mexicana* antigen. 48 hour reaction. Section, H & E. X 80.

FIGURE 8

CBA/ca mouse, 8 weeks *L. mexicana* infected. Dermal infiltrate showing abundance of eosinophils at the DHR testing site after intradermal injection of 80 µg of *L. mexicana* soluble antigen. 48 hour reaction. Section, H & E. X 60.

FIGURE 9

CBA/ca mouse, 8 weeks *L. mexicana* infected. Dermal infiltrate showing mast-cells (arrows). Challenging antigen *L. mexicana*. 48 hour reaction. Methyl-green pyronin. X 128

FIGURE 8

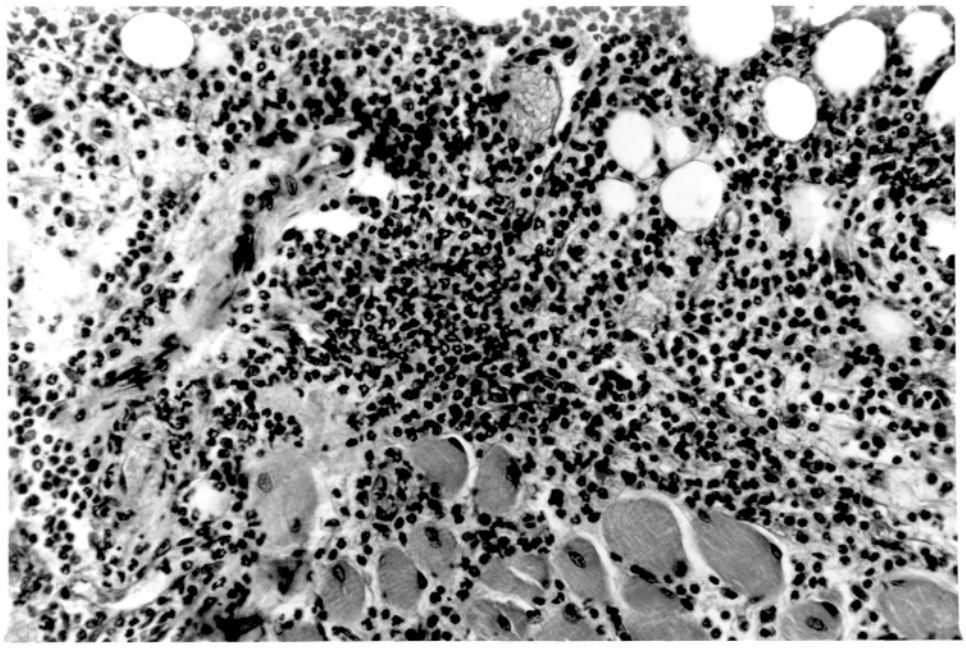
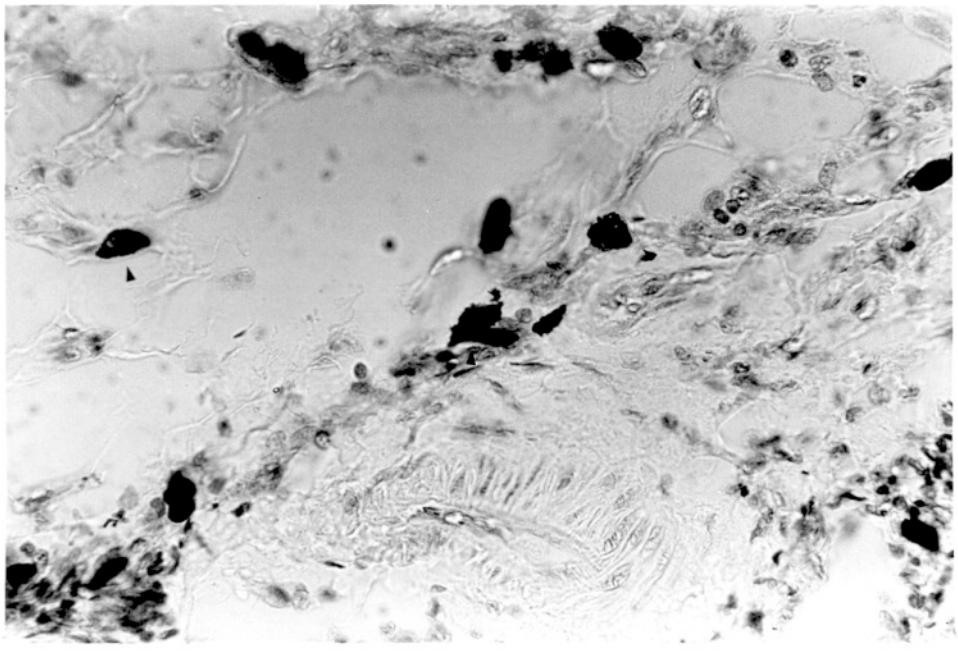


FIGURE 9



to decline and so did the infiltrate at the testing site.

