IDENTIFICATION OF *LEISHMANIA* GENES ENCODING PROTEINS CONTAINING TANDEMLY REPEATING PEPTIDES

By

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We accept this thesis as conforming to the required standard

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Date Dec. 22, 1987
In order to identify *Leishmania* proteins which may be immunologically relevant or may play a role in interactions between *Leishmania* and its mammalian host, a *Leishmania major* genomic DNA library was constructed in the vector λgt11 and screened with antibodies raised to *Leishmania major* promastigote membranes. Two recombinant DNA clones were identified which encoded repetitive sequences (Clone 20 and Clone 39). Clone 20 encoded a repetitive peptide of 14 amino acids and clone 39 encoded an unrelated repetitive peptide of 10 amino acids. Analysis of one of these clones, Clone 20, indicated that there were two RNA transcripts of 9500 and 5200 nucleotides expressed which corresponded to this clone in *Leishmania major* and *Leishmania donovani* and this expression was not stage-specific. The results of genomic DNA analysis and isolation of additional clones encoding Clone 20 sequences indicated that there were two genes which corresponded to Clone 20 in both *Leishmania major* and *Leishmania donovani* and that these genes differed from one another with respect to the number of repeats which they contained. Antibodies against the fusion protein produced by Clone 20 recognized a series of *Leishmania major* proteins of apparent mol wt 250,000. Analysis of Clone 39 indicated that there was a single transcript of 7500 nucleotides expressed which corresponded to this clone in both *Leishmania major* and *Leishmania donovani* and that there was a single gene (or two identical genes) which encoded this transcript.

The genomes of many protozoan parasites exhibit a high degree of plasticity with respect to chromosome size and number. The presence of highly repetitive regions within their DNA may be involved in maintaining this plasticity, allowing the parasite to evolve rapidly under selective pressure. Repetitive regions have been identified within many *Plasmodia* antigens and have been implicated in the ability of this parasite to evade the host immune system. The presence of *Leishmania* genes encoding proteins containing tandemly repeating peptides may indicate that these proteins play a similar role in evading the host immune system during the course of *Leishmania* infections. The possible evolution and functions of repetitive proteins in protozoan parasites is discussed.
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<td>ATP</td>
<td>riboadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>riboadenosine 5'-triphosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
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<td>DNA</td>
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<td>isopropylthiogalactoside</td>
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<td>sodium dodecyl sulphate</td>
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<td>SDS polyacrylamide gel electrophoresis</td>
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<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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VII. INTRODUCTION

*Leishmania* are obligate intra-macrophage parasites within the mammalian host. The question of how this protozoan parasite is able to successfully invade and establish an infection within the macrophage is intriguing. The macrophage is the host's primary defense against microbial invasion and it is of interest to investigate the mechanisms by which *Leishmania* are able to evade the host's defenses and establish a parasitic infection in this host cell. Progress in the study of many parasites, including *Leishmania*, has been quite slow due to many factors including unavailability of cultured forms of the parasite resulting in inadequate numbers of parasites for biochemical or immunological analysis and lack of techniques and reagents suitable for use in such studies. The discovery of methods which enabled at least some forms of certain parasites to be cultured *in vitro* and the advent of recombinant DNA and hybridoma technology has finally made the field of parasitology amenable to detailed biochemical, molecular and immunological investigations. Advances in the study of parasitology in recent years have allowed the relationships between host and parasite to be analyzed in more detail, resulting in the discovery of many unique adaptations which parasites have made to successfully invade the host and establish an infection.

Parasites of the genus, *Leishmania* (Ross, 1903) belong to:

- **Phylum:** Sarcomastigophora, Honigberg and Balamuth, 1963
- **Sub-phylum:** Mastigophora, Diesing, 1866
- **Class:** Zoomastigophorea, Calkins, 1909
- **Order:** Kinetoplastidia, Honigberg, 1963; emmend. Vickerman, 1976
- **Sub-order:** Trypanosomatina, Kent, 1880
- **Family:** Trypanosomatidae, Doflein, 1901; emmend. Grobben, 1905

(from Chang et al., 1985)

*Leishmania* are members of the order *Kinetoplastidia* and are closely related to trypanosomes, from which *Leishmania* are thought to have evolved (McGhee and Cosgrove, 1980). All kinetoplastids are characterized by having a unique organelle termed the kinetoplast. Early studies identified the kinetoplast as a darkly staining body localized at the base of the flagellar pocket (groove). It is now known that the kinetoplast is the single mitochondrion present in these organisms and that the presence of tightly packed DNA minicircles is
responsible for its characteristic dark staining property (Borst and Hoeijmakers, 1979; Silver et al., 1986).

Like many parasites, *Leishmania* is always transmitted through an insect vector, in this case the blood-sucking phlebotamine sandfly. Once transmitted to the mammalian host, *Leishmania* establish intracellular parasitism within the host macrophage, causing the disease leishmaniasis. Leishmaniasis is one of the major human parasitic diseases and it has been estimated that up to twelve million people throughout the world may be affected by this disease, mostly in developing countries (Walsh and Warren, 1979). There are 400,000 new cases of leishmaniasis reported every year (Marinkelle, 1980). Leishmaniasis is said to be a zoonotic disease: transmissible from animals to man under natural conditions. There are a large number of animals which can be infected by *Leishmania*, mainly canids, edentates and rodents, and these animals act as a reservoir for the disease in man. The effects of the disease in some animals appears to be quite cryptic. In these cases the animals act simply as carriers for the disease and do not present with disease symptoms.

The original criteria used to distinguish among different *Leishmania* species were the symptoms of the human patient and the geographical distribution of the disease. This was replaced by a more detailed system of classification based on serological differences (Adler, 1964; Greenblatt et al., 1984). The introduction of monoclonal antibodies has recently led to a resurgence of interest in serological typing of *Leishmania* strains (McMahon-Pratt and David, 1981, 1982; Handman and Hocking, 1982; Handman et al., 1984a) and Western blot techniques have meant that biochemical analysis of molecules identified using monoclonal antibodies or human immune serum is now possible (Handman, 1986; Kahl and McMahon-Pratt, 1987; Reed et al., 1987). Other biochemical techniques that have also been used include comparison of the electrophoretic mobility of isoenzymes (Gardener et al., 1974; Schnur et al., 1981) and differences in the buoyant density of nuclear and kinetoplast DNA (Chance, 1985).

More recently, molecular techniques involving the use of DNA probes derived from *Leishmania* kinetoplast DNA (Wirth and McMahon Pratt, 1982; Lawrie et al., 1985) and defined fragments of genomic DNA (Van der Ploeg et al., 1984b; Spithill and Samaras, 1985; Handman et al., 1986; Beverley et al., 1987), have been used to characterize *Leishmania* species. The
ability to genotype a *Leishmania* strain by the use of a single copy gene probe in conjunction with pulse field gradient electrophoresis (Schwartz et al., 1983; Schwartz and Cantor, 1984) and Southern hybridization techniques is especially powerful.

There are two major forms of leishmaniasis, depending on the infecting *Leishmania* species.

**I. Cutaneous leishmaniasis**: there are three types of cutaneous disease.

1. Simple cutaneous disease is characterized by local growth of parasites in macrophages of the skin, causing dermal lesions. This disease is usually self-curing.

2. Mucocutaneous disease, often occurs secondarily to a simple cutaneous infection. In this disease, the original organisms from the cutaneous lesions metastasize to the membranes of the mouth and nose where they cause destruction of mucosal and nasal tissue.

3. Diffuse cutaneous disease also occurs secondarily to a simple cutaneous infection and in this disease the organisms spread in an uncontrolled manner all over the skin.

**II. Visceral leishmaniasis:**

During the course of this disease, *Leishmania* undergo a preliminary multiplication in the dermal macrophages at the site of infection and then spread to macrophages of all the inner organs, especially the spleen, liver and bone marrow. The resulting systemic infection is usually fatal if untreated.

In Table 1 the major *Leishmania* species are shown, categorized with respect to the clinical manifestations and geographical location of each species.
### TABLE 1. Major Leishmania species causing human disease

<table>
<thead>
<tr>
<th>Disease</th>
<th>Species</th>
<th>Geographical location</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cutaneous leishmaniasis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simple</td>
<td><em>L. mexicana mexicana</em></td>
<td>Mexico, Central America</td>
</tr>
<tr>
<td>cutaneous</td>
<td><em>L. mexicana amazonensis</em></td>
<td>Brazil, Amazon region</td>
</tr>
<tr>
<td>leishmaniasis</td>
<td><em>L. mexicana pifanoi</em></td>
<td>Venezuela</td>
</tr>
<tr>
<td></td>
<td><em>L. major</em></td>
<td>Southern U.S.S.R., Middle East</td>
</tr>
<tr>
<td></td>
<td><em>L. tropica</em></td>
<td>Asia, Southern Europe, Northern and Western, Africa</td>
</tr>
<tr>
<td></td>
<td><em>L. braziliensis guyanensis</em></td>
<td>Northern, South America</td>
</tr>
<tr>
<td></td>
<td><em>L. braziliensis panamensis</em></td>
<td>Central America</td>
</tr>
<tr>
<td></td>
<td><em>L. braziliensis peruviana</em></td>
<td>Peru</td>
</tr>
<tr>
<td>Diffuse</td>
<td><em>L. mexicana amazonensis</em></td>
<td>Brazil, Amazon region</td>
</tr>
<tr>
<td>cutaneous</td>
<td><em>L. aethiopica</em></td>
<td>Ethiopia and Kenya</td>
</tr>
<tr>
<td>disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mucocutaneous disease</strong></td>
<td><em>L. braziliensis braziliensis</em></td>
<td>Western and Northern, South America</td>
</tr>
<tr>
<td><strong>Visceral leishmaniasis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. donovani</em></td>
<td>India, Africa</td>
</tr>
<tr>
<td></td>
<td><em>L. donovani infantum</em></td>
<td>Mediterranean area</td>
</tr>
<tr>
<td></td>
<td><em>L. chagasi</em></td>
<td>Northern, South America</td>
</tr>
</tbody>
</table>

(from Wirth et al., 1986)
A. MOLECULAR BIOLOGY AND GENETICS OF LEISHMANIA

Although the molecular analysis of *Leishmania* has only recently begun, already several unusual features have been discovered. The *Leishmania* genome is estimated to contain between 0.6 - 1 X 10^8 base pairs (bp) and to be composed of 62% single copy DNA (Leon et al., 1978; Villalba et al., 1982; Gibson et al., 1985; Scholler et al., 1986). Although for many years the ploidy of the kinetoplastid protozoans was unknown, current evidence indicates that the *Trypanosomatids*, including *Leishmania*, are diploid during at least one stage of their life cycle (Gibson et al, 1985; Scholler et al., 1986). *Leishmania* have an unusually high G + C content of about 60% in their genomic DNA and data from the few *Leishmania* genes which have been sequenced indicate that the G + C content is higher within coding regions than in non-coding regions (Grumont et al., 1986). It appears that there is a strong preference for the use of the bases guanine or cytosine in the first and third positions within each codon. On average, the G + C content of the first base of each codon is 60% and that of the third base is 90%, while the G + C content of the second base is only 45% (Grumont et al., 1986).

A recent advance which is important in the analysis of parasite genomes, was the development of pulse field gradient electrophoresis. This technique has been very useful for the analysis of the chromosomes of many protozoans which are small compared to the chromosomes of higher eukaryotes and do not fully condense during metaphase (Vickerman and Preston, 1970) and, therefore, can not be examined by microscopy. Early karyotype analysis of kinetoplastid protozoans was carried out using DNA from a *Trypanosoma* species (Van der Ploeg et al., 1984a,b); however, this group later carried out a comparative study using DNA isolated from several kinetoplastid protozoans, including *Leishmania* (Van der Ploeg et al., 1984c). From this study they concluded that kinetoplastid genomes appear to exhibit extreme plasticity with respect to chromosome number and size. Their analysis of the DNA from *Leishmania* species indicated that the *Leishmania* genome consisted of a minimum of 20 chromosomes within the 700 to 4,000 kilobase range. Recently the molecular karyotypes of several *Leishmania* species and sub-species have been investigated. Scholler et al. (1986) reported that *Leishmania* appear to have 26 - 33 distinct chromosomes. The plasticity of the
Leishmania genome among different species was also observed in this study. Molecular karyotyping by pulse field gradient electrophoresis has also been used in conjunction with hybridization to single copy genes (Van der Ploeg et al., 1984c; Spithill and Samaras, 1985; Handman et al., 1986). When combined, these molecular techniques allow the development of a genetic karyotype for Leishmania, which has not been possible using conventional genetic analysis.

The gene transcripts of kinetoplastids such as Leishmania, are unusual because they undergo trans-splicing (reviewed by Borst, 1986; Van der Ploeg, 1986). Early sequence analysis of trypanosomatid transcripts indicated that they contained an "extra" segment at their 5' ends which was not contiguous in the DNA and, therefore, was termed the "mini-exon". This mini-exon-derived sequence was 35 nucleotides in length and this same 35 nucleotide sequence was found at the 5' end of several different gene transcripts upstream of the translation start site. Analysis of the location of the mini-exon in the trypanosomatid genome indicated that there are about 200 mini-exon genes per nucleus, that these genes are clustered and that each gene is 1.35 kb in length. The transcript from each mini-exon gene is 140 nucleotides and the 35 nucleotide sequence which is found at the 5' end of each RNA transcript is located at the 5' end of the mini-exon transcript, bordered by a conventional eukaryotic 5' splice donor sequence. Addition of the 35 nucleotide sequence to the 5' end of the gene transcripts requires RNA splicing and, therefore, this sequence has been renamed the spliced leader sequence. The terms discontinuous transcription or trans-splicing have been used to describe this unusual method of RNA maturation. Early studies indicated that several kinetoplastid genes appeared to undergo this unusual form of post-transcriptional modification, however, further investigation into this phenomenon has led to the realization that all mRNA transcripts of kinetoplastids have the spliced leader sequence added to their 5' ends. This very unusual and unique form of mRNA maturation appears to be a characteristic of kinetoplastids and it is interesting to note that, to date, there have not been any introns found in the nuclear genes of these organisms and, therefore, the more classical cis-splicing undergone in RNA maturation of higher eukaryotes is noticeably absent.
The ribosomal RNA species from lower eukaryotes are smaller than their vertebrate counterparts. In addition lower eukaryotes possess three large ribosomal RNA species instead of the two 28S and 18S RNA components characteristic of vertebrate cells. Studies of *Leishmania* ribosomal RNA have shown that the extra ribosomal component results from a further maturation step of the 28S rRNA subunit, resulting in the production of two smaller components of 1900 nucleotides and 1600 nucleotides in addition to the normal 2100 nucleotide component (18S) (Villalba and Ramirez, 1982; Villalba et al., 1985; Campbell et al., 1987). There are 160 copies of the genes for rRNA arranged in a tandem head-to-tail arrangement within the *Leishmania* genome (Villalba et al., 1985).

Although the molecular and genetic analysis of *Leishmania* has only recently begun, several unusual features have been reported. Many of these characteristic features are shared with other related parasitic protozoans, such as the trypanosomes, and it may be that these features have evolved to enable these organisms to adapt to the parasitic lifestyle they have adopted and are important in the host-parasite relations of these organisms.
B. LEISHMANIA LIFE CYCLE

In common with many other parasites, *Leishmania* have more than one life stage and each stage is characterized by a morphologically distinct form which is found in one specific environment. The life cycle of *Leishmania* is depicted in Figure 1. Within the sandfly vector, *Leishmania* exist as motile promastigotes, while in the mammalian host, parasites transform into non-motile amastigotes which are located within host macrophages (Hoare and Wallace, 1966).

![Figure 1. Life cycle of Leishmania](image)

1. *Leishmania* Within The Sandfly

Promastigotes are cylindrically shaped organisms which possess a long anterior flagellum. Within the gut of the sandfly, promastigotes attach to the gut wall by embedding their flagella in the microvilli of the gut epithelial cells (Chang et al., 1985). Promastigotes multiply within the gut and eventually move upward towards the fly's cuticular pharynx (Bray, 1983b), where they attach tightly to the cuticle (Molyneux et al., 1975). Highly motile promastigotes eventually move into the proboscis where they can then be transferred to a mammalian host.

Studies done using *in vitro* cultured *Leishmania* have shown that there is a difference in the infectivity of log phase versus stationary phase organisms, with the stationary phase parasites being more infective in each case (Giannini, 1974; Kiethly and Bienen, 1981; Doran
and Herman, 1981; Sacks and Perkins, 1984, 1985; Franke et al., 1985). These results have been correlated with the development of the promastigotes within the sandfly (Sacks and Perkins, 1984, 1985). Biochemical analysis of the differences between infective and non-infective promastigotes have shown a difference in the polysaccharide residues present on the surface membrane (Dawidowicz et al., 1975; Doran and Herman, 1981; Wilson and Pearson, 1984; Ayesta et al., 1985; Sacks et al., 1985; Scott and Sher, 1986). In general, non-infective stages appear to have a reduced ability to bind to lectins which react with the carbohydrate residue D-galactose. Whether this change in lectin binding is due to the loss of a specific surface molecule or whether it represents a change in the overall glycosylation pattern upon differentiation of the promastigote to the infective stage has not been determined.

2. Promastigote Attachment To And Uptake By Host Macrophages

The initial interactions between the infecting promastigotes and the mammalian host are difficult to study in vivo and, therefore, information on this early stage of parasite infection has come from in vitro promastigote-macrophage studies. The first stage in Leishmania infection is the attachment and entry into the host macrophages by invading parasites. As the macrophage is generally thought to be the immune system's first line of defense against parasitic attack, it is likely that the parasite has not had to develop a complex mechanism to seek out its host target cell, but rather has adapted to take advantage of existing pathways.

The process of parasite-macrophage attachment has been studied by electron and cinemicroscopy (Chang, 1979; Pearson et al., 1983a). In these studies macrophages were observed to extend long phagocytic pseudopods in response to contact with Leishmania promastigotes. The sites of attachment of these pseudopods to the promastigotes were discrete points, separated by wide, irregular gaps. One interpretation of these findings is that these discrete points of contact may represent clustering of parasite surface molecules in response to serum factors (Pearson et al., 1983a). These observed interactions indicated that attachment and uptake of the parasite may be a receptor mediated event and that surface molecules on both the parasite and the macrophage are involved.
Studies with fresh human or guinea pig serum have shown that all *Leishmania* promastigotes fix complement via the alternative pathway, a process which occurs in the absence of antibody, and results in lysis of the majority of parasites (Mosser et al., 1985; Mosser et al., 1986). However, binding of complement factor C3 by the parasite, has also been shown to enhance parasite uptake, due to the involvement of the macrophage C3bi (CR3) receptor (Bray, 1983a; Mosser and Edelson, 1985; Blackwell et al., 1985a). Experiments carried out in the absence of serum have shown that macrophage-derived complement proteins can cause local opsonization of promastigotes, and under these circumstance there was much less damage to the promastigotes than was observed in the presence of serum, presumably due to the lack of terminal components in the complement cascade (Wozencraft et al., 1986). It appears that complement activation by promastigotes can result in lysis and destruction of the parasite or, alternatively, can result in enhanced uptake of the parasite by the macrophage. One *Leishmania* protein which has been shown to bind complement factor C3 is the major 63,000 mol wt promastigote surface glycoprotein, gp63 (Russell and Wilhelm, 1986).

The mannose/fucose receptor on macrophages has also been shown to play a role in *Leishmania* binding in both mouse and human macrophage systems. Saturation of this receptor with substrate or blocking by the addition of anti-receptor antibodies resulted in a 50% inhibition of parasite uptake by macrophages (Channon et al., 1984; Blackwell et al., 1985a; Wilson and Pearson, 1986). Studies have indicated (Blackwell et al., 1985) that the mannose-fucose receptor and the complement receptor may be functioning coordinately and that the same parasite molecule is being recognized by both receptors. The major surface protein, gp63, has been shown to contain mannan residues (Wilson and Pearson, 1986) and, therefore, may also be the main ligand for the mannose-fucose receptor.

Vlassara et al. (1985), identified a macrophage receptor with a high affinity for glucose-modified proteins (advanced glycosylation endproducts, AGE), which is thought to be involved in the uptake and degradation of senescent macromolecules. A recent report demonstrated that *Leishmania* promastigotes take advantage of this scavenger pathway and that uptake of the parasite by this macrophage receptor can be inhibited by 50% via substrate saturation. In addition, it was shown that combined treatment of macrophages with anti-CR3 receptor
antibody and substrate for the AGE-receptor resulted in a 90% overall reduction in parasite uptake (Mosser et al., 1987). It is possible, therefore, that the macrophage receptors for complement, mannose-fucose and AGE account for the major pathways of *Leishmania* uptake by macrophages, although there are other secondary routes which the parasite also appears to take advantage of.

It has been reported that all *Leishmania* species are agglutinated by naturally occurring serum antibody (Mosser et al., 1986) and there is evidence for the involvement of antibody in opsonization of *Leishmania*, perhaps resulting in uptake of *Leishmania* into the macrophages via Fc receptors on the macrophage surface (Williams et al., 1986c). Another serum component which appears to be involved in macrophage-promastigote attachment is fibronectin, a large glycoprotein which is synthesized by macrophages and for which macrophages also express a surface receptor. Fibronectin is involved with the association of certain bacteria, yeasts, viruses and spirochetes with host cells. Wyler et al. (1985) demonstrated that *Leishmania* species can bind fibronectin and proposed that *Leishmania* may use this glycoprotein as a bridge to facilitate attachment to macrophages.

The mechanisms by which *Leishmania* attach to and are taken up by macrophages is complex and it is often difficult to make comparisons between studies because different parasite strains and different macrophage sources have been used. In order to clarify the exact nature of the interactions, it will be necessary to use cloned parasite and macrophage lines, purified *Leishmania* molecules and macrophage receptors as well as monoclonal antibodies specific for both the receptors and *Leishmania* molecules which interact with them. With these types of reagents it will be possible to determine whether there are generalized mechanisms for *Leishmania* uptake by macrophages or whether *Leishmania* strain-specific or macrophage type-specific mechanisms are also present.

3. Phagosome-Lysosome Fusion

Almost all other intra-macrophage parasites have been shown to survive within the macrophage by inhibiting fusion of the phagosome with lysosomes. These include the parasitic bacteria, *Toxoplasma gondii* (Jones and Hirsch, 1972; Goren, 1977), *Chlamydia psittaci* (Friss,
1972; Wyrick and Brownridge, 1978), *Mycobacterium tuberculosis* (Goren et al., 1976), *Rickettsia prowazeki* (Walker and Winkler, 1978), *Rickettsia typhi* (Walker and Winkler, 1981), *Coxiella burnetti* (Burton et al., 1978; Hackstead and Williams, 1981), and *Legionella pneumophila* (Horowitz, 1983), spores of the microsporidian parasites, *Encephalitozoon cuniculi* (Wiedner, 1975; Niederkorn and Shadduck, 1980), and *Glugea hertwigi* (Wiedner and Sibley, 1985), and the parasitic protozoan, *Trypanosoma cruzi* (Dvorak and Hyde, 1973; Alexander, 1975). In cases where the mechanism of this inhibition has been studied it appears to be due to blockage of the phagosome acidification process (Horowitz and Maxfield, 1984; Sibley et al., 1985; Weidner and Sibley, 1985; Osuna et al., 1986). In addition to blocking phagosome acidification, *Trypanosoma cruzi* and *Rickettsia typhi* also subsequently escape into the cytoplasm where they replicate, separated from the phagosome (Nogueira and Cohn, 1976; Walker and Winkler, 1981).

The uptake of *Leishmania* by macrophages is unique as *Leishmania* do not block phagosome acidification or phago-lysosomal fusion. Instead, *Leishmania* are able to survive and replicate within the phagolysosomal compartment. In addition, fusion of occupied phagosomes with lysosomes has been observed both in vitro and in vivo (Alexander and Vickerman, 1975; Chang and Dwyer, 1976; Lewis and Peters, 1977; Brazil, 1984). The only other organisms which are thought to share this ability are the parasitic bacteria, *Coxiella burnetti* (Burton et al., 1978).

Once phagolysosomal fusion has taken place the promastigotes are exposed to the microbicidal factors of this digestive organelle. It has been well documented that up to 90% of the invading promastigotes are killed by the oxidative burst which is triggered (Murray, 1981; Pearson et al., 1983b; Channon and Blackwell, 1985a); however, it appears that there is a small proportion of promastigotes which have certain properties enabling them to survive long enough to convert to the more resistant amastigote form (Murray, 1981, 1982a; Pearson et al., 1983b). The survival frequency may actually represent the conversion frequency from promastigote to amastigote form. The factors which may be involved in the survival of *Leishmania* within the phagolysosome will be discussed in section 5.
4. Promastigote-Amastigote Conversion

The critical event in the successful parasitization of macrophages by *Leishmania* is the conversion from promastigote to amastigote. The differentiation of promastigotes into amastigotes is essential for survival of *Leishmania* within the mammalian host and precedes the intracellular multiplication of the parasite (Chang and Dwyer, 1976). The factors involved in triggering this transformation have not been fully characterized, however, one important requirement appears to be the temperature shift which occurs as the parasite is transferred from the insect (26 °C) to the mammalian host (32-37 °C). Several studies on the heat shock response of *Leishmania* have been carried out (Lawrence and Robert-Gero, 1985; Van der Ploeg et al., 1985), and it has been reported that there are both qualitative and quantitative differences in the levels of transcription of several heat shock genes between promastigote and amastigote forms. One of the characteristic heat shock-specific transcription patterns which accompanies promastigote-amastigote transformation is differential expression of the α- and β-tubulin genes (Fong, et al., 1984; Landfear and Wirth, 1984) and it appears that this differential expression may be under the control of the heat shock genes (Van der Ploeg et al., 1985). The temperature shift which occurs upon transmission of *Leishmania* from the insect vector to the mammalian host appears to play an important role in the cytodifferentiation of *Leishmania* and their adaptation to life in the mammalian host.

Many studies attempting to identify stage-specific differences have utilized techniques such as morphological, serological and biochemical comparisons. Most of the results, however, have indicated that the changes which occur between the two stages are, for the most part, quantitative rather than qualitative changes.

Pan and Pan (1986), examined promastigotes and amastigotes using electron microscopy. Their findings indicated that there were four major features which could distinguish between the fine structures of the two stages. They reported that a paraxial rod structure present in promastigotes, is absent in amastigotes. This paraxial rod structure may be important for the vigorous flagellar activity exhibited only by promastigotes. Their other findings involved differences in the shape of the flagellar pocket and in the arrangement of the subpellicular
microtubules between the two stages and the presence of large electron dense membrane bounded structures only in amastigotes.

Many investigations have been carried out to identify changes in surface antigens between the two stages, most of which have used a combination of biochemical and immunological techniques. Using serum from infected patients, Colomer-Gould et al. (1985) reported the presence of a major surface glycoprotein on the surfaces of promastigotes from various *Leishmania* species with an apparent molecular weight of 63,000 (gp63). It has been estimated that this protein accounts for approximately 1% of the total cell protein of *Leishmania* and that there are 500,000 copies per cell present on the surface of promastigotes (reviewed by Bordier, 1987). Recent studies have shown that this protein exhibits protease activity (Etges et al., 1986a) and that it is anchored to the membrane via an unusual glycolipid structure which is added to the carboxy-terminus by a series of unusual post-translational events (Etges et al., 1986b). The carboxy-terminal hydrophobic amino acids are removed and a glycolipid composed of glycosyl-inositol phospholipids whose acyl group is exclusively myristic acid and the diacylglycerol moiety of phosphatidylinositol (PI) is attached (reviewed by Cross, 1987). This appears to be the sole means of membrane attachment for this protein. The variant surface glycoproteins (VSG) of African trypanosomes are attached to the cell surface by a similar lipid-linkage (Bordier et al., 1986). It appears that the trypanosome VSG's are cleaved from the surface by an endogenous lipase activity (Fox et al., 1986); however, no such activity has, as yet, been purified from *Leishmania* (Bordier, 1987). There is still controversy over the expression of gp63 with respect to stage-specificity (Colomer-Gould et al., 1985; Bordier, 1987). One recent report correlated increased expression of the major surface antigen of *Leishmania braziliensis* promastigotes with increased infectivity (Kweider et al., 1987).

Studies using total proteins which were $^{35}$S-methionine labelled or radio-iodinated followed by Western blot analysis and/or immuno- or lectin precipitation have reported several apparently stage-specific cell surface molecules from different *Leishmania* species (Chang and Fong, 1982; Gardiner and Dwyer, 1983; Pan et al., 1984; Handman et al., 1984b; Pan, 1986; Williams et al., 1986a,c). Comparisons of the results obtained using either immunoprecipitation of radio-iodinated proteins or analysis of $^{35}$S-methionine labelled proteins by Western blots,
however, showed a significant difference in the results (Handman et al., 1984b). An explanation of this discrepancy may come from a study by Sadick and Raff (1985), who found that amastigote surface molecules appeared resistant to iodination and they concluded that this might be due to differences in glycosylation which rendered the proteins inaccessible to iodination. Many antibodies recognize carbohydrate residues rather than protein epitopes and, therefore, some of the observed differences between the two Leishmania stages as determined by serological analysis may in fact be due to changes in carbohydrate residues on the Leishmania surface. Several studies have reported changes in carbohydrate residues present on the surface as detected by lectin binding (Dawidowicz et al., 1975; Hernandez et al., 1981; Sacks et al., 1985; Wilson and Pearson, 1986).

