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MOLECULAR STUDIES USING AMASTIGOTE-SPECIFIC GENES IN *LEISHMANIA*

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Thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

Leishmania is a dimorphic parasitic protozoan which exists as a flagellated promastigote in the sandfly vector and as an intracellular amastigote in the phagolysosomal compartment of mammalian host macrophages. It is the amastigote form that is responsible for the pathology in susceptible vertebrate hosts. *Leishmania donovani* is responsible for visceral leishmaniasis, the most severe form of the leishmanial diseases. We have investigated the antibody response against an amastigote-specific protein, A2, which is developmentally expressed in *L. donovani* during promastigote-to-amastigote cytodifferentiation. A2 is conserved in *L. donovani* and *L. mexicana* species but not in other *Leishmania* species tested. We have shown that this characteristic contributes to its potential as a useful specific diagnostic antigen for visceral leishmaniasis. Developmental expression of A2 involves A2 mRNA untranslated regions (UTRs) and we have demonstrated that A2 UTRs can regulate expression of exogenous suicide genes throughout the *Leishmania* life cycle. We have shown that the A2 gene regulatory system has potential for the generation of developmentally attenuated *L. donovani* strains. Finally, we have performed a preliminary characterization of a gene, A2rel, that is tandemly associated with A2 genes in the genome. Contrary to A2 genes, the A2rel gene is well conserved in the *Leishmania* species. Although A2rel does not share sequence similarity with any known leishmanial genes characterized to date, it does appear to share characteristics with membrane-bound glycoproteins.

ABRÉGÉ

Le parasite protozoaire *Leishmania*, au cours de son cycle de transmission, se transforme du stade promastigote au stade amastigote à l'intérieur du phagolysosome de sa cellule hôte, le macrophage. *Leishmania donovani* est l'agent spécifique de la leishmaniose viscérale, une forme grave de leishmaniose, mortelle quand elle n'est pas soignée. Nous avons etudié la réponse spécifique immunitaire de l'hôte contre la protéine A2 qui est exprimée par *L. donovani* en sa forme amastigote. Les gènes A2 sont conservés chez les leishmanies appartenant aux complexes *L. donovani* et *L. mexicana*. Cette spécificité contribue au potentiel de A2 en tant qu'antigène pour le diagnostique de la leishmaniose viscérale. L'expression développementale des gènes A2 est dirigée par les séquences non-traduites situées dans la queue 3' de l'ARNm. Nous avons utilisé des séquences ADN du locus A2 pour contrôler l'expression de gènes exogènes pouvant induire une toxicité chez leishmanie. Le système génique régulatoire de A2, ainsi employé, a permis la création d'une souche atténuée de *L. donovani*, où l'expression différentielle de produits toxiques est favorisée lors de la transformation du promastigote à sa forme amastigote. Nous avons ensuite effectué une caractérisation préliminaire du gène A2rel associé à A2 sur le génome de *L. donovani* et *L. mexicana*. Contrairement à A2, A2rel est conservé chez toutes les espèces *Leishmania* investiguées. Une étude de la séquence de A2rel suggère que la protéine codée partagerait certaines caractéristiques des glycoprotéines associées aux membranes de surface.

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THESIS OFFICE STATEMENT

In accordance with the regulations of the Faculty of Graduate Studies and Research of McGill University, the following statement is included in the thesis:

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STATEMENT OF AUTHORSHIP

The work presented in Manuscripts I, II and III was performed by myself in Dr. Greg Matlashewski's laboratory. The contributions of the co-authors are described as follows:

Manuscript I - Dr. Hugues Charest, a postdoctorate fellow at the National Institutes of Health, made the anti-A2 monoclonal antibody (C9) that was used in the Western blotting studies (Figure 4). Dr. Wen Wei Zhang, a postdoctorate fellow in Dr. Matlashewski's laboratory, engineered the A2-GST fusion protein used in the Western blots and ELISAs (Figures 2). Dr. Rick Kenney, of the FDA, and Dr. Shyam Sundar, of Banaras Hindu University, are collaborators that brought back the serum samples from India and provided useful discussions.

Manuscript II - The plasmid construct containing the 3'nucleotidase/nuclease gene (Figure 1) was made by myself in Dr. Dennis Dwyer's laboratory at the Laboratory of Parasitic Diseases (NIH) with the collaboration of Dr. Alain Debrabant. Dr. Hugues Charest made the NeoPT plasmid construct used in Figure 2. Dr. Wen Wei Zhang made the pKSneo construct used as a control in Figures 5 and 6 and also collected the results presented in Figure 2.

Manuscript III - The A2rel gene was isolated by Dr. Hugues Charest but was sequenced and analyzed by Northern and Southern blot analyses by myself.

STATEMENT OF ORIGINALITY

Manuscript I:

1. Use of A2 as an antigen for the diagnosis of *L. donovani* and *L. mexicana* infections. A2 is an amastigote-specific protein and we have used it to screen a series of sera for *L. donovani* and *L. mexicana* infections. Most serodiagnostic tests use whole parasites or purified promastigote antigens.

2. Species-specificity of A2. We have demonstrated that A2 sequences are conserved in only two species of *Leishmania*, *L. mexicana* and *L. donovani*, a characteristic that adds to its potential as a serodiagnostic antigen for visceral leishmaniasis.

Manuscript II:

3. Developmental expression of negative markers in the amastigote stage. We have used the A2 gene regulatory system to differentially express, in amastigotes, genes with a toxic potential to the cells. This represents the first study in *Leishmania* where the accumulation of suicide gene products occurs in amastigotes.

4. Targeting of a suicide gene in a chromosomal locus. The herpes simplex virus thymidine kinase gene was targeted into the A2 locus by homologous recombination. This represents the first case

of integration of a gene with toxic potential into the genome of *Leishmania*.

5. Engineering of a live attenuated strain where attenuation is triggered developmentally. The expression of suicide genes, under the control of the A2 locus, has generated recombinant *Leishmania* strains which can be cultured *in vitro* as promastigotes but cannot replicate as amastigotes.

6. Use of a native enzyme of *Leishmania* as a toxic product. The *L. donovani* 3'-Nucleotidase/Nuclease is normally an externally oriented enzyme involved in the purine salvage pathway. We engineered a truncated version of the enzyme by deleting the signal peptide and anchoring sequences from the gene.

Manuscript III:

7. A2rel is unique. The amino acid sequence of the deduced open reading frame of A2rel does not share sequence similarity with any known *Leishmania* genes characterized to date.

8. Conservation of the A2rel gene. We demonstrate that A2rel, although it is linked to A2 genes on the *L. donovani* genome, is conserved in all the *Leishmania* species tested.

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LIST OF ABBREVIATIONS

AP	acid phosphatase
BMM	bone marrow-derived macrophage
CAT	chloramphenicol acetyl-transferase
CL	cutaneous leishmaniasis
DAT	direct agglutination test
DHFR-TS	dihydrofolate reductase-thymidylate synthase
ELISA	enzyme-linked immunosorbant assay
FBS	fetal bovine serum
GIPLs	glycoinositolphospholipids
GPI	glycosyl-phosphatidyl-inositol
GST	glutathione *S*-transferase
HRP	horseradish peroxidase
HSC	heat shock cognate gene
HSP	heat shock protein
HSV-TK	*Herpes simplex* virus thymidine kinase
IFAT	immunofluorescence antibody test
IFN-γ	interferon gamma
IgG	immunoglobulin G
IL	Interleukin
LPG	lipophosphoglycan
Mab	monoclonal antibody
msp	major surface protease
MVL	Mediterranean visceral leishmaniasis
NO	nitric oxide
iNOS	inducible nitric oxide synthase

ORF	open reading frame
PARP	procyclic acidic repetitive protein
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PFGE	pulse-field gel electrophoresis
PKC	protein kinase c
PNA	peanut lectin agglutinin
PYT	artificial *trans*-splicing acceptor site
SL	spliced-leader
TAFE	transversal alternating-field electrophoresis
UTR	untranslated region
VL	visceral leishmaniasis
VSG	variant surface glycoprotein
3'NT/Nu	3'nucleotidase/nuclease

INTRODUCTION

Protozoan parasites of the genus *Leishmania* (order Kinetoplastidae, family Trypanosomatidae) are responsible for a spectrum of diseases depending upon the particular *Leishmania* species responsible for the infection. Disease symptoms range from localized cutaneous lesions (*L. major*, *L. tropica*, *L. mexicana* and *L. aethiopica*) to disseminated muco-cutaneous (*L. braziliensis*) and visceral infections (*L. donovani*). The leishmaniases are transmitted by over 50 species of female sandflies of the genus *Phlebotomus*, for Old World forms, and *Lutzomyia* in the New World (WHO, 1984). The World Health Organization has targeted this group of parasites due to the high levels of morbidity and mortality associated with its diseases.

It is estimated that 20 million people are infected with various forms of leishmaniases worldwide, with 400,000 new cases being reported each year (WHO, 1993). The leishmaniases are endemic in 82 countries (Magill, 1995) but the three predominant areas are India, Africa and Brazil. Visceral leishmaniasis (VL), the most severe form of the leishmaniasis group of diseases, is considered a disease of poverty. Healthy individuals are rarely infected while individuals suffering from malnutrition, for the most part children, seem to be at higher risk of developing a severe form of the disease (Fargeas *et al.*, 1996; Cerf *et al.*, 1987). Chemotherapy regimens, using drugs such as sodium stibogluconate, pentamidine, and amphotericin B, are often long, expensive and toxic (Kar, 1995), and the treatment is increasingly inefficient, particularly in immunosuppressed patients.

VL was identified as being an opportunistic disease in AIDS patients (Rosenthal *et al.*, 1995; Alvar, 1994). Clinicians are now also being challenged with newly recognized disease symptoms, such as viscerotropic leishmaniasis caused by *L. tropica* observed in veterans of Operation Desert Storm (Dillon, 1995; Magill, 1995; Magill, 1993).

Diagnosis based on direct visualization of parasites in bone marrow or splenic aspirates, or by cultivation *in vitro* of parasites obtained in biopsy specimens, requires skilled technicians and well equipped laboratories. This type of diagnosis is difficult to perform under field conditions and the procedures used are often hazardous and invasive. Various serological tests, based on indirect detection of the presence of parasites by detecting anti-leishmanial antibodies, were developed because of the high titers of anti-leishmanial antibodies in VL cases. However, direct visualization by microscopy remains the gold standard for diagnosis. Finding new antigens for serology, in the form of recombinant proteins or purified antigens, where cross-reactivity is limited and sensitivity improved, is thus necessary (Burns et *al.*, 1993; Okong'o-Odera *et al.*, 1993b; Jaffe and Zalis, 1988a).

Understanding the molecular basis of infection in VL has been the focus of our laboratory. Recent studies have resulted in the identification of amastigote-specific genes suspected in encoding products important for the survival of the protozoan in its mammalian host (Zhang and Matlashewski, 1997; Charest *et al.*, 1996; Zhang *et al.*, 1996; Charest and Matlashewski, 1994). These studies

have also contributed to the knowledge on gene regulation in *Leishmania*.

In the present study, I continue work on the characterization of the amastigote-specific gene family A2. Chapter 1 presents a concise literature review on the biology and genetics of *Leishmania*, on the development of expression systems in trypanosomatids, and recent developments in the diagnosis of visceral leishmaniasis. In Chapter 2, I present our work on the characterization of anti-A2 protein antibodies. The A2 protein contains repetitive amino acid domains which are immunodominant epitopes and we have evaluated the diagnostic potential of this antigen. In Chapter 3, I demonstrate the feasibility of using the A2 locus to control the developmental expression of negative selective genes and to generate a live attenuated organism. Finally, in Chapter 4, I present the initial characterization of a gene, A2rel, found to be linked to the A2 gene in the *L. donovani* genome.

CHAPTER 1

Literature Review

1. Biology of the *Leishmania* parasite

Leishmania parasites oscillate between two distinct host-specific developmental stages in the course of their life cycle, offering a diversity in genetic events. Flagellated promastigotes are found in the gut lumen of the sandfly vector, while non-motile amastigotes exist intracellularly in the vertebrate host macrophages (Molyneux and Killick-Kendrick, 1987). This transition between the two forms requires means of adapting to different environments. Throughout its life cycle, the *Leishmania* parasite is confronted by hostile environments - digestive enzymes in the gut of the sandfly, the lytic complement pathway in the bloodstream of the vertebrate host, the hydrolytic enzymes and microbicidal oxidative burst in the phagolysosome of host macrophages - and it must avoid destruction in order to propagate (Beverley, 1996). *Leishmania* virulence must be closely related to the sequential events of host-parasite cellular interactions leading to the establishment of the parasite within the macrophage phagolysosomes, the most striking feature of leishmaniasis being the parasite's exclusive ability to establish in such an inhospitable environment.

1.1. Promastigote development

At least five developmental forms can be recognized in the extracellular stage of *Leishmania*: procyclic promastigotes, nectomonad promastigotes, haptomonad promastigotes, paramastigotes, and metacyclic promastigotes (Bates, 1994a). These

morphological changes occur exclusively in the gut of the sandfly. For most *Leishmania* species, the establishment of infection within the appropriate vector requires the attachment of parasites to the midgut epithelium (Molyneux and Killick-Kendrick, 1987) retaining the parasites in the gut during passage of the digested blood meal. The extent to which the parasite is able to attach to the sandfly midgut predicts the outcome of infection (Sacks *et al.*, 1994). The next step involves the detachment and anterior movement of infective forms for their migration to the mouthparts of the insect.

It was demonstrated for *L. major* and *L. donovani* that the promastigote movement to the foregut was accompanied by their differentiation (metacyclogenesis) into an infective stage, the metacyclic form (Sacks *et al.*, 1994; Sacks, 1989). Metacyclics are adapted for transmission by the sandfly vector and survival within the vertebrate host. Culture-derived metacyclic promastigotes were shown to be more resistant to intracellular killing by macrophages and extracellular killing by complement, indicating that they are particularly adapted for survival within the vertebrate host (Sacks, 1989). Development of promastigotes from a dividing non-infective stage to a non-dividing infective stage has been observed *in vivo* and *in vitro* (Sacks and Perkins, 1984). Metacyclic promastigotes found within stationary cultures can be separated from the procyclics, or non-infective forms, by their failure to bind to lectin peanut agglutinin (PNA). This indicates that metacyclogenesis is accompanied by changes in surface carbohydrates (Sacks *et al.*, 1995b; Sacks and da Silva, 1987; Sacks *et al.*, 1985).

1.1.1. Stage-specific gene expression in promastigotes

Metacyclogenesis is accompanied by the differential expression of major surface glycoconjugates. The structural and molecular changes that accompany metacyclogenesis in *L. major* include the expression of stage-specific oligosaccharides on the lipophosphoglycan (LPG), a thickness increase of the surface LPG glycocalyx and upregulation of stage-specific mRNA (Saraiva *et al.*, 1995; Sacks *et al.*, 1995b).

Coulson and Smith (1990) have isolated and characterized, by the differential screening of a cDNA library, four cDNA clones representing upregulated mRNAs in metacyclic promastigotes of *L. major*. One of the cDNA clones described, Lm cDNA 14, recognized an abundant mRNA in infective metacyclics that was barely detectable in amastigotes, while the three other clones, Lm cDNA 2, 7 and 16 also detected increased levels of transcripts in amastigotes (Coulson and Smith, 1990). Lm cDNA 16 recognises five mRNAs that are expressed differentially during the parasite life cycle. Flinn and Smith (1992), using Lm cDNA 16 to screen a genomic library, have characterized a family of five differentially expressed genes within a 10 kb region of the *L. major* genome. One of the members of this family, Gene B, is expressed exclusively in the metacyclic and amastigote stages where it localizes to the surface of the infective parasite (Rangarajan *et al.*, 1995; Flinn *et al.*, 1994). The repetitive element of the Gene B protein is related to the cell wall binding domain of the *Staphylococcus aureus* protein A (Flinn *et al.*, 1994). Lm cDNA 14, which was renamed *meta*1, was isolated from *L. major*

but was found conserved across the *Leishmania* genus. The *meta*1 gene product is found localised in the region surrounding the flagellar pocket of metacyclics (Nourbakhsh *et al.*, 1996). Brodin *et al.* (1992) have identified in *L. major*, also by the differential screening approach, full-length cDNA clones representing metacyclic promastigote-associated transcripts encoding a potential basic-zipper structural motif.

1.2. Intracellular stages

Upon inoculation into the skin of the mammalian host, promastigotes establish within the phagolysosomal vacuole of macrophages, where they transform into amastigotes and multiply (Chang and Dwyer, 1976). The transformation from promastigote to amastigote involves the loss of the flagellum and a switch to an anaerobic metabolism. During transfer of *Leishmania* parasites from the gut of their non-temperature-regulated insect vector to the macrophage phagolysosomal compartment of the temperature-regulated mammalian host, the most obvious environmental changes are an increase in temperature and a decrease in pH. Parasites which cycle between invertebrate and vertebrate hosts need to adapt for survival over a wide temperature range and other stressful environmental conditions. Heat-shock responses are thought to play an important role in parasite survival and differentiation (Shapira *et al.*, 1988).

1.2.1. Heat-shock response

The heat-shock response involves the activation of heat-shock genes and the synthesis of heat-shock proteins (HSPs) during certain stages of cell development. The environmental conditions that the parasite encounters throughout its life cycle are likely to induce the expression of stress responses. In all organisms, stress induces significant expression of HSPs. This response is the most highly conserved genetic system known, although certain features of the response may vary from organism to organism (Lindquist and Craig, 1988). All organisms examined produce proteins encoded by the hsp70 and hsp80 gene families in response to elevated temperature. It has also been found that several of the proteins induced by heat are also induced by other stresses, such as oxidative injury, chemical agents and inflammation (Lindquist and Craig, 1988). Heat-shock protein families include proteins that are expressed at low temperatures, such as most HSPs constitutively expressed by *L. major* promastigotes (Searle *et al.*, 1989) and a heat-shock cognate gene (hsc70) coding for an antigen related to the hsp70 family and constitutively expressed throughout the *L. donovani* life cycle (Rey-Ladino and Reiner, 1993; McFarlane *et al.*, 1990). These proteins are thought to play a role in cellular physiology based on their ability to form protein-protein interactions in an ATP-dependent manner (Searle and Smith, 1993). In a classical heat-shock response, members of the hsp70 gene family are expressed at higher than constitutive levels. However, when entering the mammalian host, *Leishmania* are not exposed to transient and accidental conditions,

but rather to an obligatory event in their life cycle. Searle and Smith (1993) show that an *L. major* gene product related to hsp70 (cp70.4) is expressed in the cytoplasm of both promastigotes and amastigotes under normal growth conditions. The abundance of hsp83 in *Leishmania* at lower temperatures suggest that this protein is also essential for existence under non-heat shock conditions (Argaman *et al.*, 1994). In *Leishmania*, the hsp70 and hsp83 levels do not increase significantly in response to a heat stress. Hübel *et al.* (1995) present evidence that the *Leishmania* homologue to the bacterial ClpB gene and to the yeast hsp104 gene is a true heat shock gene because it encodes a 100 kDa protein that is induced in heat-shocked promastigotes of several *Leishmania* species.

During host invasion, the stress response in the parasite is probably elicited independently of the temperature shock (Maresca and Carratù, 1992), because parasites not only adapt to higher temperatures but also to other conditions, such as hydrolytic enzyme activities and lysosomal acidic pH, against which they must protect themselves. Maresca and Carratù (1992) suggest that while in most eukaryotes thermotolerance plays an important physiological role in the maintenance of cell properties, in parasites its role must be less important because, at the onset of invasion, there is no time to develop thermotolerance. For its cellular apparatus to remain functional, the parasite may have developed a constitutive thermotolerant state before invasion. This would explain the constitutive expression of some HSPs (HSP70 and HSP83) induced prior to the morphological changes and constitutively expressed in

amastigotes (Maresca and Carratù, 1992; Searle *et al.*, 1989). Shapira *et al.* (1988) suggest that this HSP expression prior to transmission would preadapt the organism for the stress (high temperature and low pH) encountered in the mammalian host. Temperature increases may also influence stage differentiation. In axenic conditions, an increase in the promastigote culture temperature, from 26°C to 37°C, can in certain species induce morphological changes that mimic the promastigote to amastigote cytodifferentiation. During this process the cells round up, lose their flagellum and become nonmotile as well as decrease in size (Shapira *et al.*, 1988; Van der Ploeg *et al.*, 1985). Lee *et al.* (1988) have demonstrated, by using a nondifferentiating mutant strain of *L. major*, that the hsp70 gene family was not involved in this differentiation process.

1.2.2. Amastigote stage-specific gene expression

There have been few amastigote-specific genes identified to date, presumably because of the methodological difficulties associated with the culturing of intracellular stages and in obtaining viable amastigotes free of host cell contamination. However, the serial cultivation of amastigote-like organisms that resemble lesion-purified amastigotes has been described for many *Leishmania* species (Bates, 1994b; Bates *et al.*, 1992; Al-Bashir *et al.*, 1992; Rainey *et al.*, 1991; Eperon and McMahon-Pratt, 1989; Pan, 1984).

A developmentally expressed gene of *L. major* (p101/10) was identified and its corresponding transcript was found to be

upregulated by heat induction in amastigotes (Kidane *et al.*, 1989). Upregulation of cysteine proteinases was also described for amastigotes of the *L. mexicana* complex (Traub-Cseko *et al.*, 1993; Mottram *et al.*, 1992; Souza *et al.*, 1992). A large *L. chagasi* protein related to the kinesin superfamily of motor proteins was shown to be expressed predominantly by amastigotes (Burns *et al.*, 1993). The A2 protein of *L. donovani*, determined to be a virulence factor (Zhang and Matlashewski, 1997), was shown to be specifically expressed in amastigotes and represents the best available protein marker for *L. donovani* amastigotes identified to date (Zhang *et al.*, 1996).

1.3. Parasite-macrophage interaction

In the mammalian host, *Leishmania* species only replicate in macrophages. There are many factors which affect the survival of the parasite in a cell whose major function is to kill microorganisms. One of these factors is the ability of the macrophage to generate lethal oxygen radicals. The respiratory burst of the macrophage is characterized by the production of high levels of oxygen metabolites, such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), and nitrogen oxides produced through an inducible L-arginine dependent pathway, which are involved in the destruction of various microorganisms (Wilson *et al.*, 1994; Green *et al.*, 1991; Green *et al.*, 1990; Hibbs *et al.*, 1987).

Promastigotes passively invade macrophages via receptor-mediated endocytosis. A range of receptors are involved in the attachment process, including CR3 and the mannose fucose receptors (reviewed in Alexander and Russell, 1992; Chang *et al.*, 1990). Promastigotes of different *Leishmania* species were shown to activate complement, which leads to the deposition of C3 fragments on the surface of the promastigote. While promastigotes retard fusion of the phagosome with the lysosomal pocket in order to avoid hydrolytic enzyme activities (Desjardins and Descoteaux, 1997), amastigotes proliferate in a typically lysosomal acidic pH. However, *Leishmania* interfere with activation of the macrophage because the parasite cannot resist the effect of a strong nitric oxide (NO) response nor can it counteract the toxic effects of H_2O_2. *Leishmania* infection promotes macrophage dysfunction and this may be due to a reduced activation of protein kinase C (PKC), which is involved in initiation of the oxidative burst (Olivier *et al.*, 1992; Descoteaux and Matlashewski, 1989). LPG glycoconjugates, expressed on the surface of metacyclic promastigotes, have the ability to inhibit the activation of PKC (Descoteaux *et al.*, 1992), therefore regulating the expression of cytokine-inducible NO synthase (iNOS) which catalyzes the synthesis of high concentrations of NO (Proudfoot *et al.*, 1996). *Leishmania* also cause a down-regulation in the macrophage's response to stimulating lymphokines (Alexander and Russell, 1992; Mauel, 1990). IFN-γ is the predominant lymphokine that activates macrophages for the generation of toxic oxygen products and a strong NO response inducing macrophage leishmanicidal activity (Hughes, 1988; Murray *et al.*, 1983).

2. Molecular Determinants of *Leishmania* Virulence

A number of developmentally regulated molecules are expressed by metacyclic promastigotes and tissue amastigotes that promote their survival within the mammalian host (Pimenta *et al.*, 1991). Throughout the life cycle, the surface of the parasite changes. Promastigotes produce a distinct glycocalyx whose thickness is developmentally regulated, while amastigotes lack a discernable cell coat. Molecules that are expressed on, or released from, the surface of *Leishmania* appear to influence parasite survival within the host and the vector (Moody, 1993). The majority of the molecules being released from the promastigote are phosphoglycans, while two classes of GPI-anchored (glycophosphatidylinositol) membrane glycoconjugates have been identified as surface molecules (Moody, 1993). One class includes LPG, which is the major cell surface glycoconjugate of the promastigote form of *Leishmania* (Turco and Sacks, 1991), while the other class is represented by glycoproteins, the most prevalent being a 63 kDa molecule referred to as gp63 (Etges *et al.*, 1986). It appears that the expression of gp63 (Wilson *et al.*, 1989) and LPG (Shankar *et al.*, 1993; Sacks and da Silva, 1987; Handman *et al.*, 1986) may determine the ability of *Leishmania* to interact with macrophage receptors, having a major effect on parasite virulence (Chakrabarty *et al.*, 1996).

2.1. GPI-anchored proteins

The surfaces of kinetoplastid parasites, such as *Leishmania*, are dominated by GPI-anchored proteins probably due to the fact that GPI anchors permit the isolation of proteins with purely extracellular functions from the interior of the cell and allow high levels of protein packing. GPI-anchored proteins can also form glycocalyx structures by a modification of their oligosaccharide side chains (reviewed in McConville and Ferguson, 1993).

A GPI anchor-protein must contain an *N*-terminal signal sequence directing it to the lumen of the endoplasmic reticulum via the signal recognition particle, and it must also contain at its *C*-terminal a GPI-signal sequence (McConville and Ferguson, 1993). The first steps of the GPI pathway occur on the cytoplasmic surface of the endoplasmic reticulum. The GPI anchor is an efficient and stable anchor for the association of proteins with the lipid bilayer (McConville and Ferguson, 1993). Some proteins, such as the gp63 metalloproteinase, are expressed in both transmembrane and GPI-anchored forms. The transcription of genes that code for the two versions of gp63 is developmentally regulated (Medina-Acosta *et al.*, 1993). In the promastigote stage, it is expressed on the surface and contains a GPI anchor, while in the amastigote it is a non-GPI-anchored protein localized to the lysosome (Medina-Acosta *et al*, 1993, 1989). Thus, anchor type appears to regulate localization of the proteins (McConville and Ferguson, 1993).

Gp63 is also referred to as the major surface protease (msp) and it is abundantly distributed over the entire surface of both promastigotes and amastigotes (Frommel *et al.*, 1990; Medina-Acosta *et al.*, 1989; Etges *et al.*, 1986). It is present in all *Leishmania* species studied to date, however not all strains express the amastigote form. *L. major* promastigotes were shown to express a higher amount than amastigotes (Schneider *et al.*, 1992). It has been suggested that gp63 contributes to the binding of promastigotes to macrophages, by interacting directly with macrophage receptors, such as the complement receptor type 3 (CR3) (Russell and Wright, 1988; Wilson and Hardin, 1988), and a direct role for gp63 in resistance to complement-mediated lysis was demonstrated (Brittingham *et al.*, 1995). Gp63 is also thought to play a role in the survival of parasites in the macrophage phagolysosome (McGwire and Chang, 1994; Chaudhuri *et al.*, 1989). McGwire and Chang (1994) have demonstrated that when, by genetic complementation, gp63 activity was restored in gp63-deficient *L. amazonensis* variants, macrophage-binding by the parasites increased and they recovered partial intra-macrophage survival. Attenuated *L. mexicana* and *L. donovani chagasi* were shown to express decreased amounts of gp63, as compared to virulent variants of the parasites (Wilson *et al.*, 1989; Chaudhuri and Chang, 1988).