L. major derived antigen failed to elicit any DHR in any of these injected mice, and the histology at the site of antigen injection was similar to that observed in control animals, in which only congested blood vessels filled with red blood cells and some mast-cells scattered throughout the sections were detected.

V.3.1.1.2 Lymph node

In general, the lymph node draining the site where DHR developed showed hypertrophic and congested blood vessels, filled with red blood cells throughout their structures.

The paracortical area was greatly hypertrophied, and lymphocytes were not so closely packed as in the cortex. Lymphocytes undergoing transformation and immunoblasts were abundant.

The cortical area showed scanty signs of development; very rarely secondary follicles with poorly developed germinal centres were detected. An eosinophilic band consisting of cells of the macrophage-phagocytic system (histiocytes) was observed overlaying the lymphoid structure in the subcapsular area from the second week after infection. Similar types of cell also occured singly in the paracortical area. After this time, the histiocytes were observed forming aggregates. The histiocytes in these aggregates had different appearances in each experimental group. In CBA/ca mice infected with *L. major* and tested with homologous and heterologus antigens, the histiocytes were highly epithelioid and aggregated in whorls. In the

other experimental groups, the histiocytes did not aggregate in whorls, were less differentiated and contained cell debris in their cytoplasm, which was only slightly eosinophilic.

Eosinophils infiltrating the whole of the lymph node were particularly abundant in the groups exhibiting the Jones-Mote-type reaction.

Mast-cells were abundant throughout all the testing period in all the groups exhibiting non-healing lesions. These cells were observed along the marginal sinus and at the lymph node's hilus.

The medulla was in general a small area, apparently compressed by the development of the paracortex.

The lymph node from control animals and experimental groups in which a DHR was not detected only showed congestion of the vascular system.

V.3.2 ANTIBODY RESPONSE

Tables 6 and 7 show the antibody titres in the sera of CBA/ca and BALB/c mice infected with *L. major* or *L. mexicana*, using homologous and heterologous antigens in the agglutination and immunofluorescence tests.

In all the experimental groups, the kinetics of the response, as revealed by the agglutination technique, showed an early rise in the titre of antibody from the second week onwards and oscillated at high levels thereafter.

The sera of normal mice agglutinated both antigens up to

TABLE 6

ANTIBODY RESPONSE MEASURED AS DIRECT AGGLUTINATION TITRES IN POOLS OF SERA FROM CBA/ca AND BALB/c MICE AT VARIOUS INTERVALS AFTER INFECTION WITH Leishmania major AND Leishmania mexicana AMASTIGOTES.

RESULTS FROM CBA/ca MICE

	INFECTED WITH L. major		INFECTED WITH L. mexicana		UNINFECTED CONTROLS	
TEST Ag =	L. maj.	L. mex.	L. maj.	L. mex.	L. maj.	L. mex.
WEEKS						
2	1280	640	10240	640	80	80
4	1280	640	10240	640	640	160
8	5000	1280	10240	1280	20	80
12	320	320	10240	160	ND	ND
16	320	640	320	640	ND	ND

RESULTS FROM BALB/ c MICE

	INFECTED WITH L. major		INFECTED WITH L. mexicana		UNINFECTED CONTROLS	
TEST Ag =	L. maj.	L. mex.	L.maj.	L. mex.	L. maj.	L. mex.
WEEKS						
2	160	320	160	80	40	20
4	10240	80	40	40	80	40
8	2560	1280	1280	160	160	80
12	10240	160	10240	80	ND	ND
16	160	320	160	320	ND	ND

Results expressed as the reciprocals of the highgest dilution giving ++ agglutination. Pools consisted of sera from three mice. Spontaneous agglutination control negative. ND : not done.

TABLE 7

TITRES OF IgG AND IgM ANTIBODIES, MEASURED BY INDIRECT IMMUNOFLUORESCENCE TECHNIQUE IN POOLED SERA FROM CBA/ca AND BALB/c INFECTED WITH EITHER L. mexicana OR L. major

	INFECTED WITH L. major				INFECTED WITH L. mexicana			
ANTIBODY TITRED	IgG		IgM		IgG		IgM	
TESTING ANTIGEN	L. maj.	L. mex.	L. maj.	L. mex.	L. maj.	L. mex.	L. maj.	L. mex.
WEEKS AFTER INFECTION								
	CBA/ca MICE							
2	N	N	20	20	N	N	20	40
4	40	80	20	20	40	40	20	20
6	40	80	40	10	40	20	10	20
8	80	80	40	20	40	40	10	40
12	640	320	80	10	160	160	10	80
16	640	320	80	10	160	640	10	80
18	1280	160	40	10	160	640	10	80
	BALB/c MICE							
2	N	N	10	20	N	N	10	10
4	20	40	10	10	10	20	10	10
6	ND	ND	ND	ND	ND	ND	ND	ND
8	40	80	10	10	80	160	20	10
12	1280	2560	10	10	40	40	20	10
16	160	160	40	40	320	160	10	20
18	320	640	20	40	N	N	N	N

N = negative. ND = not determined. Pools consisted of sera from 3 mice.