In spite of the extensive analysis of surface antigens from either promastigotes or amastigotes it is still not clear whether many stage-specific molecules have as yet been identified. The introduction of monoclonal antibodies, the availability of correctly typed and cloned Leishmania strains and the standardization of analytical techniques should result in a more clear-cut understanding of the nature of the differences between the two Leishmania stages.

5. Survival And Replication Of Leishmania Within The Macrophage

Investigations into the factors responsible for Leishmania survival have centered on the ability of Leishmania molecules to either resist degradation by lysosomal enzymes, to inhibit the function of lysosomal enzymes or to detoxify the lysosomal reagents released by the respiratory burst. Mosser and Edelson (1987), have recently reported that the magnitude of the respiratory burst triggered in macrophages infected with Leishmania is significantly lowered when the parasites are coated in complement, perhaps because macrophage complement receptors may fail to trigger a respiratory burst (Wright and Silverstein, 1983). Therefore, complement fixation by Leishmania surface membrane proteins appears to be important not only for parasite binding and uptake, but also for intracellular survival within the macrophage.

It has been shown that Leishmania undergo selective changes in surface carbohydrates during growth and differentiation from non-infective to infective promastigote stages in vitro
(Sacks et al., 1985). Similar differences in lectin binding and surface charge between pathogenic and non-pathogenic strains of *Leishmania* have also been noted (Dawidowicz et al., 1975; Ayesta et al., 1985). Handman et al (1981), reported that unlike *Leishmania* cytoplasmic proteins, *Leishmania* membrane proteins appear to be resistant to protease digestion. Pre-treatment of *Leishmania* with tunicamycin resulting in partial deglycosylation of the surface antigens, renders *Leishmania* susceptible to intracellular degradation by macrophages (Chang et al, 1985). Tunicamycin treatment has also been shown to reduce infectivity in vivo (Nolan and Farrell, 1985). In a recent study, Kink and Chang (1987), showed that tunicamycin resistant *Leishmania* were more virulent to mice than the parental wild-type *Leishmania*. These results indicate a potentially protective role for carbohydrates in the resistance of *Leishmania* to degradation within the phagolysosome.

Biochemical investigation of *Leishmania* surface membranes has resulted in the identification of several membrane associated enzymes which may be important in *Leishmania* survival within the macrophage. One enzyme commonly viewed as a lysosomal enzyme has also been shown to be a constituent of the plasma membrane of *Leishmania*. Gottlieb and Dwyer (1981a,b) identified a membrane-associated acid phosphatase in *Leishmania* which was shown to be asymmetrically distributed on the external surface of the membrane. This acid phosphatase enzyme had a pH optimum of 5.0 and was active on a broad range of organic phosphate substrates. More detailed analysis of this enzyme activity revealed three distinct enzymes ACP-P1-3 (Glew et al., 1982; Remaley et al., 1985a). The finding that ACP-P1 can catalyze the dephosphorylation of proteins prompted Remaley et al. (1985a) to propose that this enzyme could be acting by dephosphorylating critical host enzymes involved in the defense mechanisms of the macrophage. Also the ability of this enzyme to catalyse the hydrolysis of the key glycolytic intermediate fructose 1,6-diphosphate could disturb the energy metabolism of the infected macrophage. In a further study Remaley et al. (1985b) reported that membrane-bound acid phosphatase was able to diminish receptor-mediated production of superoxide anion by neutrophils and macrophages.

Three distinct membrane-associated phospholipases have also been identified in *Leishmania*. A thermolabile phospholipase C, which converts PE into diglyceride and
ethanolamine, a phospholipase A$_2$, able to convert PE into free fatty acid and lyso-PE, and also an uncommon phospholipase A$_1$ (Dwyer and Gottlieb, 1986). As mentioned previously, Etges et al. (1986a) reported that the major surface glycoprotein of *Leishmania* (gp63) is a surface protease which exhibits pepsin-like activity and is linked to the membrane by an unusual lipid linkage, whether one of these endogenous lipases is able to act on gp63 to release it from the membrane is undetermined.

As well as possessing many cell surface proteins which appear to have adapted to cope with the hostile intracellular environment provided by the macrophage, *Leishmania* have made several physiological adaptations which enhance their ability to survive and multiply within the acidic compartment of the macrophage. Mukkada et al. (1985) found that in promastigotes metabolic activities were carried out at a pH optimum near neutral, while the pH optimum for amastigotes was between 4.0 and 5.5.

Excreted factors have also been proposed to function in *Leishmania* resistance to lysosomal degradation. An extracellular acid phosphatase activity has been detected (Gottlieb and Dwyer, 1982) which is distinct from the membrane-bound enzyme and it has been proposed that the extracellular form of this enzyme might also function by diminishing receptor-mediated production of super oxide anion by macrophages (Lovelace and Gottlieb, 1986). Reports have indicated that a polymorphic family of excreted *Leishmania* carbohydrate antigens referred to as "excreted factors" (EF) (Schnur et al., 1972; Schnur and Zuckerman, 1977; Slutzky and Greenblatt, 1982) may act directly as enzyme inhibitors. El-On et al. (1980) reported that the activity of $\beta$-galactosidase from macrophages could be inhibited by EF. Eilam et al. (1985) extended this study and showed that one protective action of EF could be correlated with the formation of an environment containing a high concentration of Ca$^{+2}$, bound to EF within the phagolysosome. Another possible mechanism by which EF may contribute to *Leishmania* protection from the lysosomal environment could be due to its strong negative charge (Schnur et al., 1972). Reports by Handman et al. (Handman et al., 1984a; Handman and Goding, 1985; Handman et al., 1986) indicate that the presence of a secreted glycoconjugate (*Leishmania*-lipopolysaccharide (L-LPS) conferred survival within macrophages. *Leishmania* possess relatively high protease activity (Coombs, 1982; Pupkis et al., 1986) and it
has been proposed that these proteases may function by producing the polyamines putrescine, spermidine and spermine which are secreted by the parasite and could antagonize the actions of lysosomal enzymes (Coombs and Sanderson (1985)).

*Leishmania* have made many physiological adaptations which ensure survival within the host macrophage. The ability of *Leishmania* amastigotes to replicate within the macrophage has been well documented (Chang and Dwyer, 1978; Nacy et al., 1981); however, it is still not known exactly how the multiplying *Leishmania* escape from infected cells to reinvade new macrophages. It is assumed that as the number of *Leishmania* increases the infected cell eventually bursts and that amastigotes are then liberated, attach to other macrophages and are taken up. Once inside the macrophage, the amastigotes initiate a new cycle of replication and reinfection.


It has been shown that amastigotes attach to macrophages and are taken up in much the same way as promastigotes as determined by light and electron microscopy (Chang and Dwyer, 1976). A study by Channon et al. (1984) reported that binding of the amastigote to the macrophage may not involve the mannose-fucose receptor, although this receptor has been shown to be important in binding of promastigotes. Mosser et al. (1985) found that amastigotes from all *Leishmania* strains studied were able to fix complement component C3, and that some of the strains were resistant to complement mediated killing. They proposed that fixation of complement is involved in re-entry of amastigotes into the macrophage.

Murray (1981), Murray et al. (1983a) and Pearson et al. (1983b) reported that amastigotes seemed to be better able to evade the triggering of a strong respiratory burst upon phagocytosis by macrophages and comparisons of the sensitivity of promastigotes and amastigotes to the effects toxic oxygen intermediates revealed that the amastigote stage is four to seven times more resistant to $H_2O_2$ *in vitro* (Murray, 1982b; Pearson et al., 1983b; Buchmuller-Rouiller and Mauel, 1986). The levels of *Leishmania* derived enzymatic scavengers of $H_2O_2$ were examined and both promastigotes and amastigotes were shown to contain large amounts of superoxide dismutase (SOD); however, the promastigote stage had much less catalase...
and glutathione peroxidase (GPO). Channon et al. (1985a,b) reported that the difference in 
$H_2O_2$ sensitivity between the two forms of the parasite was directly related to the activity of 
the enzyme catalase. Coombs (1982) reported a 20-fold increase in protease activity in 
amastigotes when compared to promastigotes and this may be only one of many of the 
qualitative changes between the two stages which play a role in the increased infectivity of 
amastigotes versus promastigotes.

7. Uptake Of Leishmania By The Sandfly And Amastigote-Promastigote Conversion

The *Leishmania* life cycle is once again initiated when a sandfly takes a blood meal 
from an infected mammalian host. The number of amastigotes ingested by the sandfly is 
unknown, but is presumed to be very small. Soon after being ingested, the amastigotes 
differentiate into promastigotes and start dividing in the fly gut.

It has been shown that there are several factors which are important in the 
transformation process; however, probably the most important is the decrease in temperature 
which occurs (Rudzinska et al., 1964). Other factors such as the presence of certain amino 
acids, glucose, and hemin are also involved (Simpson, 1968). It appears that inhibitors of 
protein or RNA synthesis are able to block transformation (Simpson, 1968); however, *de novo* 
DNA synthesis is not required (Brun et al., 1976a). It has also been reported that amastigote-
promastigote conversion can be inhibited by a host lymphocyte-derived factor and it has been 
proposed that the parasite may be prevented from transforming into the promastigote form 
while in the mammalian host due to this factor (Brun et al., 1976b; Krassner et al., 1980).
C. HOST RESPONSE TO LEISHMANIA

The ability of parasites to evade the host's immune response is of utmost importance to the survival of the parasite and its subsequent ability to establish an infection. By residing within host cells, a parasite has taken a major step in evading elimination because its presence is partially masked. However, the parasite cannot remain intracellular if the infection is to spread and invade other cells and, therefore, its presence does not go completely unnoticed by the host. The capacity of *Leishmania* to invade and replicate within host macrophages only confers limited protection from the immune response because the macrophage is a major effector cell in host protective responses. In order to enhance its ability to survive within the host, *Leishmania* actively suppress the immune responses which might bring about activation of the macrophage resulting in leishmanicidal activity. The host response to invading *Leishmania* can be very complex, involving all levels of the immune system.

There are large variations in the outcome of the disease in individuals infected with the same *Leishmania* organism. Uncommon manifestations of leishmaniasis are often due to the same parasite strains which cause "normal" disease in other individuals. These observations indicate that the genetic constitution of the host plays a role in the clinical manifestations of the disease. During the initial stages of the infection, uptake of parasites by the unactivated, resident macrophages of the skin results in killing of a high percentage of *Leishmania* promastigotes. The innate ability of macrophages to eliminate promastigotes appears to be genetically determined; however, it is also dependent on the species of infecting *Leishmania*. The subsequent ability of the host's immune system to resolve infections which overcome the host's innate resistance and do become established is dependent on the interaction of components of cell-mediated immunity with infected macrophages. The ability of these interactions to stimulate sufficient macrophage activation to kill the intracellular organisms, determines whether the parasitic infection will be successfully eliminated. In situations where this acquired immunity does not eliminate the parasite, it is usually due to absence of macrophage activation caused indirectly by suppression of cell-mediated immunity. In these cases an inverse correlation between antibody and delayed-type hypersensitivity responses is
observed, suggesting a causal inhibitory role for antibody under these circumstances. The ability of the acquired immune response to provide protection is also genetically determined and dependent on the invading *Leishmania* species. Therefore, there are two genetically determined mechanisms involved in the host response to *Leishmania*, innate resistance and acquired immunity. The mechanisms which the mammalian host invokes for defense against invading parasites and the mechanisms which the *Leishmania* has evolved to evade these same mechanisms will be discussed in this section.

1. Host Defense Mechanisms

The phagocytosis and destruction of micro-organisms is carried out by macrophages. Macrophages are derived from bone marrow cells which are present in the blood as monocytes. Upon differentiation of monocytes into macrophages these cells settle in the tissues (resident macrophages) where they constitute the reticuloendothelial system. The macrophage represents the host's first arm of defense against invading microbes and as such is non-specific and has no memory against subsequent challenge. Specificity and immunological memory are provided to the host by the T and B lymphocytes of the immune system.

Protection against organisms which are able to invade and replicate within the cells of the host is provided by cell-mediated immune responses. The lymphocytes responsible for this adaptive immunological reaction are termed T cells. The interaction of "sensitized" T cells with antigen from the infecting organism results in the delayed type-hypersensitivity response. Delayed-type hypersensitivity is a measurement of the cellular activity of interacting "sensitized" or antigen-specific T cells and phagocytes. The reaction is antigen-specific and is mediated by T cells. Functionally, delayed-type hypersensitivity results in the influx of phagocytic cells into the site of infection.

The other major adaptive immunological reaction which occurs when parasites enter the body is activation of the humoral immune response. Activation of B lymphocytes (B cells) results in their differentiation into antibody producing cells. Interactions between T and B cells of the immune system are responsible for antigen-specific regulation of the humoral immune response.
2. Experimental Animal Models

Experimental animal models have been used extensively to examine host-parasite interactions. The first animal model used to study Leishmania was the guinea pig (Bryceson et al., 1970). There were several limitations on this system including the fact that the infecting Leishmania species used, Leishmania enriettii, is non-pathogenic to man. Recently, the use of inbred mouse strains which can be infected by Leishmania species which are pathogenic to man, has replaced the use of the guinea pig (Bjorvatin and Neva, 1979; Childs et al., 1984). The use of mice has the added advantage of the advanced state of mouse immunogenetics and the availability of well-defined inbred strains. The majority of studies investigating the effects of host genetics have been done using either Leishmania major which causes the cutaneous disease in man or Leishmania donovani which causes the visceral disease in man. However, the diseases caused by experimental infection of these strains in mice often do not parallel the course of the disease in man. To overcome this problem, several primate models have recently been developed which exhibit disease pathologies which more closely resemble the situation in man (Nickol and Bonventre, 1985; Dennis, 1986; Lujan et al., 1986).

Although there are two distinct diseases in human Leishmania infections, cutaneous and visceral, as mentioned above the distinction is not so clear in animal models. However, it appears that the underlying immune responses of the host to infection by any Leishmania species are very similar and, therefore, in this discussion the majority of results described are generally applicable.

3. The Genetics and Mechanisms of Innate Resistance

Innate resistance is defined as the inherent ability of the host to resist infection by Leishmania and does not involve components of the immune system. The uptake of Leishmania by macrophages results in the triggering of mechanisms which are capable of killing up to 90% of invading promastigotes and a significant, if lower percentage, of invading amastigotes. The difference in macrophage killing of promastigotes versus amastigotes is an inherent property of the Leishmania parasite and is one the many physiological adaptations of the parasite which, as
described earlier, enables it to survive within the mammalian host. Differences in the ability of the macrophage to kill infecting *Leishmania* of different species have also been noted.

Genetic analysis of innate resistance and susceptibility to *Leishmania* infection has been investigated and several loci which are involved have been identified. Inbred mouse strains can be separated into susceptible and resistant groups (Bradley, 1977; Howard et al., 1980a). Genetic analysis has shown that susceptibility to *Leishmania major* infections in mice is due to a single autosomal, non MHC-linked gene (Howard et al., 1980c; DeTolla et al., 1981) which has been mapped to chromosome 8 and named *scl*, for susceptibility to cutaneous leishmaniasis (Blackwell et al., 1984a). The mechanism(s) of innate resistance to *Leishmania major* infections has not been established; however, it appears that the expression of the phenotype is inherent to the macrophage (Howard et al., 1980c) and must somehow result in differential control of parasite growth within the infected macrophage.

Genetic analysis of innate resistance to *Leishmania donovani* infections in mice revealed that this resistance appears to be controlled by a single gene (denoted *Lsh*) mapping to chromosome 1 in the mouse (Bradley et al., 1979; Mock et al., 1985). This gene is distinct from the *scl* gene involved with innate resistance to *Leishmania major*. It has been established that the *Lsh* gene is identical to the previously identified genes *Itv* (regulating resistance to *Salmonella typhimurium*) and *Bcg* (regulating resistance to *Mycobacterium bovis*) (Skamene et al., 1982; Plant et al., 1979; 1982; Potter et al., 1983). A study by Brown et al. (1982) indicated that the regulation of resistance to *Mycobacterium lepraemurium* is, at least in part, controlled by this same gene. Therefore, the innate resistance to several intracellular parasites which predominantly infect macrophages, is regulated by a single gene in mice. Crocker et al. (1984) reported that the expression of *Lsh* gene activity may be triggered by interaction with parasite-derived material. The *Lsh* gene, like the *scl* gene, appears to influence the inherent ability of infected macrophages to control parasite growth at the early stages of infection, prior to involvement of components of the immune response.
4. The Genetics and Mechanisms of Acquired Immunity

*Leishmania* infections which are able to overcome innate host resistance cause induction of the immune system. The host response to this stimulation of the immune system is termed acquired immunity. The genetic regulation of acquired immunity to *Leishmania donovani* infections in inbred strains of mice is complex and involves several genes. So far three loci have been identified, the *Rdl-1* gene which is linked to the major histocompatibility complex (MHC) on chromosome 17 (Ulczak and Blackwell, 1983), the *Ir-2* gene which is located on chromosome 2 and a third gene which appears to effect the ability to mount a cell-mediated immune response. This third gene has a parallel effect on *Leishmania donovani* and *Leishmania major* infections in mice (Howard et al., 1980c; Blackwell et al., 1985b).

The initial interactions between the host and the parasite occur between unactivated resident macrophages in the skin and the invading parasites. When this first line of defense does not eliminate all of the invading parasites other components of the immune system are triggered. Although macrophages are responsible for killing of *Leishmania* within the host, if the initial interaction does not result in elimination of the parasites then the macrophages appear to become unable to destroy the intracellular parasite unless they are subsequently stimulated by other components of the immune system. Cell-mediated immunity is responsible for activating infected macrophages to kill intracellular *Leishmania* and this immunity appears to be mediated by the release of soluble factors (lymphokines) by activated T cells; however, the presence of B cells also seems to be required, although antibody itself does not appear to provide protection.

The mouse strains used for experimental leishmaniasis are termed healing or non-healing strains. Upon experimental infection with *Leishmania*, healing strains develop swellings and small lesions at the site of infection but these resolve over a period of several weeks leaving the mice resistant to subsequent infection (Preston and Dumonde, 1976; Scott and Farrell, 1982). Non-healing strains (BALB/c) infected with *Leishmania major* are unable to control the parasites replication throughout the course of the infection and in untreated mice severe local lesions develop, often followed by cutaneous metastasis, and always by visceralization of the disease and death (Scott and Farrell, 1982). This characteristic "BALB/c-
type" disease is more comparable to human visceral leishmaniasis. In contrast to the situation in experimental *Leishmania major* infections, all of the non-healing strains infected with *Leishmania donovani* (C57BL/6J, BALB/c and DBA/1) eventually resolve the disease and develop acquired immunity to this parasite (Reiner, 1982; Fahey and Herman, 1985).

The importance of T cell-mediated immunity in resistance to leishmaniasis in healing mouse strains was demonstrated in early investigations by Preston et al. (1972), Skov and Twohy (1974) and Howard et al. (1980b). Examination of the mechanisms involved in the susceptibility of non-healing mouse strains to *Leishmania*, demonstrated that there was impairment of cell-mediated immunity, as measured by delayed-type hypersensitivity, which was *Leishmania*-specific and associated with the generation of a potent T suppressor cell population Howard et al., 1980b; Blackwell and Ulczak, 1984b). Thymectomy followed by bone marrow reconstitution resulted in retardation of lesion size and expression of delayed type hypersensitivity (Howard et al., 1980b). It was also shown that sublethal whole body irradiation of phenotypically non-healing mice, which impairs the generation of T suppressor cells, resulted in these mice exhibiting a healing phenotype and then developing long term protective immunity (Howard et al., 1981). Liew et al. (1982) demonstrated that T cell enriched fractions from healed susceptible mice transferred both delayed type hypersensitivity and protective immunity. These and other later studies have established that different subsets of T cells can mediated both resistance-promoting (host-protective) and disease-promoting (suppressive) effects in murine leishmaniasis (Howard et al.,1982, 1984a,b; Coutinho et al., 1984; Dhaliwal et al., 1985; Liew et al., 1985a,b; Titus et al., 1984, 1985; Behforouz et al., 1986; Milon et al., 1986; Solbach et al., 1986a,b; Dhaliwal et al., 1987).

It is still not clear what the exact mechanisms are which act to promote resistance or susceptibility to *Leishmania* infections; however, studies have demonstrated that the impairment of acquired immunity seen in non-healing mice is not due to an inherent defect in the host's immune system, because by appropriate manipulations it can be overcome, leading to healing in normally non-healing mice. It has been proposed that the mechanisms by which T suppressor cells become activated in non-healing mice may be determined by the level of antigen accumulation in infected macrophages (Preston and Dumonde, 1976; Gorczynski and MacRae,
Strains which are able to prevent antigen accumulation by limitation of initial parasite growth may avoid T suppressor cell generation resulting in a productive cell-mediated response and elimination of the *Leishmania* parasites (Howard et al. 1982; Mitchell 1984). The demonstration that there are innate differences in parasite growth within the macrophages from different strains, correlates with the proposal that the *scl* gene and the *Lsh* gene which appear to be responsible for the innate susceptibility of certain mouse strains, may control the primary innate ability of the macrophages to limit the replication of the parasite (Blackwell et al., 1984b).

T cells recognize foreign antigens presented on the surface of macrophages in the context of major histocompatibility complex (MHC) gene products. It has been shown that parasitized macrophages contribute to the immune response by processing soluble antigen from the intracellular parasite and presenting it on their surfaces (Farah et al., 1975; Berman and Dwyer, 1981; Williams et al., 1986b). T cells regulate macrophage function by releasing immunologically active factors (lymphokines) and early studies by Nacy et al. (1981) and Murray et al. (1982a) demonstrated that antigen- or mitogen-stimulated spleen cells released factors which were able to stimulate macrophages to kill intracellular *Leishmania* parasites. There are differences in the abilities of *Leishmania* infected-macrophages from healing versus non-healing mouse strains to respond to lymphokine-stimulation and local resident macrophages of the skin are better able to respond to lymphokine stimulation than incoming inflammatory macrophages (Nacy et al., 1983; Hoover and Nacy, 1984). These and other studies (Buchmuller-Rouiller and Mauel, 1986; Sharp and Banerjee, 1986) indicate that the ability of macrophages to respond to lymphokines is genetically determined and is dependent on the state of the macrophage itself. Further studies have indicated that that the major lymphokine responsible for mediating macrophage activation resulting in the destruction of intracellular *Leishmania* appears to be IFN-γ (Murray et al., 1983b; Sadick et al., 1986; Murray et al, 1987). Reiner and Finke (1983) demonstrated that T cells from non-healing *Leishmania*-infected mice were impaired in their ability to produce IL 2, the regulator of IFN-γ production, and to respond to it.
Since the activation of certain T cell functions is macrophage-dependent it is possible that the infected macrophage may be involved in the ability of the host to develop acquired immunity. Studies have identified a population of suppressor macrophages in *Leishmania major* infected mice (Scott and Farrell, 1981; Murray et al., 1986). The suppression induced by this cell population is non-specific and appears to be mediated by the production of prostaglandins. Changes in the metabolism of macrophages from non-healing mice infected with *Leishmania* have been reported (Reiner and Malemud, 1985; Farrell and Kirkpatrick (1987). Results indicate that *Leishmania* induce changes in the cycloxygenase and lipoxygenase pathways of infected macrophages resulting in the production of increased amounts of arachidonic metabolites (prostaglandins), compounds known to modulate both inflammatory and cellular immune response (Snyder et al., 1982). This increased production of prostaglandins in infected macrophages from non-healing mice can be correlated with depressed responses to non-specific mitogenic stimulation and deficient expression of MHC gene products at the cell surface (reviewed by Mitchell, 1984; Reiner, 1987; Reiner et al., 1987).

Examination of the mechanism involved in macrophage activation in murine cutaneous leishmaniasis has also led to the identification of a novel non-lymphokine cell contact-mediated mechanism. Studies by Panosian et al. (1984), Sypek et al. (1984) and Wyler et al. (1987) identified a subpopulation of T cells from healing mice convalescing from *Leishmania major* infection. These T cells were *Leishmania*-specific and could activate anti-microbial effects in *Leishmania*-infected macrophages by an apparently lymphokine-independent mechanism which requires direct cell contact.

There have been many advances made in the identification of the mechanisms involved in experimental leishmaniasis in animal models. In order to assess the importance of these findings to the course of human leishmaniasis, several investigators have attempted to correlate the findings from experimental leishmaniasis to the events which occur during infection in man. Recent studies by Jaroskova et al. (1986) and Passwell et al. (1986) demonstrated that in patients with cutaneous leishmaniasis there was a lack of helper/inducer T cells and generation of T suppressor cells and that IFN-γ also plays a central role in elimination of *Leishmania* infections in man. Murray et al. (1983a/b) showed that the activating lymphokine in human
visceral leishmaniasis appeared to be IFN-γ. Studies by Sacks et al. (1987) indicated that T cell unresponsiveness in visceral leishmaniasis was accompanied by the absence of IL 2 and IFN-γ production, was antigen-specific and that after chemotherapy, antigen-specific responsiveness was restored. Reports by Buchmuller et al. (1985) and Hoover et al. (1986) have identified a lymphokine distinct from IFN-γ which activated monocytes to kill *Leishmania donovani*.

5. Role of Humoral Immune Responses in Leishmaniasis

The role that antibody plays in immunity to leishmaniasis is controversial. Serum antibody titres are usually directly related to the severity of the disease and as the disease progresses, high levels of serum antibody, both specific- and non-specific, and high levels of circulating immune complexes are observed. It has been proposed that the antibody response may simply reflect the size and duration of the antigenic stimulation determined by the parasite load. A study by Anderson et al. (1983) examined the effects of transfer of monoclonal antibodies directed against stage- and species-specific antigens to *Leishmania mexicana* in susceptible mice which were concomitantly challenged with live parasite promastigotes. This treatment conferred complete protection, implying that antibody responses may be involved in protective immunity. This is the only recent report which provides evidence to support the idea that antibody has a protective role in determining the resistant state directly, however, there is accumulating evidence for the idea that cooperation of humoral immune responses with cell-mediated immunity might be important in determining the outcome of the disease.

Several reports have implicated B cells and/or antibody in the generation of T suppressor cells and the consequent susceptibility to disease. Sacks et al. (1984) reported that susceptible mice which had been depleted of B cells exhibited enhanced resistance to *Leishmania major* challenge and that this appeared to be due to the lack of a B cell-dependent T cell critical to the suppressor pathway. Conversely, Scott et al. (1986) reported a requirement for B cells in the generation of an effector T cell population which mediated healing of cutaneous leishmaniasis in normally resistant mice. It appears that in susceptible mouse strains, B cell depletion results in control of *Leishmania* infections by lack of induction of a T suppressor cell population, while the same treatment in resistant mice, leads to the lack of
induction of a T cell required for healing. A recent report by Ron and Sprent (1987), which demonstrated a role for B cells in controlling the clonal expansion of activated T cells supports these findings. Whether the T cell population which is absent in genetically resistant or susceptible mice depleted for B cells is the same but has different functions in the two strains of mice, or whether two different T cell populations are being affected is not clear as yet.

A study by Dennis et al. (1986) using squirrel monkeys, demonstrated that Leishmania donovani infection resulted in high antibody titres, both Leishmania-specific and non-specific. This is similar to the situation seen in infected humans. Human visceral leishmaniasis progresses in the presence of extremely high titres of specific anti-parasite antibody, the role of this antibody in protection, therefore, appears to be negligible. Barral et al. (1986), showed that in patients with acute American visceral leishmaniasis there were high levels of IgM and IgG (total and parasite-specific) in patient's sera and that this sera was able to actively suppress non-specific lymphocyte responses to mitogenic stimulation. Studies examining the role of B cells and/or antibody in human cutaneous leishmaniasis have also been carried out. Sells and Goldring (1985) found substantial amounts of Leishmania antigens internalized within plasma (B) cells found within lymph nodes of infected patients. Lynch et al. (1986) demonstrated the presence of Leishmania-specific IgE within the serum from American cutaneous leishmaniasis patients.

6. Leishmania molecules involved in host immune responses

Studies have shown that it is possible to induce protective immunity in mice with the use of killed or avirulent promastigotes from Leishmania major (Preston and Dumonde, 1976; Howard et al. 1982; Mitchell, 1984). Attempts have been made to identify specific Leishmania molecules which may help to induce resistance in the host or, alternately, which may be involved in the triggering of suppression.

Pretreatment of macrophages in vitro with purified gp63 (the major promastigote surface antigen) or with anti-gp63 antibodies inhibits promastigotes from binding to macrophages (Chang and Chang, 1986; Russell and Wilhelm, 1986). These studies indicate that immunization with purified gp63 may provide protective immunity in vivo. A study
identifying a purified Leishmania membrane glycoprotein which had been previously implicated in host-protective immunity in susceptible mice was recently reported (Kahl and McMahon-Pratt, 1987). Using monoclonal antibodies which had previously been shown to confer protection in susceptible mice (Anderson et al., 1983), this group purified a membrane glycoprotein of apparent molecular weight 46,000 which represented approximately 1% of the total promastigote membrane proteins. Experiments to assess the efficacy of this purified protein for vaccination against cutaneous leishmaniasis in animal model systems are in progress.

Scott et al. (1987) successfully immunized mice against Leishmania infection using a soluble, non-membrane fraction from Leishmania major promastigotes. The ability of soluble antigens, not derived from membranes, to provide protection against Leishmania infection indicates that cytoplasmic or nuclear proteins may also play a role in determining the host's immune response to Leishmania. The identification of the soluble molecule(s) which is responsible for mediating protection in this model will help to further define the immune mechanisms which function during Leishmania infections.