Gp63 is encoded by multiple tandemly arranged genes that range in number from six in *L. major* (Button *et al.*, 1989), to 18 or more in *L. donovani chagasi* (Roberts *et al.*, 1993). Three different classes of gp63 RNAs were found to be differentially expressed in *L. chagasi*

promastigotes. In early logarithmic phase, promastigotes express gp63 RNA of 2.7 kb, and in stationary phase RNA of 3.0 kb, while a third class is formed by 2.6 and 3.1 kb RNAs expressed constitutively throughout the promastigote growth cycle (Ramamoorthy *et al.*, 1992). These different gp63 RNAs contain unique 3'-untranslated regions. The three classes were shown to be encoded in *L. chagasi* by a cluster of four tandemly associated stationary genes (*msp*S genes), twelve logarithmic genes (*msp*L), one constitutive gene (*msp*C), followed by another stationary gene (Roberts *et al.*, 1993). *L. chagasi* amastigotes were found to express gp63 mRNAs only from the *msp*C and *msp*L class genes (Streit *et al.*, 1996). *L. chagasi* has the largest gp63 encoding gene family described to date.

2.2. Protein-free GPIs

In several trypanosomatid parasites, such as in the *Leishmania* spp., glycolipids which are not linked to protein are the major cellular glycoconjugates (McConville and Ferguson, 1993). There are two classes of free GPIs synthesized in all *Leishmania*: the polydispersed lipophosphoglycan (LPG) and the low-molecular mass glycoinositolphospholipids (GIPLs). While GIPLs are abundant in both promastigote and amastigote stages and predominantly expressed on the cell surface (McConville and Blackwell, 1991), the expression of LPGs is largely restricted to the promastigote stage. The presence of LPG was examined in the amastigotes of *L. donovani* and *L. major*, and results demonstrate that *L. donovani* amastigotes apparently cannot synthesize LPG (McConville and Blackwell, 1991), while *L.*

major amastigotes express a surface LPG which is antigenically and biochemically distinct from promastigote LPG (Glaser *et al.*, 1991). Stage-specific LPG has yet to be reported in other species of *Leishmania* (Turco and Descoteaux, 1992; Moody *et al.*, 1993).

Modifications in LPG structure accompany the process of metacyclogenesis. Adhesion of *Leishmania* promastigotes to the midgut epithelial cells of the sandfly, and release from these cells, were found to be controlled by specific developmental modifications in LPG (Pimenta *et al.*, 1992; Sacks and da Silva, 1987). LPG consists of a series of repeating saccharide units linked together by phosphodiester bonds (Turco, 1990). Stage-specific differences, observed during development of *L. major* promastigotes from the non-virulent procyclics to the virulent metacyclics, appear *in vitro* and *in vivo* in the size of the phosphoglycan chains and in the abundance of different side chains (Saraiva *et al.*, 1995; Pimenta *et al.*, 1992; Sacks *et al.*, 1990). LPG developmental polymorphism, where exposed sugars on the parasite surface are altered in a stage-specific manner, has been observed in different *Leishmania* species with different types of structural modifications occuring depending on the species (Sacks *et al.*, 1995b; Sacks *et al.*, 1994).

LPG has also been found to be involved in the attachment and entry into host macrophages by serving as a ligand, and promastigotes which lack LPG are avirulent (Glaser *et al.*, 1991; Turco, 1990; Da Silva *et al.*, 1989; Handman *et al.*, 1986). LPG may also play an important protective role for the parasite upon entry into the

phagolysosome of a host cell (Turco, 1990). Infectivity refers to the fate of intracellular promastigotes after macrophage uptake. It was proven that LPG is essential for the establishment of infection inside macrophages (Desjardins and Descoteaux, 1997; McNeely and Turco, 1990). The role of LPG in intramacrophage survival, however, may be restricted to the establishment of infection, since amastigotes synthesize little or no detectable LPG (McConville and Blackwell, 1991; Turco and Sacks, 1991). LPG repeating units may have a protective role for the promastigote during infection by scavenging toxic oxygen metabolites generated during the oxidative burst (McNeely and Turco, 1990), modulating the inducible nitric oxide synthase expression (Proudfoot *et al.*, 1996), and forming a dense glycocalyx that may protect against hydrolytic enzymes (Chang and Dwyer, 1976). LPG was also shown to be a potent inhibitor of mammalian protein kinase C (Descoteaux *et al.*, 1992; Olivier *et al.* 1992). Desjardins and Descoteaux (1997) suggest another role for LPG by showing that the repeating units enable *Leishmania* to inhibit phagosome-endosome fusion. This may represent a survival strategy for the promastigote during cytodifferentiation to the amastigote stage by preventing contact with hydrolytic enzymes.

Genetic studies have also demonstrated, by functional complementation, that LPG is a key virulence determinant. The method consists in generating non-virulent mutants and identifying the defective gene by complementation (Beverley and Turco, 1995; Ryan *et al.*, 1993). Two LPG genes were identified this way: LPG1, which represents a class of genes encoding LPG biosynthetic enzymes

(Ryan *et al.*, 1993); and LPG2 which is involved in compartmentalization and LPG assembly (Descoteaux *et al.*, 1995).

Two new members can be added to the list of structural components of the surface membrane that include GIPLs, LPG and gp63: KMP-11 and Gene B. These molecules were shown to have associations with LPG and share common properties (reviewed in Bates, 1995). KMP-11 is a major surface protein of *L. donovani* promastigotes with 1-$2x10^6$ copies per cell, but it is not known whether it is specifically expressed by metacyclics. It is thought to be involved in membrane stabilization. The Gene B protein is expressed on the surface of metacyclic promastigotes at 100,000 copies per cell, as well as on the surface of intracellular amastigotes (Rangarajan *et al.*, 1995). Gene B is also known to be present in *L. donovani*, *L. tropica* and *L. infantum*, but not in *L. mexicana* nor *L. braziliensis* (Bates, 1995).

Shankar *et al.* (1993) have shown that although both LPG and gp63 are important factors in the virulence of the parasite, reversion to virulence of attenuated *L. major* clones correlated with expression of surface LPG. Neither the degree of expression of gp63 nor the relative enzymatic activity of the molecule correlated with the level of virulence (Shankar *et al.* 1993). Chakrabarty *et al.* (1996) demonstrated, using two virulent strains of *L. donovani*, that for different pathogenic strains, the contribution of LPG and gp63 may be different.

2.3. Other virulence factors

Other possible virulence factors that have been identified in *Leishmania* include secreted acid phosphatase, cysteine proteinases, and the *L. donovani* A2 protein. Secretory antigens are likely to play a role in the survival of *Leishmania* in the host environment. LPG molecules, with (LPG) or without (PG) their glycolipid anchor, are also a major component in the cell culture supernatant.

2.3.1. Secreted acid phosphatase

The *Leishmania donovani* secreted acid phosphatase (sAP), which is a phosphomonoesterase, was characterized as a heterogeneous phosphoglycoprotein which is substituted with both *N*-linked and abundant acid-labile glycans. It appears to be the major protein secreted by *L. donovani* promastigotes during their growth *in vitro* (Bates and Dwyer, 1987) and it was also shown to be produced by amastigotes. Immunofluorescence results suggest that amastigotes secrete the acid phosphatase into the parasitophorous vacuole (Bates *et al.*, 1989). All species, with the exception of *L. major*, secrete an acid phosphatase into the culture medium (Lovelace and Gottlieb, 1986). Jaffe *et al.* (1990) demonstrated that LPG/PG and acid phosphatase share antigenic epitopes that are recognized by species specific monoclonal antibodies (mAbs) against *L. tropica* and *L. major*. The promastigote stage of *L. mexicana* secretes a phosphomonoesterase as extended filaments comprised of multiple units of the phosphoglycoprotein and non-covalently associated

proteophosphoglycans (Ilg *et al.*, 1991), while in *L. donovani* it is present as monomers and oligomers of the phosphoglycoprotein. It was suggested that the acid phosphatase, shown to be highly resistant to toxic oxygen metabolites, may promote survival of amastigotes within macrophages by dephosphorylating elements involved in lysosomal function and/or the oxidative killing mechanism (Bates and Dwyer, 1987). The proteophosphoglycans contain Ser/Thr-rich sequences representing targets for *O*-glycosylation (Wiese *et al.*, 1995; Ilg *et al.*, 1994).

2.3.2. Cysteine proteinases

Cysteine proteinases are thought to play an important role in the intracellular survival of the parasite. Homozygous null mutants for a *L. mexicana* cysteine proteinase gene (*lmcpb*) were produced by targeted gene disruption, and resulted in reduced infectivity to macrophages (Mottram *et al.*, 1996). Mutants were able to grow as axenic amastigotes, prompting Mottram *et al.* (1996) to suggest that the role of LmCPb may be in interacting with the host cell. *Lmcpb* is a stage regulated gene with high mRNA levels in amastigotes and very little mRNA in promastigotes, and its proteinase activity follows the same stage-regulated pattern (reviewed in Mottram *et al.*, 1996). This developmental regulation suggests that the enzyme may have a crucial role to play in survival of the parasite within the macrophage.

2.3.3. A2

An amastigote stage-specific gene family termed A2 was characterized in *L. donovani* and was found to code for a family of proteins ranging from 45- to 100-kDa (Zhang *et al.*, 1996; Charest and Matlashewski, 1994). The differential screening of an amastigote cDNA library identified five A2 transcripts closely related which were only expressed in the amastigote stage of *L. donovani*. A2 transcripts were also found to be present in amastigote-infected macrophages, and A2 expression could be induced in cultured promastigotes switched to conditions that mimic the phagolysosomal environment of macrophages (pH 4.5, 37C) (Charest and Matlashewski, 1994). A2 transcript accumulation in amastigotes was shown to be regulated post-transcriptionally at the level of RNA stability. Transfection assays, using a reporter gene flanked by A2 non coding regions, revealed that the 3'-untranslated region (3'-UTR) of the A2 mRNA was involved in differential accumulation (Charest *et al.*, 1996). Reporter genes targeted into the A2 locus could also be differentially expressed in the amastigote stage (Charest *et al.*, 1996).

The A2 proteins are comprised predominantly of a highly conserved repetitive element - a stretch of 10 amino acids repeated up to 45 times - and the A2 locus was estimated to be represented by at least seven genes which differ with respect to the length of the repeat region (Charest *et al.*, 1996). The repetitive sequence was found to bear striking homology (80%) with the repeated unit of an *S* antigen of *Plasmodium falciparum*, the causative agent of malaria. Another

gene was found to be closely associated to A2 on the *L. donovani* genome. Copies of this gene, which was named A2rel, alternate head to tail with copies of A2 to form a tandem repeat. In contrast to A2 mRNA, expression of A2rel transcripts remained constant in parasites that were switched to conditions known to induce A2 expression (Charest et al., 1996). The inhibition of A2 proteins, by blocking A2 mRNA expression with antisense RNA, led to A2-deficient amastigotes that were viable in culture but had difficulty multiplying in macrophages *in vitro* and *in vivo* (Zhang and Matlashewski, 1997). A2 is thus required for *L. donovani* survival in its mammalian host, and as such represents a virulence factor.

3. Other molecules expressed on the *Leishmania* surface

The importance of parasite membrane components in the uptake of the parasite by macrophages has been described in the previous sections. Of equal importance, is the role surface membrane molecules play in metabolism and nutrient acquisition.

3.1. Phosphomonoesterase activities

Leishmania donovani promastigotes were shown, by Gottlieb and his colleagues, to possess three distinct phosphomonoesterase activities localized on the external surface of the plasma membrane: acid phosphatase, 3'-nucleotidase and 5'-nucleotidase (Dwyer and Gottlieb, 1984; Gottlieb and Dwyer, 1983; Gottlieb and Dwyer, 1981).

All three phosphomonoesterase activities also occur on the surface of *L. mexicana* (Hassan and Coombs, 1987). It was suggested that the 5'- and 3'-nucleotidases may represent initiating components of the organism's purine salvage pathway (reviewed in Gottlieb, 1989) by rendering nucleotides available from the host into a nucleoside suitable for membrane transport. *Leishmania*, like other trypanosomatid flagellates, are incapable of synthesizing the purine ring *de novo* and must therefore depend on the host from which they salvage their purines (Hammond and Gutteridge, 1984).

5'-nucleotidase activity has been reported in a wide variety of prokaryotic and eukaryotic cell types (reviewed in Gottlieb and Dwyer, 1983) while 3'-nucleotidase activity has been observed in *Leishmania* spp., *Trypanosoma* spp. and *Crithidia luciliae*, but has not been demonstrated in mammalian cells (Gottlieb *et al.*, 1988; Hassan and Coombs, 1987; Gottlieb *et al.*, 1986; Dwyer and Gottlieb, 1984).

3.1.2. 3'-Nucleotidase/nuclease

The *L. donovani* 3'-nucleotidase was also shown to be a bi-functional enzyme with both nuclease and nucleotidase hydrolytic activities (Gottlieb and Zlotnick, 1987, as reviewed in Gottlieb, 1989). The 3'nucleotidase/nuclease (3'NT/Nu) is most active at pH 8.5 when assayed as a nucleotidase, and at pH 6.0 as a nuclease, and it has a preference for RNA over DNA (Gottlieb, 1989; Gottlieb and Dwyer, 1983). The *L. donovani* surface membrane 3'NT/Nu was shown to be

a glycoprotein containing exposed mannose residues (Sacci *et al.*, 1990) and this property was used in order to isolate the 43 kDa enzyme by affinity purification and proceed to its microsequencing (Debrabant *et al.*, 1995). The gene encoding the unique *L. donovani* 3'NT/Nu was isolated and characterized, and a relationship was established between the 43 kDa protein and class I single-strand-specific nucleases of fungi (Debrabant *et al.*, 1995). The presence of a homologous 3'-NT/Nu was also demonstrated in *Crithidia luciliae* and was found to dramatically increase (1000-fold) when the cells were starved for purines (Alleman and Gottlieb, 1996; Alleman and Gottlieb, 1990; Gottlieb *et al.*, 1988). Sacci *et al.* (1990) also demonstrated an increase in the level of 3'NT/Nu activity in *L. donovani* cultured in conditions of purine starvation, which on the growth curve would correspond to cells in the lag and early logarithmic phase. Alleman and Gottlieb (1996) suggest that the increased rates of purine uptake observed upon purine starvation may be due to the induction of a surface membrane transporter.

3.2. Purine transporters

The purine ring is available to the parasite as nucleobases, nucleosides, charged nucleotides or polymers in nucleic acids, and the acquisition of the purine ring depends on the transport across the parasite plasma membrane (reviewed in Cohn and Gottlieb, 1997). Although no purine transporter has been isolated in trypanosomatids, *in vivo* studies suggest that the parasite possesses transport systems to salvage purine precursors from the

environment. Depending on the study, there may be two to three transporters: Iovannisci *et al.* (1984) suggested that *L. donovani* possess a inosine/guanosine transporter and an adenosine transporter, while Baer *et al.* (1992) concluded in their study on *L. donovani* and *L. major* strains that there may be three potential transporters because of the differing adenosine transport profiles observed from strain to strain.

4. Host response to infection

4.1. Visceral leishmaniasis as an opportunistic disease

Visceral leishmaniasis (VL) is considered an opportunistic disease of immunosuppressed individuals, mainly AIDS patients. Reports of VL among HIV infected patients have increased in Mediterranian countries where *L. donovani* is endemic, but also in regions where it is not, such as Germany (Albrecht *et al.*, 1996). In immunocompromised patients, parasites normally causing cutaneous lesions may cause VL (Rosenthal *et al.*, 1995; Gramiccia *et al.*, 1992). This visceralization of parasites which are normally confined to the skin, may be due to the parasite's evasion of the dermal immunological barrier resulting from impaired T-cell function (Rosenthal *et al.*, 1995). Extrahaematopoietic localizations of amastigotes are also frequently encountered in HIV-infected patients where parasites are found at unusual sites, such as in the digestive tract, the skin and the lungs (Albrecht *et al.*, 1996; Rosenthal *et al.*, 1995). This suggests that the parasite lodges in the macrophage in

order to evade the immune system. VL is often the first severe infection to be diagnosed in AIDS patients, prompting Rosenthal *et al.* (1995) to suggest that VL should be recognized as an AIDS defining criterion in certain regions. However, VL is difficult to diagnose in patients infected with HIV because not all of the patients show the classic associated clinical features of VL (fever, splenomegaly, hepatomegaly). Classic serological methods of diagnosis are also hampered because of the difficulty in detecting anti-leishmanial antibodies.

4.2. Immune responses to *Leishmania* infections

In human infection with *L. donovani*, T lymphocytes are essential for defence against the parasite. The course of the infection is determined by lymphokine patterns produced by *Leishmania*-reactive $CD4^+$ T cells (Coffman *et al.*, 1991). This explains why patients with T-cell defects, such as those infected with HIV, are highly susceptible to an *L. donovani* infection. Recovery from leishmaniasis depends on the development of parasite-specific cellular immunity and the ability to generate sufficient amounts of Th1-type cytokines (IFN-γ, IL-2, IL-12) which lead to the activation of macrophages, whereas the predominance of Th2-type cytokines (IL-4, IL-10) is linked to progressive disease (reviewed in Albrecht *et al.*, 1996). The association between VL and HIV infections is a logical consequence of cellular immunodeficiency: T-cell depletion

and cytokine dysregulation lead to an impaired Th1-type cytokine pattern (Albrecht *et al.*, 1996).

Miralles *et al.* (1994) demonstrate that in BALB/c mice capable of controlling an *L. donovani* infection, a Th2 cell response is overshadowed by a Th1 cell-associated response. BALB/c mice are initially susceptible to experimental infection with *L. donovani*, but then develop a T-cell-dependent immune response which leads to resolution of infection (Murray *et al.*, 1991). Control of an *L. major* infection in mice is associated with the appearance of Th1 cells which contain transcripts of IFN-γ and IL-2. BALB/c mice, which are susceptible to an *L. major* infection, demonstrate expansion of Th2 cells. IFN-γ is considered to play a critical role in *L. major* infection, and failure to sustain its production in BALB/c mice after infection prevents the development of a successful host response (Tobin *et al.*, 1993). However, Tobin *et al.* (1993) and Sadick *et al.* (1990) suggest, based on studies consisting of the addition of IFN-γ in susceptible mice, that although this cytokine is necessary, it is not sufficient to modulate the course of an infection. Additional cytokines may be necessary to reverse the establishment of a Th2 response and the progression of the disease.

The dichotomy between Th1 and Th2 subsets is not as clear in the human system as in the murine model (Kemp *et al.*, 1993). However, Kemp *et al.* (1993) demonstrate that there are *Leishmania*-reactive T-cells with Th1- and Th2-type lymphokine patterns that coexist in humans after *Leishmania* infections. Individuals who, after an *L.*

donovani infection, remain asymptomatic and develop an infection which resolves without treatment, appear to have a response associated with the production of IFN-γ. In contrast, individuals who go on to develop active visceral leishmaniasis have an enhanced induction of IL-10 and/or IL-4 and suppressed IFN-γ secretion (Carvalho *et al.*, 1992; Carvalho *et al.*, 1985).

4.3. Genetic control of *Leishmania* infection

The outcome of disease is largely under genetic control. Innate resistance and susceptibility to *L. major* and *L. donovani* infections in mice is controlled by a single gene (*Lsh*) present in two allelic forms. *In vitro* experiments have demonstrated that *Lsh* is expressed by the mature tissue macrophage (Olivier and Tanner, 1987). *Lsh* is referred to as the "macrophage resistance gene" and it is identical to the genes designated *Ity* and *Bcg*, which control *Salmonella typhirium* and *Mycobacterium bovis* infections, respectively (Blackwell *et al.*, 1994; Vidal *et al.*, 1993; Alexander and Russell, 1992; Hughes, 1988). A candidate gene (*Nramp1*) for *Bcg* was isolated by positional cloning and it is thought to play a key role in the early phase of macrophage infection (Vidal *et al.*, 1993; Vidal *et al.*, 1995). The loss of natural resistance to infection of *Nramp1* null mutants was studied and results demonstrated that *Nramp1*, *Bcg*, *Ity*, and *Lsh* are the same gene (Vidal *et al.*, 1995). Nramp, the natural resistance-associated macrophage protein, is a family of integral membrane proteins which

includes Nramp1 (Gruenheid *et al.*, 1997; Cellier *et al.*, 1995; Vidal *et al.*, 1993).

5. Diagnosis of visceral leishmaniasis

Differential diagnosis of the various forms of the leishmanial diseases is hampered by the signs and symptoms that may resemble other diseases. Conclusive diagnosis usually relies on detection and culture of the parasite from biopsy samples, procedures requiring laboratory equipment and expertise in countries where facilities and resources are often limited. Indirect detection methods, based on serodiagnostic tests, have proven more convenient. These include immunodiagnosis based on the detection, in patient serum, of antibodies against the parasite antigens, or of the antigens present in circulating immune complexes.

5.1. Detection of antibodies

5.1.1. Diagnostic techniques

Many diagnostic techniques have been developed for antibody detection, the most popular being the direct agglutination test (DAT), the indirect fluorescent antibody test (IFAT), and the enzyme-linked immunosorbent assay (ELISA). The DAT consists of incubating sera with a parasite suspension and detecting agglutinating antibodies

against *Leishmania* (Sinha and Sehgal, 1994; Harith *et al.*, 1986). Since the parasites have been previously stained with Coomassie brilliant blue, the titre in the test serum is read as one dilution before that which yields a clear blue spot similar to the one generated by the negative control. Harith *et al.* (1986) developed a DAT for the diagnosis of VL and set the cutoff titre of seropositivity at 1:1600. The DAT is economical and easy to perform, however it does not allow to distinguish between early infection and after cure because of titre levels which may remain high for years after cure (Hailu, 1990). The IFAT detects circulating antibodies in patients with VL using washed formalin or acetone-fixed promastigotes as the antigen and fluorescin labelled anti-human IgG (reviewed in Kar, 1995). This test is rather ineffective with cutaneous leishmaniasis because of the low level of circulating antibodies. ELISA is more sensitive than DAT using crude promastigote antigens; in a study by Fargeas *et al.* (1996), 80% of sera from patients with kala-azar were ELISA positive using crude antigen as compared to 46% who were DAT positive. There are, however, problems of cross-reactivity and improved specificity is achieved by the use of purified and recombinant leishmnanial antigens instead of the whole organism (Fargeas *et al.*, 1996; Tebourski *et al.*, 1994; Okong'o-Odera *et al.*, 1993b; Burns *et al.*, 1993; Rachamim *et al.*, 1991; Jaffe and Zalis, 1988a, 1988b). Reed *et al* (1990) have also improved specificity of the ELISA by using enzyme-linked protein A or protein G instead of IgG. The competitive-ELISA (C-ELISA), which uses monoclonal antibodies to compete with *Leishmania* antibodies present in patient serum, was developed specifically for the serodiagnosis of human VL

(Jaffe and McMahon-Pratt, 1987). Antibodies against two *L. donovani* pure protein antigens of 70 kDa and dp72 kDa could be detected by C-ELISA at least one month before detection by ELISA and IFAT (Abranches *et al.*, 1991). The 70 kDa protein was also successfully used as a diagnostic antigen in VL serodiagnosis by Jaffe and McMahon-Pratt (1987).

DAT has been the diagnostic method of choice due to its applicability in field studies (Harith et al., 1987). It is still widely used, despite its lack of specificity caused by cross-reactive antigens. The DAT was shown to be particularly suitable for the large-scale screening of canine VL in Corsica where trypanosomiasis does not occur, eliminating the presence of those cross-reacting antibodies (Neogy *et al.*, 1992). Throughout the Mediterranean basin, *L. donovani infantum*, the causal agent of Mediterranean visceral leishmaniasis (MVL), is endemic and its reservoir's predominant hosts are dogs (Da Costa *et al.*, 1996). While IFAT has been used widely to diagnose VL, it is not efficient for the analysis of numerous samples. ELISA is more rapid and simpler, as was shown for the serodiagnosis of canine VL (Rachamim *et al.*, 1991). IFAT involves titration of each serum sample and microscopical examination, while ELISA requires a single dilution of the serum samples analysed in a microplate reader.

While diagnostic methods based on the detection of antileishmanial antibodies in serum are more sensitive than diagnosis of VL based on microscopy and culture (Fargeas *et al.*, 1996), these methods still suffer from cross-reactivity, and the lack of distinction between past

and present infection and between clinical and asymptomatic conditions. Okong'o-Odera *et al* (1993a) have developed an ELISA which detects antibodies against gp63 and permits the distinction between ongoing and previous *L. donovani* infections. Fargeas *et al.* (1996) have used five peptides derived from an *L. donovani* surface protein related to the gp63 multigene family to determine antileishmania antibodies in patient sera. These peptides, conjugated to human serum albumin, gave ELISAs that were more specific but less sensitive than ELISAs conducted with crude promastigote antigen. Interestingly, specificity and sensitivity could be increased by using a mixture of two conjugates (Fargeas *et al.*, 1996). In a study by Shreffler *et al.* (1993), a large number of VL patients had high levels of gp63-specific antibodies, while detectable levels of these same antibodies were low in cutaneous and mucocutaneous leishmaniases. This is due to higher IgG responses in VL patients as compared to patients with CL and other infections (Elassad *et al.*, 1994; Scott *et al.*, 1991).

5.1.2. Application of immunoblotting in diagnosis

The immunoblotting technique, or Western blot, is used in diagnosis by separating parasite antigens on a polyacrylamide gel, transferring them to a nitrocellulose membrane, and visualizing specific bands corresponding to antibody reactivity with parasite antigens (Da Costa *et al.*, 1996). Rolland-Burger *et al.* (1991), Mary *et al.* (1992) and Cardenosa *et al.* (1995) have looked at the immunoblot patterns of the humoral response to *L. d. infantum* antigens in order to identify

specific antigens that correspond to active disease or asymptomatic infection. While sera from patients with VL recognized many antigens, Mary *et al.* (1992) determined that the 14-16 kDa antigens had the greatest specificity (98%) and sensitivity (100%). Rolland-Burger *et al* (1991) described four other *L. d. infantum* antigens (75-, 90-, 94-, and 115-kDa) that appeared highly specific, while they did not recognize a significant reaction with the 14-16 kDa region. Cardenosa *et al.* (1995) noticed that the low molecular mass region, between 14 and 40 kDa, was very VL specific, and they described bands of 40 kDa (90% sensitivity), 33 kDa (79%), and 17 kDa (79%), while other bands had a sensitivity under 60%. A lower specificity due to cross-reactivity with trypanosomiasis was however observed. The 17 kDa band tended to disappear in treated patients, prompting Cardenosa *et al.* (1995) to suggest this band could be used for the determination of the clinical evolution of VL. Through immunoblotting, Tebourski *et al.* (1994) identified an immunodominant p32 antigen present in the membrane of *L. d. infantum* parasites. It was found that 95% of sera from patients with MVL recognized the p32 antigen by immunoblot. Cross-reactivity with trypanosomiasis was very strong, with 58% of patients with Chagas' disease that cross-reacted (Tebourski *et al.*, 1994). However, Chagas disease and MVL are not co-endemic diseases, which limits the problem of cross-reactivity.