ReSults expresed as reciprocals of highest serum dilution giving ++ immunofluorescence.

1:160. The spontaneous agglutination controls were always negative.

The immunofluorescent technique detected no significant levels of IgG antibodies before the fourth week and then only at low levels. Thereafter the antibody levels increased steadily, reaching levels comparable to those found with the agglutination technique by the sixteenth week after infection.

Low levels of IgM antibodies were detected in all experimental groups, throughout the observational period.

Promastigotes treated with normal sera or PBS instead of sera from infected mice did not fluoresce. Promastigote (Figs. 10, 11) organelles stained differentially at the flagellum, cytoplasm, nucleus and kinetoplast demonstrating successively increase of brillance.

By the end of the observations, 16 weeks after infection, all experimental groups produced similar levels of antibodies. The antibody titres detected by the immunofluorescence technique were similar when tested against either homologous or heterologous antigens. The agglutination technique, however, produced higher titres with *L. major* derived antigen in both *L. major* and *L. mexicana* infected mice.

Antibody titres and DHR responses were tested for correlation using SPEARMAN's rank correlation coefficient (SIEGEL, 1956) (TABLE 8). No correlation was found between agglutinating antibody titres and DHR in any of the experimental models. IgM antibody titres were correlated with DHR only in CBA/ca mice infected with *L. major*

TABLE 8

SPEARMAN'S RANK CORRELATION COEFFICIENTS FOR THE CORRELATIONS BETWEEN IMMUNOFLUORESCENT AND AGGLUTINATING ANTIBODIES TITRES AND DELAYED HYPERSENSITIVITY RESPONSE TO Leishmania ANTIGENS IN FOUR EXPERIMENTAL MODELS OF CUTANEOUS LEISHMANIASIS.

	IMMUNOFLUORESCENT ANTIBODY TITRES (IgM)		IMMUNOFLUORESCENT ANTIBODY TITRES (IgG)		AGGLUTINATING ANTIBODY TITRES	
DHR ANTIGEN =	L.maj.	L.mex.	L.maj.	L.mex.	L.maj.	L.mex.
INFECTION MODELS						
CBA/ca MICE L. major	0.95 *	0.36	0.82	0.11	0.26	0.00
CBA/ca L. mexicana	0.24	0.63	−0.52	−0.56	0.00	0.71
BALB/c L. major	−0.10	−0.52	0.00	−0.67	0.54	−0.13
BALB/c L. mexicana	0.65	0.00	−0.22	−0.40	0.36	0 .15

* = significant at the 0.05 probability level.

DHR ANTIGEN specifies the antigen used for the DHR tests.

FIGURE 10

Indirect immunofluorescence on *L. major* promastigotes using sera from BALB/c mice, 12 weeks infected with *L. major* (first layer, dilution 1: 640); goat anti-mouse IgG (Fc) (second layer, dilution 1:64). X 400.

FIGURE 11

Indirect immunofluorescence on *L. mexicana* promastigotes using sera from CBA/ca mice, 12 weeks infected with *L. mexicana* (first layer, dilution 1:320); goat anti-mouse IgG (Fc) (second layer, dilution 1:64). Note nuclear staining. X 100.