Whether the excreted Leishmania carbohydrate antigens referred to as excreted factor, glycoconjugate or Leishmania-LPS play a role in parasite survival within the host is unclear. Initial studies indicated that Leishmania-LPS might be involved in the attachment and uptake of the parasite by macrophages (Handman and Goding, 1985) and a later study by Handman and Mitchell (1985) indicated that vaccination with this antigen conferred protection to Leishmania major infection in susceptible mice. However, in a second study, Mitchell and Handman (1986) purified the secreted water-soluble form of this molecule and reported that it appeared to confer suppression rather than protection on vaccinated mice. In a recent study to clarify the role of Leishmania-LPS in Leishmania infection, it was reported that Leishmania-LPS is not involved in parasite attachment or uptake and that its role is to protect the parasite once it has gained entry into the cell (Handman et al., 1986). Why immunization with a molecule which apparently functions by protecting the parasite from degradation within the macrophage should be able to affect the outcome of subsequent parasite challenge is unclear. Rodrigues et al. (1986), used chemically defined glycoconjugates from Leishmania mexicana amazonensis and examined the cellular immune responses to these antigens by T cells from
infected mice. They could detect differences between the T cell responses from healing and non-healing strains; however, in both cases there was a response.

Gorczynski (1986) examined the relationship of sugar residues to protection and suppression. He reported that determinants responsible for protection in susceptible mice are dependent on N-linked sugars while those responsible for suppression are not. These results may also explain the differences seen in the immune response to the membrane-form versus the water-soluble form of *Leishmania*-LPS seen in the studies of Handman and Mitchell and perhaps indicate that the observed protection seen in these studies may in fact be mediated by the sugars present on this molecule. Sugars which are also present on other *Leishmania* molecules which interact with the directly immune system during infection.
7. Summary

The accumulating data from experimental leishmaniasis in animal models have shown that there appear to be genetically determined factors which control the resistance or susceptibility of mammalian hosts to infection with *Leishmania*. The results of studies investigating the host defense mechanisms invoked by *Leishmania* infections, indicates that the genetically determined susceptibility appears to be due to defects in macrophage function rather than inherent defects in the immune system. The level of initial replication of parasites within macrophages appears to be the major factor contributing to the subsequent ability of the immune system to respond. The initial inability of susceptible mice to control intracellular parasite replication is followed by the induction of suppressor cell mechanisms. The exact nature of the signals which trigger this suppression are not known, however, higher antigen loads in susceptible macrophages and parasite-induced changes in macrophage metabolism resulting in failure to present antigen in a manner which triggers protective T cell responses and/or in failure to respond to appropriate activation signals when given, may be some of the mechanisms involved. Studies using purified macrophage and T cell products have established the importance of macrophage expression of MHC gene products, macrophage-derived monokines, such as IL-1, and T cell-derived lymphokines, such as IL-2 and IFN-γ, in the successful activation of cell-mediated immunity resulting in parasite elimination. Parasite-modulated changes appear to affect these mechanisms.
D. RELATED PARASITES OF MAN

Parasites have adopted many strategies for successfully invading and establishing infections within their mammalian hosts. Some of these strategies are shared by parasites with little evolutionary relationship while some strategies are unique to certain types of parasites or even to a certain species within a related group. A general knowledge of some of the important aspects of other host-parasite relationships gives insights into shared strategies and functions common to *Leishmania*, and points out features which are unique.

Several parasites invade and replicate in macrophages including *Leishmania*, American trypanosomes, and leprosy bacilli. Organisms of the genus *Plasmodium* infect erythrocytes and hepatocytes. By residing within host cells during at least some stages of their life-cycle, these parasites avoid immune elimination. African trypanosomes are extracellular parasites and they have evolved the unique process of antigenic variation to avoid immune elimination. Most chronic parasitic infections also cause immune depression which contributes to the parasites ability to evade the host's immune system. For many parasites, the diseases which they cause exhibit a wide-spectrum of clinical manifestations, implying that the outcome of the disease is dependent on genetic variability in both the parasite and the host. Identification of parasite molecules is still at an early stage and has been primarily immunological or biochemical. The assignment of functional roles, however, has not been achieved for many parasite molecules.

In this chapter the related parasites listed in the table below will be discussed. Although they represent both protozoan and bacterial parasites and exist either intracellularly or extracellularly within their mammalian host, they all share certain features with the protozoan parasite *Leishmania*. 
### TABLE 2. SELECTED MAJOR PARASITES OF MAN

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Cell</th>
<th>Disease</th>
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<tr>
<td><strong>INTRACELLULAR PARASITES</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>Leishmania</em> spp.</td>
<td>Macrophages</td>
<td>Leishmaniasis (Oriental Sore, Kala Azar)</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>Macrophages, Muscle Cells</td>
<td>Chagas' Disease</td>
</tr>
<tr>
<td><em>Plasmodium</em> spp.</td>
<td>Red Blood Cells, Hepatocytes</td>
<td>Malaria</td>
</tr>
<tr>
<td><em>Mycobacterium leprae</em></td>
<td>Macrophages</td>
<td>Leprosy</td>
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<td><strong>EXTRACELLULAR PARASITES</strong></td>
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<td><em>Trypanosoma brucei</em> spp.</td>
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<td>Sleeping Sickness</td>
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1. **American Trypanosomes**

   Trypanosomes are protozoan parasites belonging to the genus *Trypanosoma*. Like other *Trypanosomatids*, they are propelled by a single flagellum during their motile stages. The genus *Trypanosoma* is part of the order *Kinetoplastidia* and, therefore, like *Leishmania* they are characterized by the presence of a kinetoplast at the base of the flagellum which contains the mitochondrial DNA. Trypanosomes are transmitted to their mammalian hosts by insect vectors and exist in more than one life stage. The genus *Trypanosoma* is divided into two divisions the *Stercoraria*, which are transmitted by feces from the vector and are characterized by the American trypanosomes and the *Salivaria*, which are transmitted via insect saliva (African trypanosomes).

a) **Life cycle of American trypanosomes**

   The trypanosome species, *Trypanosoma cruzi* is the predominant American trypanosome. The life cycle of *Trypanosoma cruzi* is shown in Figure 2. *Trypanosoma cruzi* are transmitted to mammalian hosts by blood-sucking triatomine bugs. Within the insect vector, *Trypanosoma cruzi* multiply as epimastigotes, differentiating in the hindgut and rectum into infective,
nondividing, metacyclic trypomastigotes. These infective forms are discharged by the insect feces and enter the mammalian host by contamination of wounds generated by the bloodsucking activity of the insect or through contamination of mucous membranes. Once inside the mammalian host, the parasites are phagocytosed by macrophages. Inside the macrophage the parasites are initially observed within membrane-bound vacuoles; however, they are able to lyse the vacuolar membrane and escape into the cytoplasm where they multiply as aflagellate amastigotes (Nogueira and Cohn, 1976). The amastigotes then differentiate into nondividing trypomastigotes that are released into the bloodstream where they can reinvade other macrophages or the smooth and skeletal muscle cells of the heart or circulate in the blood to be ingested by another insect vector. The bloodstream trypomastigotes appear to be less readily taken up by macrophages than the invading metacyclic trypomastigotes (Nogueira, 1986).

Studies done to investigate the attachment and interiorization of *Trypanosoma cruzi* by host cells, have indicated that the process may be receptor-mediated. Recent results by OuaiSSI et al. (1984; 1986), indicate that *Trypanosoma cruzi* is able to bind the host macrophage-derived glycoprotein, fibronectin. It appears that *Trypanosoma cruzi* binds fibronectin through the recognition site Arg-Gly-Asp-Ser and that fibronectin may, therefore, act as a bridge to mediate attachment of the parasite to the macrophage. Fibronectin has also been shown to bind to *Leishmania* species and has been implicated in their attachment to macrophages.
American trypanosomiasis (Chagas' disease) afflicts over 10 million people throughout Central and South America (Brener, 1982). The human disease is divided into three phases. Within two to three months of the initial acute phase of the infection, the majority of cases resolve to an asymptomatic chronic stage which can last for many years. The levels of parasites in the blood at this stage are very low and diagnosis is very difficult. In over 40% of cases this asymptomatic stage ends and severe enlargement and abnormalities of the heart and digestive system occur resulting in death.

b) Host response to Trypanosoma cruzi

The host immune response to Trypanosoma cruzi infection has recently been reviewed by Nogueira (1986). There has been little genetic analysis done on the host response to American trypanosomes; however, it is known that there are differences in the resistance of inbred mouse strains to experimental infection by Trypanosoma cruzi. The immune mechanisms involved in resistance to Trypanosoma cruzi infection and control of parasitemia are not well understood. The establishment of strong acquired immunity during the course of the disease, does not result in elimination of the parasites, presumably because of the ability of the parasite to escape from the phagolysosomal compartment and to reside and replicate within the cytoplasm of the macrophage. The ability of Trypanosoma cruzi to establish itself intracellularly within the macrophage seems to be dependent on the state of the macrophage. While Trypanosoma cruzi can readily infect unactivated macrophages, phagocytosis of the parasite by activated macrophages results in intracellular killing, mediated by the oxygen-dependent mechanisms produced upon macrophage activation. It has been established that antibodies play an important role in controlling Trypanosoma cruzi infection. Antibodies appear to function by increasing phagocytosis of the parasite by macrophages rather than by triggering complement-mediated lysis (Lages-Silva et al., 1987). However, the generation of antibodies has also been proposed to play a role in the pathology of the disease, by being associated with autoimmune responses that are often triggered (Szarfman et al., 1982).

The protective role of antibody is dependent on the prior activation of macrophages by cell-mediated mechanisms. The lymphokines, IFN-γ and IL 2, have been implicated in the
inhibition of intracellular multiplication of *Trypanosoma cruzi*, both within macrophages and other nonphagocytic cells, such as myocardial cells, which *Trypanosoma cruzi* is known to invade (Reyes and Chinchilla, 1987). Choromanski and Kuhn (1987) reported that deficiency in IL 2 production may play a major role in host susceptibility to *Trypanosoma cruzi* and that production of IL 2 may be regulated by infected macrophages, presumably via alterations in prostaglandin synthesis. The activated macrophage product tumour necrosis factor (TNF) has also been shown to inhibit intracellular *Trypanosoma cruzi* growth (De Titto et al., 1986). A characteristic feature of the later stages of chronic *Trypanosoma cruzi* infections is a severe non-specific immunodepression (Reed et al., 1983).

The ability of bloodstream trypomastigote forms to evade phagocytosis by macrophages is thought to be due to the production of specific "anti-phagocytic" factors (Nogueira, et al., 1981). This ability may well protect these forms of the parasite, which appear later in the infection, from being taken up by macrophages which have become activated during the course of the infection and which would now be able to mediate their intracellular destruction. There is also evidence to suggest that the parasite has evolved a proteolytic activity which is capable of cleaving the Fc portion of bound antibodies resulting in neutralization of their ability to mediate macrophage uptake (Krettli et al., 1979).

c) *Trypanosoma cruzi* antigens

One of the major surface glycoprotein which has been identified is a protein with an apparent molecular weight of 72,000 (gp72). The ability of complement to lyse the non-infective insect stage (epimastigote) of *Trypanosoma cruzi* in an antibody-independent manner via the alternative complement pathway is well established, however, the mammalian stages (trypomastigotes) of the parasite evade this lysis. Joiner et al. (1985), demonstrated that the surface antigen (gp72) is the molecule to which complement factor C3 binds and in a further study (Joiner et al., 1986), they reported that differences in the form of C3 bound to the different stages were observed and it appears that the trypomastigote gp72 molecule differs structurally from the gp72 molecule found on epimastigotes. This protein has an unusual carbohydrate composition which appears to be developmentally regulated (Schechter et al.,
1986). The apparent change in developmental glycosylation may explain the different complement factor C3 binding capacities of this molecule noted by Joiner et al. (1985; 1986). Whether regulation of binding of factor b is a general mechanism used by protozoan parasites to evade complement lysis is not known. A glycoprotein of apparent molecular weight 90,000, has been identified by Nogueira et al. (1981) which appears to be specific for the bloodstream trypomastigote stage and may be involved with the decreased interiorization ("antiphagocytosis") of these forms by macrophages, which has been observed. A glycoprotein which is expressed in all stages of Trypanosoma cruzi life cycle has been identified by Dos Reis et al. (1986) and (Scharfstein et al., 1986). This molecule is synthesized as a 57,000 molecular weight protein which is expressed on the parasite surface as a molecule of apparent molecular weight 51,000. Recently, monoclonal antibodies have been developed which are reactive with trypomastigote surface antigens of apparent molecular weight, 68,000 and 90,000/105,000 (Wrightsman et al., 1986). The 68,000 molecular weight protein appeared to be present on all stages of all Trypanosoma cruzi strains tested while the 90,000/105,000 doublet appeared to be restricted to the trypomastigote stage but is only found on certain strains.

2. African Trypanosomes

a) Life cycle of African trypanosomes.

The life cycle of African trypanosomes is shown in Figure 3. African trypanosomes are transmitted by the tsetse fly. Within the insect vector they exist as a non-infective procyclic form which replicates in the insect's midgut. These forms travel up into the salivary glands where they differentiate, first into the epimastigote stage, and then into the infective metacyclic trypomastigote stage. During the last differentiation the parasite synthesizes its unique and characteristic surface coat composed of a matrix of a single glycoprotein (Cross, 1975) and it is these forms that are inoculated into the mammalian host when the tsetse fly feeds. Upon infection the parasites differentiate into dividing, long slender bloodstream forms which also express the surface coat. The immune response of the host is largely directed against this single surface antigen. The ability of the parasite to exist within the bloodstream of the infected host, is due to the unique ability of this parasite to undergo antigenic variation
of the surface glycoprotein with which the surface coat is composed and thus the term variable surface glycoprotein (VSG). The immune response to the original VSG eliminates the majority of the parasites; however, a small population which bear a different, antigenically distinct VSG then proliferate until an immune response can be mounted against this new VSG. This cycle of parasite elimination followed by proliferation of parasites expressing a new VSG results in constantly changing antigenic stimulation to the host's immune system and the waves of parasitemia which characterize the disease. Within the bloodstream of the mammalian host another stage of differentiation occurs, transforming the long slender trypomastigotes into short stumpy forms, and it is this form which is able to survive in the insect when it is taken up into the gut of the feeding tsetse fly.

**Figure 3. Life cycle of African trypanosomes**

African trypanosomes are the causative agents of sleeping sickness. There are thought to be one million people throughout the world affected by this disease and there are 20,000 new cases reported every year (Walsh and Warren, 1979). The parasite strains which cause human sleeping sickness are both subspecies of *Trypanosoma brucei*, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, both of which were identified at the turn of the century. In humans the disease is chronic and debilitating, it is characterized by an initial
stage where the parasites are found at the site of infection resulting in local swelling, followed by a systemic stage, where the parasites are distributed by the bloodstream throughout the body and this stage is marked by episodes of severe illness and general malaise. Parasites eventually invade the central nervous system and in the final stages the patient falls into a stupor and often the cause of death is starvation. As the disease progresses there is an increased immunodepression and, therefore, at the later stages secondary infections often occur and may become fatal.

b) Host response to *Trypanosoma brucei*

Unlike the situation in leishmaniasis, mouse models for trypanosomiasis never fall into obvious resistant and susceptible categories. Almost all mice infected with African trypanosomes eventually die of the disease; however, there is a difference in the survival times of different strains. Genetic analysis has shown that this "resistance" appears to be controlled by a single autosomal gene or gene cluster (Pinder, 1984).

One of the symptoms of African trypanosomiasis is a decrease in the level of complement. It is still not known whether activation of complement by the parasites results in this hypocomplementemia, however, it has been shown that trypanosomes are able to activate both the classical and alternative complement pathways but that the parasites are resistant to lysis, because they are able to restrict the cascade beyond the establishment of C3 convertase on the parasite surface (Devine et al., 1986).

The immune system of the host plays an important role both in defense against the invading parasite and in the pathology of the disease itself (reviewed by Shapiro and Pearson, 1986). There is little evidence that cell-mediated responses play a role in controlling the disease. Instead it appears that protection is mediated through IgM specific for surface antigen. During the early stages of infection when the parasite is distributed throughout the bloodstream, there is an intense lymphocyte proliferative response, resulting in a huge increase in the amount of circulating antibody, both parasite-specific and non-specific. It appears that membrane fractions from trypanosomes are capable of triggering this polyclonal B cell activation perhaps via activation of macrophages to produce a B cell mitogen. (Grosskinsky
and Askonas, 1981; Sacks et al., 1982). Much of the antibody response appears to be T cell-independent as it occurs in T cell deficient mice (Clayton et al., 1979a,b) and appears to be directed to external antigenic determinants on the parasite VSG (Diffley et al., 1983). Pinder et al. (1986), found that the antibody response to trypanosome infection was the same in both susceptible and resistant mouse strains, but it occurred much earlier in resistant mice and was, therefore, able to control the first wave of parasitemia. The antibody response in both strains was T cell independent. The most important factor in resistance, appeared to be the earlier immune response. In the later stages of the disease in mice, there is a severe immunodepression to both parasite and non-specific antigens (Hudson and Terry, 1979) and it has recently been reported that T suppressor cells can be induced by trypanosome membrane fractions (Yamamoto et al., 1987). Although cell-mediated responses are thought to have little effect on resistance to African trypanosomes, a study by De Gee et al. (1985) indicated that the induction of IFN-γ in the resistant mice appeared to be linked to a more effective immune response to the parasite. The pathology of the disease appears to be due to the immune response to the parasite. One of the major effects of the polyclonal B cell stimulation, is the formation of autoantibodies and circulating immune complexes. These may be involved in some of the inflammatory reactions and tissue damage which are symptomatic of the disease.

c) Trypanosoma brucei antigens

The variant surface glycoproteins (VSG) are the most extensively studied of the Trypanosoma brucei proteins. The ability of African trypanosomes to undergo immunological changes has been known since the early 1900's, however, the mechanism by which this antigenic variation occurs has only recently been elucidated. It appears that a low degree of antigenic switching (10⁻⁷ to 10⁻⁶ per division) is an intrinsic genetic property of trypanosomes and that external stimuli, such as antibodies, do not influence this switching, although they do exert a selective pressure on the resulting organisms (reviewed by Borst and Cross, 1982; Van der Ploeg et al., 1982). The molecular basis for trypanosome antigenic variation has recently been reviewed by Borst and Greaves (1987). It was the analysis of VSG gene transcription which led to the discovery of the mechanisms of discontinuous transcription or trans-splicing.
(reviewed by Borst, 1986; Van der Ploeg, 1986). It now appears that all mRNA transcripts of *Kinetoplastid*ia have the spliced leader sequence sequence added to their 5' ends.

After translation, the VSG is modified by cleavage of its amino-terminal signal sequence, N-linked glycosylation and a series of unusual events at the carboxy-terminus resulting in attachment of a glycolipid structure (reviewed by Cross, 1987). The attached glycolipid is the sole means of membrane attachment and it appears that there is a specific endogenous membrane-associated enzyme with phospholipase C-like activity, which is able to cleave myristic acid to produce a soluble VSG (Ferguson et al., 1985) which is released into the bloodstream (Shapiro, 1986; Diffley and Straus, 1986). As mentioned previously, the *Leishmania* major surface glycoprotein (gp63) is also anchored to the membrane by a similar myristic acid-containing phospholipid (Etges et al., 1986b; Bordier et al., 1986).

African trypanosomes also express non-VSG proteins on their surface. Characterization of these molecules has been mainly serological although some biochemical analysis has also been carried out. Serological analysis has identified antigens which were found in all African trypanosomes tested as well as antigens which were species- and stage-specific (reviewed by Shapiro and Pearson, 1986).

3. Mycobacterium leprae

a) Life cycle of Mycobacterium leprae

Although the bacterium *Mycobacterium leprae* was identified as the causative agent of leprosy over 100 years ago, studies have been hampered by the intractability of this baccillus to propagation in the laboratory. The bacteria can not be grown *in vitro* and only replicate very slowly, doubling every 11-13 days when growing exponentially. Previously researchers were reliant on growth of *Mycobacterium leprae* within mouse footpads, which only resulted in the recovery of very small numbers of bacteria (10^6 per footpad) after 180 days of incubation. The discovery that the nine-banded armadillo is susceptible to leprosy and can become systemically infected attaining concentrations in the tissues of 10^{12} per gram, has totally changed this situation.
Leprosy is a human disease and is estimated to affect more than 10 million people throughout the world (Gaylord and Brennan, 1986). *Mycobacterium leprae* is only slightly contagious; however, the exact mechanism of infection is still not clear. *Mycobacterium leprae* is an obligate intracellular parasite of mammalian macrophages and, like *Leishmania* and *Trypanosoma cruzi*, upon phagocytosis by macrophages resists destruction and is able to proliferate within these cells. The attachment and uptake of *Mycobacterium leprae* by macrophages appears to occur through non-specific phagocytosis. It appears that lipid moieties of the bacteria may provide a general binding interaction with the macrophage and be important in this non-specific uptake mechanism (Saito et al., 1986). This contrasts with the receptor-ligand interaction by which *Leishmania* uptake occurs. *Mycobacterium leprae* was originally thought to reside primarily within the phagosomal compartment of macrophages (Hart et al., 1972); however, a recent study by Sibley et al. (1987) reported that *Mycobacterium leprae* actually prevents phagolysosomal fusion upon uptake by unactivated macrophages unlike *Leishmania*, which exist within fused phagolysosomes. The disease exhibits a wide spectrum in infected humans, ranging from tuberculoid leprosy to full lepromatous leprosy. In tuberculoid leprosy the patient develops high levels of cell-mediated immunity which kills the parasite but this secondarily also causes destruction of the peripheral nerves often leading to crippling results. In patients with full lepromatous leprosy there is almost no immune response to the parasite resulting in extensive involvement of the skin with large swellings; however, there is less peripheral nerve damage in these patients and, therefore, less severe crippling.

b) Host response to *Mycobacterium leprae*

The study of the genetic basis for resistance to leprosy is very new. The broad spectrum of the disease in man indicates that host genetic factors play an important role in the course of the infection. Normal mouse strains are not susceptible to the disease; however, comparisons of the humoral and cellular immune responses in inbred mouse strains have been made and initial results indicate that a spectrum of resistance occurs. It is likely that the trait of resistance is under polygenic control. Recent studies have shown that at least some part of
the response to *Mycobacterium leprae* in humans, appears to be MHC restricted (Modlin et al., 1986; Ottenhoff et al., 1986b).

As with most other intracellular parasites, protective immunity is mediated by T cells (Bloom and Godal, 1983), and in lepromatous leprosy there is a severe depression in *Mycobacterium leprae*-specific T cell responsiveness. There is an inverse relationship of serum antibody levels to cell-mediated immunity as in leishmaniasis; however, it is not clear whether this is due to a generalized polyclonal activation which would include *Mycobacterium leprae*-specific antibodies, but not be confined to them, or whether this response is entirely *Mycobacterium leprae*-specific. It has been known for a long time that the ability of *Mycobacterium leprae* to invoke strong immune inflammatory response results in the destruction of the bacteria but also causes much of the tissue damage which is characteristic of the disease. Like *Leishmania* this bacteria has been shown to be susceptible to killing by H$_2$O$_2$ produced by activated macrophages (Klebanoff and Shephard, 1984). Holzer et al. (1986) reported that like *Leishmania*, *Mycobacterium leprae* did not elicit a strong oxidative burst upon uptake by unactivated macrophages, and that an intact T cell IL 2/IFN-γ response was necessary for productive macrophage activation. Sibley et al. (1987) demonstrated that addition of killed bacteria or pretreatment of the macrophages with IFN-γ, resulted in lysosomal fusion and induction of the respiratory burst by macrophages upon uptake of *Mycobacterium leprae*. A study by Kaplan et al. (1986) reported that the immune defect in lepromatous leprosy probably results from a lack of response to *Mycobacterium leprae* by the patients’ T cells rather than any parasite-induced inability of the infected macrophages to respond to IFN-γ. It appears that macrophages from lepromatous leprosy patients may differ in certain characteristics from macrophages from tuberculoid leprosy patients and normal controls. There is lowered expression of Fc receptors on macrophages from lepromatous leprosy patients after exposure to *Mycobacterium leprae* and these macrophages also exhibit excessive phagocytic activity (Mistry et al., 1986). It is still not clear, however, whether these differences are intrinsic genetic properties of the patients or whether *Mycobacterium leprae* infection is responsible for their induction.
There is general agreement that lepromatous patients exhibit a complete lack of cell-mediated immune responsiveness to antigens of *Mycobacterium leprae* both *in vivo* and *in vitro*, although there are conflicting reports about the nature of this immunosuppression. There is evidence for the induction of *Mycobacterium leprae*-specific T suppressor cells, which when removed allowed *Mycobacterium leprae*-specific cell-mediated immunity to be expressed (Mehra et al., 1982; 1984; Bloom, 1986). A recent report, however, has demonstrated that the stimulation of T suppressor cells by *Mycobacterium leprae* antigens is not confined to lepromatous leprosy patients and is observed in both tuberculoid patients and normal controls as well (Prasad et al., 1987). It appears that the course of the disease is determined by ratio of T helper/T suppressor cells present in the lesions and that *Mycobacterium leprae* antigens are capable of inducing suppression in both susceptible and resistant humans. There is evidence that certain *Mycobacterium leprae* antigens are capable of stimulating both suppressor and helper T cell functions (Ottenhoff et al., 1986c; Kaplan et al., 1987) and it may be that there is a differential ability of individuals to respond to these different stimuli perhaps linked to the individuals HLA class II gene repertoire (Ottenhoff et al., 1986b).

The role of antibody in regulation of the immune response to leprosy has been difficult to determine. Recently it has been shown that the unique secreted glycolipid produced by *Mycobacterium leprae* suppresses induction of cell-mediated immunity but is also a positive regulator of IgM. Whether there is any link between the observed polyclonal stimulation and the subsequent suppression of cell-mediated immune responses is unknown, but the involvement of B cells in suppression of cell-mediated immunity has also been proposed to play a role in the course of cutaneous leishmaniasis (Scott et al., 1986). In order to examine whether antibody-mediated suppression of cell-mediated immunity is important in leprosy a satisfactory mouse model is needed.

c) *Mycobacterium leprae* antigens

Almost all known protein and glycoprotein antigens of *Mycobacterium leprae* have been shown to be serologically identical or cross-reactive with antigens of *Mycobacterium bovis* (Bacillus Calmette–Guerin (BCG) strain). It appears that one of the only antigens specific for
*Mycobacterium leprae*, is the unusual secreted, phenolic glycolipid (PGL-1) (Hunter et al., 1982). Because of its unique nature, and the large quantities of it secreted by *Mycobacterium leprae*, it was hoped that this antigen might be important in determining the outcome of *Mycobacterium leprae* infection and, therefore, this antigen and the immune responses it induces have been extensively studied. However, as previously mentioned, this antigen appears to have both suppressive and stimulatory capacities and the potential usefulness of this antigen is uncertain.