An *L. chagasi* gene was cloned, which was found to encode an immunodominant protein containing a repetitive epitope closely conserved between the Brazilian *L. chagasi,* and *L. donovani* from

Sudan (Burns *et al.* 1993) and India (Singh *et al*, 1995). rK39 is the recombinant product of the 39 amino acids repeat encoded by a kinesin-related gene of *L. donovani chagasi* which codes for a 230 kDa antigen, LcKin, found predominantly in amastigotes (Burns *et al.*, 1993). The presence of anti-rK39 antibodies was shown to be 100% correlated with VL and no cross-reactivity was observed with other leishmanial diseases or trypanosomiasis (Badaro *et al.*, 1996; Singh *et al.*, 1995; Burns *et al.*, 1993). ELISAs have demonstrated that chemotherapy had no effect on the anti-rK39 IgG titres unless the parasite burden in patients decreased (Singh *et al.*,1995). Another study has detected antibodies against rK39 in patients with subclinical infections a few months before the disease became evident, while patients with self-healing infections had undetectable levels of anti-rK39 antibodies (Badaro *et al.*, 1996). Since rK39 is found predominantly in amastigotes, and this form is responsible for pathology, high titers of anti-K39 antibodies were detected in patients with kala-azar and not in patients with subclinical infections (Badaro *et al.*, 1996). rK39 was thus shown to be a potent amastigote antigen shared by members of the *L. donovani* complex (Burns *et al.*, 1993).

5.1.3. Monoclonal antibodies

Specific monoclonal antibodies are used with Western blot and competitive-ELISA to detect circulating anti-leishmanial antibodies (Kar, 1995). Jaffe *et al.* (1984) have produced 16 species-specific monoclonal antibodies against membranes of *L. donovani* which

produce no cross-reactions with other *Leishmania* species or with *T. cruzi*. The antigens recognized by the monoclonal antibodies D-2, D-10 and D-13 were also recognized by human sera from kala-azar patients (Jaffe *et al.*, 1988b). D-13 in particular, evaluated using a C-ELISA, identifies an epitope that is species-specific and which has serodiagnostic value (White and McMahon-Pratt, 1988; Jaffe and Zalis, 1988b). More recently, it has been determined that D-2 is more species-specific than D-13 (White and Hanhman, 1991). Monoclonal antibodies have also been used in the immunoprecipitation of radiolabeled antigens or by ELISA, for the differentiation of species and subspecies, and the taxonomic classification of *Leishmania* (Sacks *et al.*, 1995a; Jaffe and McMahon-Pratt, 1983; Handman and Curtis, 1982; Handman and Hocking, 1982; McMahon-Pratt *et al.*, 1982).

5.2. Detection of circulating antigens

While rising antibody titres are significant, in many cases VL infection has a long prepatent period and diagnosis cannot be established until antibody levels are high enough. Low humoral responses can also occur, as is the case for immunocompromised individuals due to HIV infection. New diagnostic methods based on the detection of parasite DNA have been developed. These techniques are based on the hybridization of a specific DNA sequence with its complementary DNA in the parasite under test (reviewed in Barker, 1989). The polymerase chain reaction (PCR) is a valuable tool in the detection of parasite DNA or RNA in patient blood as circulating

parasites may be common in very early infection (Adhya *et al.*, 1995). The PCR technique was used to amplify kinetoplast DNA minicircles (Smyth *et al.*, 1992; Rogers *et al.*, 1990), genomic repeats specific for all causative agents of visceral leishmaniasis (Piarroux *et al.*, 1994), and the small subunit rRNA-coding region of *L. donovani* (Costa *et al.*, 1996). Detection of product is done either by sizing after gel electrophoresis or hybridization with species-specific DNA probes (Adhya *et al.*, 1995; reviewed in Wilson, 1995).

Nuzum *et al.* (1995) diagnosed 90% of confirmed kala-azar cases using *Leishmania* minicircle kDNA as the basis for the PCR primers and affinity-based hybridization analysis. *Leishmania* are members of the Kinetoplastidae order, a group which possesses a unique DNA structure, called the kinetoplast. The kinetoplast DNA is comprised of maxicircles, present in 30-50 copies, and minicircles, present in 10,000-20,000 copies (Rodgers *et al.*, 1990). The kinetoplast DNA is amplified using well conserved sequences as primers and species-specific kinetoplast probes for hybridization (Nuzum *et al.*, 1995; Smyth *et al.*, 1992; Rodgers *et al.*, 1990). In the case of immunocrompromised patients, PCR-based diagnosis appeared more sensitive than direct examination of bone marrow aspirates and cultures, and serodiagnosis by ELISA (Piarroux *et al.*, 1994).

6. Genetics of *Leishmania* and other trypanosomatids

Trypanosomatids are characterized by the presence of the kinetoplast, a modified version of the mitochondrion, which contains a large amount of DNA localized close to the base of the flagellum and organized in maxi- and mini-circles (Pays, 1993). Members of the order Kinetoplastida are parasitic protozoa that diverged early in eukaryotic evolution (Sogin *et al.*, 1989) and they exhibit many unusual features of gene organization and expression (Soto *et al.*, 1993). These features include the presence of genes organized in tandem arrays, polycistronic transcription of multicopy gene families, post-transcriptional processing by *trans*-splicing and RNA editing, and antigenic variation by DNA rearrangement (as reviewed in Flinn and Smith, 1992; Simpson and Shaw, 1989; Muhich and Boothroyd, 1988; Van der Ploeg, 1987; Borst, 1986). Trypanosomatids appear to be predominantly asexual and expand clonally, by uniparental reproduction (Tibayrenc *et al.*, 1991; Beverley *et al.*, 1988). The current general view considers *Leishmania*, and trypanosomatids in general, as diploid organisms (Pays, 1993), although some chromosomes were shown to be aneuploid (Cruz *et al.*, 1993; Lighthall and Giannini, 1992).

6.1. Gene organization and expression

Protein-coding genes of trypanosomatids are generally clustered, as seen for the Lm16 locus of *L. major* (Flinn and Smith, 1992) and

variant surface glycoconjugate (VSG) genes in trypanosomes (reviewed in Borst, 1986), among others. Many gene copies are arranged in tandem arrays interspersed by a few hundred base pairs (reviewed in Pays, 1993). However, this amplification of genes does not necessarily lead to increased expression, as demonstrated for VSG expression in *T. brucei* (Pays, 1993). The *Leishmania* gp63 is also encoded, like the VSG, by a large gene family (Webb *et al.*, 1991). The high copy number of certain genes may be linked to the antigenic nature of their products. In trypanosomes, transcription is discontinuous with the successive expression of different gene copies in order to generate antigenic variation (Borst, 1986). In other trypanosomatids, such as *Leishmania*, the simultaneous expression of members of an antigen gene family leads to the expression of antigenic variety due to sequence polymorphism of the different encoded proteins (reviewed in Pays, 1993; Webb *et al.*, 1991).

Tubulin genes are also present in multiple copies arranged in tandem repeats. *L. enriettii* has approximately 30 copies of the α-tubulin gene per cell, contained in two allelic gene clusters (Curotto de LaFaille and Wirth, 1992). Tubulins are the major protein species in promastigotes, constituting more than 10% of the total cellular protein (Fong and Chang, 1981).

6.2. Regulation of gene expression

In trypanosomatids, mRNAs are formed by the processing of polycistronic pre-mRNAs by *trans*-splicing (reviewed in Clayton, 1992 and Agabian, 1990). The first evidence of multicistronic transcription of cellular genes in trypanosomatids was provided by Johnson *et al.* (1987). They demonstrated that regulation of gene expression occurs at the level of mRNA processing taking place in the intergenic regions of the pre-mRNAs. Two cleavages occur in the intergenic region where the pre-mRNA is processed at the 3' end by polyadenylation and at the 5' end of each gene by capping with a 39 nt spliced-leader (SL) sequence, or mini-exon (Borst, 1986; Miller *et al.*, 1986). These cleavages thus result in multiple mRNAs being generated from a single transcriptional unit (Johnson *et al*, 1987; Borst, 1986). Trypanosomatids do not have introns but instead mature mRNAs are generated by the 5' capping of all mRNAs with a unique segment. Originally thought to be a process restricted to trypanosomatids, it was found that this also occurs in nematodes, but only in 10-15% of their mRNAs (Ross *et al.*, 1995; Donelsen and Zeng, 1990). The SL sequence is transcribed as part of a unique small RNA of 135-140 nucleotides. The gene that encodes the SL RNA sequence in trypanosomes is repeated approximately 200 times in the genome (Muhich and Boothroyd, 1988; Murphy *et al.*, 1986). SL RNA and structural genes are transcribed independently and are then spliced in order to form mature mRNAs (Murphy *et al.*, 1986). SL addition occurs in *trans*, which differentiates it from splicing of nuclear mRNA introns in other eukaryotic systems where splicing occurs in *cis*. The

discontinuous transcription mechanism, where the first 35 nt of each message are not encoded contiguously with the body of the message, has been demonstrated in the synthesis of *Leishmania* α- and β-tubulin mRNAs (Landfear *et al.*, 1986).

While a consensus motif exists for the *trans*-splicing acceptor site, characterized by an AG acceptor site and an adjacent upstream pyrimidine-rich region (Curotto de Lafaille *et al.*, 1992), there is no consensus motif for polyadenylation (Borst, 1986). Polyadenylation occurs at a site within 1 kb upstream of the *trans*-splice acceptor site. LeBowitz *et al.* (1993) demonstrated, using the intergenic region separating the *L. major* DHFR-TS (dihydrofolate reductase-thymidylate synthase)-coding region from the adjacent 3' transcripts, that poly(A) and *trans*-splicing are coupled, and that the poly(A) site is determined by the location of the downstream splice acceptor site.

Because of the complex life-cycle of trypanosomatids, involving a switch from the extracellular to the intracellular mode of life, or in the case of African trypanosomes a switch from bloodstream to insect form, a complete switch in metabolism requires modulated gene expression. Genes showing different regulation are often found on the same transcriptional unit and regulation of mRNA level is determined post-transcriptionally (Charest *et al.*, 1996; Hotz *et al.*, 1995; Hug *et al.*, 1993).

The differential expression of the A2 gene in *L. donovani*, while its tandemly linked A2rel is expressed constitutively, represents a good

example of post-transcriptional regulation (Charest *et al.*, 1996). It was demonstrated that A2 3'-UTR sequences, and not 5' upstream sequences, were essential for differential expression by mediating an increase in transcript stability. Both the 3'-UTR and its correct processing by *trans*-splicing were shown to be necessary for the developmental expression of the reporter gene, and induction in transfected cells was triggered by a combination of pH and temperature shifts (Charest *et al.*, 1996).

VSG and procyclin expression in *T. brucei* represent also an example of differential gene expression. In procyclic trypanosomes VSG promoters are weakly active, whereas in bloodstream trypanosomes the activity of a promoter is upregulated up to 20-fold (Rudenko *et al.*, 1994). Transcriptional control cannot however account entirely for the stage-specific expression of the units. Upstream and downstream untranslated regions of *T. brucei* were assayed with reporter constructs and were found to influence gene expression levels (Hehl *et al.*, 1994; Hug *et al.*, 1993; Jefferies *et al.*, 1991). Teixeira *et al.* (1995) also showed that the 3'-UTR was involved in regulation of amastigote-specific transcripts in *T. cruzi.* The sequence of the 3'-UTR of the mRNAs is important in determining abundance of transcripts in trypanosomatids. This was initially demonstrated by Jefferies *et al.* (1991) where reporter activity was detected in the bloodstream form of *T. brucei* when the 3'-UTR of a VSG mRNA was added downstream of the reporter gene, but led to a reduction in activity in the procyclic form. It has been suggested that a consensus motif of 16 bases in the 3'-UTR of different procyclin mRNAs is

important in regulating expression (Hehl *et al.* 1994). Berberof *et al.* (1995) analysed the 3' end region that influences gene expression of VSG and procyclin in *T. brucei*, and they delimited a 97 nt region upstream of the polyadenylation site that was necessary to confer stage-specificity to gene expression.

No RNA pol II promoters have been reported in *Leishmania* (Curotto de Lafaille, 1992) thus the 3' intergenic region plays an essential role in the accumulation of transcripts, as was demonstrated for A2 mRNAs in *L. donovani* (Charest *et al.*, 1996).

6.3. Stable transfections

DNA transfection is applied to the study of molecular function. Expression vectors were developed using untranslated regions since upstream and downstream elements flanking coding regions in polycistronic transcripts were found to affect the relative abundance of genes. The importance of these sequences was demonstrated in the expression of foreign genes in trypanosomatids using transfected plasmid constructs. This was achieved in *Leishmania* by Laban and Wirth (1989) and in *Leptomonas* by Bellofatto and Cross (1989). These systems were based on the transient expression of the chloramphenicol acetyl-transferase (CAT) gene under the control of parasite-specific sequences (Bellofato and Cross, 1989; Laban and Wirth, 1989).

Later studies have focused on constructs derived from the circular R region (bearing the DHFR-TS gene) amplified in certain lines of methotrexate-resistant *L. major* (Coburn *et al.*, 1991; Kapler *et al.*, 1990; Lebowitz *et al.*, 1990). The R amplicon is composed of a 30 kb segment of chromosomal DNA that is rearranged and joined to form an extrachromosomal circular DNA (Beverley *et al.*, 1988). In the construction of the plasmid using the R amplicon, the coding region for the DHFR-TS was replaced by the neomycin resistance gene (*neo*), which confers resistance to the aminoglycoside antibiotic geneticin (G418) (Kapler *et al.*, 1990), generating the pR-NEO plasmid which gave a correctly processed hybrid mRNA within *L. major*. Lebowitz *et al.* (1990) generated smaller derivatives of the pR-NEO, such as the pX vector which contains only 2.3 kb of *L. major* DNA and a polylinker site for the introduction of foreign genes. This vector was used to express the GP46/M.2 protein of *L. amazonensis* in *L. major* (LeBowitz *et al.*, 1990). Coburn *et al.* (1991) demonstrated that both the pR-NEO and pX vectors could be introduced into a wide range of *Leishmania* species.

Bacterial vectors were also designed with intergenic regions of the α-tubulin cluster from *L. enriettii* flanking the *neo* gene. Drug-resistant cells expressing the *neo* gene were observed, and the neo mRNA was processed using the signals present in the α-tubulin gene (Laban *et al.*, 1990). Kelly *et al.* (1992) described the construction of *T. cruzi* expression vectors that use fragments derived from flanking regions of the gGAPDH genes (glycosomal enzyme glyceraldehyde 3-phosphate dehydrogenase). They transfected *L. mexicana*

promastigotes with the pTGAN vector demonstrating that *Leishmania* are capable of expressing genes flanked by *T. cruzi* sequences. The *neo* gene was incorporated as a selectable marker. Vectors have also been developed which do not contain any *Leishmania* sequences, but which carry an artificial *trans*-splicing acceptor site flanking drug resistance genes (Papadopoulou *et al.*, 1994).

Studies have demonstrated that molecular constructs can be introduced into an infective line of *L. donovani* and can be stably maintained during the amastigote stage. The ability to introduce DNA constructs into an infective strain, and pass them through the amastigote stage, allows the exploration of expression of many parasite genes in the infectious cycle (Coburn *et al.*, 1991). Charest and colleagues (Charest *et al.*, 1996; Zhang *et al.*, 1996) have used flanking regions of the A2 locus for the developmental expression of marker genes in the amastigote stage. An A2 antisense plasmid has been developed in order to inhibit the expression of A2 mRNA thereby generating A2-deficient amastigotes which display loss of virulence (Zhang and Matlashewski, 1997).

Vectors have also been designed for the expression of negative selection markers. Lebowitz *et al.* (1992) tested the thymidine kinase gene (*tk*) of herpes simplex virus-1 in *L. major*. Expression of the *tk* gene, combined on a vector with the *neo* or hygromycin (*hyg*) resistance genes for positive selection, resulted in sensitivity of the *Leishmania* cells to ganciclovir. The expression of toxic products may be informative in some experimental situations by allowing the

overexpression of genes normally necessary only in low quantities, or by causing the expression of a protein in an inappropriate life stage (Biebinger *et al.*, 1997). Wirtz and Clayton (1995) and Bienbinger *et al.* (1997) describe the use of an inducible expression vector in trypanosomes. Their system is based on an exogenous control which depends on binding of a tetracycline-inducible repressor to operators downstream of the PARP (Procyclic Acidic Repetitive protein, or procyclin) promoter (Wirtz and Clayton, 1992). A vector, useful for the inducible expression of toxic products, has been recently designed containing two promoters: a tetracycline-inducible promoter for the expression of toxic products, and a constitutively active promoter to drive expression of the selectable marker (Biebinger *et al.*, 1997). However, expression which was uninduced by tetracycline appeared to be influenced by the 3'UTR. To test this, a VSG 3'UTR was used in the bloodstream form of *T. brucei* and resulted in induction of higher expression. Since no RNA pol II promoters have been identified in *Leishmania* (Curotto de Lafaille *et al.*, 1992), expression systems must then depend on intergenic regions to regulate the specific expression of foreign genes (Charest *et al.*, 1996).

6.4. Gene Targeting

Altering of the genome by homologous recombination is also a powerful tool for testing gene function and gene expression

(Capecchi, 1989). DNA transfection techniques permit the engineering of trypanosomatid parasites that can overexpress or lack a specific gene product (Cruz *et al.*, 1993; 1991). This approach consists of replacing genes with selectable markers, such as the *neo* and *hyg* genes, although it is limited to genes whose function is not essential during *in vitro* culture. The process, using two independent selectable markers in successive rounds to replace both alleles of an endogenous gene, is called double-gene targeting (Cruz *et al.*, 1991).

Since *Leishmania* is a functionally asexual diploid, both alleles must be modified prior to functional testing. Using knockouts that were conditionally lethal, Cruz *et al.* (1991) were able to develop a method for engineering conditionally-viable *Leishmania* by the targeted deletion of essential metabolic genes, whereby producing safe attenuated immunizing strains. Fragments of the flanking regions of the *dhfr-ts* locus have also been successfully used in order to integrate the *neo* gene into the genome of *L. major* (Cruz and Beverley, 1990). The use of a second selectable marker, such as the *hyg* gene, allowed the creation of null mutants at the *dhfr-ts* locus (Cruz *et al.*, 1991). This was achieved by two rounds of gene targeting, first with *neo*-containing and then *hyg*-containing targeting vectors. Souza *et al.* (1994) applied the concept of gene targeting to disrupt both alleles of a single-copy gene encoding a proteinase. Curotto de Lafaille and Wirth (1992) have also used double-gene targeting with *neo* and *hyg* for the complete removal of the α-tubulin gene cluster from the *L. enriettii* genome.

Double-gene targeting has also been performed on infective *Leishmania* for the development of appropriate vaccine strains. Cruz *et al.* (1993) have attempted to generate monozygous *dhfr-ts* knockouts in virulent *L. major*. Despite the success of the double gene targeting, they were unable to obtain the desired mutants. They were unable to obtain the complete inactivation of the *dhfr-ts* locus in virulent *L. major* by methods that were successful in attenuated lines: the virulent *Leishmania* seemed to always retain one or more chromosomes containing *dhfr-ts*. The authors hypothesized that if a gene is nonessential, a second round of targeting yields the desired homozygous knockout, whereas if the gene is essential, homozygous knockouts are lethal. However, *Leishmania*'s ability to undergo changes in chromosome number permits the recovery of cells simultaneously bearing wild type and both planned replacement chromosomes. This could potentially be a diagnostic criteria for essential gene function (Cruz *et al.*, 1993).

CHAPTER 2

Antibody Response Against a *Leishmania donovani* Amastigote-Stage-Specific Protein in Patients With Visceral Leishmaniasis

(MANUSCRIPT I)

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ABSTRACT

The antibody response against an amastigote-specific protein (A2) from *Leishmania donovani* was investigated. Sera from patients with trypanosomiasis and various forms of leishmaniasis were screened for anti-A2 antibodies. Sera from patients infected only with *L. donovani* or *L. mexicana* specifically recognized the A2 recombinant protein. These results were consistent with karyotype analyses which revealed that the A2 gene is conserved in *L. donovani* and *L. mexicana* strains. The potential of this antigen in diagnosis was further explored by screening a series of sera obtained from patients in regions of the Sudan and India where *L. donovani* is endemic. The prevalence of anti-A2 antibodies was determined by Western blotting for all samples. Enzyme-linked immunosorbent assay (ELISA) and an immunoprecipitation assay were also perfomed on some of the samples. Anti-A2 antibodies were detected by ELISA in 82 and 60% of the samples from individuals with active visceral leishmaniasis (kala-azar) from the Sudan and India, respectively, while the immunoprecipitation assay detected the antibodies in 92% of the samples from India. These data suggest that the A2 protein may be a useful diagnostic antigen for visceral leishmaniasis.

INTRODUCTION

Leishmania species are responsible for a wide spectrum of diseases, including self-healing skin ulcers (cutaneous leishmaniasis due to species of the *L. tropica* and *L. mexicana* complexes), mutilating lesions of the oronasal-pharyngeal mucosa (mucocutaneous leishmaniasis due to *L. braziliensis*), and fatal visceral infections (visceral leishmaniasis due to species of the *L. donovani* complex). Leishmaniasis is considered by the World Health Organization one of the six major tropical diseases of developing countries (WHO, 1993). *Leishmania* is a dimorphic protozoan which exists as a flagellated promastigote in the sandfly vector and as an intracellular amastigote in the mammalian host. The cellular transformation of the *Leishmania* parasite into an amastigote occurs within the phagolysosomal compartment of host macrophages, where it also multiplies. It is the amastigote stage which is responsible for the pathology in susceptible vertebrate hosts (for a review, see Molyneux and Killick-Kendrick, 1987).

We have previously isolated and characterized a gene, termed A2, which is specifically expressed in the amastigote stage of *L. donovani*, the causal agent of visceral leishmaniasis (Charest & Matlashewski, 1994; Charest *et al.*, 1996). We have also identified the A2 protein and shown that it is specifically expressed at high levels in amastigotes but not in promastigotes (Zhang *et al.*, 1996). The A2 gene is one of the few amastigote-stage-specific genes identified in *Leishmania* (Charest and Matlashewski, 1994).

The A2 gene product is composed mostly of a highly conserved repetitive element which has identity with an S antigen of *Plasmodium falciparum* (Charest and Matlashewski, 1994). The A2 locus is comprised of at least seven genes, which differ with respect to the lengths of sequences encoding the repeat peptide unit (Charest *et al.*, 1996; Zhang *et al.*, 1996). A2 proteins range in size from 45 to approximately 100 kDa (Zhang *et al.*, 1996) and were shown to be recognized by serum from a patient with visceral leishmaniasis (Charest and Matlashewski, 1994). Since A2 is amastigote-stage-specific and is immunogenic (Charest and Matlashewski, 1994), we investigated its potential as a diagnostic antigen.

Diagnosis of visceral leishmaniasis cannot be made solely on the basis of clinical signs and symptoms because of its resemblance to other causes of febrile splenomegaly such as malaria, typhoid fever, and tuberculosis, to name a few. Initial assessment based on symptoms is confirmed through culture of parasites from aspirates of spleen, bone marrow or lymph nodes. Methods used for the diagnosis of visceral leishmaniasis may also rely on immunological techniques which detect circulating antibodies. Serological test procedures include the direct agglutination test, which involves the detection of agglutinating antibodies against *Leishmania* (Harith *et al.*, 1986; Sinha and Sehgal, 1994); the immunofluorescence-antibody test (IFAT), with whole organisms used as antigen; the enzyme-linked immunosorbent assay (ELISA); and PCR. However, immunodiagnostic methods using whole parasites as the source of antigen are often limited by the problem of cross-reactivity between species

(reviewed in Kar, 1995). Thus, there is a need for specific antigens in diagnostic tests, particularly in the case of visceral leishmaniasis.

We report here an evaluation of patient antibody responses against the A2 protein by screening sera with a recombinant A2 protein fused to glutathione *S*-transferase (GST) in Western blot assays and ELISAs. We have also combined immunoprecipitation and Western blot analyses to screen sera. We present results which show that A2 may be a valuable diagnostic antigen for serodiagnosis of visceral leishmaniasis.

MATERIALS & METHODS

Western Blot Analyses

An A2-GST fusion protein that lacks the putative N-terminal signal sequence was produced from the pGEX-2T/A2R vector as previously described by Zhang *et al.* (1996). Recombinant protein expression was induced from the *lac* promoter by addition of IPTG (isopropyl-ß-D-thiogalactopyranoside) (Promega, Canada). Total proteins from lysed *Escherichia coli* cells were loaded on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel. Western blotting was performed with an anti-A2 monoclonal antibody (C9) (Zhang *et al*, 1996) or human immune serum at a 1:250 dilution as first antibody. A biotinylated goat anti-mouse immunoglobulin G (IgG) Fc (for the monoclonal antibody) or anti-human IgG Fc (Gibco BRL, Canada) was used as the second antibody. Bound antibodies were detected with streptavidin-conjugated horseradish peroxidase (Amersham, Canada) and 3,3'-diaminobenzidine (Sigma, Canada) in a 0.03% H_2O_2 solution.

Enzyme-Linked Immunosorbant Assay (ELISA)

The A2-GST fusion protein produced in *E. coli* cells was affinity purified according to the method described by Smith and Johnson (1988). An overnight culture of *E. coli* cells transformed with pGEX-2T/A2R was diluted 1:100 in 800 ml fresh medium and grown at 37°C for three hours, at which point protein expression was induced by addition of IPTG to a 1mM concentration. Incubation was

prolonged for 2 h. The cells were pelleted and resuspended in a 1/100 culture volume of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The cells were lysed on ice by mild sonication (four times for 20 s each) and centrifuged at 13 000 X *g* for 20 min at 4°C. The supernatants were kept and 1 volume of 50% glutathione-Sepharose beads (Pharmacia Biotech, Canada) was added; adsorption was complete after 1 h of mild agitation at room temperature. After adsorption, the beads were collected by a 500 X *g* spin for 30 s and washed 5 or 6 times with 10 volumes of PBS. Elution of the protein was performed by incubating a 10 mM solution of free reduced glutathione in 50 mM of Tris-HCL (pH 8.0) for 30 s at room temperature.

Flat-bottomed microtiter immunoassay plates (Immulon4; Dynatech Laboratories) were coated overnight at 4°C with 0.5 µg of A2-GST fusion protein or the same amount of control GST protein in 100 µl of PBS. The coating solution was flicked out of the plates (no washing), and free binding sites were blocked with 200 µl of 5% milk in PBS containing 0.05% Tween 20 (T-PBS) for 1 hr at 37°C. The plates were then washed three times in T-PBS. Sera diluted at 1:250 in 1% bovine serum albumin and T-PBS were added in 100-µl aliquots with duplicates for each sample. The plates were incubated for 1 hr at 37°C. After three washes, horseradish peroxidase-conjugated goat anti-human IgG (Biorad, Canada) diluted 1:2000 in 1% bovine serum albumin-T-PBS was added in 100-µl aliquots. The plates were washed three times. Finally, ABTS [2,2'-azinobis(3-ethylbenzthialinesulfonic acid] substrate diluted in citrate buffer and

activated with 0.01% (vol/vol) H_2O_2 was added. The reaction was left to proceed 30 min and then stopped by addition of 10 µl of a 10% SDS solution per well. The optical density of the reaction mixture was measured at 405 nm in an ELISA reader (Microplate autoreader, Bio-Tek Instruments). A reference-positive serum was used in all plates. The lower limit of positivity (cut off) was determined by the mean of negative controls plus three standard deviations.