FIGURE 10

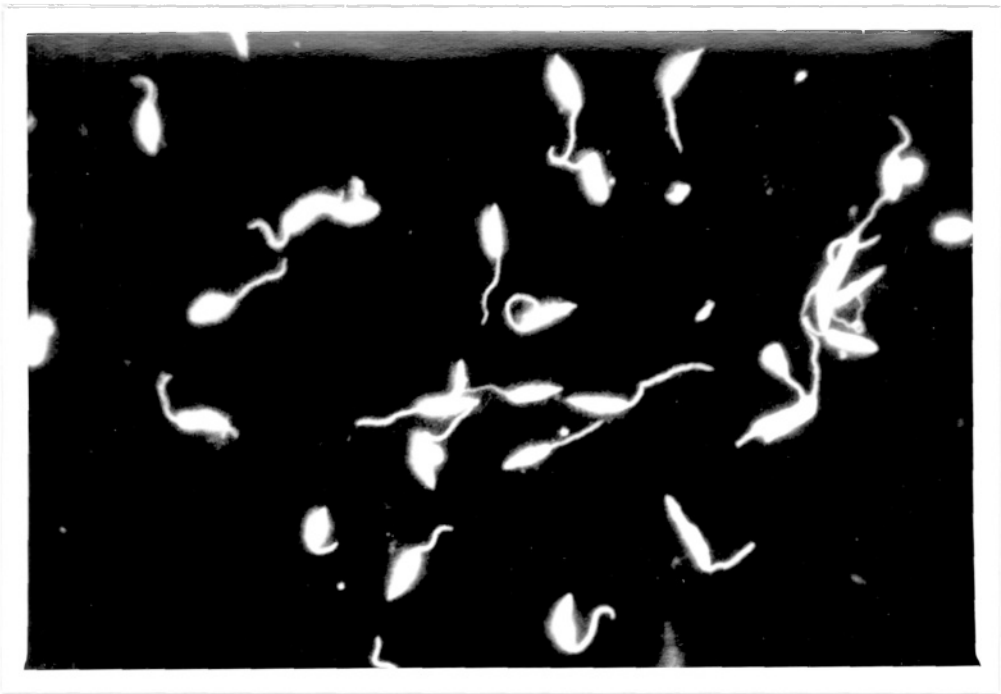


FIGURE 11

($p<0.05$), while IgG antibodies showed no significant correlation with DHR in any of the experimental groups. The value corresponding to the CBA/ca infected mice was close to significant ($rs = 0.82$). The value of the normal correlation coefficient for the same data was $r= 0.94$, (clearly significant) and although it is of little formal value due to the small numbers of observations, it would suggest that IgG antibodies might be correlated with DHR. However, a larger sample would be required to prove the correlation was significant with the less powerful SPEARMAN's rank correlation test.

V.4 DISCUSSION

Delayed hypersensitivity responses to protein antigens have been described in the literature as adopting the following forms (reviewed by TURK, 1980):

1 - Classic DHR (tuberculin-type): a long lasting state of hypersensitivity elicited following immunization with antigens incorporated in mycobacterial adjuvant. This type of reactivity reaches the maximum intensity between 24 and 30 hours, and the reaction is still marked after 48 to 72 hours. The histological picture of the tuberculin-type response consisted of a predominance of mononuclear cells with little infiltration by polymorphonuclear cells.

2 - The JONES - MOTE hypersensitivity, which is a transient response detected 5 to 10 days after sensitization with antigens incorporated in adjuvants containing no *Mycobacterium*. This form of skin reactivity reaches its maximum at 24 hours and is almost imperceptible by 48 hours.

3 - A further form of DHR is that elicited by skin contact with chemical compounds (1-fluoro-2,4-dinitrobenzene (DNFB), 2-ethoxymethylene-2-phenyl-5-oxazolone (OXAZOLONE). This reaction is similar to the JONES - MOTE-type in its kinetics, but differs from it in that the sensitivity state persists beyond 14 days. The association of JONES-MOTE and contact sensitivities with infiltration of the skin by basophilic leucocytes has been pointed out by RICHERSON et

al. 1970 and DVORAK et al. 1975. As a result of these studies, the term cutaneous basophil hypersensitivity (CBH) has been used to distinguish JONES-MOTE and chemical contact sensitivity from the tuberculin-type of delayed hypersensitivity.

In this study, the kinetics of the DHR appeared to be related to the susceptibility of mice to *Leishmania* infection. In CBA/ca mice, which were relatively resistant to infection by *L. major*, the kinetics of the DHR, tested with either the homologous or the heterologous antigen, was of the tuberculin-type. On the other hand, in mice highly susceptible to *Leishmania* infections (BALB/c and CBA/ca infected with *L.mexicana* and BALB/c infected with *L.major*), *L. mexicana* antigen elicited a DHR reminiscent of the JONES-MOTE-type, while *L. major* antigen failed to elicit any reaction in the same groups.

Tuberculosis and leprosy share with leishmaniasis the feature of having a broad spectrum of clinical forms (TURK & BRYCESON, 1971; LENZINI et al. 1977). The latter authors also studied the kinetics of DH response to PPD (purified protein derivative) antigen in patients grouped along a spectrum of clinical forms of tuberculosis. They found that patients with localized lesions, who responded well to treatment (RR patients), developed a tuberculin-type response, while patients with more disseminated forms of the disease (UI patients), developed JONES-MOTE-type responses. ALEXANDER & CURTIS (1979) showed a similar relation between DHR kinetics and clinical manifestations of disease in murine leprosy. C57Bl mice, which were

relatively resistant to infection with *Mycobacterium lepraemurium*, developed a tuberculin-type of DHR response when challenged with antigen from the same organism, while BALB/c mice which are relatively susceptible to the infection, developed a JONES-MOTE-type reaction.

The results reported here show that a similar relationship exists between the degree of resistance and the DHR in murine leishmaniasis.

Although both CBA/ca and BALB/c mice are highly susceptible to *Leishmania mexicana* and BALB/c mice are similarly susceptible to *L. major*, it is intriguing that *L. mexicana* antigen induces a JONES-MOTE-type response in *L. mexicana* infected BALB/c and CBA/ca mice and in *L.major* infected BALB/c mice while *L. major* antigen produced no response.