The techniques of recombinant DNA and monoclonal antibody production have provided a useful method for overcoming the problems of identification of immunologically relevant *Mycobacterium leprae* antigens from an organism which is refractory to *in vitro* cultivation. The cloning and expression of DNA from *Mycobacterium leprae* using the \( \lambda gt \) system has provided major advances in the study of *Mycobacterium leprae* protein antigens. Antigens expressed in the bacteriophage vector \( \lambda gt11 \), have been identified with both polyclonal and monoclonal antibodies. There appear to be three immunodominant antigens which can be identified in this way. These antigens are present in *Mycobacterium leprae* extracts as proteins of apparent molecular weights of 71,000, 68,000, and 14,000 and it appears that these proteins undergo post-translational modifications, as the molecular weights of the unprocessed recombinant proteins is often higher or lower than the corresponding mature product found in *Mycobacterium leprae*. Immunological analysis of these recombinant proteins has demonstrated that they often contain *Mycobacterium leprae*-specific epitopes as well as epitopes which cross-react with antigens from many other mycobacterial species (Buchanan et al., 1987). Studies have also been initiated to examine the ability of cloned T cell lines from *Mycobacterium leprae* vaccinated volunteers and leprosy patients to recognize recombinant *Mycobacterium leprae* antigens (Mustafa et al., 1986; Ottenhoff et al., 1986a). These studies have identified two *Mycobacterium leprae* antigens of 36,000 and 18,000 apparent molecular weight.
4. Plasmodia

a) Life cycle of Plasmodia

*Plasmodia* are parasitic protozoans which are evolutionarily unrelated to *Trypanosomatids*. There are four *Plasmodia* species which can infect humans, the two most prevalent of which are *Plasmodium falciparum* and *Plasmodium vivax*. The life cycle of *Plasmodia* is considerably more complex than that of the trypanosomatids (see Figure 4). *Plasmodia* species are transmitted by mosquitoes and cause the disease malaria. Human malaria is characterized by several stages which correspond to the life cycle of the parasite within the mammalian host. The bite of an infected mosquito inoculates sporozoite parasites into the bloodstream where they are quickly removed from the circulation by hepatocytes as they pass through the liver (usually within 20 minutes). Within the liver the parasites differentiate into merozoites and multiply, however, there are no clinical symptoms of the disease during this stage. The release of the merozoites from the liver cells initiates the clinical disease. Malaria caused by *Plasmodium falciparum* or *Plasmodium vivax* can be differentiated by the characteristics of the release of merozoites from the liver, in *Plasmodium falciparum* infection there is a single synchronous release, while in infections caused by *Plasmodium vivax* there are several successive releases at widely spaced intervals of months or even decades (relapses). The released merozoites invade erythrocytes, differentiate and proliferate within them. As proliferation proceeds within the erythrocyte the cell ruptures and releases more merozoites which invade new erythrocytes. The symptoms of malaria, anaemia, fever spikes and circulating immune complexes, are caused by the rupture of these infected cells. Infection with *Plasmodium falciparum* usually causes the death of the host and is often associated with cerebral dysfunction. This is thought to be caused by occlusion of blood flow to the brain due to the attachment of large numbers of parasite-infected erythrocytes to the capillary endothelium. *Plasmodium vivax* infection is characterized by successive relapses and chronicity.
All stages which occur within the mammalian host which have been discussed so far are asexual, and these forms can not infect the mosquito. When some bloodstage merozoites infect erythrocytes they differentiate not into asexual multiplication stages, but into sexual gametocytes. The uptake of erythrocytes which contain these sexual gametocyte stages is what leads to subsequent mosquito infection. Within the mosquito, fertilization and zygote formation occurs within the first 30 minutes after ingestion. The zygote form then differentiates and divides repopulating the mosquito salivary glands ready to initiate another mammalian infection cycle. The time from ingestion to repopulation of the salivary gland with infective sporozoites is approximately 2 weeks.

b) Host response to Plasmodia

Although there has been extensive analysis of the course of malaria in several animal model systems, the applicability of these findings to human diseases is unclear. Rodent models are available but the Plasmodia species which infect rodents are different from those which infect man and none of the rodent malarias exhibit a particularly close resemblance to any of the human diseases. The malaria diseases observed in non-human primates although caused by different Plasmodia species bear a much closer resemblance to the disease seen in man; however, these studies are still quite new.
The immune response to *Plasmodia* infections has recently been reviewed by Howard (1986) and appears to be very complex. Natural immunity to malaria in humans is acquired very slowly and progresses from early responses that are able to only partially control parasite growth and the toxic effects of blood infection, to later responses that are able to eliminate parasites from the blood. Evidence that acquired immunity requires continuous antigenic stimulation comes from observations that immune individuals who move away from areas where malaria is endemic often succumb to acute and potentially fatal infection upon their return. The *Plasmodia* have a remarkable capacity to reinitiate multiple infections in a previously immunized host. The parasite appears to be able to either suppress induction of anti-parasite immune responses or evade these responses if they develop. Both humoral and cell-mediated immune responses seem to play important roles in immunity to malaria and both types of immunity are thought to be capable of parasite destruction. The exact nature of cell-mediated immunity in malaria is not clear. The involvement of T helper cells in the induction of specific B cells responses appears to be important, but a role for cytotoxic T cells in lysis of infected erythrocytes is not established. It appears that non-specific macrophage-mediated responses may be involved in inhibition of intra-erythrocytic replication of parasites, which may be important in resolution of the disease. Protective humoral immune responses in malaria appear to function by inhibiting interaction of the bloodstream stages with the host cells which they invade. Complement does not appear to play a role in immune destruction of parasites mediated by antibodies and the role of antibody in parasite opsonization leading to cytotoxicity or phagocytosis is not clear.

The most characteristic feature of the host's immune response to *Plasmodia* infection is the massive polyclonal B cell activation which is observed. The primary malaria infection results in specific anti-*Plasmodia* antibodies but these are non-protective. The levels of these antibodies increase upon repeated exposure, however, they do not correlate with immune status. It has been proposed that the polyclonal B cell stimulation may in fact act to suppress the development of protective immunity and may contribute to its transitory nature (Anders, 1986).
c) Plasmodia antigens

The study of *Plasmodia* proteins which may be important in mediating host immune responses has resulted in the identification and characterization of a considerable number of *Plasmodia* proteins, expressed during various stages of the parasite life cycle. The technique of constructing cDNA or genomic DNA libraries in expression vectors which can then be screened using antibodies from human immune serum or serum from immunized animals, has been widely used to identify clones corresponding to *Plasmodium* antigens (Kemp et al., 1983; Anders et al., 1984; Hall et al., 1984; Langsley et al., 1985). One of the most unusual findings has been the presence of extensive regions of tandemly repeated sequences within a large number of these proteins. Several recent reviews have been published which discuss these repeats and their potential role in host-parasite interactions (Miller et al., 1986; Anders, 1986; Mitchell, 1987). The figure below shows schematic diagrams of the structures of several of these repeat antigens (see Figure 5).

![Diagram of Plasmodia antigens](image)

**CIRCUMSPOROZOITE PROTEIN (CS PROTEIN)**

**S ANTIGEN**

**RING INFECTED ERYTHROCYTE SURFACE ANTIGEN (RESA)**

**FALCIPARUM INTERSPERSED REPEAT ANTIGEN (FIRA)**

**SMALL HISTIDINE AND ALANINE RICH PROTEIN (SHARP)**

Figure 5. Schematic diagram of some repetitive Plasmodia antigens. The repetitive regions are indicated by the shaded areas. (from Anders, 1986)
Some of these antigens, such as the CS protein, the major surface protein on sporozoites (Dame et al., 1984) and the S Antigen, a soluble extracellular, heat-stable protein released from asexual stages (Cowman et al., 1985), have one extensive block of relatively homogeneous repeats flanked by non-repeat sequences. Other proteins contain more than one block of tandem repeats such as RESA, the ring infected erythrocyte surface protein (Coppel et al., 1984), and FIRA, the falciparum interspersed repeat antigen (Stahl et al., 1985). In some cases the repeated blocks are identical and in other cases the sequence of the repeats differs from one defined block to another. When the sequences from similar proteins from different *Plasmodia* species or strains are compared there are often differences in the sequences and/or numbers of the repeats and in some cases the repeated sequences exhibit no homology, although other parts of the protein are very homologous. It also appears that several of the repetitive sequences encode epitopes which are cross-reactive, both to other repeats within the same molecule and to repeat units in other distinct proteins. The predominance of these repetitive antigens, implies that they have a functional importance to the parasites survival. A mechanism by which these repeated sequences could act to divert the host's immune response away from other antigenic targets which might be important for parasite survival has recently been proposed (Anders, 1986). Anders proposed that the repetitive sequences may act to overstimulate the host immune response resulting in the observed polyclonal B cell activation, this would be especially true for repeats which are present in large numbers increasing the number of reactive epitopes in each protein. The effect of these repetitive antigens would be to inhibit the maturation of an effective B cell response by blocking the normal progression of the clonal expansion of B cells and inhibiting the production of high-affinity antibody. This hypothesis seems to explain some of the observed responses of the host's immune system to malaria. The slow development of protective immunity and its poor persistence, could be due to blockage of the normal B cell maturation process and the continued hypergammaglobulinaemia could be due to the continued proliferation of a large number of low affinity antibody producing cells in response to the constant expression of repetitive sequences some of which cross-react.
5. Summary

Both the American trypanosomes and the African trypanosomes are evolutionarily closely related to *Leishmania*. The similarities between all of these *Trypanosomatid* parasites is seen in the basic underlying biology. The unusual trans-splicing mechanism of RNA maturation is the most intriguing of these shared biological characteristics and the discovery that the major surface glycoprotein of *Leishmania* is linked to the membrane via an unusual lipid linkage also seen in the VSG proteins of the African trypanosomes may indicate that this is another feature which is common to members of the order *Kinetoplastidia*.

There are many similarities in the life cycles of *Leishmania* and the American trypanosomes and the mechanisms these parasites use to invade the mammalian host and establish an infection, but there are also several differences. The life cycle of *Trypanosoma cruzi* is similar to that of *Leishmania*; however, *Trypanosoma cruzi* also has an extracellular blood-stage form, unlike *Leishmania*. The mode of transmission from the insect vector is also different, as *Leishmania* are transmitted in a manner similar to that of the other major *Trypanosoma* division, the *Salivaria*. There are few obvious similarities with respect to life cycle or host-parasite interactions of the African trypanosomes with either *Leishmania* or *Trypanosoma cruzi*. The African trypanosomes do not have an intracellular stage within the mammalian host and they have evolved the unique capacity to undergo antigenic variation, which is the major mechanism by which they evade the hosts immune system.

Although *Mycobacterium leprae* is a bacterial parasite and, therefore, completely unrelated to the *Trypanosomatids*, like the protozoans *Leishmania* and *Trypanosoma cruzi*, it primarily infects macrophages; however, the exact location of each parasite within the macrophage is quite different. *Leishmania* are able to exist within the fused phagolysosomes, *Trypanosoma cruzi* escape from the phagosome into the macrophage cytoplasm prior to fusion of the phagosome with the lysosomal compartment and current evidence indicates that *Mycobacterium leprae* stays within the phagosomal compartment but unlike *Leishmania* inhibits phagolysosomal fusion. The importance of activated macrophages as the major effector cells in the host immune response against these parasite infections is well established. T cell-mediated immunity is responsible for this macrophage activation in each case and the same effector
lymphokines and monokines appear to be involved. The exact mechanism(s) by which cell-mediated suppression occurs during infections by these parasites is still unknown, however, it has been proposed that alterations in prostaglandin synthesis within infected macrophages resulting in suppression of normal T cell responses may be involved in both *Leishmania* and *Trypanosoma cruzi* infections. The role of antibody in *Leishmania* and *Mycobacterium leprae* infections is unclear, although in both cases it has been proposed that the induction of antibody might play a role in the observed suppression of cell-mediated immunity. Antibody production appears to play a role in host protection against *Trypanosoma cruzi* infections, perhaps because of the ability of *Trypanosoma cruzi* to exist extracellularly within the blood, unlike *Leishmania* or *Mycobacterium leprae*.

*Plasmodia* species are representative of parasitic protozoans which are unrelated to the Trypanosomatids. *Plasmodia* have a more complex life cycle existing primarily within two different types of host cells, hepatocytes and red blood cells and an effective host response to malaria infection involves both cell-mediated and humoral immunity. *Plasmodia* appear to have evolved the unique feature of expressing proteins which contain repetitive sequences to divert the normal maturation of the host's immune response and evade elimination.
E. THESIS OBJECTIVES

The protozoan *Leishmania* is a relatively simple organism which has adapted to take advantage of a parasitic lifestyle. *Leishmania* has a genome of only $10^8$ base pairs and a lifecycle with only two different stages, the promastigote which is found in the insect vector and the amastigote which is found within the mammalian host. *Leishmania* establishes an infection in the major phagocytic cell of the mammalian host, the macrophage and, although perhaps initially *Leishmania* were simply taken up by non-specific phagocytosis, *Leishmania* have evolved mechanisms to enable uptake by macrophages through specific receptor-ligand interactions. The normal destruction of invading microbes by macrophages is completed within the acidic phagolysosomal compartment and unlike the majority of intra-macrophage parasites, *Leishmania* has not evolved mechanisms to inhibit this pattern of events. Instead *Leishmania* have adapted to survive within this acidic compartment.

All mammalian parasites must be able to evade the hosts immune system if they are to survive. This can be done by masking of the parasites' presence within a host cell and/or by suppressing or otherwise interfering with the host's immune responses. By residing within the macrophage, *Leishmania* are partially masked from the host's immune system; however, *Leishmania* also appear to actively suppress the host's immune response. The mechanisms by which this suppression is brought about are still unknown. In order to understand how parasites are able to establish infections within the mammalian host, it is necessary to identify the mechanisms which they have evolved to invade and survive within specific host cells or body compartments, to establish replication and to spread throughout the body. By investigating the basic biology of parasites, mechanisms which are common to all parasites and mechanisms unique to single species are discovered. Studies done to analyze the relationships between host and parasite also lead to valuable insights into the functioning of the host's immune system. Because of its relative simplicity, *Leishmania* is an ideal candidate for this type of analysis.

In this thesis, the identification of *Leishmania* molecules which may have important functions in the establishment and/or maintenance of *Leishmania* infections within the
mammalian host is reported. Using recently developed techniques of cloning and immunodetection, three Leishmania genes encoding proteins containing repetitive regions were identified and the possible evolution and function of these molecules in Leishmania will be discussed.
VIII. MATERIALS AND METHODS

A. MATERIALS

All chemicals used were analytical or reagent grade and were purchased from Sigma Chemical Company or British Drug House Limited. All restriction enzymes were purchased from P. L. Biochemicals Incorporated and were used under the reaction conditions described by the company. In addition, the enzymes T4 DNA Ligase, E. coli DNA Polymerase I (Klenow fragment), E. coli DNA Polymerase I (Kornberg enzyme), Dnase I, Mung Bean nuclease and Exonuclease III were also purchased from P. L. Biochemicals Incorporated. RNase A, lysosyme, BSA, Proteinase K, tRNA (type X), DTT, IPTG and iodoacetamide were purchased from Sigma Chemical Company and Eco RI methylase was from New England Biolabs.

Acrylamide, Bis-acrylamide and TEMED were from Bio-Rad. Ultra Pure electrophoresis grade agarose and RNA size standards were obtained from Bethesda Research Laboratories. Nitrocellulose filters (BA85) were from Schleicher and Schuell. The nylon membranes Hybond-N and Immobilon were from Amersham Corporation and Millipore, respectively. Biotin-labelled anti-rabbit antibodies and avidin-biotin-peroxidase complexes were purchased from Vector Laboratories Incorporated. \( ^{32}P \) dNTPs (3000 Ci/mmol) were from Amersham Corporation. Deoxy NTPs and dideoxy NTPs were obtained from P. L. Biochemicals Incorporated and M13 oligonucleotide primers were synthesized on an Applied Biosystems oligonucleotide synthesizer.

B. STRAINS AND MEDIA

1. Leishmania

*Leishmania major* NIH Seidman strain (Neva et al., 1979) was kindly supplied by Dr. N. Reiner (University of B.C., Vancouver, B.C.). The *Leishmania major* promastigotes were grown at 26 °C in tissue culture medium 199 (Gibco Laboratories) supplemented with 10% foetal bovine serum (Hyclone, Laboratories Inc.). *Leishmania donovani* strain 1-S, Cl2-D (Dwyer, 1977) was generously provided by Dr. D. Dwyer (NIH, Bethesda). The *Leishmania*
donovani promastigotes were grown at 26 °C in chemically defined RE-IX medium (Gibco Laboratories).

2. Bacteria

Escherichia coli (E. coli) strains Y1090(r−) and Y1089(r−) were the restriction minus strains used as hosts for λgt11 clones (Young and Davis, 1983a). The genotype of Y1090(r−) is ΔlacU169, proA+, Δlon, araD139, strA, supF, [trpC22::Tn10], hsdR−, hsdM+, (pMC9). The genotype of Y1089(r−) is ΔlacU169, proA+, Δlon, araD139, strA, hflA, [chr::Tn10], hsdR−, hsdM+, (pMC9). Both strains were maintained on LBMgAmp plates which consisted of LB medium [10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, NaCl 5 g/l, (pH 7.5)] and 1.5% agar (Difco Laboratories) supplemented with 10mM MgCl2 and 100 μg/ml ampicillin. The strains were grown in LBMg, LB medium supplemented with 10Mm MgCl2 at 37 °C with aeration.

E. coli strains LE392 and P2392 were used as host strains for λEMBL 3 clones. The genotype of LE392 is F−, hsdR514(r−,m−), supE44, supF58, Δ(lacIZY)6, galK2, galT22, metB1, trpR55, λ− (Murray et al., 1977). The genotype of P2392 is F−, hsdR514(r−,m−), supE44, supF58, lacYI or Δ(lacIZY)6, galK2, galT22, metB1, trpR55, λ−, (P2, cox3). These strains were grown in LBMg at 37 °C with aeration and were maintained on LB plates [LB medium and 1.5% agar].

E. coli JM83 [ara, Δ(lac proA,B), rspL, φ80, lacZΔM15 (r−,m−)] was used to propagate pUC plasmids (Viera and Messing, 1982; Yanisch-Perron et al., 1985). This strain was maintained and grown in LBMgT [LBMg supplemented with 50 mg/l thymidine]. E. coli JM101 [Δ(lac proA,B), supE, thi, (r−,m−)/F', traD36, proAB, lacIqZΔM15] (Messing, 1983) was used as a host for M13mp vectors (Yanisch-Perron et al., 1985). JM101 was maintained on plates made with M9-minimal salts medium [6 g/l Na2HPO4, 3 g/l KH2PO4, 1 g/l NH4Cl, 0.5 g/l NaCl, 1mM MgSO4, 0.1 mM CaCl2, 1 mM thiamine HCl, and 0.2% glucose] and 1.5% agar and was grown in either M9-minimal salts medium or YT medium [8 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, and 5 g/l NaCl, (pH7.2)] at 37 °C with good aeration.
3. Phage And Plasmids

_Eco RI_ digested and phosphatased λgt11 vector DNA (Young and Davis, 1983b) and _Bam HI/Eco RI_ digested λEMBL3 arms (Frischauf et al, 1983) were purchased from Vector Cloning Systems (now Stratagene Cloning Systems).

### C. STANDARD TECHNIQUES

Standard techniques were carried out essentially according to the protocols described by Maniatis (1982). Both large scale and mini-preparations of plasmid DNA were carried out by alkaline lysis procedures. λgt11 phage DNA preparations (large scale and mini-preparations) were carried out by the plate lysis procedure. All phenol extractions were carried out by the addition of an equal volume of phenol:chloroform(CHCl<sub>3</sub>):isoamylalcohol (25:24:1) (v/v/v) and all chloroform extractions were done with an equal volume of CHCl<sub>3</sub>:isoamylalcohol (24:1) (v/v). Ethanol (EtOH) precipitations were performed by the addition of a tenth volume 2.5 M NaAc and two volumes of ice-cold 95% EtOH to solutions containing DNA (unless otherwise noted), incubation of the mixture at 0 °C or -20 °C for between 5 min to over night and collection of the precipitated DNA by a 30 min high speed spin in either a microcentrifuge (15,000 g) or a centrifuge (20,000 g) depending on the volume. All precipitated DNA was washed at least once with 70% EtOH prior to lyophilization and resuspension in the appropriate solution. Non-denaturing agarose/EtBr gel electrophoresis of DNA samples was performed using between 0.5% to 1% agarose gels containing 1 μg/ml EtBr in 1 x TAE buffer [40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH8.0]. One tenth volume of Ficoll dye buffer [10% Ficoll, 0.04% bromophenol Blue in 1 x TAE] was added to samples prior to electrophoresis which was carried out in 1 x TAE at between 5 - 10 V/cm using a mini-gel apparatus (Hoeffer Scientific Instruments). DNA was visualized by short wave (254 nm) or long wave (300-360 nm) UV fluorescence. All DNA ligation reactions contained 50 mM Tris (pH 7.4), 10 mM MgCl<sub>2</sub>, 50 μg/ml BSA, 10 mM DTT, 1 mM ATP, and appropriate amounts of T4 DNA Ligase and DNA as noted.
D. LEISHMANIA GENOMIC DNA ISOLATION

High molecular weight genomic DNA was isolated from log phase *Leishmania* promastigotes essentially by the method of Blin and Stafford (1976). A 2.5 l culture of *Leishmania major* promastigotes was grown to early log phase (1.5 x 10^6/ml) and the cells were pelleted at 10,000 g, 5 min, 4 °C. The pelleted cells were washed twice in 50 ml PBS [140 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4] and the final pellet was gently resuspended in 3 ml of PBS. The cells were slowly added to a freshly prepared solution of 0.5 M EDTA, 0.5% N-lauryl sarcosine, 100 μg/ml Proteinase K which had been pre-heated to 50 °C. The lysed cell mixture was incubated at 50 °C overnight with occasional swirling and then extracted twice with phenol. The aqueous DNA solution usually formed the bottom layer. The DNA was extracted a final time against CHCl_3 and dialysed extensively against TE [10 mM Tris, 1 mM EDTA (pH 7.5)]. Contaminating RNA was removed by the addition of heat-treated RNase A to a final concentration of 100 μg/ml and incubation at 37 °C for 2 hr. The RNase-treated DNA was phenol extracted, CHCl_3 extracted and dialysed as described above. The concentration of the purified DNA was determined by A_260 measurements (Maniatis et al., 1982) of a small aliquot of the DNA which had been sheared and diluted.

E. λgt11 LEISHMANIA GENOMIC DNA LIBRARY CONSTRUCTION

A λgt11 *Leishmania major* genomic expression library was constructed as described (Huynh et al., 1984; Wallis and McMaster, 1987b). Two aliquots of *Leishmania major* genomic DNA were partially digested to produce random DNA fragments with an average size of 5 kb. Each 60 μg aliquot of genomic DNA was separately digested with a previously determined amount of either Hae III or Alu I. The reactions were stopped by the addition of EDTA to 20 mM and heating to 65 °C for 10 min. The two digested DNA aliquots were pooled and fractionated by size on a calibrated S-1000 Sephacryl column (470 mm x 8 mm). The DNA was allowed to pass down the column under gravity and 400 μl fractions were collected. Aliquots (10 μl) from fractions which were estimated to contain DNA fragments in the
appropriate size range, were analyzed by agarose gel electrophoresis and those fractions containing DNA within the 4 - 7 kb range were precipitated with EtOH.

The precipitated DNA (8 μg) was resuspended in dH₂O to a concentration of 100 μg/ml as determined by A₂₆₀ measurement. *Leishmania* DNA fragments were methylated at internal *Eco RI* sites by a 20 min incubation in the presence of 50 mM Tris (pH 7.5), 2 mM EDTA, 6 mM DTT, 0.21 mM S-adenosyl methionine and 2 U of *Eco RI* methylase. The reaction was stopped by heating to 60 °C for 10 min. Carrier tRNA was added to 200 μg/ml and the methylated DNA fragments were extracted with phenol. The aqueous DNA layer was transferred to a new tube, the phenol layer was washed with an equal volume of TE and the aqueous layers were pooled. The extracted DNA was precipitated with EtOH and resuspended in a 20 μl ligation mix containing a 100-fold molar excess (2 μg) of annealed *Eco RI* linkers (Bethesda Research Laboratories) and 5 U T4 DNA Ligase. The ligation reaction was incubated overnight at 10 °C. Concatamerized linkers were removed from the *Leishmania* DNA fragments by diluting the ligation reaction 5 fold in 1 x *Eco RI* reaction buffer, heating to 70 °C for 10 min, quick cooling on ice, adding BSA to 100 μg/ml and a 30-fold excess of *Eco RI* (250 U) and incubating the reaction at 37 °C for 6 hr. The reaction was stopped by the addition of EDTA to 20 mM and heating to 70 °C for 10 min. Free linkers were removed by a second size fractionation on the S-1000 Sephacryl column as described earlier. Aliquots (10 μl) were removed from each 400 μl fraction, the size of the DNA fragments contained within each fraction was determined by agarose gel electrophoresis and fractions containing DNA within the 4 - 7 kb size range were precipitated in the presence of an equimolar concentration of commercially prepared *Eco RI* digested and phosphorylated λgt11 arms. The precipitated DNA was resuspended in 10 μl ligation mixtures containing 2.5 U T4 DNA Ligase resulting in a final DNA concentration of 330 μg/ml. The ligation reactions were incubated overnight at 10 °C.

Aliquots of the ligated DNA (1 μg) were packaged using commercially prepared *in vitro* packaging extracts (Gigapak, from Stratagene Cloning Systems) according to the protocol provided by the supplier. For each packaging reaction, a freeze-thaw extract and a sonicated extract were thawed on ice. Ligated DNA was mixed with the freeze thaw extract on ice and
to this the sonicated extract was added. The final mixture was gently mixed to avoid the generation of air bubbles and incubated at room temperature (RT; 22 °C) for 2 hr. The packaged phage were diluted by the addition of 500 μl SM [100 mM NaCl, 10 mM MgSO₄, 50 mM Tris (pH 7.5), 0.01% gelatin] and 20 μl CHCl₃. The packaged phage were pooled and stored at 4 °C.

F. PLATING OF λGT11 PHAGE, INDUCTION OF β-GALACTOSIDASE PRODUCTION AND TRANSFER OF PROTEINS TO NITROCELLULOSE FILTERS

Y1090(r⁻) cells were grown to stationary phase in LB medium supplemented with 0.2% maltose and 10 mM MgCl₂. The cells were then pelleted at 1000 g for 10 min, resuspended in 0.5 volume of 10 mM MgCl₂ and stored at 4 °C for up to 5 days. Appropriate numbers of λgt11 wild type or recombinant phage were allowed to adsorb to MgCl₂-treated Y1090(r⁻) host cells (200 μl/82 mm plate and 600 μl/150 mm plate) for 15 min at 37 °C, after which LBMMg top agarose [LBMg medium plus 0.7% agarose; 4 ml/82 mm plate and 10 ml/150 mm plate] was added and the mixture was poured onto slightly dried LBMg plates (1 - 2 days old). The top agarose was allowed to harden at RT for 5 - 10 min and the plates were incubated at 42 °C until plaques appeared (approximately 3 - 4 hrs). For "phage dot blots", MgCl₂-treated Y1090(r⁻) host cells were plated as described above but without the addition of any phage. After the top agarose hardened, 1 μl aliquots of phage were applied to the plate. Once the "phage dots" had dried the plates were incubated at 42 °C until the plaques formed. To induce transcription from the β-galactosidase gene, the plaques were then covered with nitrocellulose filters which had been soaked in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and allowed to dry, the plates were transferred to 37 °C and growth was allowed to continue for a further 2 - 4 hours. The nitrocellulose filters were then carefully peeled off and rinsed with TBS [50 mM Tris (pH 7.5), 170 mM NaCl]. The filters were either used immediately or air-dried on filter paper and stored in the dark. Stored filters were re-wetted in TBS prior to subsequent use.
G. CLEARING OF ANTIBODY PROBES

All primary antibodies used to screen DNA expression libraries and to detect proteins on Western blots, were first depleted for reactivity against bacterial host proteins and wild type λgt11 proteins. Nitrocellulose filters were prepared from 150 mm plates onto which wild type λgt11 phage had been plated at a density of $10^6$ phage per plate, sufficient to give almost confluent lysis. Primary antibodies were diluted 1/100 into TBS + 1% BSA and were incubated for 60 min with a nitrocellulose filter onto which *E. coli* Y1090(r−) and wild type λgt11 proteins had been transferred. Sequential incubation with three separate filters was sufficient to deplete the antibody of any reactivity against components of wild type λgt11 or the *E. coli* host strain. Primary antibodies which had been cleared in this way were used as probes for screening the λgt11 expression library.

H. ANTIBODY SCREENING

The *Leishmania major* genomic library was screened using the protocols described by Young et al. (1983a,b,1985a), Wallis and McMaster (1987a). Throughout the screening procedure it was important that the filters were not allowed to dry out. Each filter was treated individually in a separate petri dish, 7.5 ml of each solution was used per 82 mm filter and 15 ml per 150 mm filter. The filters were blocked to prevent non-specific binding by incubation with gentle swirling (200 rpm) for 60 min in 3% gelatin in TBS. The filters were then incubated for 60 min with primary antibody which had been depleted of reactivity against wild type λgt11 lysate. The filters were washed three times for a minimum of 5 min each in TBS, TBS + 0.05% Triton-X100, and TBS. The bound antibodies were detected by incubating the filters for 60 min with biotin labelled horse anti-rabbit antibodies, freshly diluted 1/150 into TBS + 1% BSA to give a final concentration of 5 μg/ml. The filters were washed as described above and then incubated for 30 min in freshly diluted avidin-biotin-peroxidase complexes [1/400 into TBS + 1% BSA]. The filters were washed three times in TBS alone and the bound antibodies were then detected by the addition of a freshly prepared developing solution. For 100 ml of developing solution, 75 mg 4-chloro-1-napthol was dissolved in 16.5 ml ice-cold
MeOH and just prior to use this was combined with a solution of 82.5 ml TBS to which 50 μl of 30% H$_2$O$_2$ had been added. The developing solution was incubated with the filters for 2 - 15 min depending on the strength of the reaction and the filters were then rinsed with dH$_2$O and allowed to dry. Filters were stored dry in the dark and did not lose their colour.

I. AEMBL 3 LEISHMANIA GENOMIC DNA LIBRARY CONSTRUCTION

High molecular weight *Leishmania major* genomic DNA (600 μg) was partially digested with *Mbo I* at a final concentration of 0.3 U/μg to produce DNA fragments within the size range of 9 - 23 kb. Aliquots (200 μg) were removed at 20 min, 40 min and 60 min, the reaction was stopped by the addition of EDTA to 20 mM, heating to 70 °C for 10 min and followed by EtOH precipitation. The digested DNA aliquots were resuspended in TE at a concentration of 660 μg/ml and pooled. The resuspended DNA fragments were heated to 68 °C for 10 min, quick-cooled on ice and layered onto a 38 ml sucrose gradient [10 - 40% sucrose in 1 M NaCl, 20 mM Tris (pH8.0), 5 mM EDTA prepared in a 25 mm x 90 mm polyallomer tube (Beckman)]. The DNA was size separated on this gradient by centrifugation in a SW27 rotor at 26,000 rpm at 20 °C for 24 hr (Maniatis et al. (1982)). Fractions (2.0 ml) were collected from the bottom of the gradient using a peristaltic pump (Pharmacia). The fractions were monitored by A$_{260}$ as they were collected, using a UV monitor (Pharmacia), and the fractions containing DNA were precipitated with EtOH. The fractionated DNA was resuspended in 40 μl TE to a concentration of approximately 100 μg/ml and the size of the DNA fragments in each fraction was determined by analysis of small (3 μl) aliquots of each fraction by agarose gel electrophoresis.