Immunoprecipitation Analysis

L. donovani Sudanese strain 1S2D cells engineered to overexpress the A2 protein were grown at 37°C, pH 5.5, in RPMI containing 20% fetal calf serum and G418 (100 µg/ml). A2 proteins overexpressed in *L. donovani* amastigotes were immunoprecipitated with human immune serum by a method previously described by Brandau *et al.* (1995). Briefly, 10^7 cells were harvested by centrifugation and lysed in 50 µl of solubilizing buffer (50mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, and 2.5 µg each of aprotinin, leupeptin and pepstatin per ml). Lysates were centrifuged at 13000 X *g* for 15 min at 4°C, and soluble proteins were incubated with 1µl of human immune serum for 1 h at 4°C. A 35-µl portion of protein A-Sepharose (Pharmacia Biotech) was added, and the total mix was further incubated for 2 h at 4°C. The immunoabsorbent was centrifuged and washed three times in Net Gel (150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 0.02% NaN_3, 0.25% gelatin, 50 mM Tris [pH 7.5]). Twenty-five microliters of 2X SDS sample buffer (125 mM Tris, 4% SDS, 20% [vol/vol] glycerol, 100 mM dithiothreitol, 0.005%

bromophenol blue [pH 6.8]) was added to the slurry, heated at 95°C for 5 min, and loaded on an SDS-10% polyacrylamide gel. Immunoprecipitated A2 proteins were detected by Western blot analyses with anti-A2 monoclonal antibody C9 and by the ECL Western blotting detection system (Amersham). The secondary antibody, a goat anti-mouse IgG horseradish peroxidase conjugate (BioRad), was used at a 1:3500 dilution.

Karyotype analyses

Leishmania chromosomes were separated in a 1% agarose pulsed-field grade gel (BioRad) using a BioRad DRII pulsed-field gel electrophoresis (PFGE) apparatus. *Leishmania* species used in the karyotype analyses are listed in Table 1. Separating conditions were set at 150 V with 120 s of switching time for 48 h at 14°C. Genomic DNA was obtained from *in vitro*-cultured promastigotes of the different strains listed in Table 1. Southern blot membranes of chromosomes separated by transversal alternating-field electrophoresis (TAFE) were kindly provided by Danielle Legare and Dr. Marc Ouellette, from Laval University. Chromosomes were separated according to procedures described by Grondin *et al.* (1993). All Southern blot membranes were hybridized with a probe representing the A2 protein coding region obtained from a 1.1 kb *Xba*I-*Xho*I fragment of genomic clone Geco90 (Charest and Matlashewski, 1994). Hybridization was performed at high stringency: 1M NaCl, 1% SDS, and 10% dextran sulfate for 18 h at 65°C.

Table 1. Species of *Leishmania* used in karyotype analyses.

Organism	Reference stock	Location or ATCC no.
Species used in PFGE		
Leishmania donovani complex		
1. *L. donovani*	MHOM/SD/00/1S-CL2D	Sudan
2. *L. donovani* WR657	MHOM/IN/80/DD8	India
3. *L. donovani* WR684	MHOM/ET/67/82	Ethiopia
4. *L. infantum*	MCAN/SP/00/XXX	Spain
Leishmania mexicana complex		
5. *L. mexicana*	MNYC/BZ/62/M379	Brazil
Leishmania tropica complex		
6. *L. tropica* WR683	MHOM/SU/58/OD	Sudan
7. *L. tropica* WR664	MHOM/SU/74/K27	Sudan
8. *L. major* WR662	MHOM/IL/67/Jericho11	Israel
9. *L. major*	MRHO/SU/59/P/LV39	Sudan
Species used in TAFE		
Leishmania donovani complex		
10. *L. donovani*	SF-2211	50212
11. *L. chagasi*	SF-1880	50133
12. *L. infantum*	SF-1881	50134
Leishmania mexicana complex		
13. *L. amazonensis*	SF-1878	50131
14. *L. mexicana*	SF-1911	50156
Leishmania braziliensis complex		
15. *L. braziliensis*	SF-1882	50135
16. *L. guyanensis*	SF-1871	50126
17. *L. panamensis*	SF-1913	50158
Leishmania tropica complex		
18. *L. major*	SF-1864	50122
19. *L. tropica*	SF-1876	50129
20. *L. aethiopica*	SF-1861	50119

* ATCC: American Type Culture Collection

Human Sera

Three different groups of immune sera were screened in this study. The first group was used to determine the specificity of anti-A2 antibodies, while the two other groups were used to determine the prevalence of anti-A2 antibodies in individuals with visceral leishmaniasis.

(i) Group A. Group A included sera from the Centers for Disease Control and Prevention (CDC) (Atlanta, Ga.) from patients with different types of *Leishmania* infections and *Trypanosoma cruzi* infections.

(ii) Group B. Group B included sera from the CDC collected from patients admitted to a Sudanese hospital. These were patients with kala-azar - acute visceral leishmaniasis - admitted for treatment (see Table 2). Sera were screened at the CDC by IFAT with whole parasites as antigen. Some of the sera were also subjected to a direct agglutination test. Three samples from hospital patients admitted for reasons other than leishmaniasis that tested negative on the IFAT represent the control group.

(iii) Group C. Group C included sera collected in India from patients in various stages of treatment or recovery from kala-Azar. They had been diagnosed with visceral leishmaniasis based on clinical signs of the disease (hepato-splenomegaly, fever, weight loss, hair loss, hypergammaglobulinemia) and on parasite culture of splenic and/or bone marrow aspirates. Samples were divided into 2 categories: (a) patients with active disease and (b) patients in healing and remission from whom blood was drawn at different times after treatment. For

most of the patients, a standard intravenous antimony treatment of 20 mg/kg of body weight/day for 30 days was used. Five samples from a control group representing unexposed hospital workers with no history of visceral leishmaniasis were used as negative controls.

We deemed important the use of endemic control sera for this study. Clearly we did not expect to observe anti-A2 antibodies in individuals not infected with *L. donovani*, since the A2 genes are present only in *L. donovani* and *L. mexicana* species, as demonstrated in Fig. 1.

RESULTS

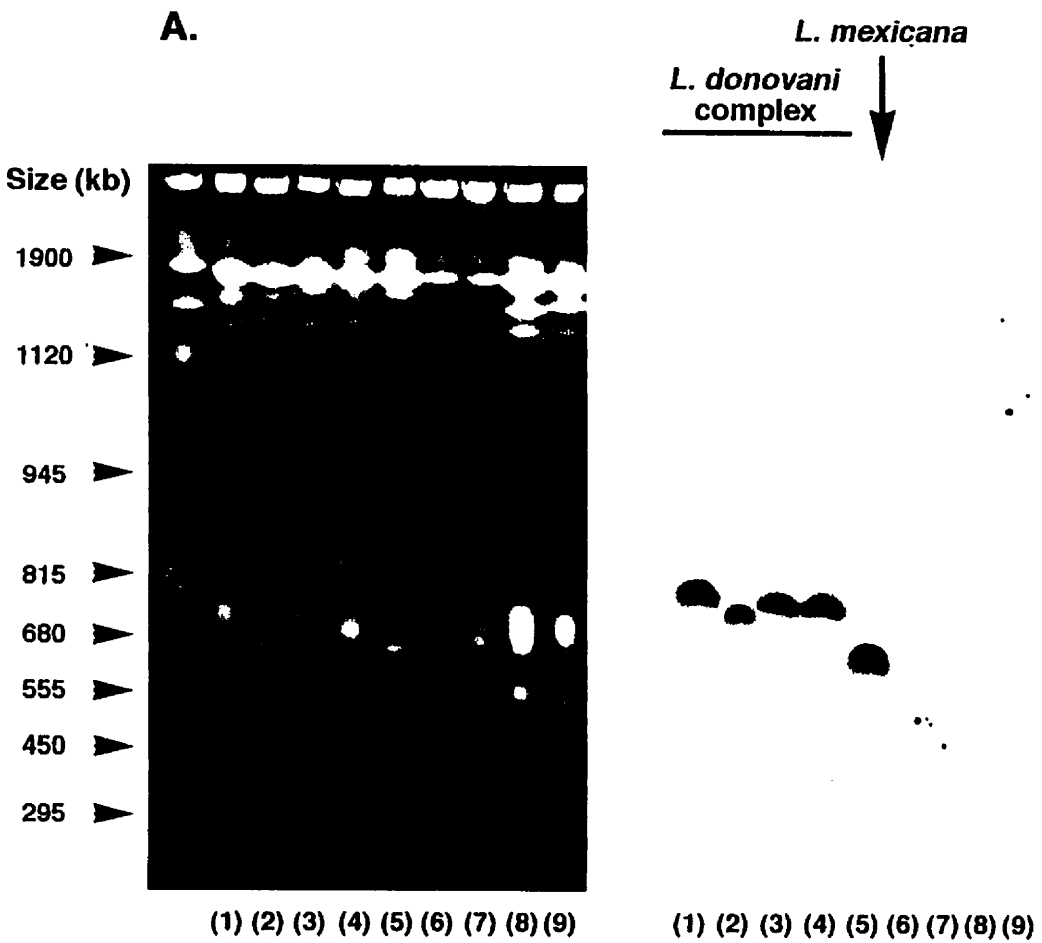
Karyotype Analyses of A2 Genes

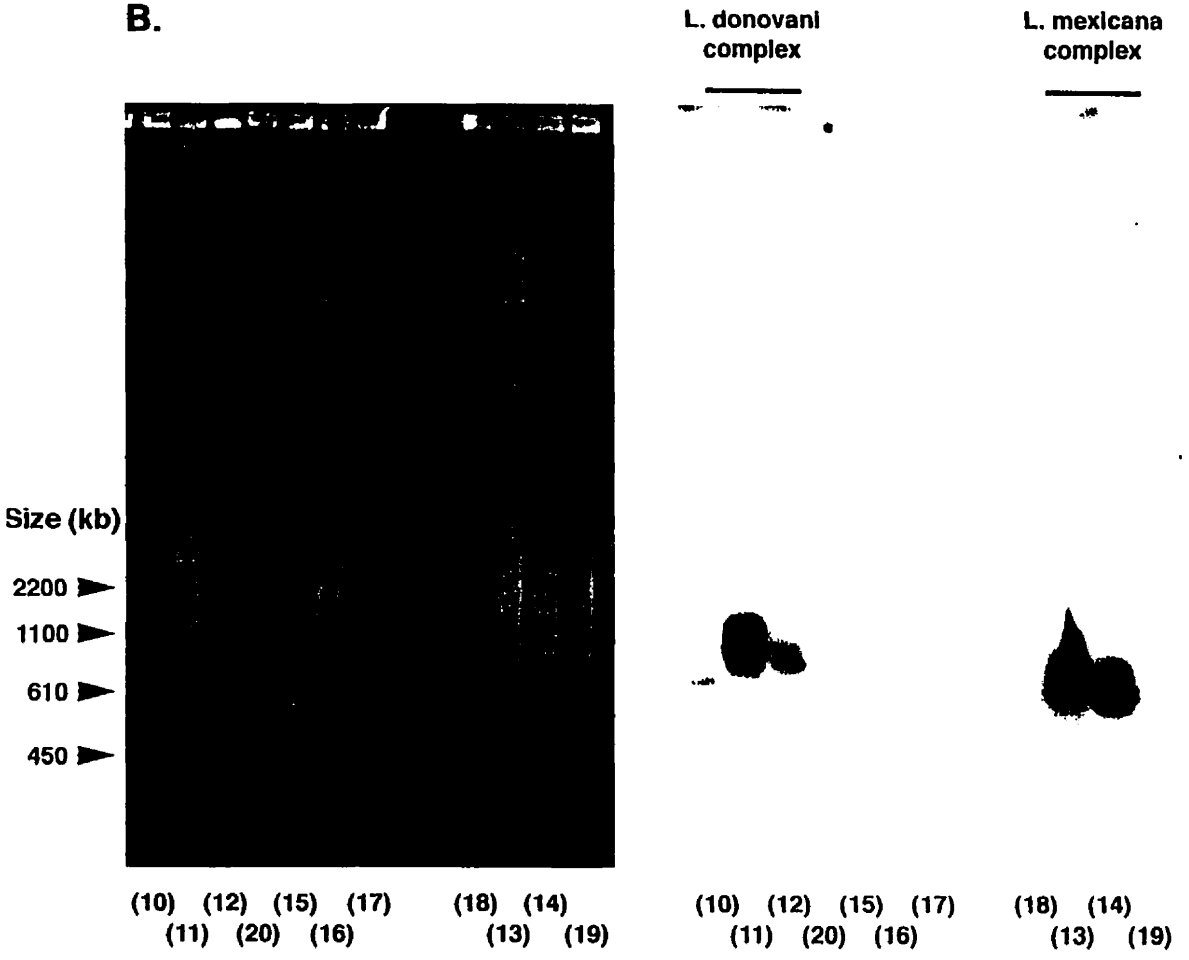
The A2 genes were originally isolated from *L. donovani donovani* (Charest and Matlashewski, 1994). To determine if the A2 gene is conserved in other *L. donovani* subspecies and *Leishmania* species, karyotype analysis of *Leishmania* chromosomes was performed. Analyses revealed that the A2 genes were conserved only in species from the *L. donovani* complex (New World and Old World visceral agents) and *L. mexicana* (New World cutaneous agent) (Fig. 1). The A2 gene was not detected in the *L. tropica* complex (*L. major*, *L. tropica* and *L. aethiopica*) nor in the *L. braziliensis* complex. These data show that the A2 genes are specific for *L. donovani* and *L. mexicana*. It is possible that the A2 genes have diverged in the other species to such an extent that they do not hybridize with the A2 probe at the high-stringency conditions used.

Specificity of the Anti-A2 Antibody Response

Because of the specificity of the A2 gene to visceral agents and its absence in most cutaneous species, we determined whether there was a similar specificity in the anti-A2 antibody response. Western blot analyses of immune sera against a recombinant A2-GST fusion protein was performed. Figure 2 shows an example of the Western blot procedure used to screen the sera samples. Total proteins from *E. coli* cells expressing the recombinant A2-GST fusion protein (Fig.

Figure 1. Karyotype analyses detection of the A2 gene in *Leishmania* species associated with pathology in humans. Chromosomes were separated by PFGE (**A**) or TAFE (**B**). Equal loading was verified by agarose gel staining. Southern blot membranes were hybridized at high stringency with a probe representing the A2 protein coding region (a 1.1 kb *Xba*I-*Xho*I fragment purified from a genomic clone). Lanes: 1, *L. donovani*; 2, *L. donovani* WR657; 3, *L. donovani* VR684; 4, *L. infantum*; 5, *L. mexicana;* 6, *L. tropica* WR683; 7, *L. tropica* WR664; 8, *L. major* WR662; 9, *L. major*; 10, *L. donovani*; 11, *L. chagasi*; 12, *L. infantum*; 13, *L. amazonensis*; 14, *L. mexicana*; 15, *L. braziliensis*; 16, *L. guyanensis;* 17, *L. panamensis*; 18, *L. major*; 19, *L. tropica*; 20, *L. aethiopica* (see Table 1 for details of *Leishmania* spp. used). Molecular weights were determined with yeast chromosomes.


A.
L. mexicana
L. donovani complex
Size (kb)
1900
1120
945
815
680
555
450
295
(1) (2) (3) (4) (5) (6) (7) (8) (9)
(1) (2) (3) (4) (5) (6) (7) (8) (9)


B.
L. donovani complex
L. mexicana complex
Size (kb)
2200
1100
610
450
(10) (12) (15) (17) (18) (14)
(11) (20) (16) (13) (19)
(10) (12) (15) (17) (18) (14)
(11) (20) (16) (13) (19)

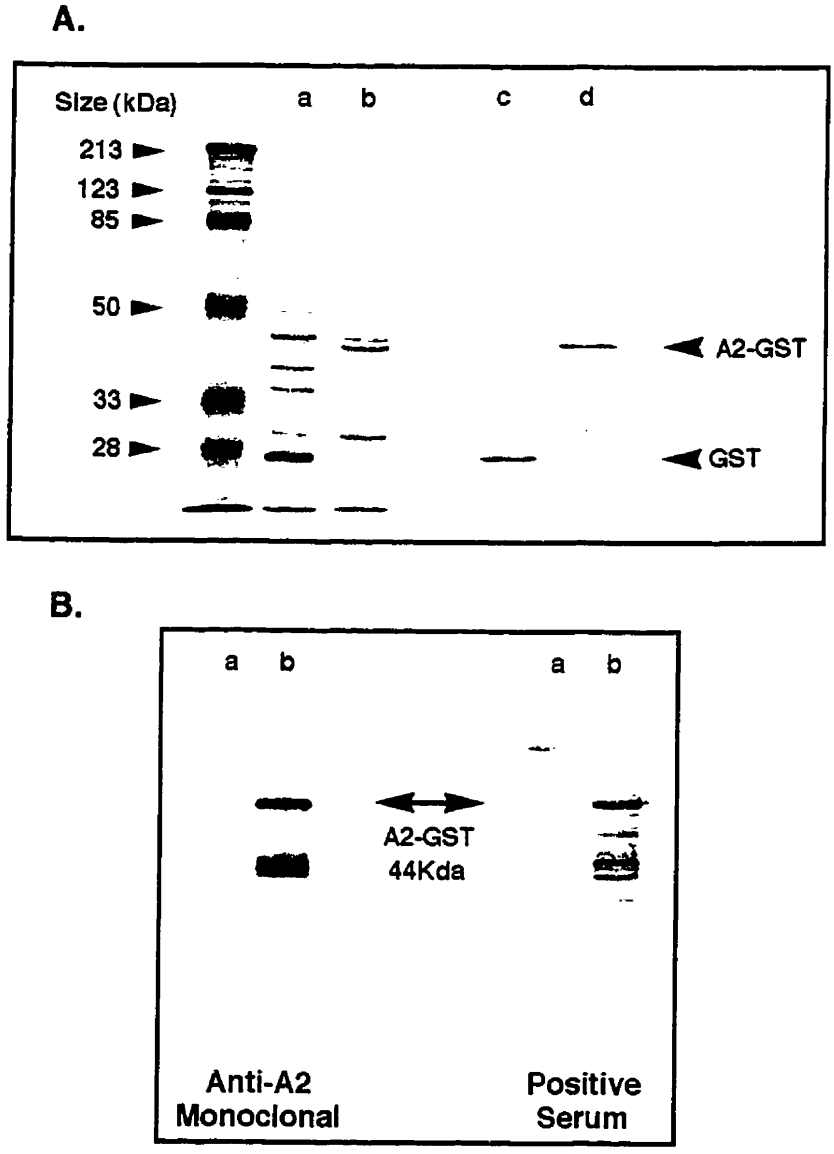


Figure 2. (A) Coomassie blue staining of total proteins from *E. coli* transformed with the GST-expressing pGEX-2T plasmid alone (lane a) and the pGEX-2T plasmid containing the insert A2 DNA (lane b). Lanes c and d contained 2 μg each of purified GST and A2-GST fusion protein, respectively. (B) Western blot analyses of total proteins present in lanes a and b were performed with a monoclonal antibody against A2 and a positive immune serum from a visceral leishmaniasis patient, as indicated.

2A, lane a) and control recombinant GST alone (lane b) were subjected in parallel to SDS-polyacrylamide gel electrophoresis. Purified versions of the recombinant proteins were also run in parallel (Fig.2A, lanes c and d). The GST protein alone runs at 22 kDa, while the GST-A2 fusion protein is at 44 kDa. As positive controls for Western blotting (Fig. 2B), A2 was detected with the anti-A2 monoclonal antibody C9 (Zhang *et al.*, 1996) or immune serum which was previously shown to react strongly against *L. donovani* antigens from a young Iranian patient suffering from visceral leishmaniasis (Charest and Matlashewski, 1994). Figure 2B demonstrates that this Western blot analysis could be used to identify sera containing anti-A2 antibodies.

Sera from patients with *T. cruzi*, *L. tropica*, *L. braziliensis*, *L. mexicana* or *L. donovani* infections were next screened for reactivity against the recombinant A2 protein. Positive samples displayed a pattern similar to that for the positive control of Fig. 2B (positive serum). Anti-A2 antibodies were detected only in individuals infected with *L. donovani* or *L. mexicana*. Five of seven of *L. donovani*-infected individuals and 3 of 5 *L. mexicana* cases were positive for anti-A2 antibodies. Individuals infected with *T. cruzi* (four samples), *L. tropica* (four samples), and *L. braziliensis* (two samples) were all negative for anti-A2 antibodies. Therefore, there was no cross-reactivity to trypanosome or other *Leishmania* infections. These results are consistent with the karyotype analyses, which revealed that A2 genes were conserved only in *L. donovani* and *L. mexicana* strains.

Prevalence of Anti-A2 Antibodies in *L. donovani* Infected Individuals

Based on the results of Western blot screening for anti-A2 antibody response (above), we examined the prevalence of anti-A2 antibodies in sera from a larger population of *L. donovani*-infected patients. In Western blot analyses, a positive response against the A2 recombinant protein was detected in 41% of *L. donovani*-infected individuals in the Sudan and 48% in India, as well as in 33% of individuals in a posttreatment stage (Table 2). In ELISAs using the purified recombinant A2-GST and GST as a negative control (as shown for the Coomassie Blue staining in Fig.2A), 18 of the 22 Sudanese sera tested and 15 of the 25 Indian sera tested from infected individuals showed IgG reactivity with the recombinant A2 above the cutoff level (Table 2 and Fig. 3), while there was no reactivity with GST alone (data not shown). This corresponds to, respectively, 60 and 82% of positive cases detected. As shown in Fig. 3B, patients in posttreatment were separated according to the time at which serum samples were collected after treatment. Group III represents patients up to 12 months after treatment, and Group IV represents patients from 24 to 106 months after treatment. Surprisingly, one patient who had been treated for visceral leishmaniasis more than 8 years before (106 months) tested positive in the ELISA (Fig. 3B).

Immunoprecipitation analysis was also performed to examine reactivity against conformational epitopes on A2. It was thought that

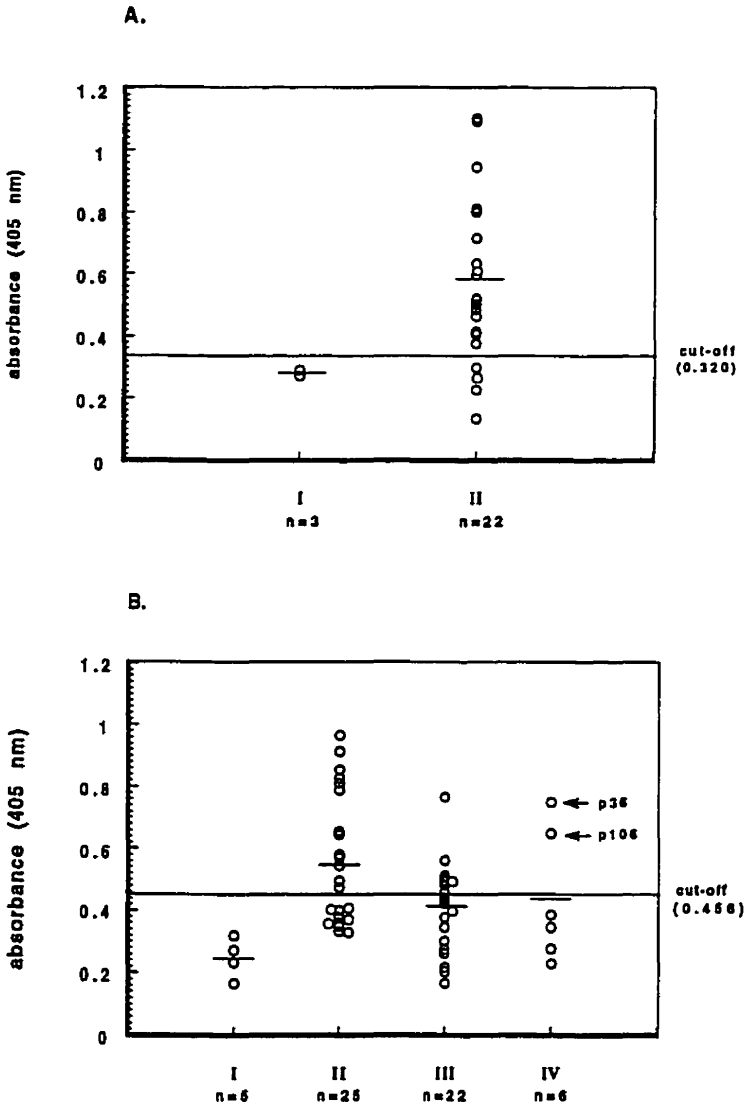


Figure 3. ELISA of the IgG antibody response of visceral leishmaniasis patients and other groups against the *L. donovani* A2 protein. The horizontal lines mark the arithmetic mean of each group. The cutoff values were obtained by calculating the means of the negative control serum samples plus 3 standard deviations. (**A**) Sudanese samples (group B): I, control sera that tested negative on IFAT for leishmaniasis from patients in a Sudanese hospital; II, sera from patients with active disease. (**B**) Indian samples (group C): I, control sera from unexposed hospital workers; II, sera from patients with active disease; III, sera collected from treated patients up to 12 months posttreatment; IV, sera collected from treated patients more than 12 months posttreatment. p36 and p106 correspond to serum samples collected from patients respectively 36 and 106 months, respectively, after treatment.

this assay might provide better sensitivity for detecting anti-A2 antibodies. Total solubilized proteins derived from cultured *L. donovani* were immunoprecipitated with immune human sera, and the immunoprecipitated proteins were subjected to SDS-PAGE. A2 proteins in the immunoprecipitates were then identified with the anti-A2 monoclonal antibody by Western blot analysis. Figure 4 shows representative data for several samples. Figure 4, lane a, shows the pattern of the various A2 proteins present in lysates of *L. donovani* cells which overexpress two A2 protein species at 53 and 33 kDa. When both the 53- and 33-kDa bands were observed, a sample was considered positive for anti-A2 antibodies. Identification of the 33-kDa band was particularly helpful since, in some samples, the antibody heavy chain tended to comigrate with the 53-kDa band, overshadowing its effect. Figure 4, lane b, shows a pool of five negative-control serum samples. Lanes c and d represent samples from two individuals in posttreatment in which sample c was negative and sample d was positive for anti-A2 antibodies. Lanes e through h are from four individuals with kala-azar and range from weakly positive (lane h) to strongly positive (lane f). Immunoprecipitations were performed only with the sera collected in India (group C). Ninety-two percent of active disease cases tested positive for anti-A2 antibodies by this analysis (Table 2) as opposed to 48% positive with Western blotting only and 60% with ELISA only. These data demonstrate that the immunoprecipitation analysis was the most sensitive approach for identifying anti-A2 antibodies. This also indicates that many of the antibodies produced against A2 recognize conformational epitopes.

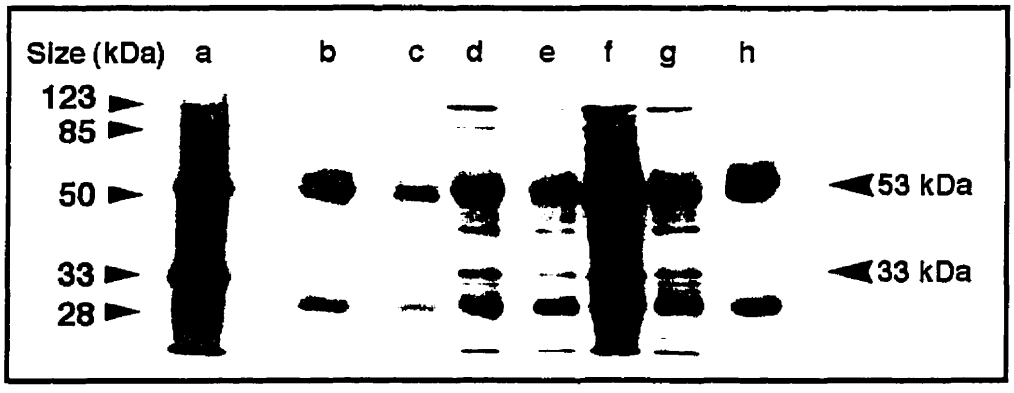


Figure 4. Immunoprecipitation analysis of sera from group C. Immunoprecipitated A2 protein was detected by Western blot analyses with the anti-A2 monoclonal antibody. Lane a, total cell lysate of *L. donovani* amastigotes; lane b, pool of sera from healthy individuals (control); lanes c and d, sera from individuals in posttreatment; lanes e through h, sera from individuals with active disease. The 53- and 33-kDa A2 proteins are indicated.