The paradoxically greater and earlier tuberculin-type DH reponse of CBA/ca mice, infected with *L. major* to the heterologous *L. mexicana* antigen instead of the homologous antigen, correlates well with the ability of *L. major* infection to protect both mice and man against *L. mexicana* infection, even before the development of homologous protection (ADLER & GUNDERS, 1964; ALEXANDER & PHILLIPS, 1978).

This is surprising, as CBA/ca mice are normally totally susceptible to *L. mexicana*. Perhaps an *L. major* specific antigen initiates the immune response through antigen presentation by Ia positive cells. Once the cell mediated response is in progress, other T lymphocyte subpopulations such as T_{DH} or T cytotoxic cells can

recognise antigens common to *L. mexicana* and *L. major*.

The immune response is currently conceived as the resulting balance of effector and suppressor mechanisms (TURK & PATH, 1976). According to this conception, JONES-MOTE and tuberculin-types of DHR responses are manifestations of increasing degrees of cell mediated immunity. Such a gradient of responses could be exemplified by the results of LENZINI et al. (1977) who found that the response to treatment in patients of tuberculosis, producing a JONES-MOTE-type response to PPD, was better than those anergic to the antigen, though not as good as those giving a tuberculin -type response. Similarly, ALEXANDER (1978) showed a strong correlation between resistance to infection and the magnitude of JONES-MOTE-type reaction in BALB/c mice infected with *M. lepraemurium*, while the greatest resistance to infection was in those animals producing a tuberculin-type response.

The concept of DHR being the result of a steady-state equilibrium between effector and suppressor effects appears to be further supported by the fact that cyclophosphamide (CY) pretreatment can transform a JONES-MOTE response in the direction of tuberculin-type response in several experimental systems (TURK, 1975; ALEXANDER, 1978;). Similarly, there is a diminution and an eventual disappearance of the JONES-MOTE-type response shown by the susceptible mice in this study.

The change from a JONES-MOTE to a tuberculin-type of response observed after CY pretreatment is believed to be

due to removal of suppressor cells, which may be either B lymphocytes (TURK, 1975) or T lymphocytes (ASKENASE et al. 1975; GILL & LIEW, 1978).

PRESTON et al. (1978) and ARREDONDO & PEREZ (1979), on the other hand, have proposed that suppressor T cell populations might be responsible for the impairment of DHR observed in CBA mice infected with massive inocula of *L. major* and in BALB/c mice infected with *L. mexicana* respectively. Such a view is supported by the finding of HOWARD et al. (1980 a) that inhibition of the DHR response in the *L. tropica*-BALB/c system was accompanied by the emergence of a T suppressor cell population.

Work on systems other than murine leishmaniasis have produced evidence that high doses of antigen can induce suppresor cells *in vitro* (KONTIAINEN & FELDMAN, 1976), and that supressor T cells induced in that manner can inhibit the induction and expression of DHR in mice (LIEW, 1977).

Several results support the view that an excess of antigen may be a possible cause of immunodepression in leishmaniasis:

a - In human diffuse cutaneous leishmaniasis (DCL), abundant parasites coincide wth immunodepression (reviewed by PRESTON & DUMONDE, 1975).

b - Intravenous injection of soluble antigen increases the severity of subsequent infection by *L. enriettii* in the guinea-pig (BRYCESON et al. 1970)

c - DCL can be induced in guinea-pigs by large inocula of *L. enriettii* (BRYCESON et al. 1974).

d - PRESTON & DUMONDE (1976) reported an inverse relationship between infecting dose and DHR in CBA mice infected with *L. major*.

This evidence suggests that in the present study, the growing number of amastigotes seen in the lesions as the infection progressed may have played a role in the decline of DHR response in the highly susceptible mice.

Other authors have proposed that blocking antibodies or immune complexes may perpetuate the cutaneous disease (GARNHAM and HUMPHREY, 1969; BRYCESON et al. 1972; ALEXANDER & PHILLIPS, 1978; MITCHELL et al. 1981), but no evidence has been put forward as yet to confirm this view.

Results of DHR are frequently reported only as a 24 or 48 hour measurements. The work of ALEXANDER & CURTIS (1979) on murine leprosy and my own results in this study, however, show the importance and potential prognostic value of kinetic studies. They could be used for treatment evaluation, especially in DCL, or to estimate the degree of resistance to infection in population studies.

Dissociation between DHR and protective immunity has been recently reported in murine leprosy (LOVIK & CLOSS, 1983; ADU et al. 1983). Similarly, in murine leishmaniasis ALLISON (quoted by MAUEL & BEHIN, 1974) reported that C3H mice reinoculated with *L. major* after recovering from a primary infection, developed ulcerative non-healing lesions, which were accompanied by DHR. These facts might question the association that has always been implied between protective immunity and DHR in *Leishmania* infection. However, elicitation of DHR in *Leishmania*

infections is generally speaking indicative of a good prognosis, and so far, the only readily available tool existing to evaluate levels of resistance to the infection in population studies.