Commercially prepared *Bam HI/Eco RI* digested AEMBL 3 arms were mixed with the sized *Leishmania* DNA fragments at an equimolar ratio in a 6 μl ligation reaction containing 3 U T4 DNA Ligase. The reaction was incubated at 4 °C overnight and the ligated DNA was packaged as described above. The library contained 1.2 x 10^6 recombinants. The library was amplified by plating the recombinant phage at a density of 2 x 10^4 phage per 150 mm plate (as described below) and elution of the lysed phage into SM buffer [100 mM NaCl, 50 mM Tris
(pH 7.7), 10 mM MgSO$_4$]. The amplified phage library contained $2.7 \times 10^{11}$ recombinants and was stored in 0.3% CHC$_3$ at 4 °C in the dark.

J. PLATING OF $\lambda$EMBL 3 PHAGE

The protocol used to plate $\lambda$EMBL 3 phage was identical to that used to plate $\lambda$gt11 except that the host strains used were LE392 or P2392. $\lambda$EMBL 3 phage were incubated at 37 °C and the plaques took between 5 - 6 hr to appear.

K. PURIFICATION OF DNA USING GENECLEAN

1. Purification Of DNA From Solution

DNA was purified by binding to Glassmilk (Geneclean from BIO 101 Incorporated) according to the protocol given by the manufacturer. Solutions containing DNA were diluted by the addition of 2.5 volumes of a saturated NaI solution (provided by the company) and to this appropriate amounts of the Glassmilk suspension were added (5µl to solutions containing 5 µg or less and an additional 1 µl for every 0.5 µg of DNA above 5 µg). The mixture was then incubated on ice for 5 min to allow DNA to bind and bound DNA was pelleted by centrifugation in a microcentrifuge at 15,000 g for 10 - 20 sec. The pellet was resuspended and washed in 250 µl ice-cold NEW wash solution three times [NEW wash was a Tris/EDTA solution in 50% EtOH provided by the company]. Bound DNA was eluted by resuspending the washed, pelleted material in a small volume (5 - 50 µl) of dH$_2$O, heating the solution to 50 °C for 3 min and spinning at 15,000 g in a microcentrifuge for 30 sec. Purified DNA was recovered in the supernatant. This purification procedure routinely resulted in yields of 80 - 90% and DNA purified in this way could be used directly in enzyme reactions, hybridizations and ligations.

2. Purification Of DNA From Agarose Gels

DNA fragments fractionated by agarose gels electrophoresis were purified from the agarose using Geneclean. The position of the DNA fragment was determined by UV fluorescence and a slab of agarose containing the desired DNA fragment was excised from the
gel. The weight of the agarose slab was determined, 2.5 volumes of NaI solution was added and the mixture was incubated at 50 °C for 5 - 15 min to dissolve the agarose. The protocol described for purification of DNA from solution was followed after this point except that it was not necessary to add any more NaI solution.

L. NICK TRANSLATION OF DNA PROBES

All DNA fragments which were labelled by nick translation were purified from agarose gels by using Geneclean as described above. DNA probes which had been radioactively labelled by nick translation (Melgar and Goldwaith, 1968; Rigby et al., 1977; Maniatis et al., 1982) were used to isolate clones by plaque or colony hybridization and to identify hybridizing DNA fragments in Southern blot analysis or RNA transcripts on Northern blot analysis. A 25 μl reaction was used to label 300 ng of DNA. Each 25 μl nick translation reaction contained: 50 mM Tris (pH7.5), 5 mM MgCl₂, 1 mM DTT, 80 μg/ml BSA, 20 μM dGTP, 20 μM dTTP, 1 μM dATP, 1μM dCTP, 0.2 mM CaCl₂, 5 ng/ml DNase I, 35 μCi [α-32P] dATP, 35 μCi [α-32P] dCTP, 10 U DNA Polymerase I (Kornberg enzyme) and 300 ng DNA. The reaction was incubated for 60 min at 15 °C. After incubation, DNA probes were purified and unincorporated radioactivity was removed using Geneclean as described above. Labelled DNA probes prepared in this way had an average specific activity of 10⁸ cpm/μg. Probes were denatured by heating in a boiling water bath for 5 min and quick-cooling on ice for 5 min.

M. PLAQUE AND COLONY BLOTTING

1. Plaque Blotting

Two recombinant lambda libraries in the vectors λgt11 and λEMBL 3, were screened by plaque hybridization (Benton and Davies, 1977; Maniatis et al., 1982) under identical conditions. The phage were plated at progressively lower densities throughout the screening process, the density of plating used was 4 x 10⁴ plaques/150 mm plate for first screens, 10³ plaques/82 mm plate for second screens and 10² plaques/82 mm for third screens. The plaques were transferred to Hybond-N nylon membrane discs by carefully placing the membranes on
the agarose surface and allowing the phage to adsorb for 1 - 5 min. The membrane and agar plate were marked using a sterile needle to ensure correct orientation of the plaques. The membranes were removed and the phage were denatured by placing the membranes, phage side up, on a pad of Whatman 3MM paper soaked with 1.5 M NaCl, 0.5 M NaOH for 7 min and neutralized on Whatman 3MM soaked with 1.5 M NaCl, 0.5 M Tris (pH7.2), 1 mM EDTA for 3 min. The neutralization step was repeated with a fresh pad of soaked Whatman 3MM. The membranes were washed in 2 x SSC [300 mM NaCl, 30 mM Na3citrate] and transferred to filter paper to air dry, phage side up. The membranes were then wrapped in Saran Wrap and placed phage side down on a UV transilluminator for 2 - 5 min to covalently cross-link the DNA to the nylon membranes. The membranes were then hybridized according to the hybridization protocol described below.

2. Colony Blotting

A recombinant plasmid library in the vector pUC19 was screened by the high density colony hybridization method (Hanahan and Meselson, 1980; Maniatis et al., 1982). Colonies were plated at a density of 2 x 10^4 colonies per 150 mm LBAmp plate [LB + 1.5% agar supplemented with 100 μg/ml ampicillin] and following growth for 14 hr at 37 °C pre-wet and numbered Hybond-N membranes were placed on the agar surface and gently pressed down with a sterile spreader. Orientation holes were marked on the membranes and the plates. After 5 min the membranes were removed and placed colony side up on Whatman 3MM paper. Duplicate membranes were prepared by placing a second, numbered Hybond-N membrane on top of the original membrane and pressing the two membranes together between glass plates. Duplicate membranes were orientated with needle marks and the membranes were then carefully peeled apart and placed, colony side up, on fresh LBAmp plates. Both the original plates and the new plates with the Hybond-N membranes were incubated at 37 °C for 5 hr. Colonies which had been transferred to Hybond-N membranes were lysed and denatured as described for plaque blotting except that the membranes were washed with extra care after neutralization to ensure that all bacterial debris was completely removed. For second and third screens, colonies were plated at densities of 10^3 and 10^2 colonies per 82 mm plate, respectively,
and duplicate membranes were not made. The DNA was fixed to the membranes by UV cross-linking as described above and the membranes were hybridized under the conditions described below.

N. HYBRIDIZATION PROTOCOL

This protocol was used for plaque and colony hybridizations as well as for Southern and Northern hybridizations. Hybond-N membranes were pre-hybridized and hybridized in hybridization solution: 6 x SSC [0.9 M NaCl, 90 mM Na₃citrate], 5 x Denhardt's [0.1% (w/v) Ficoll, 0.1% (w/v) polyvinyl pyrrolidone, 0.1% (w/v) BSA], 0.5% SDS and 100 µg/ml carrier herring testes DNA (sheared and denatured). Pre-hybridizations were carried out for a minimum of 60 min using 0.2 ml of hybridization solution per cm² of membranes. Hybridizations were carried out for a minimum of 12 hr using the same volume of hybridization solution to which heat-denatured, radioactive, nick translated probes had been added at concentrations of 50 - 100 ng/ml. For both pre-hybridizations and hybridizations, membranes were incubated in sealed plastic bags at 68 °C, submerged in a dH₂O water bath. Following hybridizations, membranes were washed sequentially at 68 °C in large volumes of 2 x SSC for 15 min (repeated once), 2 x SSC, 0.1% SDS for 30 min and finally 0.1 x SSC [15 mM NaCl, 1.5 mM Na₃citrate] for 10 min. The membranes were then wrapped in Saran Wrap and exposed to either Kodak XAR-5 or XRP-1 film at 4 °C with or without an intensifying screen depending on the amount of radioactivity bound.

O. SOUTHERN ANALYSIS

Southern analysis (Southern, 1975; Smith and Summers, 1980; Maniatis et al., 1982) was carried out on DNA samples which had been digested with a variety of restriction enzymes and subjected to agarose gel electrophoresis. After electrophoresis the DNA fragments in the gel were nicked by submerging the gel in 0.25 M HCl for 15 min. The gel was then denatured and neutralized by sequential submersion for 30 min in the same solutions used for plaque and colony blotting. Southern blotting of DNA samples from agarose gels to Hybond-N membranes
was carried out by either unidirectional transfer, with a buffer reservoir of 20 x SSC [3 M NaCl, 0.3 M Na₃citrate], or bidirectional transfer, with no buffer reservoir. Transfers were allowed to proceed for a minimum of 12 hr. After blotting the membranes were washed in 2 x SSC, air-dried and DNA fragments were cross-linked by UV transillumination for 2 - 5 min. The membranes were hybridized, washed and set up with film by the hybridization protocol described above.

P. LEISHMANIA RNA PREPARATION

Total RNA was isolated from *Leishmania* promastigote and amastigote forms using cesium chloride gradients as described by Chirgwin et al. (1979). *Leishmania* amastigote RNA was provided by T. Ma (U.B.C., Vancouver, B.C.). *Leishmania* promastigotes from a 500 ml log phase culture (2 x 10⁷ cells/ml) were pelleted at 5,000 g at 4 °C for 5 min. The cells were washed twice in 40 ml 150 mM NaCl, 0.1% DEP. The final pellet was resuspended in 10 ml of a freshly made solution of 7.5 M Gn-HCl, 0.5% N-lauryl sarcosine, 10 mM DTT. In order to reduce the viscosity, the lysed cell-solution was passed five times through a 18G1/2 needle using a sterile syringe. Aliquots (2 - 2.4 ml) of the lysed cell-solution were layered on to 3.0 ml CsCl cushions [5.7 M CsCl, 25 mM Na citrate (pH 5)] prepared in 63 mm x 12.5 mm cellulose nitrate tubes (Beckman). Centrifugation was carried out in a SW50.1 rotor at 36,000 rpm for 20 hr at 20 °C. After centrifugation, the supernatant was carefully removed using a sterile Pasteur pipette and the transparent RNA pellet was resuspended in 300 μl of TE + 0.1% SDS. The RNA was extracted twice with an equal volume of phenol and once with CHCl₃. The organic layers were washed with TE + 0.1% SDS after each extraction so as to minimize RNA loss. RNA was precipitated from the aqueous phase by standard EtOH precipitation at -20 °C, resuspended in 800 μl 7.5 M Gn-HCl, 0.5% N-lauryl sarcosine, 10 mM DTT and precipitated by the addition of 0.5 volume (400 μl) of 95% EtOH followed by incubation at -20 °C. The RNA was pelleted a final time and resuspended in 50 μl DEP-treated dH₂O and the yield was estimated by A₂₆₀ measurement. The RNA was stored in aliquots at -70 °C.
Q. NORTHERN ANALYSIS

RNA samples were fractionated on 1% agarose - formaldehyde gels in 1 x MOPS buffer [50 mM morpholinopropanesulphonic acid (pH 7.0) (MOPS), 1 mM EDTA] (Lehrach et al., 1977; Maniatis et al., 1982). Following electrophoresis for 60 min at 30 V and 90 min at 100 V, the gel was stained in the dark in 5 μg/ml EtBr in dH₂O for 2 min and then destained in dH₂O in the dark for 2 - 3 hr or until ribosomal bands could be visualized by UV fluorescence. After this point the protocols used for Southern and Northern analysis are identical except the RNA was always transferred from the gel to Hybond-N membranes by unidirectional transfer.

R. SUBCLONING OF DNA FRAGMENTS

1. DNA Preparation And Ligation

DNA fragments from the originally identified phage or plasmid clones were subcloned into the plasmid pUC19 for restriction analysis or into the phage M13mp18 or M13mp19 for sequence analysis. DNA fragments to be subcloned were isolated from agarose gels using the Geneclean procedure described earlier. Plasmid DNA or phage M13 replicative form DNA were prepared for subcloning by digestion with the appropriate restriction enzyme(s) followed by phenol and chloroform extractions and EtOH precipitation. Plasmid intermolecular ligations were performed in 20 μl reaction volumes containing vector DNA at a final concentration of 10 μg/ml and a 3 - 5 -fold molar excess of insert DNA. M13 intermolecular ligations were performed in 20 μl reaction volumes containing 0.8 pmole/ml vector and 4 pmole/ml insert. T4 DNA Ligase (2.5 U/ 10 μl reaction) was added and the reactions were incubated at 10 °C overnight.

2. Transformations

Plasmid or M13 DNA was introduced into appropriate host bacterial cells that had been made competent by incubation in CaCl₂ at 4 °C (Dagert and Ehrlich, 1979). An overnight culture of bacteria was diluted 100-fold in culture medium (LB or YT) and incubated with
shaking at 37 °C until it reached an OD₆₆₀ of 0.2 - 0.3. The cells were collected by centrifugation at 1000 g for 10 min, resuspended gently in 0.5 volume of ice-cold 50 mM CaCl₂ and kept on ice for 30 min. The cells were then collected by a second centrifugation as described above, resuspended in 0.1 volume of ice-cold 50 mM CaCl₂ and either used immediately (JM101 cells for M13 transformations) or kept overnight at 0 °C (JM83 cells for pUC19 transformations).

For plasmid transformations, 200 µl of JM83 CaCl₂-treated cells were added to a 5 - 50 µl solution containing DNA and the mixture was incubated on ice for 20 min. The cells were then heat shocked at 37 °C for 5 min, 2 ml of LB medium was added and the cells were incubated, without shaking, at 37 °C for 60 min. Aliquots of the transformation mix (100 µl) were plated on LBAIX plates [LB plates supplemented with 100 µg/ml ampicillin, 2mM IPTG and 235 µg/ml X-gal] and incubated at 37 °C for 12 - 16 hr.

Transformations with M13 DNA were carried out by mixing 300 µl of freshly prepared CaCl₂-treated JM101 cells with 12.5 µl of a ligation reaction and incubating the mixture on ice for 40 min. The cells were then heat shocked at 42 °C for 2 min. A warmed (42 °C) mixture of 4 ml YT + 0.6% agarose supplemented with 2.5 mM IPTG, 250 µg/ml X-gal and 200 µl of exponential JM101 cells was added to the heat shocked cells and they were plated on YT plates and incubated at 37 °C for 8 - 16 hr.

S. DNA SEQUENCE ANALYSIS

1. Preparation Of M13 Deletion Clones

The 3.4 kb Eco RI insert from Clone 20 was isolated and ligated to Eco RI digested M13mp18 replicative form DNA as described (RF-20). Sets of deletion clones were prepared with Exonuclease III (Exo III) and Mung Bean nuclease as described by Henikoff (1984). RF-20 DNA (20 µg) was digested to completion with SphI to create linear DNA molecules with 4-bp 3' protrusions at each end which were protected from Exo III digestion. The digested DNA was EtOH precipitated, resuspended in Bam HI reaction buffer and digested to completion with Bam HI to produce RF-20 DNA with a 4-bp 5' protrusion at one end which was accessible to
Exo III digestion. The DNA was precipitated again and resuspended at a concentration of 100 μg/ml in 66 mM Tris (pH 8), 0.66 mM MgCl₂. An aliquot (10 μg) was incubated with 750 U Exo III at 37 °C. Aliquots (5 μl) were removed at 35 sec intervals, added to tubes containing 15 μl of a preheated (70 °C) solution of 200 mM NaCl, 5 mM EDTA (pH8) and incubated at 70 °C for 10 min. DNA was precipitated by the addition of 3 volumes of 95% EtOH and incubation at -20 °C for 2 hr. The precipitated DNA aliquots were resuspended in 100 μl 30 mM NaAc (pH 4.6), 50 mM NaCl, 1 mM ZnCl₂, 5% glycerol and 70 U Mung Bean nuclease/ml. The DNA was incubated at 37 °C for 30 min, brought to neutral pH by the addition of 6 μl 500 mM Tris (pH 8), 125 mM EDTA and phenol extracted. The phenol layers were rinsed with 30 μl TE and the pooled aqueous phases from each time point were precipitated with EtOH. Each DNA aliquot was resuspended in 20 μl dH₂O and 10 μl of DNA from each time point was self-ligated in a 50 μl ligation reaction containing 10 U of T4 DNA Ligase at 10 °C, overnight. Ligation mix aliquots (20 μl) were used to transform CaCl₂-treated JM101 cells as described above. The extent of deletion was determined by agarose gel electrophoresis of a 250 ng sample of DNA from each time point, taken prior to ligation, and comparison of average deletion clone length with the parental clone and M13mp18 wild type vector.

2. Preparation Of M13 Template DNA

Single-stranded M13 template DNA was isolated by infecting 1.2 ml of early log phase JM101 grown in 2 x YT with a single M13 plaque. The cells and phage were incubated at 37 °C with very vigorous shaking for 4 - 6 hr. Cells were pelleted by centrifugation at 15,000 g for 2 min, 1.0 ml of the phage supernatant was added to 250 μl 20% PEG, 2.5 M NaCl and the mixture was incubated at RT for 15 min. The PEG-precipitated phage were pelleted by centrifugation at 15,000 g for 10 min, the pellet was dried by inversion, the phage were resuspended in 100 μl TE, phenol extracted twice and template DNA was precipitated by the addition of 0.5 volume 7.5 M NH₄Ac and 2 volumes of EtOH. Purified template DNA was resuspended in 40 μl TE and stored at -20 °C.
3. DNA Sequencing Techniques

M13 single-stranded template DNA was sequenced by the dideoxy chain termination method described by Sanger et al. (1977) and Messing et al. (1983). Aliquots (5 µl, approximately 1 µg) of template DNA were annealed with 0.1 pmole M13 universal sequencing primer in 7 µl reactions containing 10 mM Tris (pH 8), 7 mM MgCl₂, by heating the reactions to 75 °C and allowing them to cool to RT. After annealing, 1 µl of 15 µM dATP and 1.5 µl [α³²P] dATP were added to the reactions. The annealed DNA/dATP mixes were then aliquoted (2 µl for A/T and 2.5 µl for G/C) into tubes labelled C, T, A or G which contained 1.5 µl of the appropriate dNTP/ddNTP termination mix, described below:

C : 0.005 mM dCTP, 0.1 mM ddCTP, 0.1 mM dGTP, 0.1 mM dTTP
T : 0.005 mM dTTP, 0.5 mM ddTTP, 0.1 mM dGTP, 0.1 mM dCTP
A : 0.1 mM ddATP, 0.075 mM dTP, 0.075 mM dGTP, 0.075 mM dCTP
G : 0.005 mM dGTP, 0.2 mM ddGTP, 0.1 mM dCTP, 0.1 mM dTTP

DNA Polymerase I (Klenow fragment) was diluted to 1 U/µl in 10 mM Tris (pH 8), 10 mM DTT, 100 µg/ml BSA and 1 µl (1 U) was added to each tube. The reactions were incubated at 30 °C for 15 min. At that time, 2 µl of a chase solution (250 µM dATP, 0.5 U/µl DNA Polymerase (Klenow fragment) was added to each tube and the reactions were incubated for a further 15 min at 30 °C. The reactions were stopped by the addition of 5 µl formamide-dye stop mix [98% deionized formamide, 10 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol] and placing them on ice. The DNA was denatured by heating the reactions to 95 °C for 3 min and quick-cooling on ice for 10 min. Sequencing gels were either 8% or 6% polyacrylamide, 8 M urea, 0.5 x TBE [0.5 x TBE = 45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, (pH 8)]. Gels were pre-run for 15 min at 35 W (constant power) in 0.5 x TBE. Aliquots (3 µl) from each reaction were loaded on to the pre-run gels and electrophoresis was carried out in 0.5 x TBE at 35 W (90 min for 8% gels and 120 min for 6% gels). Gels were dried on Whatman 3MM paper using a heated, vacuum gel dryer and were exposed to XRP-1 film for 12-16 hr at RT.
DNA sequencing gels were read using a digitizer from Science Accessories Corporation. Sequence data was stored and analyzed using the computer programs of Staden (1982) and Delaney (1982).

**T. LEISHMANIA TOTAL PROTEIN PREPARATION**

Total *Leishmania* promastigote proteins were prepared by pelleting and washing $10^8$ log phase (2 x $10^7$ cells/ml) promastigotes, followed by resuspension in 5% SDS and boiling for 10 min. The boiled cell mixture was reduced and alkylated by the addition of DTT to 50 mM, heating for 10 min at 90 °C, followed by the addition of iodoacetamide to 200 mM and incubation at RT for 40 min. The reduced and alkylated proteins were dialysed against a 500-fold volume of 0.1% SDS, to remove excess DTT and iodoacetamide, and aliquots were stored at -20 °C.

**U. PREPARATION OF PROTEIN LYSATES FROM INDUCED λGT11-RECOMBINANT LYSOGENS**

1. Lysogeny Of Y1089(r~) Host Cells

Y1089(r~) cells from an overnight culture grown in LBAmn medium supplemented with 0.2% maltose, were pelleted and resuspended in 0.5 volume of 10 mM MgCl$_2$. The density of cells was determined by absorbance at OD$_{600}$ and phage adsorbtions were set up using a multiplicity of infection of 5. The phage cell mixture was incubated at 37 °C for 20 min and then was diluted in LB medium to enable plating of approximately 200 cells/82 mm LBAmn plate. The plates were incubated at 32 °C overnight. Lysogenic bacterial colonies were identified by comparison of colony growth at 32 °C and 42 °C (lysogens are unable to grow at the higher temperature).

2. Protein Lysate Preparation

Protein lysates were prepared from lysogenic bacterial colonies as described by Young et al. (1983a). Cultures (200 ml) of the Y1089(r~) lysogens were grown at 32 °C with shaking to an absorbance of 0.5 at OD$_{550}$ and induced for phage production by temperature shift to 44 °C.
for 20 min. IPTG was then added to 10 mM, to induce β-galactosidase production, the temperature was reduced to 37 °C and incubation was continued at this temperature for 60 min. Cells were harvested by centrifugation at 37 °C, the pellet was resuspended in 0.01 volume (2 ml) TBS and quick-frozen in a dry ice/EtOH bath. The cells were lysed by warming the frozen mixture to 50 °C. The viscosity of the lysed cell mixture was reduced by incubation with 100 μg/ml DNase I and RNase A at 37 °C for 20 min followed by 10 - 20 min sonication in a high intensity bath sonicator (Laboratory Supplies Company Incorporated). The sonicated lysate was centrifuged at 15,000 g for 5 min and the supernatant was transferred to a new tube. The insoluble pelleted material was solubilized by resuspension in 0.001 volume (200 μl) TBS + 5% SDS and boiling for 10 min. The pelleted material was also reduced and alkylated as described for Leishmania total protein preparation. Both the supernatant and pelleted fractions were stored at -20 °C.

V. SDS-PAGE ANALYSIS

SDS-PAGE was carried out using standard techniques (Laemmli, 1970). Gels were 0.75 mm thick and were made up of a lower separating gel [7 - 10% acrylamide, 375 mM Tris (pH 8.8), 0.1% SDS] and an upper stacking gel [3% acrylamide, 125 mM Tris (ph 6.8), 0.1% SDS]. Protein samples were prepared for electrophoresis by the addition of an equal volume of dye buffer [250 mM Tris, 5% SDS, 15% glycerol, 1% DTT (freshly added), 0.1% bromophenol blue, (pH 6.8)] and boiling for 5 min. Electrophoresis was carried out at 15 mA for 60 min in a mini-protein gel apparatus (Hoeffer Scientific Instruments), using running buffer consisting of 25 mM Tris, 192 mM glycine, 0.1% SDS, (pH 8.3). After electrophoresis, the gel was either stained with Coomassie blue protein stain [0.3% Coomassie blue in 50% MeOH, 10% acetic acid] for 60 min and destained in 10% acetic acid for 60 - 90 min to visualize the protein bands or the proteins in the gel were electroblotted to nylon membranes by Western transfer for immunodetection, as described below.
1. Electroblotting Of Proteins From SDS-Polyacrylamide Gels

The electrophoretic transfer of proteins from polyacrylamide gels to membranes was carried out as described (Towbin et al., 1979; Gershoni and Palade, 1983; Renart and Sandoval, 1984). This technique has been termed "Western" blotting (Burnette, 1981). Protein samples were separated by SDS-PAGE as described above. After electrophoresis, gels were equilibrated for 15 - 30 min in electrophoretic transfer buffer [25 mM Tris, 192 mM glycine, 0.1% SDS, (pH 8.2) in 15% MeOH]. Immobilon nylon membranes were pre-wetted in 100% MeOH for 1 - 2 sec, rinsed in dH₂O and equilibrated in transfer buffer for 10 - 15 min. Once wetted it was important to keep the Immobilon membranes from drying out, if the membranes did become dry they had to be re-wetted and rinsed as described. A trans-blot apparatus (Hoeffer Scientific) was set up as described by the manufacturer and the proteins were electroblotted onto Immobilon membranes at 10 mA, overnight. After transfer, membranes were rinsed with TBS and either air-dried and stored, or used immediately for immunodetection. The gels could be stained with Coomassie blue at this point to ensure that protein transfer had progressed to completion.

2. Immunodetection Of Proteins Transferred To Nylon Membranes

Immunodetection of proteins transferred to Immobilon membranes was carried out according to the protocol given by the manufacturer. Membranes were re-wetted if necessary, as described above, and then treated as described for antibody screening except that the Immobilon membranes were blocked by incubation in 5% BSA in TBS at 37 °C for 60 min, all washes were done using 0.1% BSA in TBS for 5 min, three times and all antibody incubations were carried out for 2 hr.

3. Preparation Of Anti-Fusion Protein 20 Antibodies

Phage lysates were prepared from Clone 20 lysogens as described. The fusion protein from Clone 20 lysogens was contained in the solubilized, reduced and alkylated pelleted material and this was used to immunize a rabbit to produce anti-fusion protein 20 antiserum.
Rabbits were immunized with 3 bi-weekly injections of approximately 500 μg Clone 20 fusion protein preparation in complete Freund's adjuvant and bled 2 weeks following the last immunization.

4. Affinity Purification Of Anti-Fusion Protein 20 Antibodies

Antibodies were affinity purified by binding and elution from strips of membrane onto which fusion protein 20 had been blotted. Protein lysate from Clone 20 lysogens was separated by SDS-PAGE and electroblotted onto nylon membranes. The region of the membrane which contained fusion protein 20 was then excised and these strips were blocked using 3% gelatin in TBS. Anti-fusion protein 20 antibody was affinity purified by diluting the rabbit serum 1/100 in TBS + 1% BSA, incubating it with the pre-blocked membrane strips for 60 min, removing unbound antibody, washing the membrane strips sequentially with TBS, TBS + 0.05% T-X100, 50 mM Tris (pH 7.5), and TBS and eluting antibody from the membrane strips by incubation with 0.15 M NaCl, 50 mM diethylamine, 10 mM NaN₃, (pH 11.2), 1% BSA for 10 min with constant agitation. The eluted antibody was equilibrated to pH 7 with 1 M Tris (pH 7) and was further depleted of non-specific phage lysate reactivity by the addition of wild type λgt11 bacterial lysate. This affinity purified antibody was used to detect proteins on Western blots, as described above.
IX. RESULTS AND DISCUSSION

A. LEISHMANIA MAJOR GENOMIC DNA EXPRESSION LIBRARY CONSTRUCTION AND SCREENING

Many of the problems associated with studying the biochemistry and molecular biology of parasites, such as the inability of many parasites or certain life stages to be grown in vitro or the instability of many parasitic proteins, can be circumvented by the cloning and expression of parasite genes in the bacterium, \textit{Escherichia coli} (McIntyre et al., 1987). The introduction of techniques using antibody probes to identify DNA sequences which correspond to protein-encoding genes, has been a major advance in the field. It means that genes can be identified solely by virtue of the immunogenicity of the proteins they encode. The vector \(\lambda\text{gt}11\) has several useful properties which make it a good choice for the construction of recombinant DNA expression libraries (Young and Davis, 1983a). \(\lambda\text{gt}11\) was constructed to allow insertion of foreign DNA fragments of between 0.1 to 8 kilobases in length. The resulting recombinant phage can then be propagated in host cells as single-copy genomic inserts. This enhances their stability and allows repression of foreign genetic information which may be detrimental to host cell growth. The vector is inducible and responds by a rapid increase in copy number and high level transcription of the foreign DNA. Both the vector \(\lambda\text{gt}11\) and the host strains constructed for its propagation have features which minimize the degradation of the foreign protein produced. The single cloning site within \(\lambda\text{gt}11\) is situated in the 3' end of the \(\beta\)-galactosidase gene and, therefore, induction of this gene results in production of a \(\beta\)-galactosidase fusion protein with the foreign DNA-encoded moiety at the carboxy terminus. The production of such fusion proteins has been shown to result in enhanced stability (Young and Davis, 1983b). The host cells used to propagate \(\lambda\text{gt}11\)-recombinants have been engineered such that they are defective in protein degradation and this also contributes to the stability of foreign proteins produced. The stability of the recombinant proteins is necessary for the initial detection of appropriate positive clones and also for any subsequent isolation of the corresponding fusion proteins from these clones.
For higher eukaryotes with large genomes (>10^9 base pairs) and the presence of large intervening regions of noncoding DNA sequences (introns) separating regions of coding sequence (exons) within a single gene, expression library protocols have been based on the production of cDNA to be used as the inserted fragments. Because proper expression of foreign DNA in λgt11 depends on the orientation and reading frame of the inserted DNA with respect to those of the β-galactosidase gene, only one sixth of the recombinants will represent productive β-galactosidase fusion proteins which correspond to proteins expressed in the foreign organism. The necessity of using cDNA from organisms with larger and more complex genomes to construct expression libraries is obvious; however, there is also the disadvantage of unequal representation within the library, biased towards sequences from the more abundant mRNAs.