Table 2. Comparison of numbers of samples positive for anti-A2 antibodies using three different assays.

Serum group	Clinical condition	No. of positive samples/no. tested (%) by: Western blotting	ELISA	Immuno-precipitation
B	Active disease	13/32 (41)	18/22 (82)	[a]ND
	Controls	0/3 (0)	ND	ND
C	Active disease	13/27 (48)	15/25 (60)	25/27 (92)
	Posttreatment	9/27 (33)	10/27 (37)	18/27 (67)
	Controls	0/5 (0)	ND	0/5 (0)

[a] ND, not done

DISCUSSION

Karyotype analyses of species from the four *Leishmania* complexes (*L. donovani*, *L. mexicana*, *L. tropica*, and *L. braziliensis*) revealed that A2 genes are conserved in *Leishmania* strains of the *L. donovani* and *L. mexicana* complexes but not in strains of the *L. tropica* or *L. braziliensis* complexes. From a phylogenetic point of view, the conservation of A2 genes in both *L. donovani* and *L. mexicana* establishes an interesting link between these two groups, etiological agents of different types of leishmaniasis. *L. donovani* and its subspecies are responsible for visceral leishmaniasis in the Old and New World, while *L. mexicana* is an exclusively New World species that causes cutaneous lesions.

Since the expression of A2 is amastigote specific and this stage is associated with pathology in humans, we were interested in examining the antibody response against the A2 protein. We demonstrated by immunoblotting that the A2 antibody response was specific for patients with visceral leishmaniasis and *L. mexicana* infections and that there was no cross-reactivity with sera from individuals with other parasitic diseases. The anti-A2 antibodies were not detected in patients with cutaneous leishmaniasis caused by *L. major*, *L. tropica*, or *L. braziliensis*, nor in individuals with non-leishmanial diseases. This was consistent with the karyotype data showing that the A2 genes were conserved in *L. donovani* and *L. mexicana*.

Patients infected with *L. donovani* were screened for anti-A2 antibodies by using a recombinant A2 as well as native A2 proteins. Immunoprecipitation of A2 protein and then detection of immunoprecipitated A2 with anti-A2 monoclonal antibody identified anti-A2 antibodies in 92% of patients with active visceral leishmaniasis. The Western blotting alone and the ELISA using the recombinant A2 were less sensitive (48% and 60%, respectively). This result shows that A2 holds considerable potential as an antigen in serodiagnosis and that further studies should focus on the development of A2 as a serodiagnostic antigen for visceral leishmaniasis.

A large number of patients potentially infected with *L. donovani* can be tested by serodiagnosis, replacing techniques that rely mainly on the identification of parasites from tissue biopsy. The A2 antigen can be added to the list of parasite antigens potentially useful for specific immunodiagnosis of visceral leishmaniasis. This list includes native proteins such as gp63, which can permit a distinction between ongoing and previous infections to be made (Okong'o-Odera *et al.*, 1993), a p32 membrane protein of *L. donovani infantum* promastigotes which is suitable for the specific diagnosis of Mediterranean visceral leishmaniasis (Tebourski *et al*, 1994), and the 70- and 72-kDa proteins purified from *L. donovani* promastigotes (Jaffe and Zalis, 1988a; Jaffe and Zalis, 1988b), among others. Recombinant proteins have also proven useful, among them rK39, a recombinant protein that contains a 39-amino-acid repeat part of a 230-kDa protein predominant in *L. chagasi* tissue amastigotes (Burns

et al, 1993), and recombinant gp63 antigens from *L. chagasi* and *L. donovani* (Shreffler *et al.*, 1993).

Contrary to proteins such as gp63, A2 is specific to *L. donovani* and *L. mexicana*, which eliminates misdiagnosis due to cross-reactivity. A2 also has two characteristics that resemble the *L. chagasi* 230-kDa protein whose 39-amino-acid repeat was shown to be a useful antigen for serodiagnosis of visceral leishmaniasis (Burns *et al.*, 1993): A2 is amastigote stage specific, and it contains a repeat sequence. rK39 was shown to be an early surrogate marker for disease progression in visceral leishmaniasis, and rK39 seroactivity correlates with active disease. Ninety-eight percent of active disease cases were detected with this marker (Badarò *et al.*, 1996). As shown in the present study, high levels of anti-A2 antibodies also occur in cases of acute visceral leishmaniasis, demonstrated by the detection of 92% of individuals with active disease by using the A2 antigen.

A2 is a protein that is developmentally expressed in the amastigote stage of the parasite, the stage at which the *Leishmania* organism is in the phagolysosomal compartment of host macrophages. As previously suggested (Badarò *et al.* 1996), during the acute phase of the disease the host may produce specific antibodies, including the A2 and K39 antigens, against replicating *Leishmania*. We suggest that A2 could be employed in conjunction with the recently developed rK39 antigen in the serodiagnosis of visceral leishmaniasis. It could, for example, be used as a second antigen when positive results are equivocal with the rK39 antigen.

The anti-A2 monoclonal antibody which was raised against a recombinant A2 protein (Zhang *et al.*, 1996) was also shown in this study to be capable of reacting strongly on Western blots with the A2 protein. The A2 monoclonal antibody may be useful for distinguishing *L. donovani* and *L. mexicana* from other *Leishmania* species. Monoclonal antibodies have previously been used in serodiagnostic assays. For example, Jaffe and McMahon-Pratt (1987) have developed a competitive serodiagnostic assay for visceral leishmaniasis using species-specific *L. donovani* monoclonal antibodies. The assay is based on the specific inhibition of monoclonal antibody binding to a crude parasite homogenate by serum from patients with visceral leishmaniasis. Monospecific antibodies are also suited for the taxonomic identification of *Leishmania* species (Jaffe *et al.*, 1984; Pan and McMahon-Pratt, 1988) and several species-specific leishmanial proteins have been identified with monoclonal antibodies (Jaffe *et al.*, 1984; Jaffe and Zalis, 1988a). The anti-A2 monoclonal antibody could, for example, be used to differentiate between visceral leishmaniasis due to *L. donovani* or *L. tropica* infections. In a recent study (Sacks *et al.*, 1995), *L. tropica* was found to visceralize in some individuals, confirming that *L. tropica* is a coendemic agent of visceral leishmaniasis in India. The same visceralizing effect of *L. tropica* was observed in soldiers returning from Operation Desert Storm (Magill *et al.*, 1993).

In summary, we have examined the antibody response against the amastigote-specific antigen A2, which is present in members of the

L. donovani and *L. mexicana* complexes. The antibody response to the *L. donovani* and *L. mexicana* complexes was specific enough to hold potential in serodiagnostic assays for visceral leishmaniasis. A2 is one of the only *L. donovani* amastigote-specific markers identified to date, and we suggest that it may prove valuable in a serodiagnostic test that uses a spectrum of antigens specific to different stages of the *Leishmania* parasite and/or to different species.

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CONNECTING STATEMENT TO MANUSCRIPT II

In Manuscript I, we have evaluated the diagnostic potential of an amastigote stage-specific protein, A2. Recalling that the amastigote is the parasite stage responsible for pathology, it follows that people with acute visceral leishmaniasis have higher levels of replicating amastigotes in their lymphoid tissues than individuals with subclinical infections. Individuals with visceral leishmaniasis would therefore be expected to develop antibodies against the A2 protein. It has been shown that recovery from leishmaniasis leads to solid immunity against subsequent reinfections, hence vaccination using attenuated *Leishmania* strains is a particularly interesting approach for induction of long term protection. The possibility of controlling parasite growth in its pathological form, the amastigote, would lower the inherent risk of using live parasites, an important parameter towards the development of safe recombinant vaccine strains. With this aim in mind, in Manuscript II we have explored the possibility of using *Leishmania donovani* A2 regulatory sequences to differentially express toxic gene products, specifically in the amastigote form of the parasite.

CHAPTER 3

Inducible Expression of Suicide genes in *Leishmania donovani* Amastigotes

(MANUSCRIPT II)

ELODIE GHEDIN, HUGUES CHAREST, WEN-WEI ZHANG,
ALAIN DEBRABANT, DENNIS DWYER and GREG MATLASHEWSKI

ABSTRACT

The inducible A2 gene regulatory system was used to differentially express, in amastigotes, heterologous genes with a toxic potential. DNA constructs were made which encoded a truncated version of the *Leishmania* 3'-nucleotidase/nuclease targeted for expression to the cytoplasm, and the herpes simplex virus thymidine kinase gene. These genes were introduced between A2 non-coding regulatory sequences which allow upregulation specifically in amastigotes. Developmental expression in amastigotes induced the accumulation of toxic products which affected cell replication and viability *in vitro* and *in vivo*. This study demonstrates that the A2 gene regulatory system can be used to create an *L. donovani* line in which attenuation is developmentally regulated when the parasite differentiates from promastigotes to amastigotes. A *Leishmania* strain attenuated in this manner may be used in studies examining immune responses and/or in the development of a live vaccine strain.

INTRODUCTION

The *Leishmania donovani* A2 gene family is amastigote stage-specific (Charest and Matlashewski, 1994) and is essential for survival of this parasitic protozoa in its mammalian host (Zhang and Matlashewski, 1997). We have demonstrated that the A2 gene regulatory system represents an excellent model to study the regulation of gene expression in *Leishmania* cells (Charest *et al.*, 1996). The developmental expression of A2 gene transcripts and protein products is induced in *in vitro* cultured promastigotes by a combination of pH and temperature shifts, conditions associated with the passage of the parasite from the insect vector to the phagolysosome environment inside the mammalian macrophage cell (Charest and Matlashewski, 1994; Zhang *et al.*, 1996). A2 gene expression is post-transcriptionally regulated and selectable marker transcripts carrying the A2 mRNA 3'-UTR display amastigote specific expression in transfected *Leishmania* cells. Reporter genes integrated into the A2 chromosomal locus by homologous recombination displayed the same pattern of developmental expression as the A2 gene (Charest *et al.*, 1996).

In this study, we have used the A2 gene regulatory system to differentially express, in amastigotes, genes with a toxic potential to the parasite. This allowed us to test the potential of the A2 regulatory sequences in concert with toxic products for the generation of a developmentally attenuated *L. donovani* strain. The suicide genes tested in our constructs were the herpes simplex virus

thymidine kinase gene (*hsv-tk*) previously used by LeBowitz *et al.* (1992) as a negative selectable marker in *L. major*, and a truncated version of the *L. donovani* 3'-nucleotidase/nuclease (3'NT/Nu) which is an externally oriented surface membrane enzyme also present in related trypanosomatid protozoa. The expression of the herpes virus *tk* gene transfected in promastigotes results in sensitivity to nucleoside analogues, such as the anti-herpes drugs ganciclovir (GCV) and acyclovir. Thymidine kinase activity is not detected in *Leishmania* (Krenitzky *et al.*, 1979). The 3'NT/Nu is capable of hydrolyzing 3'nucleotides and nucleic acids (Gottlieb *et al*, 1986; Dwyer and Gottlieb, 1984; Gottlieb and Dwyer, 1983) and is therefore thought to be involved in the salvage of host derived purines (Gottlieb, 1989). *Leishmania* are incapable of synthesizing purine rings *de novo* (Hammond and Gutteridge, 1984). We have generated a truncated version of the *3'nt/nu* gene expressing a protein which would not be targeted to the secretory pathway but remain in the cytoplasm. The assumption was that it would therefore exert its nucleotidase/nuclease activity intracellularly and hence be toxic to the parasite.

Both *tk* and the truncated *3'nt/nu* were put under the control of the A2 3'-UTR and stably introduced into *L. donovani* promastigotes by transfection. Developmental expression from both episomal and integrated constructs was verified in *in vitro* differentiated amastigotes. We demonstrate that, as expected, the pattern of expression of these toxic genes, under the control of A2 gene sequences, was higher in amastigotes, but was not completely

amastigote-specific. Nevertheless, we show that the truncated 3'nucleotidase affected negatively growth of the parasite in amastigote conditions and TK induced sensitivity of amastigotes to the nucleoside analogue. Promastigotes, in contrast, were relatively unaffected by the expression of the exogenous genes.

These data show that by expressing toxic gene products under the control of the A2 gene regulatory system, it was possible to generate *Leishmania* in which attenuation was developmentally regulated by the differentiation from promastigotes to amastigotes. Such attenuated *L. donovani* lines may be used in studies examining host immune responses during initial infection with a live strain and subsequent resolution of the infection when the developmentally attenuated parasite is cleared from the mammalian host. The inducible expression of toxic gene products may also be used as a component in the development of safe and effective vaccines where a combination of approaches are considered.

MATERIALS & METHODS

Leishmania strain and culture medium

Promastigotes and amastigotes of a cloned line of *Leishmania donovani* (1S, clone 2_D (Dwyer, 1977) from the 1S-strain (Stauber, 1966), WHO designation: MHOM/SD/62/1S-cl2_D) were cultured as follows. Promastigotes were grown at 26°C in M199 medium with glutamine (Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% defined heat-inactivated fetal bovine serum (FBS; HyClone laboratories Inc., Logan, UT) and 25 mM HEPES (*N*-2-Hydroxyethyl Piperazine-*N*'-2-Ethanesulfonic acid, ICN Biomedicals Inc., Aurora, OH) at pH 6.8. Axenic amastigotes were grown at 37°C in RPMI-1640 medium with glutamine supplemented with 20% FBS and 20 mM MES (2-(*N*-morpholino)-ethanesulfonic acid, (Sigma Chemical Co., St-Louis, MO) at pH 5.5 (Joshi *et al.*, 1993).

Promastigotes used in Figure 2 are a non infectious laboratory strain of the 1S2D *L. donovani donovani* strain. They were cultured in RPMI-1640 medium buffered with 20 mM HEPES to pH 7.3, and supplemented with 10% FBS. Promastigotes were cultured at 27°C in G418-free medium for three days and then midlog phase cultures were diluted 1:20 in 10 ml of fresh medium containing G418 concentrations ranging from 0 to 500 µg/ml.

Amastigotes used in Western and Northern blot experiments were obtained by growing promastigotes to late log phase (10^7 cells/ml),

harvesting them by centrifugation (1,500 X g, 10 min), and resuspending them in supplemented RPMI-1640 at 37°C. Cells were harvested 12-18 hours later.

Cell growth assays of TK transfectants were performed in media supplemented with a water-buffered ganciclovir sodium solution (Cytovene; Hoffman-La Roche, Ontario).

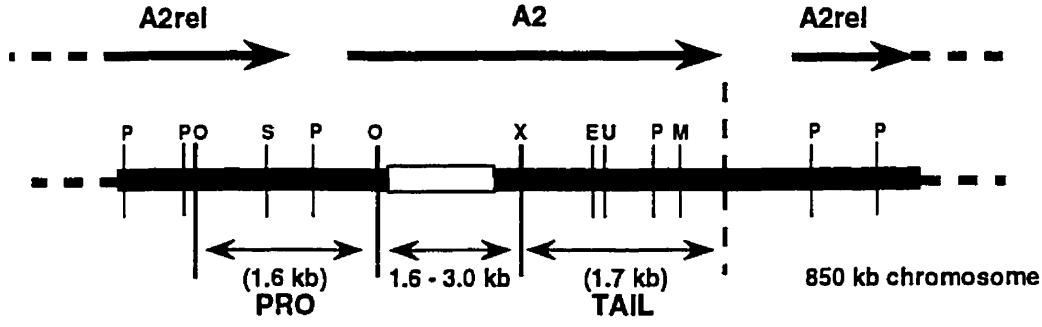
Nucleic Acids Preparations and Analyses

Total RNA was extracted from promastigotes and amastigotes by the phenol-chloroform-guanidium isothiocyanate method usind TRIzol (Life Technologies, Inc.). DNA samples were prepared from promastigotes using the "mini-prep" procedure of Medina-Acosta and Cross (1993). Southern and Northern blot hybridizations were carried out in 1M NaCl, 1% SDS, 10% dextran sulfate for 18h at 65°C. DNA probes were prepared from agarose gel-purified fragments labeled with [α-^{32}P]dCTP (ICN; 3,000 Ci/mmol) by nick translation.

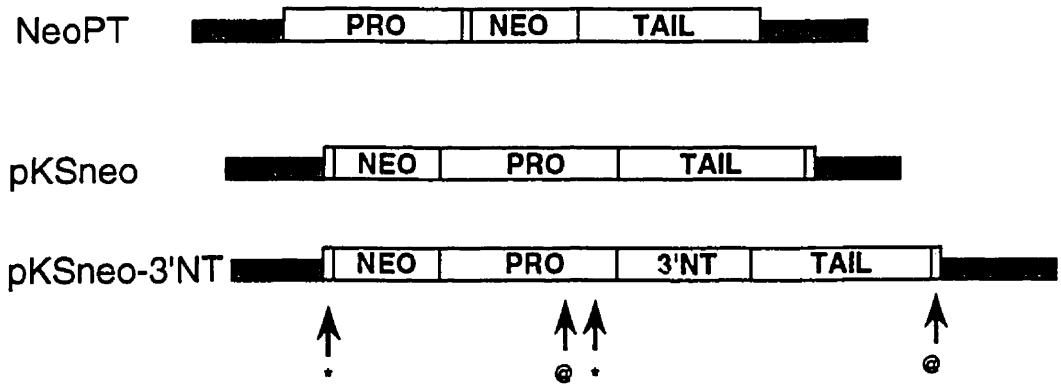
Plasmid Constructs

NEOPT and pKSneo - The NEOPT plasmid was designed to allow the differential expression of the *neo* gene in amastigotes (Charest *et al.*, 1996). The construct (Fig.1A) carries the Pro (P) element which represents the A2/A2rel intergenic region and part of the upstream

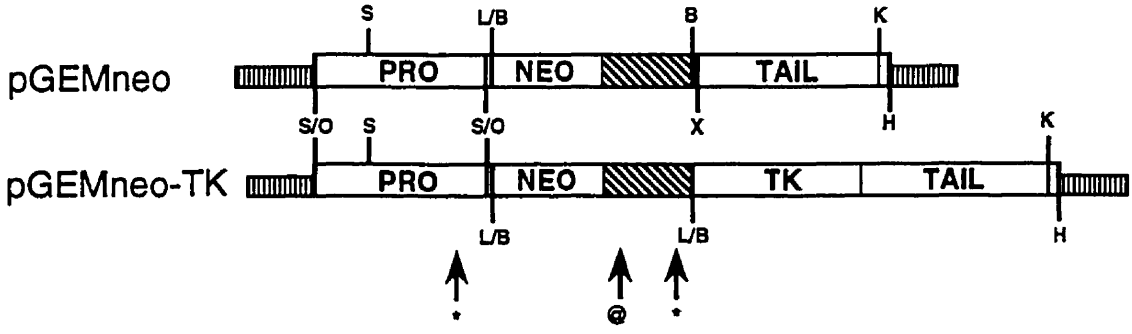
Figure 1. Schematic representation of the constructs used for upregulation of foreign gene expression in amastigotes. *Upper panel*, Schematic outline of the A2/A2rel genomic organization on a 850 kb chromosome. Arrows delineate the A2 and A2rel mature mRNA encoding regions. The open box represents the repeated region of the A2 open reading frame. This genomic map also shows the upstream (PRO) and downstream (TAIL) untranslated sequences from the A2 gene. *Panels A and B*, Schematic representation of the various constructs. The outlined sequences were inserted into the plasmid vectors pBluescript-KS or pGEM-11zf. The pBluescript backbone is represented by a dark gray area and the pGEM backbone by a hatched area. Synthetic *trans*-splicing and poly-adenylation acceptor sites (92 bp, pyt), represented by a light gray area, was inserted at the end of the TAIL, except in the case of the NEOPT plasmid where it is located before the NEO. The diagonal hatched area in *Panel B* represents the α-tubulin intergenic region from *L. enriettii* which provides *trans*-splicing and polyadenylation acceptor sites. (*) *Trans*-splicing acceptor site; (@) Poly-adenylation site. B, *Bam*HI; E, *Eco*RI; F, *Sfi*I; H, *Hind*III; K, *Kpn*I; L, *Bgl*II; M, *Sma*I; O, *Xho*I; P, *Pst*I; S, *Sal*I; U, *Stu*I; X, *Xba*I.


A2rel
A2
A2rel
P
PO
S
P
O
X
EU
P M
P
P
(1.6 kb)
PRO
1.6 - 3.0 kb
(1.7 kb)
TAIL
850 kb chromosome

A.


NeoPT
PRO
NEO
TAIL
pKSneo
NEO
PRO
TAIL
pKSneo-3'NT
NEO
PRO
3'NT
TAIL

B.


S
L/B
B
K
pGEMneo
PRO
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TAIL
S/O
S
S/O
X
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K
pGEMneo-TK
PRO
NEO
TK
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L/B
L/B
H

gene coding sequence (A2rel), a synthetic 92 bp *trans*-splicing element (pyt), the neomycin resistance gene, and the Tail (T) element which represents the 3'-UTR of A2. Construction of the NEOPT vector was described in Charest *et al.* (1996). The construction of the pKSneo plasmid was described in Zhang *et al.* (1996). Contrary to the NEOPT plasmid, the *neo* gene is inserted upstream from the Pro element instead of between the Pro and the Tail (3'-UTR of A2). The Pro element provides a polyadenylation site (Fig.1A) in 3' for the *neo* gene, while the *trans*-splicing in 5' is controlled by a pyt element. Thus, the *neo* gene mRNA derived from pKSneo is not differentially regulated in promastigotes and amastigotes. This vector served as a backbone for making the construct expressing 3'NT/Nu (pKSneo-3'NT).

pKSneo-3'NT - A truncated version of the 3'-Nucleotidase/Nuclease gene (*3'nt/nu*) was amplified by a polymerase chain reaction (PCR) from plasmid Cl-2 (Debrabant *et al.*, 1995) which contains a 4.5 kb fragment carrying the *3'nt/nu* coding sequence (1.4 kb). The sense primer was designed so that the leader sequence would not be present on the amplified product. The antisense primer was designed for exclusion of the hydrophobic *C*-terminal domain which is used to anchor the protein to the surface membrane. The sense oligo (5'gactccatgg **atg** acgctcctcagtactgtcgca3') created an initiation codon, while the antisense oligo (gacttgatca **cta** cacggcgacaatcgccgtcac) created a stop codon. The PCR product (1.1 kb) was first inserted in the pCRII cloning vector (T/A cloning system, Invitrogen) and then

subcloned using the *Spe*I and *Xba*I restriction sites from the pCRII vector into the *Spe*I site of the pKSneo vector (pKSneo-3'NT; Fig.1A).

Constructs for targeting *tk* into the A2 locus

pGEMneo and pGEMneo-TK- The pGEMneo plasmid was designed for the integration of the *neo* gene into the A2 genomic locus but in such a way that it is not under the control of the A2 regulatory sequence. The pGEMneo-TK construct was designed so that both *neo* and *tk* are targeted into the A2 locus but only TK expression is regulated by the A2 non-coding sequences. The *neo* gene was amplified from the pALTneo vector along with an α-tubulin intergenic region from *L. enriettii* which provides a *trans*-splicing acceptor site and a polyadenylation acceptor site (Laban *et al.*, 1990) (Fig.1B). The following primers were used: Sense, 5'- gg **agatct** acctttctctcttccgac -3' (bases in bold represent a *Bgl*II site); Antisense, 5'- tacgactcactatagggc -3'. The "*neo*-intergenic region" (*Neo*-IG) product (1.8 kb) was subcloned *Bgl*II/*Bam*HI in the *Bam*HI site of vector pGEM-11zf (Promega, Canada). The Pro and Tail elements were inserted on either side of the *neo*-intergenic or *neo*-intergenic-*tk* sequences to permit targeting by homologous recombination (Fig. 1B). For the pGEMneo construct, the Tail was subcloned directionally at the *Xba*I and *Hind*III restriction sites. For the pGEMneo-TK plasmid, the *tk* gene was first inserted upstream from the Tail element on a KS pBluescript (Stratagene, Canada) (KSTail plasmid). The 1.3 kb *tk* coding region was amplified from the pFG5 vector

which contains the *tk* gene (Colbere-Garapin *et al.*, 1979). The following primers were used: Sense, 5'- gg **tctaga** cgcttaacagcgtcaaca -3'; Antisense, 5'- gg **tctaga** ggtctcggtggggtatcg -3' (bases in bold represent a *Xba*I site). The PCR product (1.3 kb) was first inserted in the pCRII cloning vector and then subcloned with the *Xba*I restriction sites from the pCRII vector in front of the Tail element. The plasmid created, KS/TK-Tail, was digested *Bgl*II/*Hind*III (the *Bgl*II site is located upstream from the tk initiation codon) and the TK-Tail element was subcloned *BamHI*/*Hind*III at the 3' end of the pGEM construct (Fig.1B). pGEMneo and pGEMneo-TK were linearized with *Sal*I and *Kpn*I, and fragments were purified from an agarose gel. Linear *Sal*I/*Kpn*I fragments containing either the *neo* and intergenic region alone (pGEMneo) or the *neo*, intergenic and *tk* genes (pGEMneo-TK) flanked by 1.0 kb of the Pro and the complete Tail sequences (1.8 kb) were excised from pGEMneo and pGEMneo-TK plasmids and 20 µg of the agarose gel-purified DNA was electroporated into promastigotes.

Transfections

Transfections and selection of clones were performed as described in Charest *et al.* (1996). Mixed populations of recombinant promastigotes were used for experiments with episomal copies of the truncated *3'nt/nu* and *neo* genes. Recombinant promastigotes carrying *neo* and *tk* genes targeted into the chromosomal A2 gene

cluster were selected with minimal doses of G418 (10 µg/ml) and clonal populations were established by limiting dilutions.

Protein Analyses

Promastigotes and amastigotes washed three times by centrifugation at 4°C in 10 mM Tris, 150 mM NaCl, pH 7.5 buffer, were resuspended at a concentration of $5x10^8$ cells/ml in 2XSDS sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% [vol/vol] glycerol, 100 mM dithiothreitol, 0.005% bromophenol blue). Protein samples, denatured by boiling for 10 min, were separated on a 10% SDS-PAGE (Laemmli, 1970).

The 3'nucleotidase activities of the native 3'NT/Nu and the truncated 3'NT/Nu were visualized in the SDS-PAGE gels by *in situ* staining using 3'AMP as a substrate and malachite-green as the staining agent (Zlotnick *et al.*, 1987). The 3'NT/Nu proteins were detected in Western blot analyses with a rabbit polyclonal antiserum and a goat anti-rabbit IgG horseradish peroxidase conjugate (BioRad) as the secondary antibody (as described in Debrabant *et al.*, 1995). Bound secondary antibodies were detected using the ECL Western blotting detection system (Amersham) or with streptavidin-conjugated horseradish peroxidase (Amersham) and 3,3'-diaminobenzidine (Sigma) in a 0.03% H_2O_2 solution.

Infection of Macrophages in Culture

Mouse bone marrow cell preparations and infections with *L. donovani* cells were performed as described in Moore *et al.* (1993). Late log-phase promastigotes were used to infect cultures of macrophages at a ratio of 20 parasites per macrophage for 24 hours at 37°C, after which non-phagocytosed parasites were eliminated by low speed centrifugation and washing infected cells with phosphate buffered saline (PBS). Infected cells were incubated at 37°C in RPMI supplemented with 10% FBS and intracellular infection levels were evaluated daily by cytospin and Giemsa staining.

Infections in Mice

Infections were performed as described in Zhang and Matlashewski (1997). In brief, female BALB/c mice (Charles River, Canada) 6-8 weeks old were injected via the tail vein with 1.5 X 10^8 wild type or transfected promastigotes. Four weeks post-infection, mice were examined for *L. donovani* parasite burden by counting the number of amastigotes in imprints of the liver stained with Giemsa. Liver parasite burdens, expressed as Leishman-Donovan units (LDU), were calculated as the number of amastigotes per 1000 cell nuclei X liver weight (g) (Murray *et al.*, 1989).