The histological study at the sites of antigen inoculation in the mice tested for DHR showed a mononuclear infiltrate in all instances. However, in mice giving a JONES-MOTE-type response, there were also abundant eosinophils and mast-cells which, unlike the case of the mice producing a tuberculin-type response, persisted during the whole of the observation period. The abundance of polymorphonuclear leucocytes is more akin to an Arthus-type reaction (which is a type of immediate hypersensitivity response dependent on the formation of Ag-Ab complexes), than to typical DHR.

An abundance of granulocytes at the sites of DHR testing has also been observed in other studies involving *L. mexicana* infected C3H mice (GRIMALDI & MORIEARTY, 1981). It would appear unlikely that the reaction observed was in fact an Arthus reaction in view of the lack of correlation between the DHR reaction and the antibody levels, which was also observed by GRIMALDY & MORIEARTI in the study just mentioned. It is thought that the abundance of eosinophils is a characteristic feature of the hypersensitivity reaction in mice. CROWLE (1975), reviewing the literature on DHR response of these animals, found that, compared with man and guinea pigs, mice showed less induration at the site of inoculation and granulocytes were often a prominent feature of the histology, especially 24

hours after the inoculation of the antigen. For example, in mice the DHR to polysacharides although showing abundant mononuclear cells, is dominated by polymorphonuclear leucocytes, while in the guinea pig, the same antigen elicited an infiltrate consisting predominantly of mononuclear cells. This response was certainly not an Arthus reaction since it could not be transferred by serum and was transferred by cells.

The nature of the antigen must also play a role, since several particulate antigens, such as *Salmonella sp.* and LCM virus, induce DHR in mice in which granulocytes are not predominant (CROWLE, 1975). It is interesting to note in this respect that the lesions in mice infected with *L. mexicana*, unlike those infected with *L. major*, showed abundant eosinophils.

The presence of mast-cells in the antigen inoculation sites and lymph nodes of the animals tested for DHR is a further indication of the JONES-MOTE character of the response. As PADAWAR (1963) noted, rats and mice lack detectable circulating basophils while having abundant mast-cells. The fact that they are capable of mounting cellular responses in the absence of basophils is interpreted as supporting the view that mast-cells replace basophils in the cutaneous basophilic hypersensitivity (CBH) of mice and rats. Mast-cells indeed resemble basophils in morphology, granule content and in their pharmacological and immunological properties. This view is shared by DVORAK & DVORAK (1975).

The changes and the differential degree of development

observed in the paracortical area of the draining lymph nodes observed in this study during the evolution of the infection (see Chapter II) confirm the cellular nature of the reaction and match the kinetics of the reaction.

In relation to humoral response, although the kinetics of the response of agglutinating antibodies was different from that of the immunofluorescent antibodies, there were no differences between the infection models, and by the end of the observations the titres of both types of antibodies were similar. These results agree with those of OLOBO et al. (1980) who found no differences in the kinetics and antibody levels in strains of mice with different degrees of susceptibility to infection with *L. major*. This would indicate that the humoral response plays no important role in protective immunity. However, other evidence suggests that antibodies may play a role in protective immunity:

PRESTON et al. (1972) and PRESTON & DUMONDE (1976) working with *L. major* infected CBA mice, showed:
a - That immune-serum enhances the protective capacity of immune-peritoneal cells in immunotransfer experiments.
b - That thymectomy, which depresses cell mediated immunity, manifested by failure of DHR, also depresses immunofluorescent antibodies.

POULTER (1980) studying *L. enriettii* infected guinea pigs demonstrated that: a - Antibody levels increase when healing commenced and were highest in convalescent animals. b - The serum from recently challenged

convalescent guinea pigs had a protective effect in passive recipients, which was made evident by the smaller number of parasites present in the tissues of the recipient animals, as compared to the number of parasites observed in the tissues of similarly infected controls.

In this study, the correlation found between IgM and probably IgG antibodies detected by immunofluorescent technique and DHR, in the *L. major* CBA/ca model, would seem to support the association between fluorescent antibodies and cell mediated immunity found by PRESTON et al. (1972) in the same infection model.

Promastigotes of *Leishmania* are agglutinated by the serum of uninfected animals of several species (ULRICH et al. 1968; REZAI et al. 1969, 1975; SCHMUNIS & HERMAN, 1970). Lysis was rare in the experiments by ULRICH and coworkers, but SCHMUNIS & HERMAN found lytic activity in all sera tested.