The smaller genomic size of many parasites and the apparent lack of introns within many of their genes has generated interest in the feasibility of construction of genomic DNA expression libraries from these organisms. McCutchan et al. (1984) produced Mung Bean nuclease-digested *Plasmodium* genomic DNA fragments to construct a λgt11 expression library. The *Plasmodium* genome is estimated to be approximately 10^8 base pairs and is estimated to have a dA.dT content greater than 80% (Corcoran et al., 1986). The ability of Mung Bean nuclease to produce random fragments of DNA is dependent to some extent on the sequence and conformation of the substrate DNA. Young et al. (1985a,b) used randomly-sheared genomic DNA from the bacteria *Mycobacterium tuberculosis* and *Mycobacterium leprae*, which have genomes of approximately 10^6 base pairs, to produce genomic DNA expression libraries in λgt11.

Although it is possible to grow the promastigote stage of *Leishmania* parasites *in vitro* and to obtain limited quantities of amastigotes from experimentally infected animals, molecular and biochemical studies on *Leishmania* proteins have been limited. The major reagents available to investigators studying parasites have been parasite-specific antibodies and the techniques of expression library construction has meant that molecular analysis of parasite proteins and their corresponding genes is now possible. Since an expression library based on genomic DNA fragments represents the total content of the parasite's repertoire of proteins, it
overcomes problems associated with stage specific expression which would be inherent in cDNA-derived libraries and yet the use of stage-specific antibodies allows detection of stage-specific proteins within the library if desired. The *Leishmania* genome is estimated to contain $10^8$ base pairs and there have been no introns reported in the few *Leishmania* genes which have been sequenced. The number of *Trypanosoma* genes which have been sequenced is more extensive and there have been no introns found to date in these organisms either.

In order to identify *Leishmania* proteins which may be immunologically relevant, a *Leishmania major* genomic DNA expression library was constructed using the vector λgt11. This library was then screened with polyclonal anti-*Leishmania major* membrane antibodies.

1. *Leishmania major* Genomic DNA Expression Library Construction

*Leishmania major* genomic DNA was prepared and random DNA fragments with an average size of 5 kilobases were generated by carrying out two separate partial digestion with either restriction enzyme *Alu I* or restriction enzyme *Hae III*, both of which recognize 4 base pair sequences and which generate blunt-ended fragments. The digested DNAs were pooled and fractionated by size on a S-1000 Sephacryl column and *Eco RI*-linkers were ligated to DNA fragments predominantly within the 4-7 kilobase range. Excess linkers were removed by *Eco RI* digestion followed by a second S-1000 column size fractionation. DNA fragments were precipitated in the presence of an equal molar concentration of commercially prepared *Eco RI* digested, phosphorylated λgt11 arms. The precipitated DNA was resuspended in a solution containing ligation reaction components to give a final DNA concentration of 330 ng/μl. Ligated DNA was packaged into phage particles *in vitro* using commercially prepared packaging extracts and the packaging efficiency was $2.5 \times 10^6$ packaged phage per μg of vector DNA. A total of 900 ng of genomic DNA inserts were used to produce a library which contained $24 \times 10^6$ plaque forming units and less than 1% of these were non-recombinant phage as assessed by the colour of plaques on IPTG/X-gal plates. The average size of the inserted DNA was 4 kilobases and the library, therefore, contained the equivalent of 150 *Leishmania major* genomes oriented in frame with the β-galactosidase *Eco RI* cloning site. The library was not amplified to avoid unequal amplification.
2. Production of Antibodies Raised Against Leishmania Major Membranes

The original anti-*Leishmania major* membrane antibodies used to screen the *Leishmania major* genomic DNA expression library were kindly provided by Dr. Terry Pearson of the University of Victoria. *Leishmania major* promastigotes were grown to log phase and harvested by low speed centrifugation. The promastigotes were washed, resuspended and sonicated. The sonicate was centrifuged at 12,000 x g for 5 minutes and the supernatant was then layered over a 30% sucrose cushion, centrifuged at 60,000 rpm and purified membranes were removed from the top of the cushion. From 4 litres of cells (50 X 10^9 promastigotes) 13.2 mg of crude membranes were isolated. Two rabbits were immunized with the crude membrane preparation. The rabbits were primed by subcutaneous and intramuscular injection of the membranes (250 µg) in complete Freund's adjuvant and boosted in a similar fashion after 5 weeks and 10 weeks with the same membrane preparation (250 µg) in incomplete Freund's adjuvant. At week 14 the rabbits were bled and 300 ml of polyclonal anti-*Leishmania major* promastigote membrane serum was recovered.

3. Screening of Leishmania major Genomic DNA Expression Library

The *Leishmania major* genomic library was screened with the anti-membrane antibody as described in the Materials and Methods. In the first screen a total of 10^6 phage were plated on 10 X 150 mm plates at a density of 10^5 phage per plate. From this first screen, approximately 100 positives were identified and picked. Forty of the first screen positives were rescreened at a lower density of approximately 1000 plaques per 82 mm plate. Eighteen of these initial positives were identified as positives in the second screen, however, it was apparent at this point that three of these positives stained much more darkly than the other positives. Figure 6a shows sections from second screen filters on to which plaques from a negative control clone (1), a moderately staining clone (2) and a darkly staining clone (3) had been transferred. A third screen was carried out on all eighteen second screen positives by plating at a density of 100 plaques per 82 mm plate and nine positives were identified; three very darkly staining, three moderately darkly staining and four very faintly staining. The six most darkly staining positives were plaque purified and the three very darkly staining positives
were further characterized. These were clones 15, 20 and 39. Phage dot blots of plaque purified phage from a negative control clone (1), a moderately staining clone (2) and a darkly staining clone (3) were carried out as described in the Materials and Methods and the results are shown in Figure 6b.

4. Initial Characterization Of Positive Clones

a) Preliminary restriction analysis of λgt11 clones

Phage DNA was prepared from the three positive λgt11 clones. Eco RI digestion of the recombinant DNA resulted in the release of the DNA insert. Analysis by agarose gel electrophoresis showed that Clones 15 and 20 contained inserts which were of the same size (3.5 kb) and the insert from Clone 39 was 2.0 kb.
b) Subcloning of inserts into pUC19 and restriction analysis

The cloned Eco RI inserts were isolated and subcloned into the plasmid pUC19 as described in Materials and Methods. Further restriction mapping indicated that the inserts from Clones 15 and 20 were identical, and only Clone 20 was analyzed further. As can be seen in Figure 7, within Clone 20 there were unique sites for the restriction enzymes Xho I, Cla I, Bgl II and Pvu II and within Clone 39 there were unique sites for the restriction enzymes Hind III and Bam HI. The orientation of the fragments with respect to the β-galactosidase gene of λgt11 is shown in Figure 7. The orientation was determined by restriction analysis of the two clones and wild type λgt11 and comparison of fragment lengths with respect to known restriction sites within the two cloned inserts.

(a)

Lac Z

--------- 3.45 kb

(b)

Lac Z

--------- 2.0 kb

Figure 7. Restriction Endonuclease Maps of Recombinant λgt11 Phage Clone 20 and Clone 39.
The transcription direction of the lacZ gene of β-galactosidase is shown by the arrow. The length of the inserts is given and the restriction endonuclease recognition sites are shown.
(a) Clone 20. (b) Clone 39.
B. CHARACTERIZATION OF CLONE 20

1. Sequence analysis of the insert from Clone 20

The \textit{Eco RI} insert from Clone 20 was subcloned into the vector M13 mp19 in both orientations and the DNA sequence of the first 200 base pairs from each end was determined as described in the Materials and Methods. The DNA sequence from the end of the insert adjacent to the 5' end of the \(\beta\)-galactosidase gene in the original \(\lambda\)gt11 clone was particularly striking. A sequence of 42 base pairs was tandemly repeated throughout the 200 base pairs of DNA which was sequenced and the pattern of repeats could be seen to extend past the point at which the sequencing gel could be clearly read. Sequence analysis indicated that there was a recognition site for the restriction enzyme \textit{Pst I} within each of the repeats. Restriction analysis of the Clone 20 insert using the enzyme \textit{Pst I}, indicated that approximately 1 kb of the insert DNA contained this repeat sequence and, therefore, there were apparently 20 to 25 copies of this repeat within the clone. Further sequence analysis was carried out by preparing exonuclease III deletion clones as described in the Materials and Methods. A total of 14 different repeats were sequenced and the compiled data can be seen in Figure 8. Four separate M13 clones were sequenced. The first sequence (CLONE 1) represents the sequence derived from original Clone 20 insert and the sequence below it (CLONE 2) is from a time=0 deletion clone. The second group of repeats was sequenced from a deletion clone (CLONE 3) which initiated within the middle of the repeats and the third group of repeats was sequenced from a deletion clone (CLONE 4) which initiated within the repeats but extended out of the repetitive region. Within the repetitive sequence there was a run of 26 consecutive base pairs in which there was no variability at all. The bases which are conserved in all the repeats are marked with an asterisk above the first line of sequence data. The conserved \textit{Pst I} site is in the centre of this region and is found in every repeat which has been sequenced. The recognition sites for the enzymes \textit{Hae III} and \textit{Pst I} are underlined. The end of the repetitive region is shown at the bottom of the figure and the bases which share sequence identity when aligned with the repeats are marked by asterisks.
Four separate M13 clones were sequenced using the dideoxynucleotide chain terminating method (Sanger et al., 1977; Messing et al., 1983). The recognition sequences for the restriction endonucleases \textit{Hae III} and \textit{Pst I} are underlined and the nucleotides which are conserved in every repeat are marked with an asterisk above the top line of the sequence. Nucleotides which do match the consensus repeat sequence in the 3' non-repeat region are marked by an asterisk under the last line of the sequence. CLONE 1 is the insert from \textit{Agt11} Clone 20. CLONES 2, 3 and 4 are Exo III nuclease deletion clones prepared as described by Henikoff (1984).
Sequence analysis of deletion clones which initiated further from the cloning site, enabled the determination of the end of the repeats and the start of a non-repetitive 3' coding sequence. The non-repeat sequence extended another 177 base pairs and terminated in a TAG stop codon allowing the reading frame and predicted amino acid sequence to be determined. In Figure 9, a restriction map of the Clone 20 insert is shown (Figure 9a) along with the results of the sequence analysis of the deletion clones which extended 3' from the repeats (Figure 9b) and a hydrophobicity plot of the coding region (Figure 9c). The sequence has been aligned in Figure 9b so as to line up the last few repeats with respect to the start of the non-repeat 3' sequence which follows. The restriction sites \textit{Pvu II} and \textit{Sal I} are underlined and labelled. In the non-repetitive sequence which occurs 3' to the repeats there is a potential glycosylation site, Asn-Val-Thr (Spiro, 1973; Wagh and Bahl, 1981) and there is a carboxy terminal hydrophobic tail which can be seen in the hydrophobicity plot (Figure 9c).

The positions which vary at the DNA level are indicated by superscripted numbers in Figure 9b. All the positions which are variable, vary only with respect to one other nucleotide in each case, for example at variable position 1 only an A or a G are found. Variable positions 1 and 5 do not result in any substitutions at the amino acid level. Variation at position 2 results in either a G or a C at the third position in the codon and leads to either a Lys or an Asn residue at this position; a Lys residue was present in 5 out of the 13 repeats sequenced and a Asn residue was present in 8 of the 13 repeats. Both Lys and Asn are polar residues which are usually found on the surface of protein structures. The variable positions 3 and 4 both affect the same codon and result in either a Leu or a Val residue at this position; each occurs with approximately equal frequency (6 and 7 occurrences out of 13, respectively). The presence of Leu or Val at this position represents a very conservative change because both amino acids have very similar properties; both are hydrophobic amino acids that can be found either externally or internally, within the protein structure. Although there are 5 variable DNA positions, these result in variability at the amino acid level in only 2 adjacent amino acid positions (marked with an asterisk in Figure 9b) and neither change would be likely to disrupt any secondary structure which might be involved. Secondary structure analysis (Garnier et al., 1978) of the coding sequence from Clone 20 indicated that the repeated region was 100%
helical and the non-repetitive region was 65% extended chain, 18% reverse turn, 13% helical and 4% coil. A hydrophobicity plot (Kyte and Doolittle, 1982) of the sequenced repeats and the non-repetitive region is shown in Figure 9c. The repeats can clearly be seen to be hydrophilic, while the non-repetitive region is mixed.

The sequence and restriction analysis of this insert indicated that Clone 20 contained a 1.2 kilobase open reading frame adjacent to and in frame with the β-galactosidase gene of λgt11 as indicated in Figure 9a. This open reading frame contained 20 to 25 repeats followed by 177 bp of non-repeat sequence as shown in Figure 9b.
Figure 9. Restriction Endonuclease Map, DNA Sequence, Predicted Amino Acid Sequence and Hydrophobicity Plot of the Repetitive Region of Clone 20.

(a) Restriction endonuclease map of the insert of Clone 20. The restriction endonuclease recognition sites, insert length and direction of transcription of the insert from the Lac Z gene of λ-gt11 are shown. The repetitive region is shaded in, the non-repetitive 3' region is denoted by the stippled area and the position of the stop codon is denoted by the boxed TAG. The fragments which were isolated and used as probes in Northern and Southern analysis are also shown.

(b) DNA sequence and predicted amino acid sequence of the coding region from Clone 20. The repetitive regions are underlined, as are the recognition sites for the restriction endonucleases Pvu II and Sal I. The variable nucleotide positions within the repeat sequences are denoted by superscripted numbers and the variable amino acid positions are marked with asterisks above the first line of the sequence. A putative N-glycosylation site is marked (■).

(c) Hydrophobicity plot of the coding region of Clone 20 (Kyte and Doolittle, 1982).
2. RNA Analysis

In order to formally establish that the DNA present in Clone 20 encoded a transcribed *Leishmania* gene, Northern blot analysis was carried out by separation of total promastigote RNA by agarose gel electrophoresis followed by transfer to nylon membranes and hybridizing with radioactively labelled DNA fragments as described in the Materials and Methods. A 1.25 kb *Eco RI/Sal I* fragment was isolated from the original insert. This fragment extended from the cloning site through the repetitive coding sequence and into the untranslated region beyond the stop codon (Probe A in Figure 9). This probe was then used to identify the corresponding *Leishmania* RNA transcript.

![Figure 10. Northern Analysis of Leishmania major and Leishmania donovani RNA Hybridized with a Repetitive Probe from Clone 20.](image)

Total RNA from *Leishmania major* and *Leishmania donovani* promastigotes was fractionated on a 1% agarose–formaldehyde gel, transferred to a Hybond–N membrane and hybridized with Probe A which had been nick-translated to a specific activity of $10^8$ cpm/µg. Size standards were BRL RNA standards.

(a) *Leishmania major* RNA (20 µg). The film was exposed for 5 hr without intensifying screens.
(b) *Leishmania donovani* RNA (20 µg). The film was exposed for one week without intensifying screens.

As shown in Figure 10a total *Leishmania major* RNA hybridized with Probe A identified 2 transcripts of 9,500 and 5,200 nucleotides. To determine whether these transcripts were species-specific, total *Leishmania donovani* promastigote RNA was analyzed and the results are shown in Figure 10b. These results indicate that in the promastigote stage of both *Leishmania* species, *Leishmania major* and *Leishmania donovani*, there are 2 transcripts of 9,500 and 5,200 nucleotides expressed which correspond to the DNA contained within Clone 20. There was a large difference in intensities between the two autoradiograms shown in Figure 10; the autoradiogram of the *Leishmania donovani* RNA had to be exposed for a much longer period of time (one week) compared to the autoradiogram of the *Leishmania major* RNA (5 hr). This may represent differences in the level of expression or sequence identity between the
transcripts of the 2 species. The presence of two transcripts corresponding to Clone 20 insert DNA, may indicate the presence in the *Leishmania* genome of either 2 genes each with a transcript of a different size, or a single gene which produces a transcript which is post-transcriptionally modified. There is a slight difference in the intensities of the 2 bands in both species on Northern blot analysis, with the larger band being darker in each case. This might represent a difference in expression of transcripts from 2 genes or a difference in the post-transcriptional modification pathway of some transcripts from a single gene. Alternately, this difference in intensity might represent differences in the number of repeats within each transcript or the hybridization affinity of the 2 transcripts for the probe being used.

A 500 bp *Pvu II/Bgl II* DNA fragment was isolated from Clone 20 which contained approximately 100 base pairs of the 3' non-repetitive sequence and extended 3' through the stop codon for another 400 base pairs (see Figure 9, Probe B). In Figure 11 the results of hybridizing this non-repetitive probe to *Leishmania major* total promastigote RNA (Lane a) and *Leishmania donovani* total promastigote RNA (Lane b). The same 2 transcripts are once again identified using this probe; however, in this case the difference in intensity between the transcripts from a single strain is not so noticeable. Although these blots have been exposed for 2 weeks, the signal produced is much reduced due to the lack of a repetitive region within this probe. The similarity of intensities of the 2 bands when probed with a non-repeat probe, supports the idea that if there are 2 genes in the *Leishmania* genome which correspond to Clone 20, that the difference in intensity of the 2 RNA transcripts which is observed when a repetitive fragment is used as a hybridization probe is not due to differential expression and that differences in the sequence or number of the repeats contained in one gene compared to the other is a more likely explanation. There is also a significant difference in the signals produced from the 2 strains, again indicating a possible difference in the sequence identity or level of expression between the 2 strains with respect to these transcripts.
Figure 11. Northern Analysis of Leishmania major and Leishmania donovani RNA Hybridized with a Non-Repetitive Probe from Clone 20.
Total RNA from Leishmania major and Leishmania donovani promastigotes was fractionated on a 1% agarose-formaldehyde gel, transferred to a Hybond-N membrane and hybridized with Probe B which had been nick-translated to a specific activity of $10^8$ cpm/µg. Size standards were BRL RNA standards. The film was exposed for one week without intensifying screens.
(a) Leishmania major RNA (40 µg).
(b) Leishmania donovani RNA (40 µg).

In order to determine whether the expression of gene 20(s) was stage-specific, Leishmania donovani promastigote and amastigote RNA was analyzed on a Northern blot using Probe A (Fig. 9a) as a hybridization probe. The results can be seen in Figure 12. Although there is a large difference in the intensities of the bands in the two lanes, the same RNA transcripts which correspond to Clone 20 are expressed in both the promastigote (Lane a) and amastigote (Lane b) stages of Leishmania donovani.

Figure 12. Northern Analysis of Leishmania donovani promastigote and amastigote RNA Hybridized with a Repetitive Probe from Clone 20.
Total RNA from Leishmania donovani promastigotes and amastigotes was fractionated on a 1% agarose-formaldehyde gel, transferred to a Hybond-N membrane and hybridized with Probe A which had been nick-translated to a specific activity of $10^8$ cpm/µg. Size standards were RNA standards purchased from Bethesda Research Laboratories. The film was exposed for 10 days with intensifying screens.
(a) Leishmania major RNA (40 µg).
(b) Leishmania donovani RNA (40 µg).

Both of the transcripts which correspond to Clone 20 were expressed in both Leishmania major and Leishmania donovani promastigotes and in both promastigote and amastigote stages of Leishmania donovani. Therefore, the gene (or genes) corresponding to Clone 20, do not exhibit species- or stage-specific expression.
3. Genomic Analysis

Genomic DNA from *Leishmania major* was used in Southern blot analysis in an attempt to determine whether there were two Clone 20-like genes present in the *Leishmania* genome. *Leishmania major* genomic DNA was digested to completion with various restriction enzymes, separated by agarose gel electrophoresis, transferred to nylon membranes and hybridized with radioactively labelled DNA probes as described in the Materials and Methods.

Figure 13 shows the results from a typical Southern blot using *Leishmania major* genomic DNA hybridized with Probe A from Clone 20 (see Figure 9a). For each restriction digest there were two genomic DNA fragments which hybridized to this probe even though this probe did not contain sites for any of the restriction enzymes used. There is also a difference in the intensity of the bands on the Southern for each restriction digest. The two possible explanations for this are either the presence of two genes containing repeats in the *Leishmania* genome or the presence of a second group of repeats within one gene located 5' to the repeats identified in Clone 20. The difference in intensity may indicate a difference in the sequence of the repeats or in the number of repeats present within each region or gene.

**Figure 13.** Southern Hybridization of *Leishmania major* Genomic DNA with a Repetitive Probe from Clone 20.
Genomic DNA (7.5 μg) from *Leishmania major* was digested to completion with *Bam HI, Sal I*, and *Bgl II* alone or in combination (as indicated), fractionated on a 1% agarose gel, transferred to a Hybond-N membrane and hybridized with Probe A which had been nick-translated to a specific activity of $10^8$ cpm/μg. Size standards were *Hind III* digested λDNA fragments.
To further examine whether there were two genes corresponding to Clone 20 in *Leishmania major* and whether the results were the same in *Leishmania donovani*, both Probes A and B (see Figure 9a) were hybridized to Southern blots of *Leishmania major* and *Leishmania donovani* genomic DNA. The genomic blots in Figure 14a were hybridized with Probe A and the blots in Figure 14b were hybridized with Probe B. In each case both probes hybridized to identical genomic DNA fragments and the same light and dark fragment patterns are seen. The most likely explanation for the presence of two fragments in the Southern blots, therefore, is the presence of two separate genes in the *Leishmania* genome, differing with respect to the number of repeats within each gene or the sequence of those repeats. These two genes will be referred to as gene 20.1 (the gene corresponding to Clone 20 and the darker bands on genomic analysis) and gene 20.2 (corresponding to the lighter bands on genomic analysis).

When the two species are compared, the intensities of the bands detected using the repetitive Probe A (Figure 14a), are very similar in each digest. This indicates that the overall difference in intensity seen in RNA analysis of the transcripts from the two species probably reflects a difference in the levels of expression of these transcripts between *Leishmania major* and *Leishmania donovani*, rather than differences in sequence identity. Comparison of the bands detected using the non-repetitive Probe B (Figure 14b), demonstrates that this probe hybridizes strongly to the bands corresponding to gene 20.1 in *Leishmania major* but much less strongly to the bands corresponding to gene 20.2 in *Leishmania major* and to the bands corresponding to both genes in *Leishmania donovani*. Probe B was isolated from a clone corresponding to gene 20.1 (Clone 20) and, therefore, the sequence identity of this probe with gene 20.1 is 100%. The less intense hybridization patterns seen on hybridization of this probe to the other bands, indicates that the sequence identity is reduced and, therefore, the non-repetitive 3’ regions of these genes are not completely identical.
Figure 14. Southern Hybridization of Leishmania major and Leishmania donovani Genomic DNA with Repetitive and Non-Repetitive Probes from Clone 20.

Genomic DNA (7.5 µg) from Leishmania major and Leishmania donovani was digested to completion with Bam HI and/or Xho I (as indicated), fractionated on a 1% agarose gel, transferred to a Hybond-N membrane and hybridized with either Probe A or Probe B which had been nick-translated to a specific activity of $10^8$ cpm/µg. Size standards were Hind III digested λDNA fragments.

(a) Hybridized with Probe A.
(b) Hybridized with Probe B.
4. Isolation of Additional Clones Encoding Clone 20 Repeats

The DNA insert from Clone 20 represented the 3' end of the gene and the cleavage site which produced the fragment occurred within the repetitive region. It was, therefore, of interest to identify additional DNA clones which extended the sequence 5' from the repeats or ideally to identify a clone which contained sequences both 5' and 3' to the repeats. Also because of the evidence indicating that there were two genes corresponding to Clone 20 within the Leishmania genome, a clone corresponding to the second gene might be identified. A cDNA library, the original λgt11 library and a second genomic library constructed in λEMBL 3 were all screened with DNA probes from Clone 20.

a) Screening of a Leishmania major cDNA library

The entire insert fragment from Clone 20 was used as a hybridization probe to screen a Leishmania major cDNA library which was kindly supplied by Dr. L. Button. The library was constructed by insertion of cDNA which had been ligated to Bgl II adapters into the Bam HI site of the plasmid vector pUC19. The library was plated on 6 X 150 mm plates at a density of $10^5$ colonies per plate and duplicate filters were made of each plate. After amplification with chloramphenicol, the filters were probed with the 3.5 kb insert from Clone 20 which had been radioactively labelled by nick translation as described in the Materials and Methods.

A single cDNA clone, cl, which mapped to the 3' end of Clone 20, was isolated. A restriction map of this clone was determined and is shown in Figure 15a along with the overlap with Clone 20. The solid arrows in Figure 15a indicate the extent of sequencing of this area in Clone 20 and Clone cl. The sequence data are shown in Figure 15b. Clone cl corresponded to another gene unrelated to gene 20.1 which mapped to the 3' end of the original λgt11 Clone 20. The gene corresponding this cDNA (gene cl) initiates within the original λgt11 Clone 20, 200 bp from the 3' cloning site. Clone cl overlaps Clone 20 by 150 bp and extends 650 bp further in a 3' direction.
Figure 15. Restriction Endonuclease Map, DNA Sequence and Predicted Amino Acid Sequence of Clone 20 and the Overlapping cDNA Clone, c1.

(a) Restriction endonuclease map of the overlapping Clones 20 and c1.

The restriction endonuclease recognition sites, insert lengths of both clones and the fragment from Clone c1 which was used as a hybridization probe are shown. The repetitive region in Clone 20 is denoted by the shaded area. The area of overlap of the two clones is denoted by the stippled area. The region which was sequenced and is shown in Figure 15b is denoted by the heavy arrows. The putative start codon for gene c1 is boxed and the direction of transcription is shown by the dotted arrow.

(b) DNA sequence and predicted amino acid sequence of gene c1. The recognition sites for the restriction endonucleases *Pst I*, *Sal I* and *Hae III* are underlined, as is the putative *Trypanosomatid* trans-splicing recognition sequence. The start of Clone c1 and the end of Clone 20 are shown.

Analysis of the sequence 5’ to the ATG start codon of the c1 gene, indicated that it contains a region which corresponds to the recognition sequence for the addition of the *Trypanosomatid* spliced leader RNA. The recognition sequence for spliced leader addition is
located within 100 bp of the initiating Met and consists of a C + T rich region followed 1 to 11 nucleotides downstream by an acceptor A-G splice site (Tschudi et al., 1985; Van der Ploeg, 1986). In Figure 15b a sequence which corresponds to this recognition site is underlined. This region is 45 bp upstream from the initiator Met and consists of 9/11 C + T followed 2 and 5 nucleotides downstream by the dinucleotide AG. It is likely that the spliced leader RNA is added to the pre-mRNA at this point. RNA analysis using Clone c1 indicated that the single transcript from this gene was 1350 nucleotides in length (Figure 16). The sequence derived from Clone c1 was compared with sequences in the existing databases and there were no significant similarities.

Figure 16. Northern Analysis of Leishmania major RNA Hybridized with Probe C from Clone c1.
Total RNA from Leishmania major promastigotes (20 µg) was fractionated on a 1% agarose-formaldehyde gel, transferred to a Hybond-N membrane and hybridized with Probe C which had been nick-translated to a specific activity of 10^6 cpm/µg. Size standards were BRL RNA standards. The film was exposed with intensifying screens for ten days.

Figure 17 shows the results of genomic Southern analysis using the insert from Clone c1 (Probe C) as a hybridization probe. In Figure 17a the restriction enzymes used each cut within Probe C (except for Bam HI) and the fragment sizes correlated with those predicted from the restriction map and extended the map further 3' (see Figure 21a). In Figure 17b genomic DNA from Leishmania major and Leishmania donovani digested with enzymes which do not cut within Probe C were hybridized to this probe and the results indicate that in both Leishmania major and Leishmania donovani there was a single copy of this gene or if there were two copies that they were identical with respect to their restriction maps using the enzymes shown.

The blot used in Figure 17b was initially used for hybridization analysis using Probe A (see Figure 14a). The probe was then removed and the blot was reprobed with Probe C. Comparison of Figures 9a and 12b indicates that Probe C is hybridizing to bands which are
identical to the dark bands seen in Figure 14a, except for the *Leishmania major*, *Xho I* digests, which is expected as there is an *Xho I* site separating the two Probes. These results may mean that Probe C is only linked to gene 20.1, which corresponds to Clone 20 and contains the larger number of repeats.

![Southern Hybridization of Leishmania major and Leishmania donovani Genomic DNA with Probe C from Clone c1.](image)

(a) Genomic DNA (7.5 µg) from *Leishmania major* was digested to completion with *Pvu II*, *Pst I*, *Bam HI*, and *Sal I* alone or in combination (as indicated), fractionated on a 1% agarose gel, transferred to a Hybond-N membrane and hybridized with Probe C which had been nick-translated to a specific activity of $10^8$ cpm/µg. Size standards were *Eco RI/Bam HI* digested λDNA fragments.