RESULTS

Neomycin (G418) resistance of neo transfectants.

In order to assess the possibility of using the A2 gene regulatory sequences to developmentally express heterologous genes in *Leishmania*, we first analyzed the effect of gene copy number on basal levels of expression in the promastigote stage. Since transfections were performed in promastigotes, it was first necessary to establish basal expression levels of exogenous genes when in single copy (targeted) or in a multicopy plasmid (episomal). For these experiments, the *neo* gene, under the control of the A2 sequences, was used both as a selectable marker and as a reporter gene. The effect of the differential expression of NEO was monitored indirectly by calculating the growth of promastigotes cultured in the presence of increasing concentrations of the drug G418. Figure 2 shows the resistance to G418 of *Leishmania* promastigotes transfected with the NEOPT plasmid (episomal) and of a targeted clone (R2) carrying the *neo* gene in the A2 genomic locus. Minimal doses of G418 were used for selection of R2, since we were aware that A2 expression was very low in the promastigote stage. R2 and NEOPT are described in Charest *et al.* (1996). R2 promastigotes, which could slowly multiply in medium containing up to 100 μg/ml of G418, carry only one copy of integrated *neo* (Fig.2, *middle panel*). However, growth was inhibited at 150 μg/ml. In contrast, promastigotes carrying the *neo* gene in episomal vectors could multiply in culture medium containing up to 500 μg/ml without a drastic impact on growth rates. Wild type 1S2D

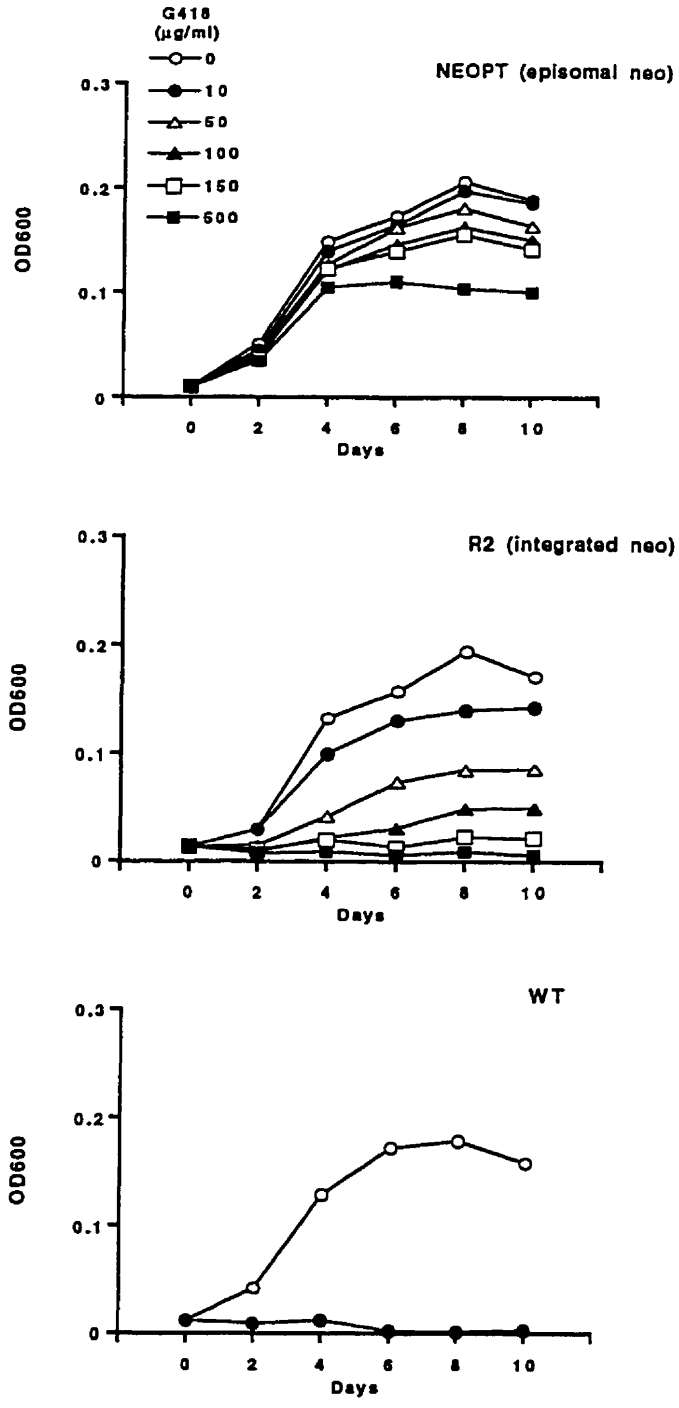

Figure 2. Neomycin resistance of neo transfectants. G418 resistance of targeted clone R2, NEOPT plasmid transfected cells, and wild-type 1S2D promastigotes. Optical densities at 600 nm were measured every other day for 10 days.

promastigotes were extremely sensitive to G418 (Fig.2, *lower panel*); the EC50 was estimated to be 4 μg/ml. The basal level in promastigotes of exogenous gene expression, under the control of the A2 gene regulatory system, is lower from targeted constructs than from plasmid constructs presumably because of the difference in copy number: the targeted construct contained one copy of integrated *neo* while episomal vectors are usually present in multiple copies. Because in our constructs the *neo* gene was also used as the selectable marker, increased G418 pressure will tend to drive the replication of the *neo*-containing episomes. This may account in part for the higher viability of plasmid-transfected promastigotes at higher G418 concentrations.

Differential expression of the truncated 3'NT/Nu from an episomal construct.

We first tested the A2 gene regulatory sequences for the expression of toxic gene products from an episomal construct. We used as a potential toxic gene a truncated version of the *L. donovani* 3'NT/Nu where the *N*-terminal signal sequence and the *C*-terminal anchor sequence were removed from the *3'nt/nu* sequence (pKSneo-3'NT). Our assumption was that the 3'NT/Nu activity expressed in the cytoplasm would be toxic to *Leishmania*.

Figure 3 shows truncated 3'NT/Nu expression at different G418 concentrations (from 0 to 400 μg/ml) in cells transfected with

pKSneo-3'NT. Amastigote RNA was obtained by switching promastigotes to amastigotes in conditions that were previously shown to increase expression of A2 mRNAs (Charest and Matlashewski, 1994), in order to increase truncated 3'NT/Nu expression in amastigotes. The size of the 3'NT/Nu transcript (2.5 kb) derived from the pKSneo-3'NT plasmid was consistent with RNA processing taking place at the artificial *trans*-splicing acceptor site located at the 3'end of the Tail element (pyt). As expected, overall truncated 3'NT/Nu mRNA expression was higher in amastigotes than in promastigotes, and increased accordingly with the G418 selection pressure. The densitometric analyses, using NIH image version 1.61 software, also revealed that truncated 3'NT/Nu transcripts accumulated at a 8-fold higher level in amastigotes grown in 400 μg/ml than in 25 μg/ml of G418. As shown in Figure 3, there was a stronger induction of truncated 3'NT/Nu transcripts in amastigotes than in promastigotes. We determined by densitometric analysis that this induction was on average 5-fold higher in amastigotes than in promastigotes.

Western blot analyses were performed to determine if protein accumulation was consistent with transcript expression. As seen in Fig. 4A, the endogenous 3'NT/Nu was present at 43 kDa while the truncated 3'NT migrated at 38 kDa, as expected. Clearly, there was a higher level of the plasmid-derived truncated 38 kDa 3'NT/Nu, than the endogenous 43 kDa protein. There was an increase in truncated

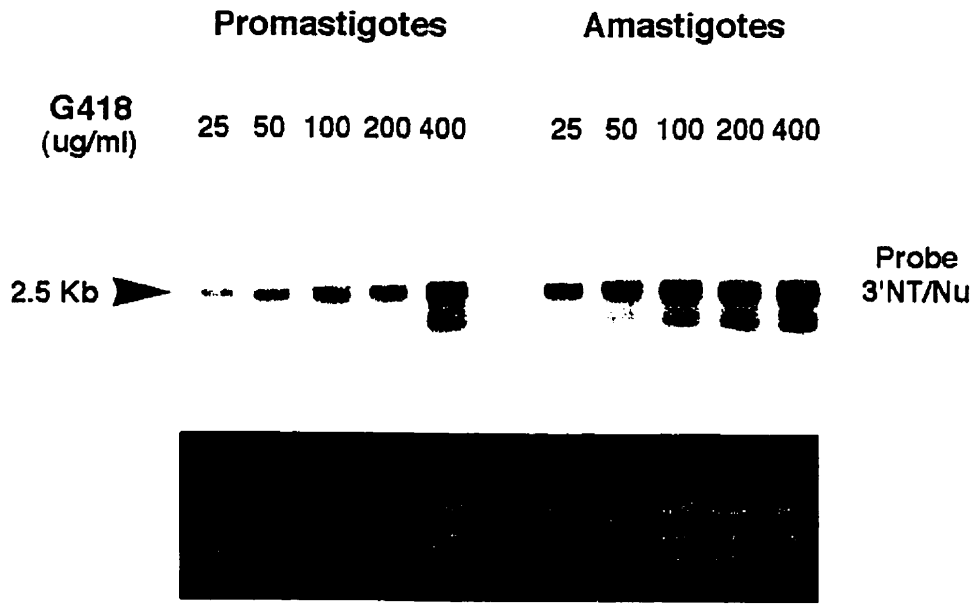

Figure 3. Northern Blot Analyses; Differential expression of the truncated 3'NT/Nu and increased accumulation through selection pressure. Promastigotes were cultured in media at increasing concentrations of G418. Total RNA was extracted from log-phase promastigotes. Amastigote RNA was obtained by switching log-phase promastigotes to amastigote culture conditions and extracting RNA 12 hours later. Membranes were hybridized with the 1.1 kb gel-purified PCR product of the 3'NT/Nu. Equal loading was verified by staining the denatured RNA with ethidium bromide in the agarose gel prior to Northern blot (shown on the bottom panel).

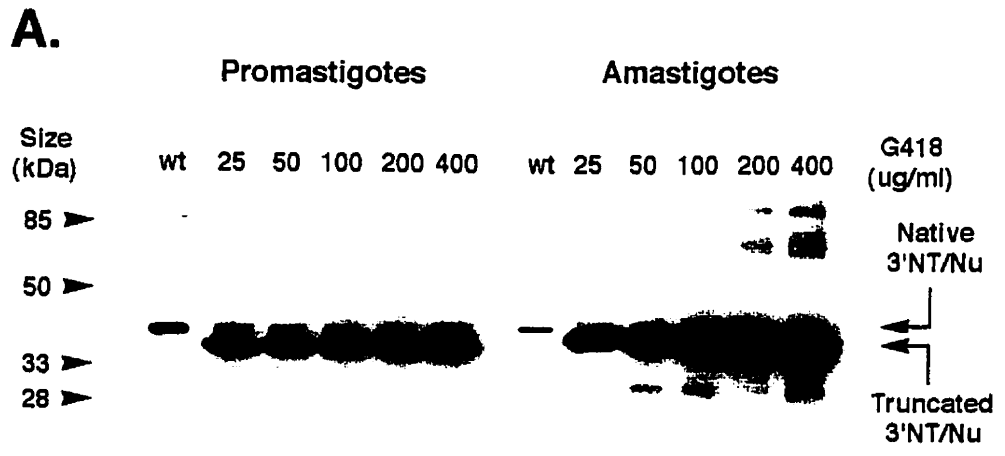

Figure 4. Truncated 3'NT/Nu protein expression and activity. (A) Protein accumulation of the truncated 3'NT/Nu. Western blot analyses of 2.5 μg of total protein obtained from lysed cells. Total protein was obtained from lysed log-phase promastigotes. Amastigote proteins were obtained by switching log-phase promastigotes to amastigote culture conditions and lysing cells 12 hours later.

38 kDa protein production in both promastigotes and amastigotes associated with increasing amounts of G418 in culture medium, demonstrating that increasing the selective pressure for the neomycin resistance gene product, derived from the plasmid, results in increased levels of the truncated 3'NT/Nu product. This also shows that there was overall more truncated 38 kDa 3'NT/Nu in amastigotes than in promastigotes.

The nucleotidase activity of the truncated 3'NT/Nu in promastigotes and amastigotes was determined by staining renatured proteins for 3'nucleotidase activity using 3'-AMP as a substrate. Figure 4B, left panel, shows the levels of endogenous and truncated 3'NT/Nu proteins expressed in promastigotes and amastigotes. Fig. 4B, right panel, shows the activity stain for 3'nucleotidase activity. The Western blot clearly indicates the overexpression of the truncated 3'NT/Nu in the amastigote stage, when compared to the promastigotes stage. Enzyme activity of the truncated 3'NT/Nu was only detected in the transfected amastigotes, but not in promastigotes (Fig. 4B, lower right panel).

The data show that there was a basal level of truncated 3'NT/Nu expression in promastigotes, however the activity in this stage was too low to be detectable. In contrast, amastigote expression of the truncated enzyme was detected on the activity gels, indicating that the truncated 3'NT/Nu was active in transfected amastigotes.

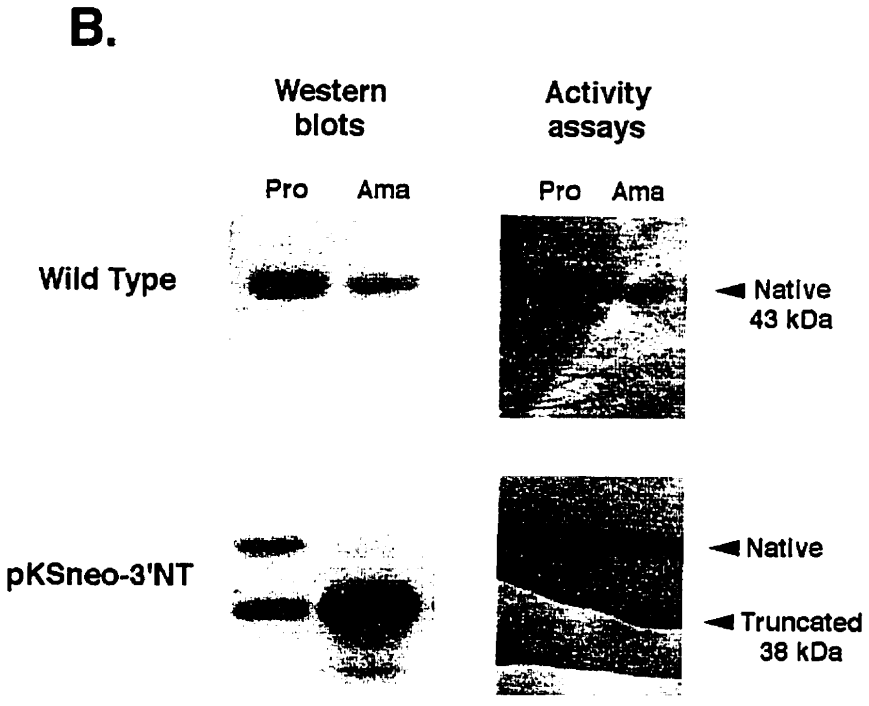

Figure 4. **(B)** Total proteins from promastigotes and amastigotes were subjected to SDS-PAGE, Western blot analysis (left panel) and a gel activity assay (right panel). 3'NT activities were visualized in these gels by *in situ* staining using 3'AMP as a substrate and malachite-green as the staining agent. The native 3'NT/Nu is present at 43 kDa while the truncated 3'NT/Nu migrates at 38 Kda. Note: Truncated 3'nucleotidase activity was only detected in amastigotes.

Induction of truncated 3'NT/Nu leads to reduced amastigote viability.

We next determined whether accumulation of truncated 3'NT/Nu was toxic to the cells. Promastigotes and amastigotes, transfected with the pKSneo-3'NT plasmid, were grown in their respective media supplemented with increasing concentrations of G418 to increase expression of truncated 3'NT/Nu. In a preliminary experiment, *Leishmania* transfected with the pKSneo plasmid were grown in increasing concentrations of G418 (25 to 400 μg/ml) to determine whether the effects on cell growth were not due to G418. Since no difference in cell growth was observed, pKSneo-transfected promastigotes and amastigotes were grown at 25 μg/ml, as represented in Figure 5. As shown in Fig. 5A, promastigotes transfected with the pKSneo-3'NT plasmid were unaffected by the truncated 3'NT/Nu enzyme, presumably because of its low activity (as previously shown in Fig. 4B). There was no difference in cell growth between control pKSneo promastigotes grown at 25 μg/ml G418 and pKSneo-3'NT promastigotes grown at increasing G418 concentrations (Fig. 5A). In contrast, when amastigotes were cultured in media containing increasing concentrations of G418, an increase in the truncated 3'NT/Nu resulted in reduced viability of the amastigotes (Fig. 5B). Even at a low G418 concentration (25 μg/ml), pKSneo-3'NT amastigote cell growth was significantly reduced. This demonstrates that the presence of 3'NT/Nu was toxic when amastigotes were grown in culture.

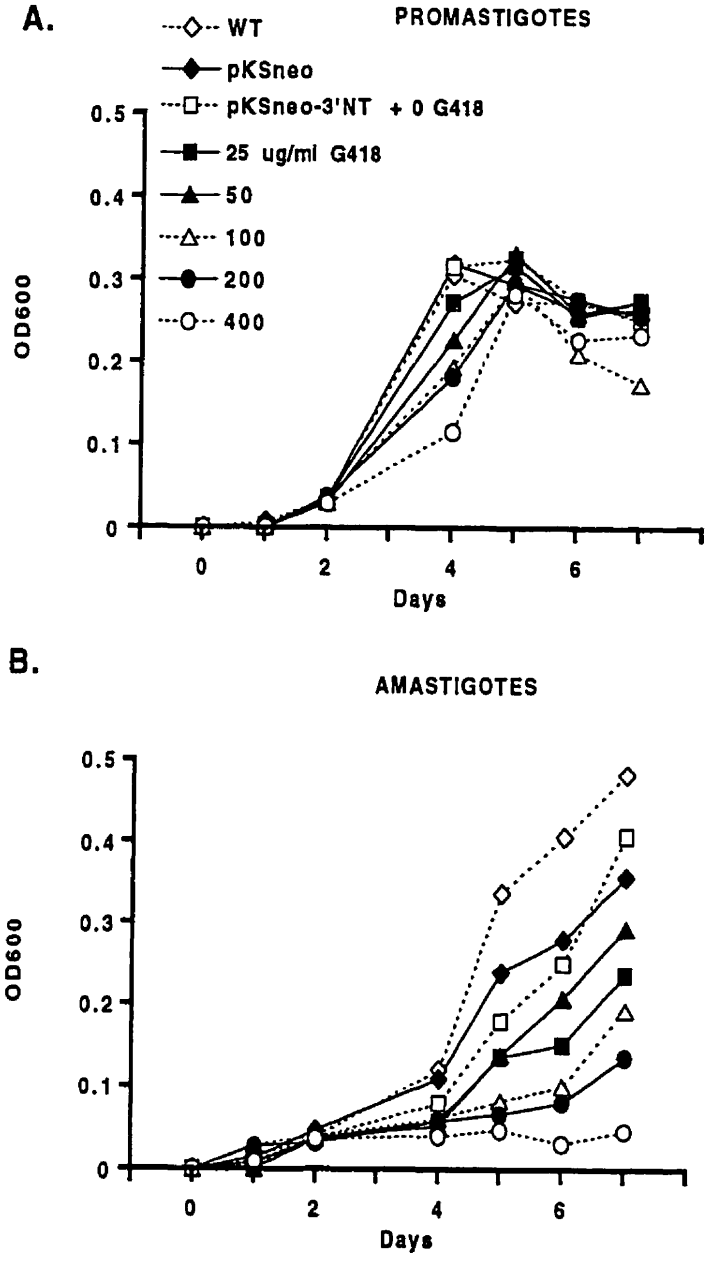

Figure 5. Effect of the truncated 3'NT/Nu on cell growth. pKSneo-3'NT transfectants were grown at increasing G418 concentrations. Optical densities at 600 nm were measured every day, with the exception of day 3. WT, wild-type cells; control vector, pKSneo transfectants grown in 25 μg/ml G418.

We next examined amastigote survival in bone marrow macrophages (BMM) which is the normal environment for amastigotes. Mouse BMM were infected for 24 h with transfected promastigotes previously cultured in increasing G418 concentrations, and proliferation of the resulting amastigotes within macrophages were monitored over a 5-day period. The percentage of infected macrophages for pKSneo (72%) and pKSneo-3'NT transfectants (65-82%) at day 0 indicated that there was no significant difference in the ability to enter macrophages. Figure 6A demonstrates that truncated 3'NT/Nu-expressing amastigotes were less viable in macrophages than control pKSneo transfected amastigotes. These results reflect what was observed in the *in vitro* cell growth studies (Fig. 5), where cell growth was hindered by increasing levels of the truncated 3'NT/Nu expression in amastigotes. Macrophage infection levels were also reduced: at day 5, 100% of macrophages were infected with pKSneo amastigotes (control), 82% with pKSneo-3'NT amastigotes from promastigotes grown in G418-free medium, with a drop to 48% for cells previously grown in G418 25 μg/ml (data not shown).

The ability of pKSneo-3'NT transfected promastigotes to establish an infection was determined *in vivo* using transfected promastigotes to initiate an infection in BALB/c mice. Promastigotes were previously grown in different concentrations of G418 before injection through the tail vein of the mice. Four weeks post-infection, mice were examined for parasite burden by counting amastigotes from liver impressions. Values, given as Leishman-Donovan units (LDU), show

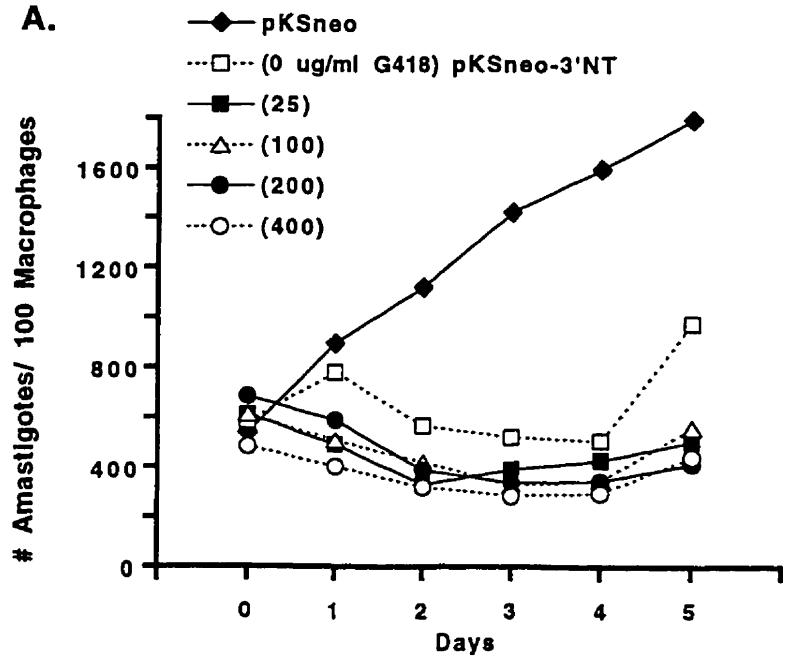

Figure 6. (A) Infection and growth rates of pKSneo-3'NT transfected *Leishmania* amastigotes in macrophages *in vitro*. Promastigotes transfected with the pKSneo control vector were grown in 25 µg/ml G418; pKSneo-3'NT were grown in concentrations of G418 ranging from 0 to 400 µg/ml G418. Late log-phase promastigotes were used to infect macrophages.

that there is no significant difference in the parasite burden between mice infected with wild-type promastigotes, promastigotes transfected with the pKSneo control plasmid, and promastigotes tranfected with the pKSneo-3'NT plasmid and grown without G418 (Fig. 6B). However, differences become apparent for transfected promastigotes previously cultured in 100, 200, and 400 μg/ml G418. The parasite burden of mice was affected by the levels of truncated 3'NT/Nu expressed in amastigotes with high levels corresponding to a decreased parasite burden, indirectly showing that amastigote proliferation decreased *in vivo* (Fig 6B). These results are consistent with the *in vitro* macrophage infection experiments showing that expression of 3'NT/Nu decreased viability in amastigotes.

Targeting of a suicide gene into the A2 coding locus.

We also tested developmental expression of a gene, with toxic potential, from an integrated construct targeted into the A2 locus. Targeting strategies using the truncated *3'nt/nu* gene did not result in correct integration of the gene. Southern blot assays revealed that the truncated *3'nt/nu* gene had integrated at the A2 locus and at another site, and Northern blot assays failed to demonstrate a developmental expression of the truncated 3'NT/Nu (data not shown). In a concurrent experiment, the HSV thymidine kinase gene was also targeted, successfully, to the A2 locus, and subsequent studies were therefore carried out on these *tk* gene-containing *Leishmania* cells.

B.

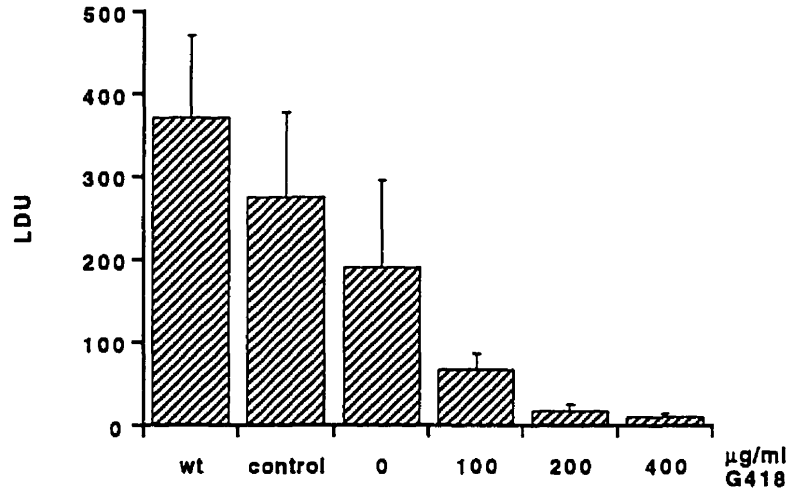

Figure 6. **(B)** Liver parasite burdens of mice infected with wild-type and transfected *Leishmania* cells grown at different G418 concentrations (0, 100, 200, 400 µg/ml). pKSneo control transfectants were grown in 25 µg/ml G418. Mice were injected with promastigotes via the tail vein ($1.5x10^8$ promastigotes/mouse, n=3/group). Four weeks post-infection, parasite burden was determined by counting amastigotes from liver impressions. Liver parasite burdens are expressed as Leishman-Donovan units (LDU) calculated as the number of amastigotes per 1000 macrophage nuclei X liver weight (g). The means per group are shown.

The strategy used to insert both the *neo* and *tk* genes into the A2 locus by homologous recombination is outlined in Fig 7A. The Southern blots confirmed the correct insertion of the *neo* and *tk* genes (Fig 7B). The *Pvu*II site within the *neo* sequence created *Pvu*II/*Mlu*I fragments of different lengths depending on the presence of the *tk*. In both the R.tk and R.ctl clones, double digests generated a 2.6 kb fragment visible on the membrane when hybridized with the Pro probe, confirming the localization of the *Neo* downstream from the Pro sequence. *Mlu*I or double digests generated a 3.8 kb fragment from R.tk probed with a 0.9 kb *Pst*I TK fragment or the A2 Tail sequence (1.7 kb *Xho*I/*Xba*I), while it generated a 4.2 kb fragment from R.ctl probed with the Tail. These results confirmed the localization of the *neo*, intergenic (IG) and the *tk* sequences upstream from the 3'UTR in the genome.