The existing reports in relation to this matter using normal mice sera are contradictory: all the authors mentioned above except SCHMUNIS & HERMAN, (1970), failed to demonstrate agglutination of promastigotes of *L. donovani, L. enriettii and L. major*. PRESTON et al. (1972) also reported no agglutination of *L. tropica* promastigotes by normal CBA mice serum. However, BRAY, 1983 (personal communication) has observed agglutination and eventual lysis of *L. major* and *L. mexicana* promastigotes. BRAY pointed out that, for lysis to occur, the complement content in the serum must be carefully preserved.

In this study, *L. mexicana* and *L. major* promastigotes were agglutinated, but not lysed by normal sera from all the mice strains used.

The conflicting reports may be due to differences in age of the cultures, factors contained in the blood of cultures, which may interact with the sera tested and the reagents used to grow the parasites, (ULRICH et al.1968) and due to the processes used in the preparation and conservation of the complement in the serum (methods of preparation often inactivate complement). To get lysis mouse serum must be used within hours of collection or within 2 weeks of freezing in liquid nitrogen (BRAY, personal communication).

The origin of natural antibodies is still to be determined. The following possibilities have been suggested: a - immunization following subclinical infection, b - exposure to heterogenic antigens and c - genetic determination of antibodies (REZAI et al. 1975).

V.5 Conclusions

This study showed:

1- The existence of an spectrum of DHR to *Leishmania* antigen which correlated with the clinical forms observed in the different infection models.

Mice exhibiting non healing-lesions, challenged with *L. mexicana* antigen produced a JONES-MOTE-type DHR, which was followed by desensititation of the response to the parasite antigen after the eighth week of infection. *L. major* antigen elicited no response in mice with non-healing

lesions.

Mice exhibiting self-healing lesions on the other hand produced a tuberculin-type response when challenged with both, *L. major* and *L. mexicana*, which was maintained until the end of the observations.

2- The study illustrated the usefulness of the kinetics study in DHR testing. It is suggested that they may be of value in evaluating treatment and in estimating the degree of resistance to infection in population studies.

3- The study of the humoral response by the determination of agglutinating and immunofluorescence antibody titers did not reveal any differences between the experimental groups exhibiting different susceptibility to *Leishmania* infections.

CHAPTER VI

CONCLUDING REMARKS

Cutaneous leishmaniasis presents a spectrum of disease (TURK & BRYCESON, 1971) in which, according to current evidence, the outcome of the infection depends on the nature and potential of the infecting organisms and in the form and extent of the host's immunological response which in turn depends on its genetic background.

The results presented in this thesis confirm once again that the infection of different strains of mice with different strains of leishmania parasites resemble the clinical features of the disease in humans, and confirm the relationship between clinical patterns, histological features and cellular responses.

Infection of CBA/ca mice with *L. major* paralleled the course of simple sore infection in man while CBA/ca infected with *L. mexicana*, and BALB/c infected with *L. major* or *L. mexicana* reproduced non healing forms of the disease.

The histopathology of the lesions varied according to the clinical course of the infection. Mice whose lesions eventually healed presented an inflammatory infiltrate consisting mainly of lymphocytes and cells of the phagocytic system in developmental stages ranging from monocytes to macrophages and epithelioid cells, in them

only a small proportion of the macrophages showed intracellular amastigotes and the parasitic load was moderate. As the infection evolved, parasites became scanty, the cells of the infiltrate gradually disappeared and the lesion eventually healed.

In mice exhibiting non-healing lesions, during the first week after infection, the infiltrate was roughly similar to that of the mice whose lesions healed during the first weeks afer infection, but more macrophages were found parasitized and the load of parasites was notably greater. As infection progressed, lymphocytes became scanty and were only found in the periphery of the lesions. Eosinophils were particularly abundant during the whole of the infection.

At the peak of development of the lesions, by the end of the observations, the inflammatory infiltrate consisted of masses of heavily parasitized macrophages, and lymphocytes were absent or very scanty.

The histopathological changes observed in the draining lymph nodes of the different experimental groups reflected the immunological status of the host. The cortex (B dependent area) which is the zone associated with the changes in the humoral response presented well developed germinal centers in all the infection models, but the paracortex (T dependent area) which is the area associated with changes in the cellular response was particularly hyperthrophic in mice exhibiting self-healing lesions and DHR of the tuberculin-type, supporting the current concept that immunity in leishmaniasis is predominantly cell

mediated.

In the mice whose lesions did not heal, the paracortical area exhibited a transient moderate development, which coincided with a transient DHR of the JONES-MOTE-type.

All the infection models showed an infiltration of the draining lymph nodes by cells of the histiocyte macrophage serie, whose appearance varied with the degree of resistance to the infection exhibited by the host: in the experimental infections modelling a high degree of resistance (CBA/ca infected with *L. major*), highly differentiated histiocytes with epithelioid appearance were observed, while in the remaining models, which showed a low resistance to the infection, the lymph nodes were infiltrated by histiocytes that became more and more undifferentiated with the progress and aggravation of the lesions, so that by the end of the observation period, most of the lymphoid tissue was replaced by vacuolated and heavily parasitized macrophages.