(b) Genomic DNA (7.5 µg) from *Leishmania major* and *Leishmania donovani* was digested to completion with *Bam HI* and/or *Xho I* (as indicated), fractionated on a 1% agarose gel, transferred to a Hybond-N membrane and hybridized with Probe C which had been nick-translated to a specific activity of $10^8$ cpm/µg. Size standards were *Hind III* digested kDNA fragments.

b) Rescreening of λgt11 library

The *Leishmania major* genomic DNA library was plated on 6 X 150 mm plates at a density of 4 X $10^4$ plaques per plate. Once the plaques appeared, the DNA was transferred to nylon membranes and probed with radioactively labelled Probe A from Clone 20 as described in the Materials and Methods. From this first screen, 60 positives were identified and on second and third screening 30 of the plaques were isolated. Miniprep DNA isolated from these phage was digested with the restriction enzymes *Eco RI* and *Sal I* and analyzed by Southern
hybridization using Probe A. Many of the phage isolated contained fragments which hybridized to the repetitive probe but which were smaller in length than the similar fragment from the original Clone 20. Presumably, these represented insert fragments which had been cut at \textit{Hae III} sites 3' to that used to produce Clone 20. Several other phage contained fragments with no \textit{Sal I} sites and, therefore, did not extend downstream of the repeats. The largest insert fragment from one of these phage (Clone 5B) was isolated and subcloned into a plasmid for further analysis.

The restriction map of Clone 5B is shown in Figure 18a. Southern analysis of this clone indicated that there were only a few repeats present at one end of the clone. Two fragments from this clone, Probe D and Probe E shown in Figure 18a, were isolated and the first few hundred base pairs of sequence from the \textit{Eco RI} cloning sites was determined (as shown by the arrows in Figure 18a). The sequence data is shown in Figure 18b. Probe D was a 250 bp \textit{Eco RI}/\textit{Bam HI} fragment which contained an open reading frame which read 5' to 3' from the \textit{Eco RI} site. The sequence of this region did not contain any of the 42 bp repeats characteristic of Clone 20. The 225 bp of sequence from Probe E which was determined indicated that this region contained at least three repeat sequences and these were almost identical to the repeats found in Clone 20. The sequence of Probe E shown in Figure 18b is 5' to 3' towards the \textit{Eco RI} cloning site and the repeats are lined up to correspond with the line-up used to display the repeats from Clone 20, which were aligned according to the end of the repeats. The line-up of the repeats indicates that they must either end half way through a repeat unit in Clone 20 or start half way through a repeat in Clone 5B. The sequence corresponding to the \textit{Pvu II} site nearest to the 3' end of Clone 5B and the typical \textit{Pst I} and \textit{Hae III} recognition sequences found within the repeats are underlined. The two nucleotides which do not conform to the repeat consensus sequence are marked by an asterisk and in each case these changes result in a change to the predicted amino acid sequence at these sites. In both cases the amino acid is changed to the small neutral amino acid Ala and, therefore, probably does not result in any disruption of the secondary structure of the repeats.
Figure 18. Restriction Endonuclease Map, DNA Sequence and Predicted Amino Acid Sequence of the ends of Clone 5B.

(a) Restriction endonuclease map of Clone 5B. The restriction endonuclease recognition sites, insert length and direction of transcription of the insert from Clone 5B are shown. The repetitive region is denoted by the shaded area and the fragments which were isolated and used as probes in Northern and Southern analysis are also shown.

(b) The DNA sequence and predicted amino acid sequence of Probe D from Clone 5B. The extent and direction of sequencing is shown by the arrow. The sequence of Probe D is shown 5' to 3' reading towards the internal Bam HI. The recognition site for the restriction endonuclease Bam HI is underlined.

(c) The DNA sequence and predicted amino acid sequence of Probe E from Clone 5B. The extent and direction of sequencing is shown by the arrow. The sequence of Probe E is shown 5' to 3' reading towards the end of the insert. This represented the only open reading frame and corresponded to the direction of transcription of the repetitive sequence obtained from Clone 20. The repeats are underlined and displayed to correspond with the format used for the repeats of Clone 20, which were aligned with respect to the end of the repeats. Recognition sites for the restriction endonucleases Pvu II, Pst I and Hae III are underlined and the two nucleotides which do not conform to the consensus repeat sequence are marked with an asterisk.
Genomic Southern analysis using these probes indicated that Probe D cross-hybridized with Probe B. Figure 19 shows the results of Southern analysis of *Leishmania major* genomic DNA hybridized with either Probe B from Clone 20 (Figure 19a) or with Probe D from Clone 5B (Figure 19b). The same bands which hybridize with Probe B hybridize to Probe D with the exception of the small *Sal I* fragment indicating that the region of cross-hybridization does not extend beyond the *Sal I* site in Probe B. The bands which cross-hybridize with Probe D (in Figure 19b) are less intense than the same bands hybridized with Probe B (in Figure 19a). There are also a series of additional bands in Figure 19b, which hybridize with more intensity to Probe D. Computer analysis of the sequences from Probe D and Probe B identified several regions which were similar, although there were no regions of perfect sequence identity. The regions of sequence similarity ranged from matches of 10/11 to 13/17 bp.

![Image](image-url)

**Figure 19. Southern Hybridization of Leishmania major Genomic DNA with the Non-Repetitive Probe B from Clone 20 and Probe D from Clone 5B.**

Genomic DNA (7.5 μg) from *Leishmania major* was digested to completion with *Bam HI* and/or *Xho I* and *Sal I* alone (as indicated), fractionated on a 1% agarose gel, transferred to a Hybond-N membrane and hybridized with either Probe B or Probe D which had been nick-translated to a specific activity of $10^8$ cpm/μg. Size standards were *Hind III* digested λDNA fragments.

(a) Hybridized with Probe B.

(b) Hybridized with Probe D.
c) Screening of a Leishmania major genomic DNA AEMBL 3 library

In order to isolate a genomic clone which extended through the repeats, duplicate filters from the *Leishmania major* genomic DNA AEMBL 3 library were screened using the 800 base pair insert fragment from Clone c1 (Probe C, see Figure 15) and the 300 base pair fragment, Probe D, from Clone 5B. Seven 150 mm plates containing $2.5 \times 10^4$ AEMBL 3 recombinant plaques were screened in duplicate as described in the Materials and Methods. Hybridization with Probe C identified 12 very weakly positive clones on the first screen and hybridization with Probe D identified 10 faint positives and 4 dark positives. None of the positives appeared to hybridize to both probes and second screening of the positives identified using Probe C were negative. The positives which hybridized with Probe D were screened a second time using the repetitive fragment from the original Clone 20, Probe A, and two positives were identified which were plaque purified.

Large scale DNA preparations were carried out on these two positives, AEMBL#1 and AEMBL#2, and the DNA was analyzed by Southern hybridization. Both of these clones corresponded to the *Leishmania* gene 20.2 (corresponding to the lighter bands on genomic DNA analysis). Figure 20 shows Southern analysis of AEMBL#1 DNA hybridized with either Probe A (Figure 20a) or Probe B (Figure 20b). Identical bands are identified in each lane using these two probes with additional bands also present in all the lanes which contain *Sal I* digests in Figure 20b. The restriction map derived from Southern analysis of this clone and genomic analysis corresponding to gene 20.2 is shown in Figure 21b below.
Figure 20. Southern Hybridization of λEMBL#1 DNA with Repetitive and Non-Repetitive Probes from Clone 20.

λEMBL#1 DNA (5 μg) was digested to completion with Bam HI, Xho I, Sal I, and Bgl II alone or in combination (as indicated), fractionated on a 1% agarose gel, transferred to Hybond-N membranes and hybridized with either Probe A or Probe B which had been nick-translated to a specific activity of 10^8 cpm/μg. Size standards were Hind III digested λ DNA fragments.
(a) Hybridized with Probe A.
(b) Hybridized with Probe B.
5. Restriction Map Of Clone 20 Genes

The data compiled from Southern hybridization analysis of the various recombinant clones containing Clone 20 repetitive regions in conjunction with genomic Southern analysis is shown in Figure 21. Figure 21a shows a genomic restriction map corresponding to the original Clone 20 (gene 20.1). The direction of transcription reads from left to right and was determined from the reading frame of Clone 20. Clone 20, Clone cl and the probes derived from them are shown and the area corresponding to the repeats is shaded in. The restriction fragment lengths used to determine the restriction map were calculated from the previously described Southern hybridizations of this region (Figures 8, 9, 12 and 14). The bracketed Xho I restriction site signifies that this site appears to cut only when the DNA is digested with the restriction enzyme Xho I in conjunction with a second restriction enzyme which cuts nearby. The secondary structure of this region may not allow access to the Xho I restriction site unless this region has been made accessible by previous digestion with an enzyme which cuts nearby. The only time this Xho I site was seen was when a Xho I/Bgl II double digestion was carried out. The sites for these two enzymes are quite close together (only 300 bp apart).

Figure 21b shows the restriction map of the Clone 20 gene which contains fewer repeats (gene 20.2) and corresponds to Clone 5B and the two λEMBL3 clones. The direction of transcription is from right to left. This correlates with the direction of transcription of Clone 20 with respect to the open reading frame within the repeats. Clone 5B, Clone λEMBL#1, Clone λEMBL#2 and the probes derived from them are shown and the area corresponding to the repeats is shaded in. The restriction fragment lengths used to determine the restriction map were calculated from the previously described Southern hybridizations of this region (Figures 8, 9, 14 and 15).
(a) GENE 20.1

Figure 21. Composite Restriction Maps for Gene 20.1 and Gene 20.2.
The restriction maps were compiled from Southern hybridization analysis of the various recombinant clones in conjunction with genomic Southern analysis (see Figures 8, 9, 12, 14 and 15). The direction of transcription in each Figure is from left to right and was determined for gene 20.1 by analysis of the direction of transcription of the insert from the original λgt11 clone, Clone 20 and subsequent sequence analysis of the 3' coding region of gene 20.1. The presumed direction of transcription of gene 20.2 is from left to right, based on sequence analysis of the repeats from gene 20.2 and their orientation within the insert from Clone 5B. The probes which were used for the Southern analysis are shown as are the various clones from which they are derived. The repetitive regions within Clones 20 and 5B are denoted by the shaded areas. The sites for the recognition sequences for the restriction endonucleases are shown and below the restriction map the fragments from each digest which correspond to the restriction map are shown. The conserved Sal I and Pvu II sites are indicated by asterisks.
(a) Gene 20.1. The bracketed Xho I site denotes a site which is only cut during double digests with Bgl II (see text).
(b) Gene 20.2.
Comparison of the restriction maps for the two genes indicates that the only similar restriction enzyme sites are the *Sal I* and *Pvu II* which are marked with an asterisk in Figure 21. The two sites are located in the non-repeated 3' coding region of gene 20.1 and the stop codon for gene 20.1 translation is located within this region. It can be seen that the region 3' to the *Sal I* site at the 3' end of both genes differ completely. Genomic *Leishmania major* DNA hybridized with Probe C (Clone c1) demonstrated that this probe only hybridized to the darker bands corresponding to gene 20.1 (Figure 17b) and there was no hybridization of this probe to Clone λEMBL#1 (data not shown). Although the sequence of the repeats in both genes is identical, gene 20.1 appears to contain a larger number of repeats and the regions 5' to the repeats in the two genes also differ substantially. The two genes appear to share sequence identity only within the repetitive region and the 3' non-repetitive coding region following it.
6. Protein Analysis

The antibody used to screen the λgt11 expression library was a polyclonal serum directed against *Leishmania* membranes and, therefore, was not useful in identifying the specific *Leishmania* protein(s) encoded by the genes corresponding to Clone 20. In order to produce a monospecific antisera which would identify the corresponding Clone 20 protein(s), lysogens of the λgt11 Clone 20 phage were prepared in the host Y1089(r-) as described in the Materials and Methods.

a) Analysis of the lysate from induced Clone 20 lysogens

Lysates prepared from Y1089 lysogens were analyzed by SDS-PAGE. Usually λgt11 β-galactosidase-fusion proteins can be isolated from the supernatants of lysate preparations; however, in some cases the fusion protein which is produced can be insoluble (Stanley, 1983) and, therefore, is present in the pellet. An example of SDS-PAGE analysis stained with Coomassie blue of wild type λgt11 lysate supernatants, both induced and uninduced, and the pelleted material and supernatant from induced Clone 20 lysates is shown in Figure 22a. In Lane 1 the supernatant from an IPTG-induced wild type λgt11 lysate is shown. In Lane 2 the supernatant from an uninduced wild type λgt11 lysate is shown. The effect of induction on the expression of the 116,000 mol wt β-galactosidase protein (marked with an arrow) on wild type λgt11 lysogens can clearly be seen. In Lane 3 the pelleted material from induced Clone 20 lysogens is shown and in Lane 4 the supernatant from an induced Clone 20 lysogen is shown. The β-galactosidase-fusion protein produced by induction of Clone 20 lysogens (fusion protein 20; fp20 in Figure 22) is a large protein of 220,000 mol wt which is present almost entirely in the pelleted material, indicating that it is insoluble. The wild type β-galactosidase which is produced in these cells is already one of the largest proteins in the cell and the large increase in size alone, caused by the addition of a further 1.2 kilobases of coding sequence, may be responsible for the insolubility of this fusion protein. The amino acid sequence of the additional protein may result in insolubility of the fusion protein; however, analysis of the hydropathy of the repetitive regions indicated that they were hydrophilic and, therefore, it would be expected that the repetitive region would be soluble. The predicted size of a protein
produced by fusion of another 1.2 kilobases of coding sequence onto the β-galactosidase gene is approximately 156,000 mol wt, however, as can be seen in Figure 22b, the size of fusion protein 20 (Lane 1) is clearly much larger than this. The standards in Figure 22b are in Lane 2 myosin (210,000 mol wt) and bovine serum albumin (68,000 mol wt), in Lane 3 β-galactosidase from an induced wild type λgt11 lysate (116,000 mol wt) and in Lane 4 non-reduced IgG (150,000 mol wt). By comparison with these standards, fusion protein 20 was estimated to have a mol wt of 220,000. It has previously been reported that the presence of repeats within a protein may cause it to run anomalously (usually as a larger protein) on SDS-PAGE (Dame et al., 1984; Cowman et al., 1985; Wellems and Howard, 1986), and it appears that this is the case for fusion protein 20. The problem of the insolubility of the Clone 20 fusion protein could not be overcome by the addition of the detergents Triton-X 100, sodium deoxycholate or SDS to 1%. The fusion protein required complete reduction, alkylation and boiling for 5 minutes in 5% SDS for solubilization.

Figure 22. SDS-PAGE Analysis of Fusion Protein 20.
Proteins were fractionated on 7% SDS-polyacrylamide gels and visualized by staining with Coomassie blue. (a) Lane 1 and 2 contain, respectively, the supernatants from IPTG-induced and uninduced wild type λgt11 lysates. Lanes 3 and 4 contain, respectively, the pelleted material and the supernatant from IPTG-induced Clone 20 lysates. (b) Lane 1 contains the pelleted material from an IPTG-induced Clone 20 lysate. Lane 2 contains the standards, myosin and bovine serum albumin. Lane 3 contains the supernatant from an IPTG-induced wild type λgt11 lysate. Lane 4 contains non-reduced IgG.
Western blot analysis of proteins separated by SDS-PAGE and transferred to nylon membranes resulted in a darkly staining high molecular weight smear and staining of smaller bands, presumably degradation products. Figure 23 shows a Western blot analysis of the pelleted material from an IPTG-induced lysate from Clone 20 (Lane 1) and the supernatant from an IPTG-induced lysate from wild type λgt11 (Lane 2) screened with depleted anti-\textit{Leishmania major} membrane antibodies. Although there are a number of bands detected by the antibody in Lane 1, an equivalent amount of fusion protein separated by SDS-PAGE and stained with Coomassie blue was a barely detectable single band. The bands with apparent mol wts lower than 220,000, presumably represent discrete fusion protein degradation products. The strength of reactivity to this protein present in the anti-\textit{Leishmania major} membrane antibodies is not surprising considering the reason that this clone was chosen for further analysis was based on its darkly staining properties in the initial screening procedure.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig23.png}
\caption{Western Analysis of Fusion Protein 20 using Anti-\textit{Leishmania major} Membrane Antibodies.}
Proteins from the pelleted material from an IPTG-induced Clone 20 lysate (lane 1) and the supernatant from an IPTG-induced wild type λgt11 lysate (Lane 2) were fractionated by 7% SDS-PAGE, transferred to an Immobilon nylon membrane and screened with anti-\textit{Leishmania major} membrane antibodies which had been depleted of reactivity to λgt11. The molecular weight size bar on the left was estimated from protein size standards which were also run on the gel.
\end{figure}

\textbf{b) Preparation of anti-fusion protein 20 rabbit antibodies}

The insoluble fraction from the Clone 20 lysate was reduced, alkylated and boiled in 5% SDS. A rabbit was immunized with this partially purified fusion protein 20 preparation in complete Freund's adjuvant and was boosted after two weeks and four weeks with the same preparation. The resulting antibodies were exhaustively cleared of reactivity to wild type λgt11 lysate proteins by binding to and elution from strips of nitrocellulose onto which fusion protein 20 had been blotted. They were further depleted by the addition of wild type λgt11 lysate.
c) Identification of Leishmania proteins corresponding to Clone 20

These affinity-purified anti-fusion protein 20 antibodies were used to identify the corresponding *Leishmania* protein(s). The results of a Western blot analysis of total *Leishmania major* proteins which were reduced and alkylated, is shown in Figure 24. Lanes 1 and 4 contain induced Clone 20 lysate, Lanes 2 and 5 contain induced wild type λgt11 lysate and Lanes 3 and 6 contain total *Leishmania major* proteins which have been reduced and alkylated. The anti-fusion protein 20 antibodies recognize a series of protein bands of apparent molecular weight 250,000 (Lane 3). The prior addition of Clone 20 lysate to the antibodies, completely blocked this reaction against *Leishmania* proteins (Lane 6) and greatly reduced the reaction with Clone 20 lysate (Lane 4). The prior addition of wild type λgt11 lysate had no effect (Lanes 1 and 3).

![Figure 24. Western Analysis of Total Leishmania major Proteins.](image)

Proteins from the pelleted material from an IPTG-induced Clone 20 lysate (Lanes 1 and 4), the supernatant from an IPTG-induced wild type λgt11 lysate (Lanes 2 and 5) and total *Leishmania major* proteins which had been reduced and alkylated (Lanes 3 and 6) were fractionated by 7% SDS–PAGE, transferred to an Immobilon nylon membrane and screened with anti-fusion protein 20 antibodies which had been depleted of reactivity to λgt11. The molecular weight size bar on the right was estimated from protein size standards which were also run on the gel. Lanes 1, 2 and 3 were screened with anti-fusion protein 20 antibodies which had been depleted of reactivity to λgt11. Lanes 3, 4 and 5 were screened with anti-fusion protein 20 antibodies which had been depleted of reactivity to λgt11 and which were incubated with the pelleted material from IPTG-induced Clone 20 lysates prior to use.
The recognition of the *Leishmania* proteins was greatly enhanced by the reduction and alkylation of the proteins prior to electrophoresis and transfer. This may be due to the fact that the antibodies were raised against reduced and alkylated fusion protein and may only recognize epitopes on similarly denatured Leishmania proteins.

The minimum length of an RNA transcript required to encode an unmodified protein of 250,000 mol wt is 7500 nucleotides. The smaller RNA transcript (5200 nucleotides) which hybridizes to Clone 20 DNA, could encode an initial translated protein product with a maximum size of 173,333 mol wt; however, because of the repeats, this protein may run anomalously on SDS-PAGE as has been demonstrated for fusion protein 20. Alternatively, if the initial translated protein product were modified by the addition of carbohydrate chains, the smaller transcript could produce a group of larger mature proteins of 250,000 apparent mol wt. The larger RNA transcript (9500 nucleotides) which hybridized with Clone 20 DNA might encode a protein larger than 250,000 mol wt which is then processed to produce a group of smaller proteins within the 250,000 mol wt range. It is also possible that the larger 9500 nucleotide transcript contains 2000 nucleotides of untranslated sequence. The transcribed gene (gene c1) is approximately 2 kb downstream of the stop codon for gene 20.1 and Northern analysis demonstrated that this gene produces an independent transcript of 1350 nucleotides. This indicates that the "extra" nucleotides which may be present on the 9500 nucleotide transcript are not due to this transcript encoding a polycistronic message containing sequences corresponding to the downstream gene, c1. The multiple bands detected on the Western blot probably represent slight differences in glycosylation or some other post-translational modification such as processing and, therefore, two different genes which produce two different transcripts could produce proteins with similar very large molecular weights. Northern analysis of the RNA transcripts corresponding to Clone 20 DNA indicated that it is unlikely that the smaller transcript is a processed form of the larger transcript. The band corresponding to the larger transcript identified by hybridization of total Leishmania major promastigote RNA with a repetitive probe from Clone 20 is more intense than the smaller transcript (Figure 10a). However, when the same hybridization analysis is carried out using a non-repetitive probe from Clone 20 the intensity of both bands is identical (Figure 11a).
and similar results using the two probes to identify DNA fragments corresponding to Clone 20 DNA on Southern analysis indicate that the difference between the two transcripts is due to a difference in the number of repeats between the two transcripts and not a difference in their level of transcription. If the smaller transcript was a processed form of the larger fragment it seems unlikely that there would be a difference in the number of repeats within the two transcripts. Therefore, it appears that both gene 20.1 and gene 20.2 are transcribed; however, it is possible that one of the transcripts is not translated to produce a protein product.

d) Attempted localization of Leishmania proteins corresponding to Clone 20

*Leishmania major* promastigotes were incubated with the affinity-purified anti-fusion protein 20 antibodies, washed, incubated with fluorescent-labelled second antibodies, washed and examined by fluorescence microscopy or analyzed in a fluorescence activated cell sorter. No significant surface labelling of the *Leishmania major* promastigotes could be detected. This may be because the antibody only reacts with protein that is completely denatured or that the methods used for detection were not sensitive enough. Alternatively it may be that the proteins encoded by gene 20(s) are not expressed on the surface. Although the antibodies used to identify Clone 20 were raised against *Leishmania major* membranes, this does not rule out the possibility that the *Leishmania* proteins which correspond to Clone 20 are located intracellularly or secreted.
C. CHARACTERIZATION OF CLONE 39

1. Sequence Analysis Of The Insert From Clone 39

The original insert from Agt1l Clone 39 was subcloned into the vector M13 mp19 and the DNA sequence of the first 130 base pairs from the end of the insert adjacent to the 5' end of the β-galactosidase gene was determined. In Figure 25, a restriction map of the 2.0 kb insert from Clone 39 is shown (Figure 25a) along with the sequence data (Figure 25b) and a hydrophobicity plot (Figure 25c). In Clone 39 there was also a sequence which was tandemly repeated throughout the region sequenced and which extended further, past the point at which the gel could be clearly read. As seen in Figure 25b, in Clone 39 this repeat sequence was a 30 base pair unit which was entirely different from the sequence found in Clone 20. Comparison of the sequence from the 4 repeat units which were sequenced indicated that there were 3 variable positions at the DNA level (denoted by superscripted numbers), however, from the predicted amino acid sequence it can be seen that these result in only one variable position at the protein level (denoted by an asterisk). Variable position 1 changes the third base in a codon and does not result in an amino acid change. Variable positions 2 and 3 are adjacent and affect the first and second codon positions resulting in an amino acid substitution at this site. The change from a Leu residue (hydrophobic, non-polar) to a Gln residue (neutral, polar) is not a very conservative change; however, it would probably not disrupt any secondary structure that the protein adopts. Since there were only 4 repeat units sequenced and since the extent of the repeats within the protein is unknown it is difficult to truly assess the relevance of any of these variations in sequence. Secondary structure analysis of the repeats indicated that they were 100% helical as are the repeats of Clone 20 and a hydrophobicity plot shown in Figure 25c indicates that the repeats are hydrophilic also as in Clone 20.
Figure 25. Restriction Endonuclease Map, DNA Sequence, Predicted Amino Acid Sequence and Hydrophobicity Plot of the Repetitive Region of Clone 39.

(a) Restriction endonuclease map of the insert of Clone 39. The restriction endonuclease recognition sites, insert length and direction of transcription of the insert from the Lac Z gene of λgt11 are shown. The repetitive region is denoted by the shaded area. The fragment which was used as a probe in Northern and Southern analysis is shown.

(b) DNA sequence and predicted amino acid sequence of the first few hundred base pairs of the insert from Clone 39. The repetitive regions are underlined. The variable nucleotide positions within the repeat sequences are denoted by superscripted numbers and the variable amino acid position is marked with asterisks above the first line of the sequence.

(c) Hydrophobicity plot of the repeat sequence of Clone 39.

2. RNA Analysis

Sequence analysis of the repeats from Clone 20 and Clone 39, showed that they were entirely different and Southern analysis of plasmids containing these inserts indicated that there was no cross-hybridization observed between the inserts from the two clones; however, it was
possible that the two inserts corresponded to different regions of the same gene. To determine whether Clone 39 corresponded to an expressed gene and whether Clones 20 and 39 encoded different proteins, the DNA insert from Clone 39 (Probe F, see Figure 25a) was used as a hybridization probe in Northern analysis of total promastigote RNA from *Leishmania major* and *Leishmania donovani*. In Figure 26a, a Northern analysis of *Leishmania major* promastigote RNA hybridized with Probe F is shown. This probe hybridized to a single RNA transcript of 7,500 nucleotides, indicating that Clone 20 and Clone 39 correspond to different *Leishmania* genes. In Figure 26b a comparison of *Leishmania major* (Lane 1) and *Leishmania donovani* (Lane 2) promastigote RNA is shown and, although the hybridization signal is much weaker in the *Leishmania donovani* lane (Lane 2), the insert from Clone 39 hybridized to a RNA transcript of the same size in both species. The differences in signal intensity may reflect differences in sequence between the genes from the different species or differences in the level of expression of this gene between the two species.

![Figure 26. Northern Analysis of Leishmania major and Leishmania donovani promastigote RNA Hybridized with a Repetitive Probe from Clone 39.](image)

3. Genomic Analysis

*Leishmania major* genomic DNA was used on Southern blot hybridizations using Probe F from Clone 39. The results from a typical Southern blot using Probe F are shown in Figure 27. The results indicate that there is a single gene, or possibly two identical genes, in the *Leishmania* genome which correspond to Clone 39. Genomic blots carried out with DNA digested with the restriction enzymes *Pvu II*, *Pst I*, *Sal I* and *Bam HI*, all resulted in high
molecular weight fragments (greater than 9 kilobases) which hybridized to Probe F. Although there is a Bam HI site within Probe F, this restriction site splits the probe into two fragments only one of which contains the repeats and at this exposure only fragments which hybridize to the repeats are visible.

4. Protein Analysis

Despite repeated attempts, no fusion protein could be identified from lysogenic cultures of Clone 39. It has been reported that certain fusion proteins are unstable in such lysogenic cultures (Stanley, 1983) and this is presumably the reason for the inability to identify the fusion protein encoded by this clone.

In an attempt to overcome this problem and to identify the Leishmania protein encoded by gene 39, a peptide was synthesized by R. Olafson (University of Victoria) which corresponded to the protein sequence of the repeat unit as shown in Figure 25b. This sequence was Glu-Ala-Glu-Glu-Ala-Ala-Arg-Leu-Gln-Ala. This peptide was coupled to BSA and used to immunize a rabbit. The resulting antibodies were tested for reactivity against the Clone 39 lysate and total Leishmania major proteins; however, no protein was identified using this antibody. By only using a single repeat "unit" as the immunizing peptide it is possible that the immunodominant epitope of the repeats was not correctly exposed. It would be better to use a longer peptide consisting of several repeat units and perhaps include adjacent non-repetitive sequence as well, to ensure that all available epitopes were exposed. The sequence from Clone
which was determined represented only a small portion of the insert and it is also possible that the 4 repeats which were sequenced were highly unrepresentative of the consensus repeat sequence.

D. SUMMARY

A *Leishmania major* genomic DNA library was constructed in the vector λgt11. Screening of this library with antibodies raised to *Leishmania major* promastigote membranes identified two recombinant DNA clones encoding repetitive sequences (Clone 20 and Clone 39). Clone 20 encoded a repetitive peptide of 14 amino acids and clone 39 encoded an unrelated repetitive peptide of 10 amino acids. Analysis of one of these clones, Clone 20, indicated that there were two RNA transcripts of 9500 and 5200 nucleotides expressed which corresponded to this clone in *Leishmania major* and *Leishmania donovani* and this expression was not stage-specific. The results of genomic DNA analysis and isolation of additional clones encoding Clone 20 sequences indicated that there were two genes which corresponded to Clone 20 in both *Leishmania major* and *Leishmania donovani* and that these genes differed from one another with respect to the number of repeats which they contained. Antibodies against the fusion protein produced by Clone 20 recognized a series of *Leishmania major* proteins of apparent mol wt 250,000. Analysis of Clone 39 indicated that there was a single RNA transcript of 7500 nucleotides expressed which corresponded to this clone in both *Leishmania major* and *Leishmania donovani* and that there was a single gene (or two identical genes) which encoded this transcript.
X. GENERAL DISCUSSION

This thesis describes the identification of *Leishmania* genes encoding proteins containing tandemly repetitive peptides. The function of these proteins in *Leishmania* is unknown; however, comparison of these *Leishmania* proteins with repetitive proteins from other organisms may indicate possible functional similarities or differences and give clues as to the selective pressures which may have resulted in the evolution of these molecules in the protozoan parasite *Leishmania*.

A. REPETITIVE PROTEINS IN HIGHER EUKARYOTES

There are a number of examples of genes from higher eukaryotes which contain tandem repeats within the coding regions. These include collagen genes (Yamada et al., 1980; Monson et al., 1982), proline-rich salivary proteins (Bennick et al., 1982; Kaufman et al., 1982; Zeimer et al., 1984) and wheat endosperm protein genes (Okita et al., 1985).