Northern blots of the TK targeted clone (R.tk) and of the control clone (R.ctl), which only contains the *neo* gene, demonstrate that induction of TK transcripts was upregulated in the amastigote stage of the R.tk clone (Fig 7C). The TK transcript length (2.8 kb) was consistent with processing occurring at the 3'end of the A2 Tail sequence and within the α-tubulin intergenic region. As expected, the NEO transcript is not differentially regulated (see Fig 1B). Its size (1.4 kb) corresponds to *trans*-splicing occurring in the Pro region and the α-tubulin intergenic region providing a polyadenylation site.

We next performed cell growth assays on promastigotes and amastigotes grown in media containing increasing concentrations of

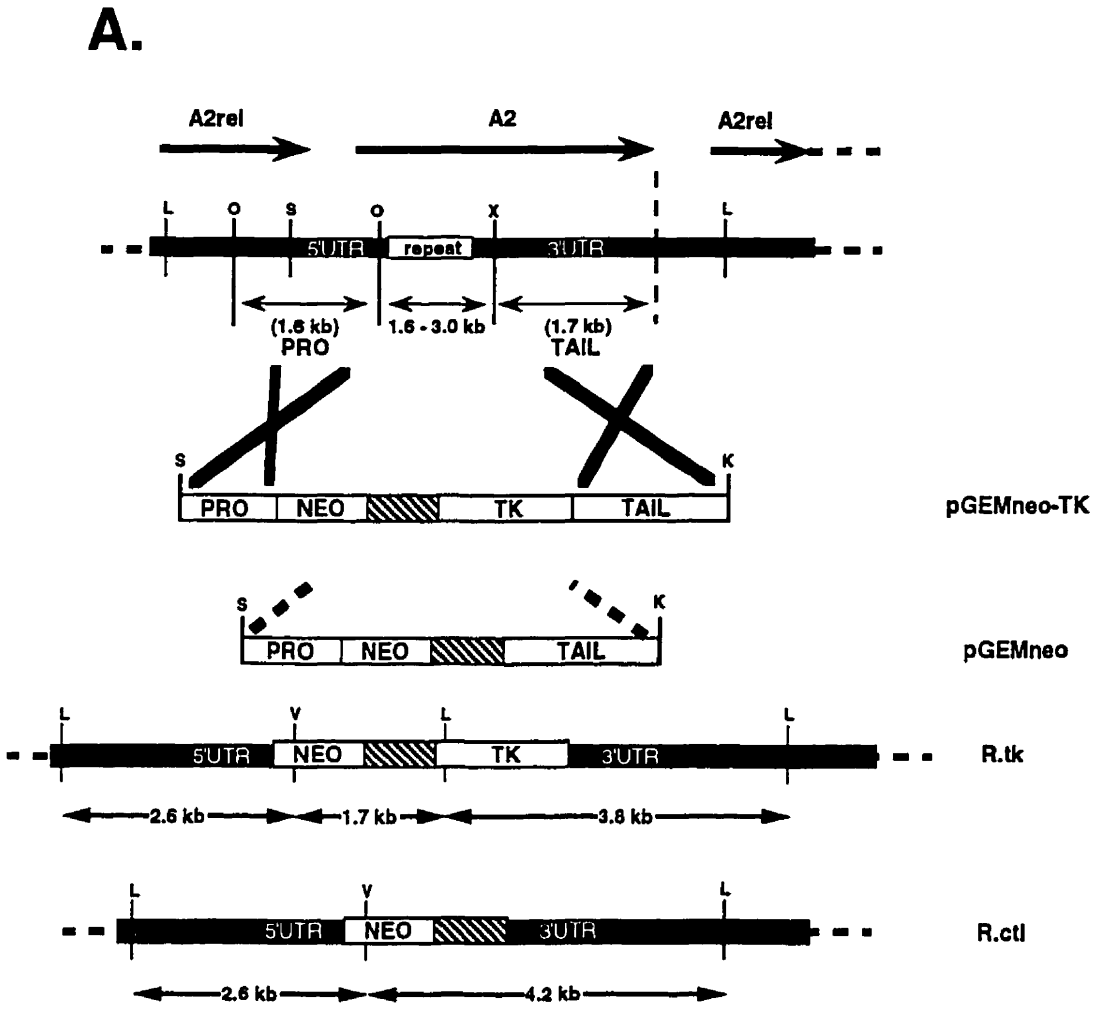

Figure 7. (A) Targeting strategy of the linearized pGEMneo (control) and pGEMneo-TK plasmids. S, *Sal*I; K, *Kpn*I; L, *Mlu*I; V, *Pvu*I. The *open box* represents the repeated section of the A2 coding region.

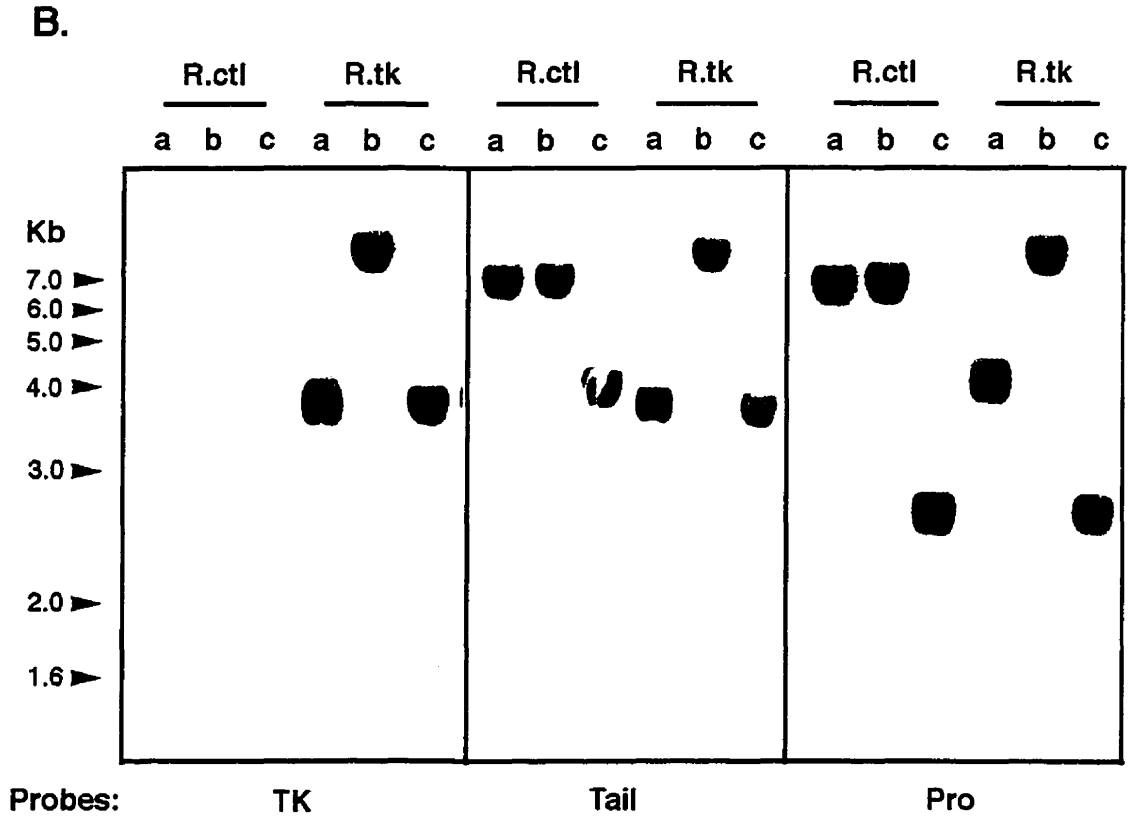

Figure 7. (B) Southern blot analyses of digested genomic DNA from selected recombinant clones, R.ctl and R.tk. Ten micrograms were loaded in each lane. a, *Mlu*I digest; b, *Pvu*II digest; c, *Mlu*I/*Pvu*II double digest. Probes: Pro, 1.6 kb *Xho*I fragment; Tail, 1.7 kb *Xho*I/*Xba*I fragment; TK, 0.9 kb *Pst*I fragment. Refer to Results section for details and rationale.

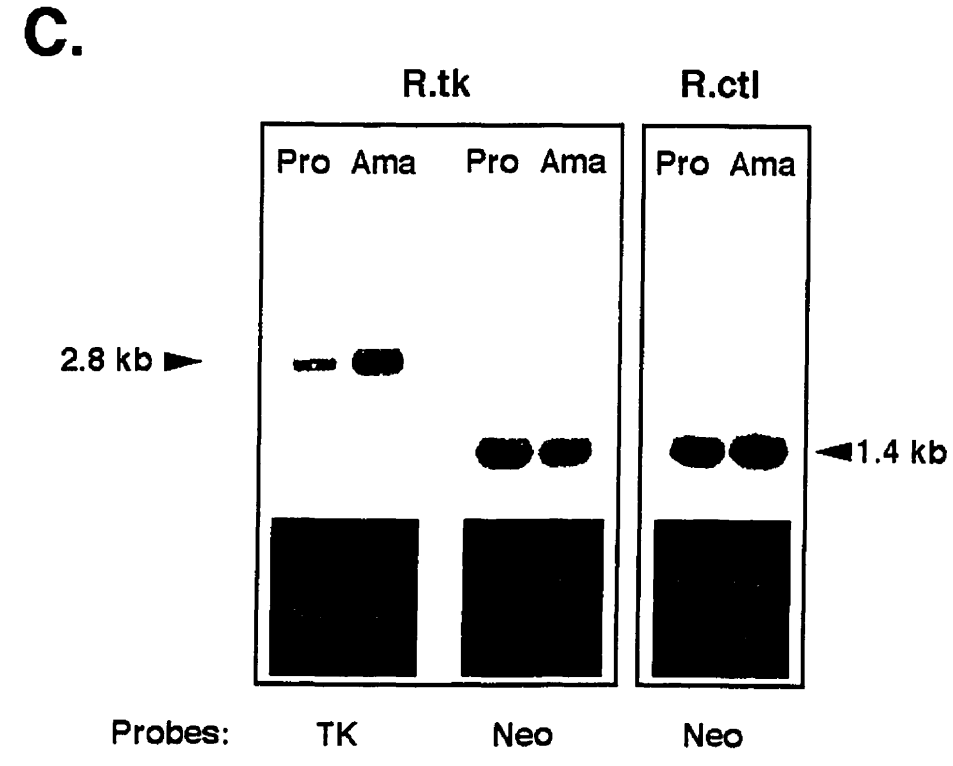

Figure 7. (C) Northern blot analyses of total RNA from promastigote and amastigote recombinant clones. R.tk, recombinant clone with the targeted *tk*; R.ctl, control clone which only contains the targeted *neo* gene. RNA samples (10 μg) were subjected to Northern blot analyses with the following probes: TK, 0.9 kb *Pst*I fragment from the *tk* gene; *neo*, 0.8 kb PCR fragment amplified from the pALTneo vector. The bottom panels show the total RNA present in each lane.

ganciclovir, which is converted to its toxic form in the presence of active TK. *L. donovani* lines were inoculated into media containing the indicated amounts of ganciclovir and allowed to grow until control cultures, in the absence of ganciclovir, had reached late log phase ($1x10^7$ cells/ml) (Fig 8A). The EC_{50} was defined as the concentration of ganciclovir which reduced the cell numbers by 50%, measured when the drug-free control culture was in late log phase, as previously described (Ellenberger and Beverley, 1989).

Wild-type promastigotes and amastigotes were unaffected by ganciclovir (Fig 8A). In contrast, both R.tk promastigotes and amastigotes were sensitive to ganciclovir but at different levels. Since TK is developmentally expressed at higher levels in amastigotes, amastigote TK transfectants were more sensitive than promastigote TK transfectants. EC_{50}s of 4.5 μM for amastigotes and 25μM for promastigotes (Fig 8A) indicate that the TK gene is overexpressed by approximately 5-fold in amastigotes.

In vitro infection studies in bone marrow-derived macrophages were performed with the R.ctl and R.tk clones (Fig 8B). Mouse BMM were infected for 24 h with R.ctl and R.tk promastigotes. Macrophages infected with R.tk were incubated in medium supplemented with increasing concentrations of ganciclovir. Infection of macrophages and growth rate of amastigotes within macrophages were monitored over a 5-day period. At day 0, there was an average of 450 amastigotes per 100 infected macrophages for R.ctl and 650 for R.tk clones. At day 5 there were approximately 750 amastigotes per 100

A.

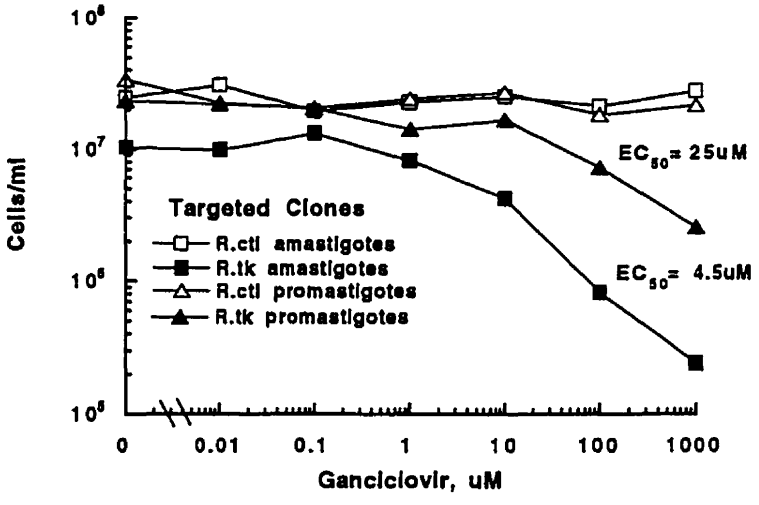

Figure 8. (A) Drug sensitivity of TK transfectants. R.tk and R.ctl clones were inoculated as promastigotes and amastigotes into media at a density of $2x10^5$ cells/ml. Different amounts of ganciclovir were added to the media at the indicated concentrations. Cells were allowed to grow until cultures in the absence of ganciclovir had reached late-log phase (approximately $1x10^7$ cells/ml). The EC_{50} was defined as the concentration that reduced the cell numbers by 50%, measured when the drug-free culture was in late-log phase.

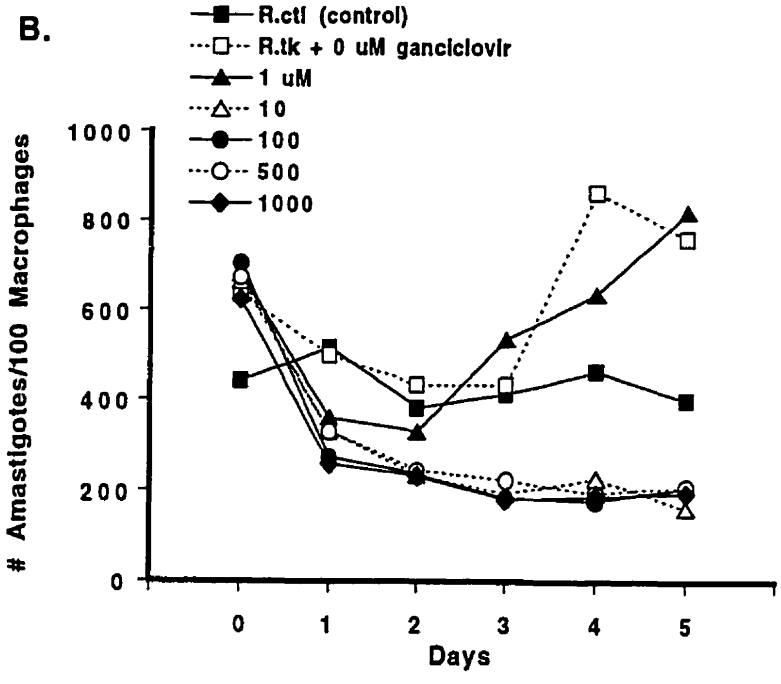

Figure 8. (**B**) Infection and growth rates of R.tk amastigotes in macrophages *in vitro*. Late log-phase promastigotes were used to infect macrophages which were then incubated with the addition of ganciclovir at different concentrations (0, 1, 10, 100, 500, 1000 μM). Amastigote infection levels in macrophages were evaluated daily. At day 0, cell samples were collected before the addition of ganciclovir.

macrophages for R.tk in drug-free medium, and 800 amastigotes per 100 macrophages in 1 μM ganciclovir. At 10 μM of ganciclovir, the growth rate decline was drastic, with levels of 180 amastigotes per 100 infected macrophages, as compared to the drug-free control (R.tk-infected macrophages grown without ganciclovir) and the cells in 1 μM ganciclovir (Fig 8B). At day 5, R.ctl infection levels stabilized at 400 amastigotes per 100 macrophages, suggesting that the R.ctl transfectants were not as infective as the R.tk transfectants grown in the absence of ganciclovir, and the reason for this is unknown.

These data show that promastigotes which carried the *tk* gene in the A2 genomic locus were relatively unaffected in culture by the presence of ganciclovir, until high concentrations of drug were reached. In contrast, when promastigotes were switched to the amastigote stage, either in culture or in the macrophage phagolysosomal compartment, an increased sensitivity to ganciclovir was induced. This demonstrates that negative selection genes can be targeted into the A2 locus for the developmental upregulation of toxic products.

DISCUSSION

We have demonstrated in this study the feasibility of using the A2 gene regulatory system to generate live attenuated *L. donovani* strains where attenuation is developmentally regulated. We have performed this by modulating expression of toxic products encoded by genes placed under the control of the A2 gene regulatory system, developmentally regulated during promastigote-to-amastigote differentiation. While promastigotes were shown to be largely unaffected by the low expression of toxic gene products, cell growth of amastigotes, which differentially express the toxic products, was hindered, and this was also reflected by a decreased survival in macrophages. We have also shown by gene replacement that the A2 locus can control the expression of negative selection genes, such as the *hsv-tk* gene.

We have analyzed two different suicide gene systems involving episomal and targeted constructs. Both suicide genes were placed under the control of the A2 UTRs in recombinant *L. donovani* cells. In our first construct, we have inserted between two A2 non-coding sequences a truncated version of the 3'nucleotidase/nuclease, a native *Leishmania* enzyme, to be developmentally expressed in amastigotes. The 3'nucleotidase/nuclease is an externally oriented surface membrane enzyme responsible for the hydrolysis of 3'nucleotides and single strand nucleic acids. The native surface membrane 3'nucleotidase/nuclease is a glycoprotein containing exposed mannose residues (Sacci *et al.*, 1990). By truncating its signal

sequence, the 3'NT/Nu did not enter the secretory pathway where its normal processing would occur, and this appeared to decrease its enzymatic activity. This was consistent with the observation that 3'NT/Nu expressed in *E. coli* had a 50-fold lower activity than the enzyme expressed in *Leishmania*, suggesting that post-translational modifications of the protein (e.g. glycosylation) are involved in its activity (Debrabant *et al.*, unpublished data). Clayton and Michels (1996) have also showed in trypanosomatid cells that some native products, in this case the phosphoglycerate kinase gene of bloodstream trypanosomes, may be toxic to the cells if not expressed at their usual site. In their study, the enzyme was induced in the cytoplasm of the organisms when it is normally contained in the glycosome.

The truncated *3'nt/nu* gene was on an episomal construct and increased expression was achieved by increasing the selection pressure with G418. Truncated 3'nucleotidase activity was detected in amastigotes, while there was no detection of enzyme activity in promastigotes. This resulted in reduced *Leishmania* amastigote viability. Truncated enzyme activity was shown to be lower than when in its native form, however amastigote cell growth was hindered even at low levels of truncated 3'NT/Nu accumulation. This may be due in part to amastigotes being more sensitive to truncated 3'NT/Nu than promastigotes. We have also demonstrated that a decreased cell growth of amastigotes in culture translated into a decreased replication of amastigotes in macrophages and in BALB/c mice.

The truncated 3'NT/Nu expressed in this study was on an episomal construct and not targeted into the A2 locus. Although Coburn *et al.* (1991) have demonstrated that transfected DNAs can be maintained in the absence of G418 pressure *in vivo*, transfectants would be expected to eventually begin to lose their plasmids, increasing infection levels because of the loss of the toxic gene. Clearly, long term studies require the integration of the gene into the genomic locus. We have attempted such an integration with the truncated *3'nt/nu* but we have not succeeded in obtaining correctly targeted clones. We were more successful, however, with the targeting of the *tk* suicide gene into the A2 genomic locus. LeBowitz *et al.* (1992) and Muyombwe *et al.* (1997) describe the expression of the *hsv-tk* gene on episomal vectors in *L. major* for the creation of ganciclovir-sensitive strains.

We have shown by gene replacement with the *hsv-tk* gene that the A2 chromosomal locus can control the expression of negative selection genes. The fact that R.tk amastigotes, which contain the targeted *tk* in the A2 locus, were affected in their replication rate at a low ganciclovir concentration (10 μM), suggests that even at a low copy number, the *tk* gene renders the amastigotes sensitive to ganciclovir. By generating ganciclovir-sensitive *Leishmania* through targeting, we have also been able to quantitatively test the A2 locus.

Regulating expression of genes with a toxic potential in the form of the parasite responsible for pathogenesis establishes a cell line which is attenuated. The particularity of using the A2 gene regulatory

system is that attenuation would be developmentally regulated. This type of cell line may have different applications. For example, it may be used in *L. donovani* infection models to illustrate certain aspects of the immune response during infection, under conditions where the infection is not lethal. It may also have a role to play, along with other genetic modifications (such as the removal of virulence genes), in the development of a live attenuated vaccine. Even in the case of cutaneous leishmaniasis where infection, once resolved, confers solid immunity to reinfection, there is no safe and effective vaccine. There always exists the risk of an attenuated mutant reverting back toward virulence. The safest live *Leishmania* vaccine could be one that involves a combination of approaches, such as the inactivation of certain virulence genes, such as A2 (Zhang and Matlashewski, 1997), the deletion by gene targeting of essential metabolic genes, such as the DHFR-TS (Titus *et al.*, 1995), and the developmental expression of toxic products, as shown in this study.

ACKNOWLEDGMENTS

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CONNECTING STATEMENT TO MANUSCRIPT III

A2 and A2rel genes are associated in *L. donovani* on a 850 kb chromosome, with copies of A2 alternating head to tail with copies of A2rel. We have shown in Manuscript I that A2 gene sequences are conserved in species of the *L. donovani* and *L. mexicana* complexes, and that this characteristic contibutes to its serodiagnostic potential. In Manuscript III, we investigated whether A2rel sequences were also restricted to these two *Leishmania* species. In Manuscript II, we used part of the A2rel coding region for the construction of plasmids. This A2rel sequence was included with the 5'UTR of A2 as part of the Pro element which provided polyadenylation and *trans*-splicing sites necessary for the correct processing of mRNA transcripts. The Pro was also used, in conjunction with the A2 3'-UTR, for the targeting of exogenous genes in the *Leishmania* genome. In Manuscript III, we provide the predicted amino acid sequence of A2rel.

CHAPTER 4

A Constitutively Expressed *Leishmania* Gene Linked to an Amastigote-Stage-Specific Gene

(MANUSCRIPT III)

ELODIE GHEDIN, HUGUES CHAREST and GREG MATLASHEWSKI

Leishmania parasites, in the course of their life cycle, differentiate from promastigotes in the sandfly vector to amastigotes in the mammalian host. The promastigote to amastigote cytodifferentiation takes place in the host macrophage phagolysosome, where the parasite resides and multiplies (Chang and Dwyer, 1976). Upon inoculation into the mammalian host, the parasite is exposed to harsh environments such as the ones generated first by the serum lytic activity (Hoover *et al.*, 1984) and then, in the host-cell phagolysosomes, by hydrolytic enzyme activities as well as the production of active oxygen derivatives (Wilson *et al*, 1994). *Leishmania* survival depends on its ability to enter into and to down-regulate certain functions of its host cell, to resist toxic agents and to express metabolic enzymes optimally active at low pH (Beverley, 1996; Green *et al*, 1991; Mauël, 1990; Mukkada *et al.*, 1985).

Our laboratory has been studying the molecular basis of the promastigote to amastigote transformation by isolating and characterizing amastigote-specific genes as well as genes closely linked to these (Zhang and Matlashewski, 1997; Zhang *et al.*, 1996; Charest *et al.*, 1996; Charest and Matlashewski, 1994). The A2 gene represents one of the first amastigote stage-specific genes characterized in *Leishmania*. The A2 protein was identified and it was found to be specifically expressed in amastigote-like cells (Zhang *et al.*, 1996). The A2 protein was also shown to be a virulence factor since it is required for amastigote survival in the mammalian host (Zhang and Matlashewski, 1997). In characterizing the A2 locus, another gene, termed A2rel, was found to be tandemly associated

with the A2 genes on the genome (Charest *et al.*, 1996). Because of the importance of A2 in amastigote survival (Zhang and Matlashewski, 1997), it was of interest to examine the A2rel gene which is closely associated with the A2 gene. Figure 1A shows the arrangement of the A2rel gene with copies of A2 genes on a genomic clone (Geco90) revealing that the A2rel gene is tandemly repeated with the A2 gene. We first compared the expression pattern of the A2rel and A2 genes in cultured promastigotes and amastigotes. Previous studies using cultured promastigotes switched to amastigote conditions, mimicking the transfer from the insect vector to the phagolysosomal compartment of the mammalian host macrophage cell, demonstrated that expression of A2 mRNAs was induced within 10 hours while the expression of A2rel remained constant during differentiation from promastigotes to amastigotes. As shown in Fig. 1B, while A2 transcripts can be induced by temperature and pH shifts mimicking the passage from the insect vector to the phagolysosomal compartment of the macrophage cell, levels of A2rel transcripts remain relatively constant (Charest *et al.*, 1996; Charest and Matlashewski, 1994). However, in these studies, amastigote-like cells were generated by switching late log-phase promastigotes to low pH and high temperature conditions overnight but could not be cultured for longer periods or passaged as amastigotes. The particular clone used in the study presented here (*L. donovani donovani* Sudanese strain 1Scl2D) can be cultured and passaged as promastigotes and amastigotes *in vitro* and retains its infectivity. As shown in Fig.1B, and concordant with the studies mentioned, A2 transcripts (3.5 kb) accumulate in cultured amastigotes at a higher

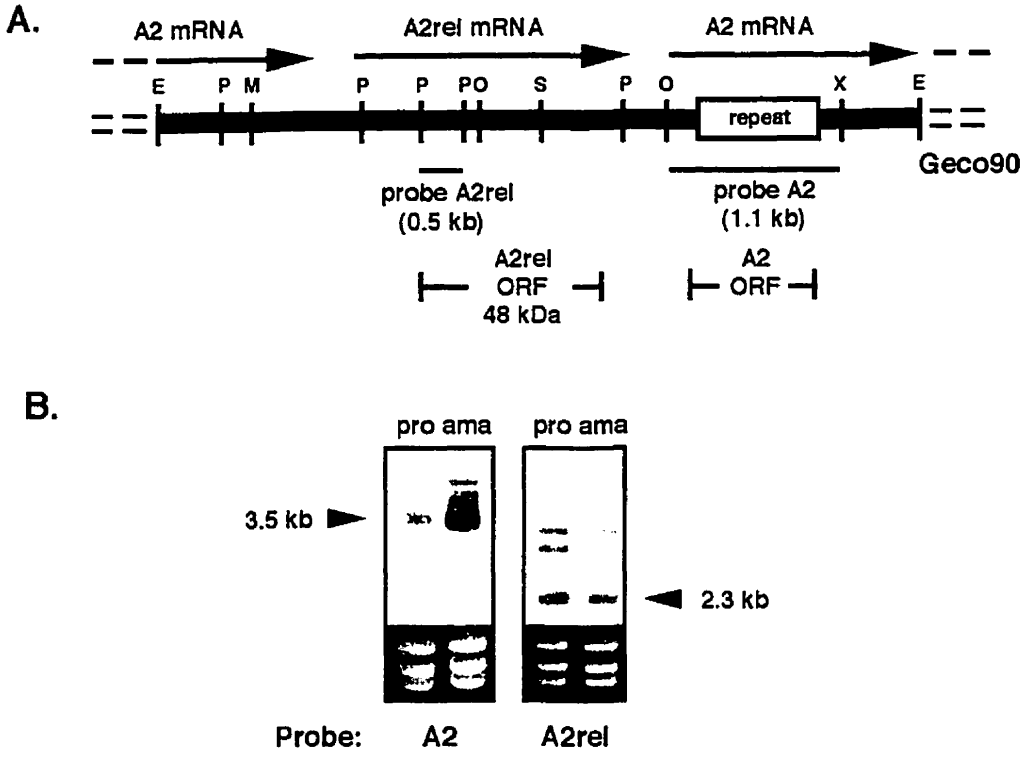

Figure 1. (**A**) Genomic arrangement of A2 and A2rel genes and localization of the A2rel open reading frame (ORF). The genomic clone Geco90 is derived from the *L. donovani infantum* Ethiopian LV9 strain (Charest and Matlashewski, 1994). This clone carries parts of two A2 gene copies and the coding region of the A2rel gene. A 1.3 kb open reading frame for A2rel was found between the two A2 gene copies. P, *Pst*I; O, *Xho*I; S, *Sal*I, E, *Eco*RI; M, *Sma*I; X, *Xba*I. The open box represents the repeated region within the A2 gene. (**B**) Northern blot analysis; A2 and A2rel gene expression in *Leishmania* promastigotes and amastigotes. *L. donovani* Sudan strain 1Scl2$_D$ cells were grown as promastigotes at 26°C, pH 6.8 in M199 medium containing 10% FBS, or as amastigotes at 37°C, pH 5.5 in RPMI supplemented with 20% FBS. Total RNA was extracted from promastigotes and amastigotes cultured as above for three days.

level than in promastigotes while A2rel transcript levels (2.3 kb) are similar in both stages of the parasite. The two higher molecular weight A2rel transcripts could derive from alternate *trans*-splicing and polyadenylation.