Thus, this study reports for the first time (to the best of my knowledge) a correlation between the degree of DHR and the level of of resistance to the infection in murine leishmaniasis: mice exhibiting self-healing lesions developed a tuberculin-type response when challenged with both *L. major* and *L. mexicana* antigens, while mice presenting-non healing lesions responded only to *L. mexicana* antigen and then with a transient JONES-MOTE-type DHR which was followed by anergy after the 12 weeks of the infection.

The role of the humoral response in protective immunity

is not clear as there were no differences in the levels of antibodies detected by agglutination and immunofluorescence between mice showing healing and non-healing lesions.

The immunopathological study demonstrated some new facts to account for the development of the lesion and the changes taking place in the draining lymph node in the course of murine cutaneous leishmaniasis:

Antigen, antibody and C3 complement factor were generally found associated in cells and structures of skin lesions and draining lymph nodes in all the experimental groups, and although the immunological significance of the finding of these immunoreactants in skin structures could not be established with certainty from the observations made in this study, their coincidence in the same structures at the same time suggests that they are complexed to each other. Such complexes could be the product of complexing of *Leishmania* antigen, specific antibody and complement or, could result from a cross reaction of complement fixing *Leishmania* antibody with tissue antigenic determinants. The fact that antibody specific to *Leishmania* does not react with normal skin tissue would make the second possibility less likely, unless, the hypothesized cross reaction were taking place between antibody to leishmania antigens and tissular determinants which are hidden in normal tissues but become exposed in the lesions, it would seem therefore that more work directed to the elution of the complexes found at these levels, their subsequent analysis on appropriate systems and the investigation of immune-complexes in the

circulation or deposited on other tissue structures is required before this point could be settled.

The deposition of a similar material in muscles on the other hand, would seem to indicate that muscles and *Leishmania* parasites share some antigenic determinants, since my experiments showed that antibody specific to *Leishmania*, from both immunized rabbits and infected mice, bound to the same muscle structures in uninfected mice. A similar phenomenon has been demonstrated in Chagas disease (COSSIO, 1974) and human visceral leishmaniasis (SZARFMAN, 1975).

Regarding the cells of the infiltrate:

Macrophages and reticular shaped cells resembling Langerhans cells were found presenting antigen in the skin infiltrate of all the experimental models with the exception of BALB/c mice infected with *L. mexicana*. These findings provide *in vivo* evidence in support of the existence of antigen presenting cells demonstrated *in vitro* by the experiments of HANDMAN et al. (1979) and BERMAN & DWYER (1981).

Eosinophils were found in the lesions of all infection models but were particularly abundant in those producing non-healing lesions. These cells were stained in their cytoplasm by *L. mexicana* antigen conjugated to FITC and by anti-mouse IgG FITC congugate, in all experimental groups. In BALB/c mice infected with *L. mexicana*, eosinophils contained simultaneously antibody, specific to *Leishmania* and complement, in mice of the same strain infected with *L. major*, eosinophils contained at

the same time *Leishmania* antigen and antibody. These findings in addition to the well known ability of eosinophils to phagocytose immune complexes (LITT 1961, 1964; HOUBA, 1976), suggest that these cells are doing just that in the lesions, which should not be surprising in view of the abundant evidence of the presence of immune complexes in other cells and structures of the infiltrate. However, as discussed in Chapter III, eosinophils can be non-especifically stained by FITC conjugates, and as it is difficult to differentiate with certainty between specific and non-specific staining, the possibility of an non-specific staining cannot be ruled out conclusively.

The immunofluorescence study in lymph nodes demonstrate in all the cases association of *Leishmania* antigen, antibody and complement in an intercellular pattern of staining in cortical and paracortical areas and as a lineal deposition in the sinus system. After reviewing the literature I would be inclined to conclude that the intercellular pattern of deposition could be associated with follicular dendritic cells in the cortical areas and with interdigitating cells in the paracortical areas. The deposits in the sinus system seem to be related to macrophages lining the structure.

Finally, experiments of cellular disagreggation performed in skin lesions and draining lymph nodes in order to identify lymphocyte subpopulations showed that B cells bearing IgG predominate over B cells bearing IgM. As the percentage of lymphocytes bearing *Leishmania* specific antibody was always greater than that of

lymphocytes bearing IgM, the specific antibody must have been associated with IgG. However, from the experiments performed in lymph nodes it was also observed that the percentage of lymphocytes bearing IgG substantially exceeded the percentage of lymphocytes bearing specific antibody, suggesting the occurence of polyclonal stimulation. T cells exhibited the Lyt 1^+ phenotype in susceptible as well as resistant mice, thus confirming the results reported by HOWARD et al. (1981), LOUIS et al. (1981) and LIEW et al. (1982).

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