Collagen proteins are proline-rich, extracellular, structural proteins which contain large, repetitive triple helical regions. The collagen genes are part of a multigene family, all of which contain blocks of the repetitive sequence, Gly-X-Y. The original unit appears to be a 54 bp region which is repeated 19 times and in the collagen genes this block, or multimers of it, are found in separate, tandemly repeating exons. It is thought that the genes have derived from duplication and multiple unequal crossing over at non-coding regions between homologous collagen genes (Yamada et al., 1980); however, the presence of several multimers of the repeat exons indicates that retrotransposition resulting in the production of genes lacking intervening sequences in certain regions may also have played a role in the formation of this repetitive, multigene family (Monson et al., 1982).

Studies have shown that there are several families of proline rich proteins which are expressed in the saliva of humans, and rats (Kaufman et al., 1982; Zeimer et al., 1984). These molecules have a high affinity for calcium phosphate and, therefore, are thought to play a role in dental repair. Sequence analysis indicates that these molecules are comprised, in part, of highly conserved, proline-rich, tandemly repeating sequences of approximately 20 amino acids.
Comparisons of the sequences of similar genes from rat and human indicate that the repetitive regions have probably arisen by a process of duplication, recombination and divergence. In the rat there are six tandemly repeated regions while in two similar human proteins there are one and three repeats.

Wheat endosperm express a polymorphic family of repetitive storage proteins called the gliadins (Okita et al., 1985). There are estimated to be up to 40 different gliadin proteins which can be divided into two closely related families, the $\alpha$- and the $\beta$-gliadins, and a less homologous family, the $\gamma$-gliadins. The overall structure of the $\alpha$-/ $\beta$-gliadins and the $\gamma$-gliadin proteins and genes are very similar. In the proteins several domains contain repetitive regions but the majority of differences between the different families are localized to one domain. Although in all cases the repeats are rich in proline and glutamic acid residues, in both $\alpha$- and $\beta$-gliadins the tandem repeat in this domain consists of a highly conserved dodecamer, while in the $\gamma$-gliadins this sequence is not conserved and the repeats of different $\gamma$-gliadins differ with respect to sequence, length and number. Comparison of similar genes from barley indicates that these gene families have evolved from a common ancestor in the Triticeae by duplication and divergence caused by unequal crossing over during general recombination or by slippage and mispairing during replication/repair.

The repetitive sequences in proteins of higher eukaryotes function to provide structural and binding regions. The collagen genes, the genes for the proline-rich salivary proteins and the gliadin genes all form part of multigene families which appear to have evolved by a process of duplication from a single gene followed by divergence via gene conversion (Baltimore, 1981), retrotransposition (Deninger and Daniels, 1986), and/or unequal crossing over events (Smith, 1976). The initial evolution of a single repetitive region within a gene is still unclear, but may have occurred through mechanisms such as slippage or mispairing during replication and/or repair (Maresca et al., 1984).
B. REPETITIVE PROTEINS IN OTHER PARASITES

1. *Schistosoma mansoni*

*Schistosoma mansoni* are multicellular parasites which are transmitted to man by infected snails. Within humans, the schistosome parasites first reside within the bloodstream and then migrate to the liver where the male and female worms mate and produce large numbers of eggs. Bobek et al. (1986) reported the sequence of a cDNA which is specific for mature female schistosome worms. The predicted amino acid sequence from this cDNA contained a stretch of 13 amino acids which is tandemly repeated twice and several other smaller repeats that are rich in glycine. This protein showed a high degree of similarity with egg shell proteins from the silk moth and is proposed to play a role in egg shell development in the female worm. Genomic analysis indicated that there were 1-5 copies of this gene (or related genes) per haploid schistosome genome unlike the multigene families of egg shell proteins which are found in the silk moth.

2. *Toxoplasma gondii*

*Toxoplasma gondii* is an obligate, intracellular protozoan parasite. Studies using monoclonal antibodies have indicated that the major surface protein of this parasite (a protein with an apparent mol wt of 30,000; p30) contains two or more identical epitopes (Rodriguez et al., 1985; Santoro et al., 1987).

3. The Trypanosoma

Recently the identification of two trypanosome proteins encoding repetitive regions has been reported. Peterson et al. (1986) isolated a λgt11 clone which contained sequence corresponding to a major surface antigen of *Trypanosoma cruzi*. Sequence analysis of this clone indicated that it encoded a protein which contains a 9 amino acid sequence that is tandemly repeated five times and is flanked on either side by a degenerate repeat sequence. RNA analysis indicated that the insert hybridizes to a single transcript which is expressed only in the trypomastigote stage. Trypomastigotes are found both in the vector and within the mammalian host bloodstream and are the only stage able to directly penetrate host cells. The protein
encoded by this clone is thought to play a role in the interiorization of trypomastigotes in mammalian cells. The role of the repeats within this protein is unknown but it is possible that they may be involved in the attachment and uptake of trypomastigotes by host cells and/or they may have some other, as yet, unidentified role.

Roditi et al. (1987) identified a *Trypanosoma brucei* cDNA clone encoding a 131 amino acid protein containing the dipeptide repeat, Glu-Pro, tandemly repeated 22 times within the central portion of this protein. This protein is expressed only during the procyclic (insect) stage of the parasite. Sequence analysis indicated that the protein might be a surface protein and this has subsequently been shown to be the case (T. W. Pearson, personal communication). This protein is encoded by a single RNA transcript and genomic analysis of *Trypanosoma brucei* DNA from different isolates indicates that the genes encoding this polypeptide appear to be prone to rearrangement. The insect stage-specific expression of this protein in *Trypanosoma brucei* and its location on the cell surface may mean that this protein has a role in the survival of the parasite within the insect gut. It is possible that this protein could be involved in the attachment of the parasite to the microvilli of the insect gut wall.

Murphy et al. (1987) reported the presence of an unusual repeated element within the genome of *Trypanosoma brucei* and related African trypanosomes. This repeated element is approximately 5 kb in length and there are up to 400 copies per diploid genome. These trypanosome repeated sequences (TRS) are bordered by direct repeats of about 4 bp and are flanked by either half of a previously described trypanosome transposable element (Hasan et al., 1984). TRS are transcribed and the transcription is developmentally regulated. The open reading frame within each repeat encodes a 1650 amino acid polypeptide which shows similarity to reverse transcriptase. The structural and transcriptional characteristics of these elements suggest that they are transposable and are related to the retroposon family of repeats. These repeated elements have not been found in other *Kinetoplastidia* such as *Trypanosoma cruzi* or *Leishmania*, indicating that their presence and amplification may be a recent event. Murphy et al. proposed that these elements may play a role in the continuous rearrangement characteristic of the trypanosome genome, by aiding in the dispersion of genetic information.
The genomes of the American trypanosomes are known to exhibit a high degree of variability in DNA content (Dvorak, 1984). This is also true in the African trypanosomes and in these trypanosomes there also appears to be a large number of translocation events associated with the rearrangements resulting in antigenic variation (Van der Ploeg et al., 1984a/b). The plasticity of the trypanosome genome may be due to differences in the amount and type of repetitive DNA contained in different species and strains. The constant rearrangement and plasticity of the trypanosome genome appears to be functionally important to the survival of the parasite and it may be that the mechanisms which generate and maintain repetitive sequences within the genome are important for this and are, therefore, under positive selection pressure.

4. Plasmodia

Repetitive sequences are a characteristic feature of many Plasmodia proteins, as discussed in the Introduction. Antibodies from immune serum have been widely used to identify clones which encode Plasmodia proteins and this has no doubt biased the selection of clones to favour those which contain repetitive epitopes. However, the presence of repeats in almost all Plasmodia proteins so far identified, implies that there is a selective pressure acting on the parasite to produce proteins which contain repeats. Several groups have analyzed the genes encoding specific repetitive antigens from different Plasmodia species or strains in order to investigate the possible mechanisms which have led to the evolution of such a high number of repetitive proteins in this parasite. Galinski et al. (1987) compared the sequences of the genes for the CS (circumsporozoite) protein from six different strains of Plasmodium cynomolgi (a simian Plasmodia). The CS protein is the major surface protein of the sporozoite stage, it contains a central region of tandemly repeated amino acids and, within each protein, the repeats are highly conserved (Dame et al., 1984). The rate of divergence of the repeats among the different strains was higher than the rate of divergence for other coding and non-coding parts of the gene. The repeats differed from one another with respect to sequence, number and size. Galinski et al. concluded that evolution was acting to preserve the repetitiveness of the locus rather than any particular epitope-containing sequence. They proposed that the
maintenance and evolution of the repeats was the result of a mechanism acting directly on the DNA sequence to constrain the internal divergence of the repeats, thereby, promoting their rapid divergence between different strains or species. This mechanism would maintain the repeat sequence by eliminating mutations or by spreading them throughout the repeats. Similar mechanisms have been proposed to function to maintain other tandem repeats such as satellite DNA, which cannot be selected for at the protein level (Singer et al., 1982; Maresca et al., 1984). The *Plasmodia* proteins gp195 (the major surface antigen of the merozoite stage),FIRA (a surface glycoprotein of 300,000 mol wt which is found on all blood stages), RESA (the ring infected erythrocyte surface antigen) and the S antigen (a soluble, secreted 250,000 mol wt protein) all appear to exhibit a higher degree of similarity among the non-repetitive regions of their corresponding genes from different species or strains than among the various repeat sequences in each gene (Webber et al., 1986; Lyon et al., 1987; Stahl et al., 1985; Coppel et al., 1984; Cowman et al., 1985). Mechanisms such as unequal homologous recombination (Smith, 1976), generation of deletions (Efstratiadis et al., 1980), gene conversion (Baltimore, 1981) and multiple replication initiation (Roberts et al., 1983) have all been proposed to act to generate this diversity and yet to maintain uniformity amongst the repeats within a single protein or region of that protein. The level of sequence identity among similar genes from different strains or species of *Plasmodia* indicates that most of the different alleles appear to have evolved from a single ancestral gene but the lack of similarity among genes encoding different repeat molecules does not indicate that they have evolved from a single ancestral "repetitive" gene (Cowman et al., 1985).

A recent report by Wellems and Howard (1986) compared two homologous genes encoding distinct histidine-rich proteins (HRPs) from a cloned isolate of *Plasmodium falciparum*. Both proteins are expressed throughout the erythrocytic stages of the *Plasmodia* lifecycle and at least one of the proteins is released as a soluble protein from infected erythrocytes. Both genes are found in a single parasite and, therefore, do not represent variants of a single gene from different parasites. The sequence of the repeats in both genes although very similar, is different in a single nucleotide resulting in a change in the corresponding amino acid sequence of the repeats. This change is found in almost every repeat of one gene. In one
gene there is also a difference in the number of repeats. It appears that these two genes were produced by a process of duplication and divergence from a common ancestral gene. The function of these proteins is unknown, however, another histidine-rich protein produced by \textit{Plasmodia}, the cell surface Knob protein, is associated with cytoadherence of infected erythrocytes to endothelium.

Two recent reports have identified a repetitive antigen from \textit{Plasmodium falciparum} that is similar to the heat shock protein 70 (hsp 70) family. The initial report by Bianco et al. (1986) reported that this protein was an abundant, soluble cytoplasmic protein of 75,000 mol wt which showed a high degree of sequence identity with the \textit{Drosophila melanogaster} hsp 70, except for a 60 bp region encoding tandem repeats, which appeared to have been "inserted" into the gene. A second paper by Ardeshir et al. (1987) identified a similar molecule which they reported to be on the surface of the parasite. The location of this protein, therefore, appears to be questionable and whether it is located on the surface or whether it is a cytoplasmic protein which becomes adhered to the surface upon cell lysis is unknown. Southern analysis indicated that the gene encoding this protein appears to be part of a multigene family which is highly conserved among different \textit{Plasmodia} species. The site of the repeats in the molecule is such that their presence would not interfere with the proposed ATPase domain of hsp 70, however, the possible function of repeats in this protein is unknown.

Several recent studies have reported the occurrence of antigenic variation of at least some of the repetitive \textit{Plasmodia} surface proteins (gp195, S antigen, HRP (Knob) protein and a merozoite surface protein of 140,000 mol wt) during the course of infection of monkeys with cloned \textit{Plasmodium knowlesi} parasite lines (Handunnetti et al., 1987; Klotz et al., 1987). Early studies in which such antigenic variation was noted (McBride et al., 1982; David et al., 1985) were done with uncloned parasites and, therefore, it was thought that the occurrence of a new antigen type during secondary waves of infection was caused by the growth of minor variants to which the host immune system had not responded during the initial parasitemia. Later studies using cloned parasite lines have demonstrated that the malaria parasite can undergo antigenic variation and like the African trypanosomes the variant antigenic types appear to be expressed in a sequential order (Handunnetti et al., 1987).
The *Plasmodia* genome exhibits considerable polymorphism among different species or strains with respect to the sizes of homologous chromosomes (Kemp et al., 1985). Unlike the situation in the African trypanosomes, rearrangements of the *Plasmodia* genome do not appear to be associated with transposition of large segments of DNA from one chromosome to another, but rather seems to involve large scale deletions and/or amplifications of DNA (Corcoran et al., 1986). Chromosome polymorphisms are found in natural *Plasmodia* populations and in lines which have been cultured for many generations in the laboratory. Although there is evidence for the occasional loss of coding sequences the majority of the DNA which is deleted is presumed to consist of repetitive non-coding sequences (Corcoran et al., 1986), such as the 21 bp *falci* element described by Rao et al. (1986). The role the plasticity of the *Plasmodia* genome and the frequency with which karyotype changes accompany phenotypic changes are unknown; however, the large differences in size which can be observed between homologous chromosomes from different isolates may influence the genetic crossing which would occur between these karyotypically different parasites (Corcoran et al., 1986).

Although it has been assumed that the main effector arm of the host immune response to *Plasmodia* is mediated through antibody binding to parasites, thereby inhibiting their invasion of hepatocytes and erythrocytes, recent studies have demonstrated that cell-mediated immunity may, in fact, play a more important role in protection than was originally thought. An investigation of the role of cell-mediated immunity in one murine *Plasmodia* model, indicated that resolution of the infection was antibody-independent and mediated by T cells (Cavacini et al., 1986). Studies done to examine the effectiveness of subunit vaccines derived from the repetitive epitopes of the CS protein demonstrated that although there was a large humoral response by the vaccinated mice, the amount of protection was minimal (Egan et al., 1987). Vaccination of mice with attenuated sporozoites resulted a strong cell-mediated response in addition to a large humoral response and, in this case, the protection which was achieved was very good. There have also been two other studies investigating the efficacy of vaccination of synthetic peptides derived from *Plasmodium falciparum* antigens (CS protein, RESA and gp195). In one study using *Aotus trivirgatis* monkeys, some of the synthetic peptides used provided partial protection against subsequent challenge of live *Plasmodium falciparum*;
however, the peptide corresponding to the RESA-derived octapeptide repeat was not protective
(Patarroyo et al., 1987). In another study, a synthetic peptide corresponding to a trimer of the
CS repeat sequence, Asn-Ala-Asn-Pro, was used to vaccinate 35 human volunteers (Herrington
et al., 1987). In a second human vaccine trial, 15 volunteers were immunized with an
Escherichia coli-derived protein which consisted of 30 of the 4 amino acid repeat sequence
from the Plasmodium falciparum CS protein described above linked to 32 amino acids from the
tetracycline gene, read out of frame (Ballou et al., 1987). In both studies, there was no effect
of boosting and only one volunteer developed a high titre antibody response against the
immunized protein and only this volunteer was resistant to a subsequent challenge of
Plasmodium falciparum sporozoites. These studies indicate that although protection can be
achieved by vaccination and there is a correlation between high antibody titre and protection,
the synthetic or recombinant proteins which were used were not sufficiently immunogenic to
provide wide-scale protection in the population, probably due to a lack of T-cell stimulating
epitopes. Several recent studies have identified human T cell clones which are Plasmodium
falciparum-specific and MHC restricted (Chizzolini and Perrin, 1986; Good et al., 1987), and
these are being used to investigate the role of T cell-mediated responses in human malaria and
the ability of different Plasmodia antigens and epitopes to induce T cell responses. Although
the function of repetitive regions within Plasmodia proteins is unknown, it has been proposed
that they may play a role in interfering with the maturation of the hosts humoral immune
response to the malaria parasite (Anders, 1986). The recent studies which demonstrate that
cell-mediated immunity rather than humoral responses may be important in the development of
host protective responses during natural infections, supports the idea that the huge antibody
response which is observed may in fact not be involved in protection during infection and may
actually be detrimental to the host. The finding that certain repetitive sequences when used as
vaccines can, in a few cases, induce protective immunity mediated via antibodies, could be due
to the ability of the humoral immune response in these individuals to mature and develop high
affinity antibodies to a single repetitive element unimpeded by the interference of cross-
reacting epitopes.
C. POSSIBLE EVOLUTION OF REPETITIVE PROTEINS IN LEISHMANIA

It appears that there is a positive selection for genome plasticity in many protozoan parasites including *Leishmania* (Van der Ploeg et al., 1984c; Scholler et al, 1987). This plasticity may have resulted in special mechanisms to enhance the generation and maintenance of repeats within the parasite genome. It is possible that these repetitive regions may have at some point become integrated into coding regions where they did not interfere with the functional capacity of the encoded protein and were thus not selected against. The evidence for retrotransposon-like elements within the trypanosome genome (Hasan et al., 1984; Murphy et al., 1987) is perhaps one mechanism by which such an integration event could occur. The presence of a *Plasmodium* protein which closely resembles other eukaryotic hsp70 proteins, which has a repetitive region apparently inserted into a site which does not interfere with a proposed functional domain (Bianco et al., 1986; Ardeshr et al., 1987), might be an example of such an event. Alternatively, or perhaps in addition, mechanisms such as multiple replication initiation (Roberts et al., 1983) might generate repeats by acting directly on existing coding sequences resulting in amplification of limited regions of sequence. If there are special mechanisms for maintaining and generating repeat sequences within the genome at the DNA level (Maresca et al., 1984), these same mechanisms would still act on repeats within coding regions; however there would, in addition, be a further selection of these sequences phenotypically, at the protein level. The presence of repetitive regions within parasite proteins could be advantageous in amplifying ligand-receptor interactions such as attachment of the parasite to the gut wall within the insect or to specific cell types within the mammalian host. Repetitive regions which are able to interfere with the development of a functional immune response by the mammalian host would also be positively selected for. The duplication of a complete gene containing repetitive sequences would allow one gene to diverge quite rapidly from the other as the selective pressure acting to maintain the functional capacity of the second gene would be removed. The presence of two related HRP proteins within a single malaria parasite (Wellems and Howard, 1986) appears to have occurred through such a process of duplication and divergence. Such duplications and divergence could lead to the generation of a
completely different gene encoding a protein with a new function or could lead to the
generation of different related loci whose expression could be developmentally regulated. This
could be the mechanism which generated the limited antigenic variation found in *Plasmodia*
and the extensive antigenic variation observed in the African trypanosomes.

The genes encoding repetitive antigens of *Leishmania* which are described in this thesis
may have evolved by one of the mechanisms described above. Unlike related *Plasmodia*
repetitive proteins, the two genes corresponding to Clone 20, gene 20.1 and gene 20.2, share a
high degree of sequence identity at both the nucleotide and amino acid levels; however, the two
genes seem much less similar in the other regions of the gene (as determined by restriction
mapping). Other than two apparently conserved *Pvu II* and *Sal I* sites immediately downstream
of the repeats, the restriction maps of the two genes are very different. This lack of similarity
extends to the region downstream of gene 20.1 which contains a coding sequence corresponding
to the unrelated gene, gene cl. Gene cl is apparently absent from the downstream region of
gene 20.2 based on Southern analysis of genomic DNA from both *Leishmania major* and
*Leishmania donovani* and on analysis of λEMBL 3 clones which correspond to gene 20.2. The
regions upstream of the repeats in the two genes may share some sequence identity as Probe D,
isolated from the 5' region of Clone 5B, appeared to hybridize to two upstream bands in
genomic Southern analysis. However, this analysis was complicated by the cross-hybridization
of Probe D to non-repetitive downstream sequences present in both genes. It seems unlikely
that these two genes represent two polymorphic alleles and that *Leishmania* are heterozygous at
this locus, unless the two homologous chromosomes differ extensively in this region. RNA
analysis of the transcripts corresponding to the two gene 20s, indicated that the level of
expression of the two transcripts was very similar. If these two transcripts are encoded by two
separate genes which differ markedly it would be assumed that the two transcripts would be
translated into two distinct proteins. Although the anti-fusion 20 antibodies recognized a set of
approximately six high mol wt proteins, it was not obvious that there were two unrelated
proteins (or groups of proteins) being produced. This may mean that, although there is a large
difference in the size of the transcripts, differential translation of the two transcripts may
result in two very similar sized proteins or that post-translational modifications, such as
glycosylation, may result in the mature proteins having comparable sizes. In order to assess whether glycosylation is responsible for the multiple bands detected on Western analysis of total *Leishmania* proteins screened with the anti-fusion protein 20 antiserum, it will be necessary to deglycosylate total *Leishmania* proteins by tunicamycin or endoglycosylase treatment and repeat the Western blot screening. If the two genes do not represent polymorphic alleles, then it is possible that they were produced by duplication and divergence from one gene or that the repeats from one gene have inserted into a second unrelated gene, perhaps by transposition of the repeats into this gene or by unequal crossing-over or gene conversion via homologous regions at the C termini of the two proteins. In order to analyze further the relationship between these two genes, it will be necessary to determine the sequence of the regions flanking the repeats in both genes and to isolate probes upstream and downstream of the repeats which do not cross-react with other regions of the gene and which might hybridize with only one of the RNA transcripts. The presence of repeats in an unrelated *Leishmania* gene, gene 39, indicates that the two repetitive gene 20s are not unique in the *Leishmania* genome and that perhaps additional genes encoding repetitive peptides will be found in *Leishmania*. In order to analyze further the gene corresponding to Clone 39, it will be necessary to determine the sequence flanking the repeats and to isolate probes which do not contain repetitive regions.
D. POSSIBLE FUNCTION OF REPETITIVE PROTEINS IN LEISHMANIA

The presence of repetitive regions within several *Leishmania* genes is strikingly similar to the situation in many *Plasmodia* genes. Both organisms are protozoan parasites that have a predominantly intracellular location within the mammalian host, although the life cycle of the malaria parasite is considerably more complex. This similarity suggests that the repetitive regions may have similar functions in both parasites. In malaria, the repetitive regions of *Plasmodia* proteins are immunodominant, and it has been proposed that the large polyclonal B cell response characteristic of malaria infections, which includes both *Plasmodia*-specific and non-specific antibody, is not host protective due to interference by the repetitive regions with the normal maturation of the antibody response. In *Leishmania* infections, chronicity is usually accompanied by high levels of antibody both *Leishmania*-specific and non-specific, which are induced by parasite antigens but exhibit no host protective function and may, in fact, be a major cause of some of the clinical symptoms associated with leishmaniasis (Galvao-Castro et al., 1984; Carvalho et al., 1985; Bohme et al., 1986). In chronic infections the expression of *Leishmania* proteins which contain repetitive regions may play a role in this polyclonal stimulation. If this is true, then the two distinct protozoan parasites, *Leishmania* and *Plasmodia*, have evolved similar mechanisms for immune evasion.

Although many of the *Plasmodia* repetitive antigens are known to be surface proteins, the location of one recently reported repetitive protein, the HSP 70 like *Plasmodium falciparum* protein, has been reported to be both cytoplasmic and on the surface. The interaction of cytoplasmic and nuclear proteins with the immune system has been demonstrated, one primary example being the nuclear protein (NP) of the influenza virus (Townsend et al., 1984; 1985). Recently, a repetitive protein encoded by an Epstein-Barr virus was reported (Speck et al., 1986). This protein appears to be a nuclear protein and has been shown to react with human immune serum. Interestingly, in addition to the 66 amino acid sequence which is tandemly repeated seven times within this protein, there are also six repeats of the tripeptide which have been shown to be involved with the interaction of proteins with the cell surface receptor for fibronectin. The results from this study indicate that this protein may be located both within
the nucleus and be associated with the cytoplasmic membrane or secreted where it can interact with host cells, including cells of the immune system. Bohme et al. (1986) demonstrated that soluble *Leishmania* antigens secreted into the culture supernatant (EF) were mitogenic to B cells and caused the production of antibodies, some of which exhibited autoantibody specificity. A recent study by Scott et al. (1987) reported the induction of protective immunity against cutaneous leishmaniasis in a murine model using a soluble extract of non-membrane derived proteins from *Leishmania major* promastigotes. Although both humoral and cell-mediated immune responses were induced by vaccination of this extract, the induction of *Leishmania*-specific antibodies did not appear to be involved in protection. Protection in this model appeared to be mediated by the stimulation of *Leishmania*-specific T cells to produce macrophage activating lymphokines. This study and the others described above demonstrate that soluble cytoplasmic or nuclear proteins can influence the interaction of the host immune system with invading parasites or viruses. Therefore, the ability of the *Leishmania* repetitive proteins encoded by the genes which were identified in this thesis to interact and influence the hosts immune response to infection by *Leishmania*, may not be dependent on the localization of these proteins to the cell surface.

Sacks et al. (1984) examined the role of B cells and/or antibodies in immunity to cutaneous leishmaniasis. They reported that in normally resistant mice, immunity was independent of antibody. However, in susceptible mice, B cells and/or antibodies were required for the suppression of cell-mediated immunity characteristic of the disease. The evidence that B cells or antibody appear to be involved in the generation of T cell suppression in leishmaniasis may mean that the polyclonal B cell stimulation observed in chronic leishmaniasis may not only interfere with the maturation of the humoral immune response, but may also play an active role in the suppression of protective cell-mediated responses. Milon et al. (1986) proposed that *Leishmania major*-specific T cells present in excess at the site of infection could participate directly in the development of cutaneous lesions by continuously recruiting phagocytes. This would result in increased numbers of host cells able to support parasite growth. They propose that a possible mechanism to explain why these infected macrophages are unable to receive activating T cell signals could be due to the formation of
circulating *Leishmania* antigen/immune complexes. Immune complexes have been shown to impair macrophage functions such as lymphokine-induced expression of MHC Class II molecules (Virgin et al., 1985). Caulfield and Schaffer (1987) demonstrated that specific immunosuppression was induced by immune complexes formed in antibody excess *in vivo*. It was shown that immune complexes which are formed in antigen excess are immunogenic but immune complexes formed in antibody excess are not immunogenic and they appear to suppress the antibody responses in an antigen-specific manner. This suppression appears to be mediated by T suppressor cells (Caulfield et al., 1983). Antigen/antibody complexes (immune complexes) therefore have potent immunoregulatory properties and perhaps their normal *in vivo* role is to regulate the immune response, stimulating it when antigens first invade the body and the antibody/antigen ratio is low, and suppressing this response when antibody/antigen ratios are high. Immune complexes have been shown to reduce resistance to the intra-macrophage parasite, *Listeria monocytogenes* (Virgin et al. 1984) and to play a role in the immunosuppression observed in the parasitic infection filariasis (Karavodin and Ash, 1980). The presence of immune complexes has been demonstrated in leishmaniasis and schistosomiasis (Carvalho et al., 1983). Perhaps in susceptible mice there is a large initial accumulation of antigen due to parasite replication, leading to activation of antibody with poor maturation of the response. The high antibody titre might then lead to immune complex formation resulting in suppression. Perhaps *Leishmania* and other protozoan parasites, such as *Plasmodia*, have taken advantage of this putative immune system feedback mechanism in order to bring about immune suppression. It will be interesting to carry out immunological assays to determine the effect of immunization of the *Leishmania* repetitive antigens on subsequent challenge of *Leishmania* in mouse strains with different susceptibilities. The repeats may provide host protection, as it appears some of the repeats from *Plasmodia* do, they may have little or no effect or they may induce host suppression and be parasite protective.

Although the presence of repetitive peptides is characteristic of many *Plasmodia* proteins, recent studies have also reported the presence of repetitive regions within two *Trypanosoma* proteins and within one protein of *Toxoplasma gondii*. The *Trypanosoma brucei* repetitive protein which has been reported is insect stage-specific (Roditi et al., 1987) and,
therefore, this protein can not be involved with interactions with the host immune system. Perhaps because *Trypanosoma brucei* has evolved the mechanism of antigenic variation of its surface coat proteins and because *Trypanosoma brucei* is located extracellularly within the mammalian host, there is little selective pressure for this parasite to evolve additional mechanisms to interfere with the host immune system. The *Trypanosoma cruzi* repetitive protein is expressed in the trypomastigote stage, which is found both within the insect and within the mammalian host. Although the protein which contains the repeats is proposed to function in attachment of the parasite to mammalian host cells, the role of the repeats themselves has not been investigated. Polyclonal B cell stimulation is a characteristic feature of Chagas' disease (Minoprio et al., 1986) and it is possible that the repeats may be involved in this. The *Leishmania* repetitive proteins described here are expressed in the promastigote stage of the parasite and at least one of these proteins appears to be expressed in the amastigote stage as well. It is possible that these proteins are involved in the attachment of the parasite to host macrophages or, during the promastigote stage, they may be involved in the attachment of the parasite to the insect gut wall.

It will be interesting to determine the role of the *Leishmania* repetitive proteins and to see whether the presence of repetitive proteins is characteristic of other related parasites such as the protozoan *Trypanosoma cruzi* or perhaps the bacterial pathogen *Mycobacterium leprae* both of which are intra-macrophage parasites and appear to share many other features with *Leishmania*. 
XI. LITERATURE CITED