We have recently shown that A2 genes are not well conserved among *Leishmania* species and that they are only detected in strains of the *L. donovani* and *L. mexicana* complexes (Ghedin *et al.*, 1997). We therefore determined how well the A2rel gene was conserved among *Leishmania* species. As shown in Fig.1C, karyotype analyses extended to several species reveal that only subspecies of the *L. donovani* complex or of the *L. mexicana* complex carry copies of the A2 gene (Figure 1C; Ghedin *et al.*, 1997). In comparison, A2rel is conserved in the four groups responsible for pathology in humans including subspecies of the *L. tropica* complex (Fig.1C & 1D) and members of the *L. braziliensis* complex (data not shown). We also observe by Southern blot analysis of DNA derived from three geographic isolates of *L. donovani* (Sudan, Ethiopia and India) and single isolates of *L. donovani infantum*, *L. major* and *L. tropica*, that contrary to the A2 gene, the A2rel is well conserved in *Leishmania* species.

We next determined the nucleotide and amino acid sequences of the A2rel gene and the amino acid sequence is shown in Fig.2A. The complete sequence of the A2rel gene was obtained by sequencing a 2.3 kb fragment from a genomic clone derived from the *L. donovani*

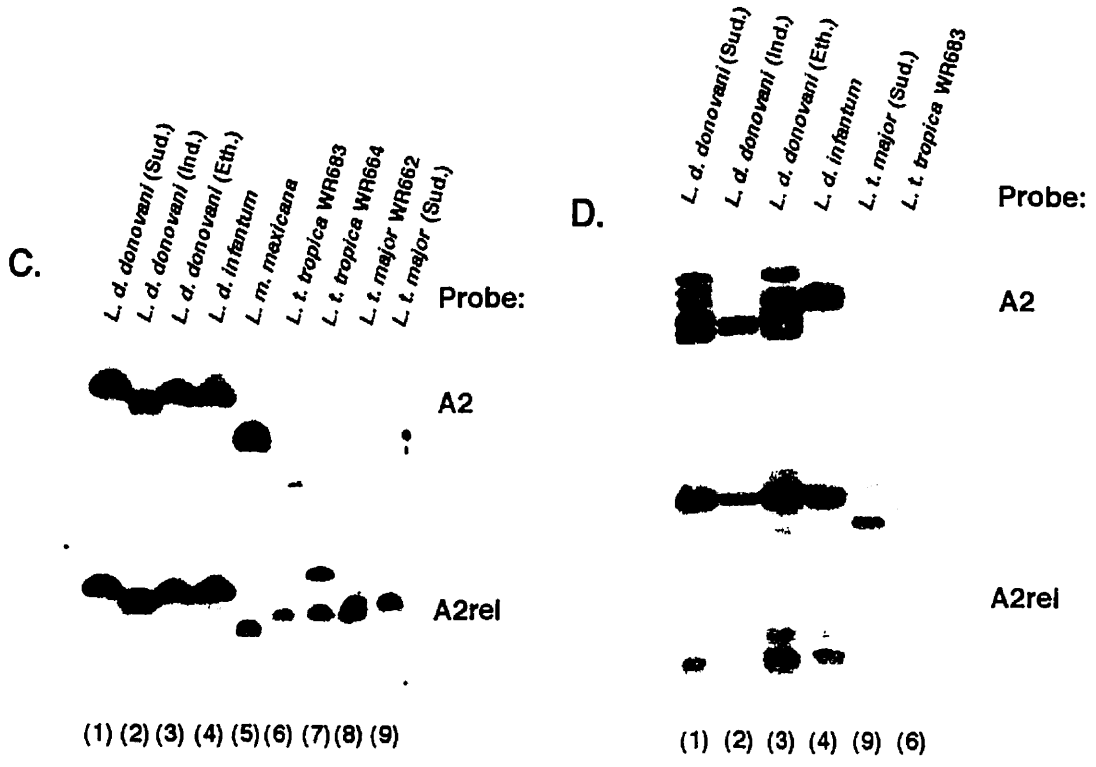

Figure 1. (**C**) Karyotype analysis; Chromosomes of the *L. donovani* complex: *L. d. donovani* from Sudan (1), WR657 from India (2), WR684 from Ethiopia (3), and *L. d. infantum* from Spain (4); of the *L. mexicana* complex: *L. m. mexicana* from Brazil (5); and of the *L. tropica* complex: *L. t. tropica* WR683 (6) and WR664 (7) from Sudan, *L. t. major* WR662 from Israel (8) and *L. t. major* from Sudan (9) were separated by pulsed field gel electrophoresis (PFGE). Southern blot membranes were hybridized at high stringency with a 0.5 kb *Pst*I probe representing part of the A2rel coding region or a 1.1 kb *Xba*I/*Xho*I fragment (see Fig.1A) representing the A2 protein coding region. (**D**) Southern blot analysis of DNA isolated from some of the above strains. Genomic DNA was digested with *Mva*I and restriction fragments were separated on a 0.7% agarose gel. Southern blot membranes were hybridized as described for the karyotype analysis.

A.

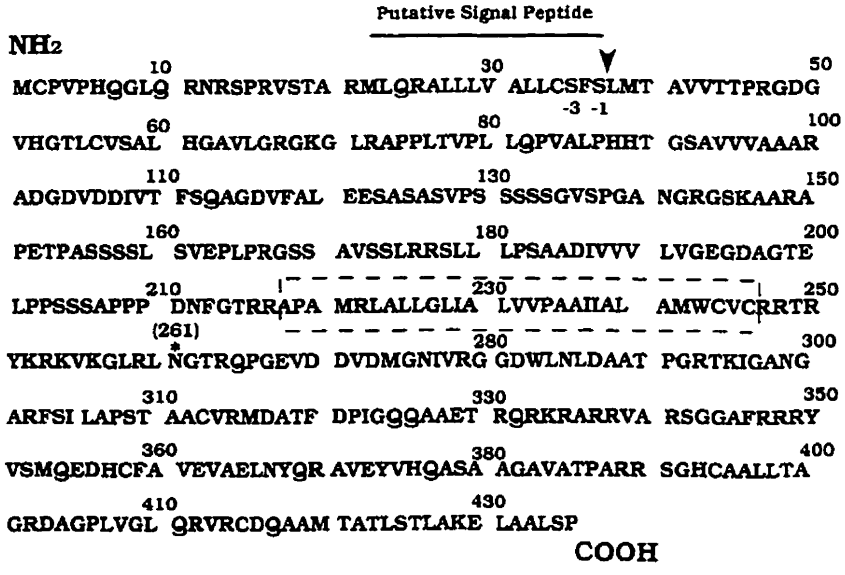

Figure 2. (A) Amino acid sequence derived from the A2rel open reading frame. A transmembrane domain, represented by the dashed box, appears between amino acids 218 and 246. A motif search revealed a potential N-glycosylation site at 261.

infantum Ethiopian LV9 strain (Charest *et al.*, 1996). Sequencing was carried out on deletion clones obtained by subcloning fragments into the KS-pBluescript plasmid in opposite directions and subjecting them to limited Exonuclease III digestion. The sequence was obtained by using overlapping deletion derivatives. We detected an open reading frame of 1308 bp which potentially codes for a protein with an expected molecular weight of 48 kDa. However, the sequence contains three in-frame start codons and there appears to be a signal peptide starting at the second methionine (Figure 2A). It has the characteristic tripartite domain structure of eukaryotic signal peptides: a positively charged n region, a hydrophobic stretch, and the c region containing polar amino acids (von Heijne, 1990). A potential cleavage site, conforming to the (-3,-1) rule, appears at a.a. 37: residues in positions -1 and -3 are small and uncharged (Ser), while a large and charged residue appears at position -2 (Phe) (von Heijne, 1983). A2rel was predicted by the Kyte-Doolittle hydrophilicity plot (Kyte and Doolittle, 1982) and the TMAP program (EMBL, Heidelberg; Persson and Argos, 1994) to possess 2 transmembrane domains (Fig.2B) with a major one between amino acids 218 and 246, suggesting that A2rel may be a membrane bound protein.

The A2rel sequence was subjected to different database searches to identify homologous genes and its best score was obtained with the US7 genes of herpes simplex virus type 1 and type 2 (HSV-1, HSV-2) which code for glycoprotein I. The FASTA output (SWISS-PROT Database search by the EMBL FASTA server; Pearson and Lipman,

B.

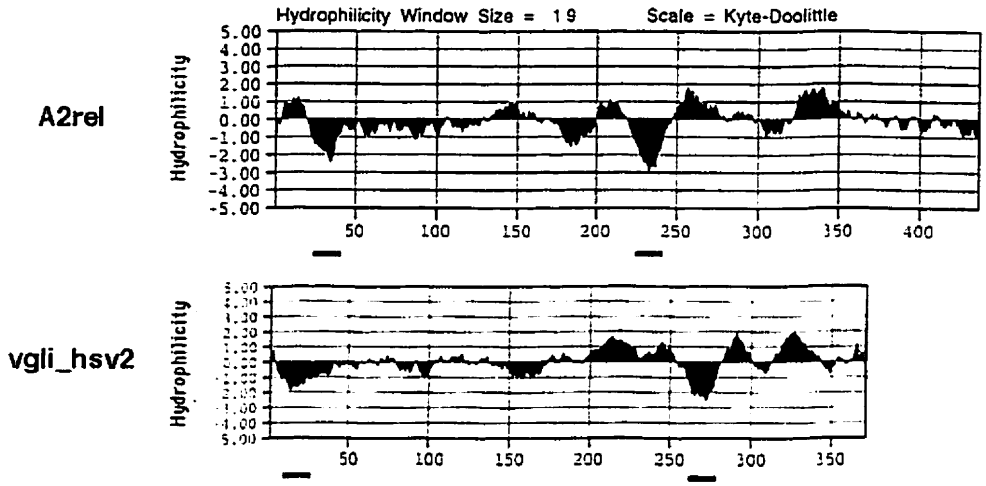

Figure 2. (B) Kyte-Doolittle hydrophilicity plots of A2rel and glycoprotein I of HSV2 (vgli_hsv2); Hydrophobic areas are represented below the axes. Potential transmembrane domains are indicated by *black bars* drawn below each plot.

1988) gave a 25% identity of the A2rel amino acid sequence with the US7 gene product (glycoprotein I) from HSV-2 in a 264 aa overlap and from HSV1 in a 175 aa overlap. The amino acid alignment of A2rel and HSV-2 glycoprotein I, and the identification of transmembrane domains suggest that A2rel and HSV-2 glycoprotein I have a similar transmembrane topology. Herpes simplex virus glycoprotein I is thought to influence interactions between cells at the plasma membrane. Disruption of the US7 (gI) gene resulted in pronounced attenuation of the HSV-1 virus which was rapidly cleared from the inoculation site (Balan *et al.*, 1994), revealing that the HSV-1 US7 gene is required for viral virulence. Further experiments are needed to determine if A2rel is also required for *Leishmania* virulence.

Genes clustered in tandem arrays may carry similar and complementary functions. Determining the function of the A2rel encoded product may help in defining the A2 gene function for *L. donovani* amastigotes during the course of the infection. This may be important given that A2 represents a virulence factor required for infection of the mammalian host (Zhang and Matlashewski, 1997). Although it is clear that there is no homology between A2 and A2rel genes, it will be important to determine what role A2rel may play in virulence and experiments are underway to address this issue.

ACKNOWLEDGMENTS

We wish to thank Dr. Greg Pogue for generously donating the Southern blot membranes of Fig. 1D. Research at the Institute of Parasitology is partially funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Fonds pour la formation de chercheurs et l'aide à la recherche du Québec (FCAR). EG holds a PhD Studentship from FCAR and GM holds an MRC Scientist Award.

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APPENDIX

TO MANUSCRIPT III

The complete nucleotide sequence of A2rel, corresponding to the length of its mRNA, is presented in Figure 1 of this Appendix. The sequence is also available on GenBank™, accession number AF016403.

Figure 2, and this section, summarize our preliminary efforts to produce antibodies directed against the A2rel protein. In initial characterization experiments, we have raised a polyclonal antibody against a recombinant A2rel protein fused to β-galactosidase which was synthesized in *E. coli*. Mice were immunized with the purified A2rel/β-galactosidase fusion protein and their serum was tested for the presence of anti-A2rel antibodies. A pool of mouse sera was used on Western blot to detect an A2rel/GST fusion protein, which would run at around 75 kDa: The complete A2rel open reading frame corresponds to a 48 kDa protein and the GST is a 27 kDa protein. The polyclonal serum was also used to search for the native A2rel protein in *Leishmania* cells (Figure 2). The antisera reacted with an A2rel/GST fusion protein of 65-70 kDa (lane b) (the lower bands represent degradation products), which suggests that the predominant fusion protein produced in *E. coli* was initiated at a start codon downstream from the first ATG of the A2rel open reading frame. Reactivity, however, was very weak with whole cell lysates obtained from promastigotes (lane c) and amastigotes (lane d). We expected the native A2rel protein to run between 44 and 48 kDa. The A2rel protein was also produced using an *in vitro* transcription translation reaction (TNT system, Promega). The deduced open reading frame was amplified by PCR and subcloned into a pBluescript

KS plasmid to synthesize a S^{35}-Methionine-labelled A2rel protein from the T7 promoter (Figure 3). A protein of around 48 kDa was synthesized, indicating that the deduced open reading frame does not contain stops. A p53 constructed into a pBluescript SK vector was used as a positive control for the reaction. The mouse polyclonal, as well as the serum from an individual with visceral leishmaniasis, failed to recognize the A2rel protein produced *in vitro* (data not shown).

Because of our failed attempts with the mouse polyclonal, and the low volumes of polyclonal serum available, we had decided to generate a rabbit polyclonal antiserum. The affinity purification of a A2rel/GST fusion protein generated a cleaved product of 30 kDa. The next attempt was in obtaining a A2rel recombinant protein bound to a histidine affinity tag (QIAexpress system; QIAGEN, Canada). The A2rel sequence was subcloned via *BamH*I into the pQE-30 expression vector and all transformed *E. coli* cells tested by the miniprep method carried the insert in the wrong orientation, suggesting that the A2rel product was lethal to the cells.

Future studies on A2rel require the generation of a polyclonal or monoclonal antibody that will permit the detection of the protein in its native form. The technical problems that have been so far encountered are presently being addressed in Dr. Matlashewski's laboratory. The study of A2rel and of its association with A2 may provide more information on virulence in *Leishmania*.

```
(1)
AGCACCGCTATCAAAGGCTGACCGCAGCGCCTGTACACGGCGGCCGCCAGTAGGCCGTCCCACTGC
TTGATACACCGCTTACCCTTCCCTCTATCCCCCCCACACACACACTGCAGCGATAGGCTTCGGCA
TCCGGCAACTCGCATACACGCGCGGATACGCGGGGAGAAGTCGAAGCCGCCCGCGGAGTAATGCG
TGCAAAGCGCATCCGCAGCTCATTGGCCGAGCGCATCGAGGACGCGTGCGGTGCGCCTTCAGCAA
CCGCAAACAGAGGAAAACACACGAGGGCGTCATTTGAGTGTGCGCGCACGTTTGCGTGGTGATGC
TGGGAGAGTGAGAGGGGGAGGGGGAGTGCGCTGTGTGCTTCGTACACCACCGTCTCTCTTCTCTGC
TGGCGCCGCGCATCATCGGCACTGGAAGAGGGGTTGCGCCTCTTTTTCTTTCCCGCGTGCGCTGCCT
TCACCCCTTCTGCCTGTGGTGTCTGTGCCGCTGTCCCGGCACACGCCCACAGACGTTTCTTCCCGGCT
GTTTCCCTCTTTAGTGCGGCGGCAATCTTGCTGCGAGCCCTTACGCACGAGCAATGATAGGTACGC
CCACATAAGCATAACC

                1  ATG TGT CCC GTG CCT CAT CAA GGG CTG CAG CGC AAC CGC AGT CCT CGC GTC TCC ACG GCG CGA
                   M  C  P  V  P  H  Q  G  L  Q  R  N  R  S  P  R  V  S  T  A  R
64      ATG CTT CAA CGG GCG CTG TTG CTT GTG GCC CTG CTG TGC AGT TTC TCA CTT ATG ACG GCG GTG GTG ACG ACA CCC CGC
        M  L  Q  R  A  L  L  L  V  A  L  L  C  S  F  S  L  M  T  A  V  V  T  T  P  R
142     GGC GAC GGT GTT CAT GGC ACT CTT TGC GTC TCT GCC TTA CAC GGC GCT GTA TTG GGG AGG GGG AAG GGC CTA CGC GCG
        G  D  G  V  H  G  T  L  C  V  S  A  L  H  G  A  V  L  G  R  G  K  G  L  R  A
220     CCA CCG CTC ACT GTG CCC TTG CTG CAG CCT GTA GCG CTA CCG CAC CAT ACG GGT TCC GCT GTT GTG GTG GCC GCC GCG
        P  P  L  T  V  P  L  L  Q  P  V  A  L  P  H  H  T  G  S  A  V  V  V  A  A  A
298     CGG GCT GAC GGC GAT GTT GAC GAC ATC GTC ACT TTT TCG CAA GCC GGC GAT GTG TTC GCC TTG GAG GAG AGC GCT TCT
        R  A  D  G  D  V  D  D  I  V  T  F  S  Q  A  G  D  V  F  A  L  E  E  S  A  S
376     GCA TCC GTG CCG TCT TCC TCG AGC TCC GGC GTC AGC CCA GGC GCT AAC GGG AGG GGC AGC AAA GCA GCA AGG GCT
        A  S  V  P  S  S  S  S  S  G  V  S  P  G  A  N  G  R  G  S  K  A  A  R  A
451     CCA GAG ACC CCG GCT TCC TCG TCG TCG TTG TCC GTA GAG CCG TTG CCG CGG GGG TCG TCC GCG GTG TCA TCG TTG AGG
        P  E  T  P  A  S  S  S  S  L  S  V  E  P  L  P  R  G  S  S  A  V  S  S  L  R
529     AGG TCG CTG TTA TTG CCC TCA GCG GCC GAC ATT GTT GTC GTC TTG GTC GGT GAA GGC GAC GCG GGC ACA GAA CTG CCC
        R  S  L  L  L  L  P  S  A  A  D  I  V  V  V  L  V  G  E  G  D  A  G  T  E  L  P
607     CCC TCA TCG TCA GCA CCC CCT CCC GAC AAC TTC GGC ACG CGC CGC GCA CCT GCG ATG CGC CTC GCT CTC CTC GGT CTC ATC
        P  S  S  S  A  P  P  P  D  N  F  G  T  R  R  A  P  A  M  R  L  A  L  L  G  L  I
688     GCC CTC GTG GTG CCC GCT GCT ATC ATC GCC CTT GCC ATG TGG TGC GTC TGC CGC CGC ACG CGG TAC AAG CGA AAG GTA
        A  L  V  V  P  A  A  I  I  A  L  A  M  W  C  V  C  R  R  T  R  Y  K  R  K  V
766     AAG GGC TTG CGT CTC AAC GGC ACT CGT CAA CCC GGC GAG GTG GAT GAC GTG GAC ATG GGC AAC ATT GTC AGG GGC
        K  G  L  R  L  N  G  T  R  Q  P  G  E  V  D  D  V  D  M  G  N  I  V  R  G
841     GGC GAT TGG CTG AAC CTC GAC GCT GCC ACC CCA GGC AGA ACT AAG ATC GGC GCC AAC GGC GCG CGT TTC TCC ATT TTG
        G  D  W  L  N  L  D  A  A  T  P  G  R  T  K  I  G  A  N  G  A  R  F  S  I  L
919     GCG CCG TCG ACC GCT GCT TGC GTT CGC ATG GAC GCC ACG TTC GAC CCG ATC GGG CAG CAG GCC GCG GAG ACG CGG CAG
        A  P  S  T  A  A  C  V  R  M  D  A  T  F  D  P  I  G  Q  Q  A  A  E  T  R  Q
997     CGC AAA CGT GCC CGG CGG GTG GCT CGT TCC GGA GGA GCG TTC CGC CGT CGC TAC GTC AGC ATG CAA GAG GAT CAT TGC
        R  K  R  A  R  R  V  A  R  S  G  G  A  F  R  R  R  Y  V  S  M  Q  E  D  H  C
1075    TTT GCG GTT GAG GTG GCG GAG CTC AAC TAC CAG CGT GCC GTG GAA TAC GTT CAT CAA GCG TCA GCC GCC GGC GCG GTC
        F  A  V  E  V  A  E  L  N  Y  Q  R  A  V  E  Y  V  H  Q  A  S  A  A  G  A  V
1153    GCG ACA CCT GCT CGG CGG AGC GGC CAC TGT GCT GCT CTC TTG ACG GCC GGT CGC GAC GCA GGG CCG CTG GTG GGT CTC
        A  T  P  A  R  R  S  G  H  C  A  A  L  L  T  A  G  R  D  A  G  P  L  V  G  L
1231    CAG CGG GTA CGG TGC GAT CAG GCA GCC ATG ACG GCT ACG CTG AGC ACG CTT GCG AAG GAG CTG GCT GCA CTG TCG CCG
        Q  R  V  R  C  D  Q  A  A  M  T  A  T  L  S  T  L  A  K  E  L  A  A  L  S  P
1309    TAG
        *

            CGCGATGCGAGACGTATCGAGCGCATGCCAGTGGGGTGCTGGGCAACACTTGGCGGCG
1370    CGTGGCTCTTTCTCACTGGTTTGGGCACGCCCACGCTGTGGGTGTGTACCTGTCTGCCCTGCC
1433    GCTCTCTCTCCTGATCTCTCGTCCTGGCAACAACAACACACCGCGAATGAGTTGCCCACAAG
1495    CCATCTGCAGTCCGCACGCGCGTCCGGGCCTTGCGGTGTGGATGCGAGCATCGCGCATTGC
1556    CGGCCACCGCCGCGTGCCAAAAGCACACACATCGCTCCCCCTCACGTGGTGAGCGTCTGCGC
1618    GTGTGTGTCTGTGTGCATAAATGCGTACGTGTGCGCTCGGTCCACGGTCACCATCGCGCCCCG
1681    CGGCATCGGTGCTCGCACCTTTCCATTCTCTCCGT
                                                                  (2324)
```

Figure 1. Nucleotide sequence of the A2rel gene carrying the coding region and the deduced protein sequence. The first two nucleotides, AG, correspond to the *trans*-splicing acceptor site. The A of the initiation codon ATG was given number +1. Numbers in parenthesis represent the complete nucleotide sequence. Underlined nucleotides correspond to *Pst*I sites.

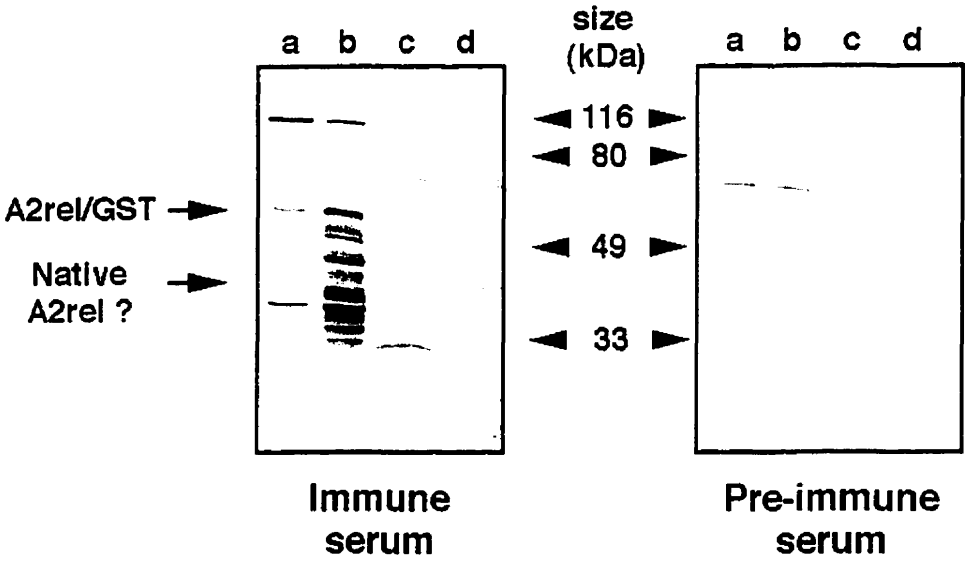

Figure 2. Western blot analysis of the A2rel protein expressed in *E. coli* and in *Leishmania* cells. Total cell lysates from control *E. coli* which were transformed with the pGEX-2T vector (lane a), *E. coli* expressing the A2rel/GST fusion protein (lane b), and from *L. donovani* Sudanese 1S2D strain promastigotes (lane c) and amastigotes (lane d) were run on a 10% SDS-PAGE. The A2rel/GST fusion protein was obtained by subcloning a PCR-amplified 1.0 kb fragment, from the genomic clone GECO90 (Charest and Matlashewski, 1994), into the pGEX-2T vector which contains the glutathione *S*-transferase gene (GST). Western blot analysis was performed using a 1:100 dilution of the mouse polyclonal (immune serum) or serum from pre-immunized mice (pre-immune serum). The polyclonal antisera was generated by immunizing mice subcutaneously with 50 μg of a purified A2rel/β-galactosidase fusion protein expressed in *E. coli* from the recombinant plasmid. The recombinant protein was produced by subcloning a 1.2 Kb *Pst*I fragment of gene A2rel into a lacZ fusion vector (pUR291), tranforming *E. coli* cells with the plasmid and inducing protein production from the lac promoter with 0.1M IPTG. The recombinant protein was purified by electroelution from a 10%-SDS gel. The first arrow indicates the A2rel/GST fusion protein in lane b; the second arrow points to where the native A2rel should migrate (between 44 and 48 kDa) in lanes c and d.

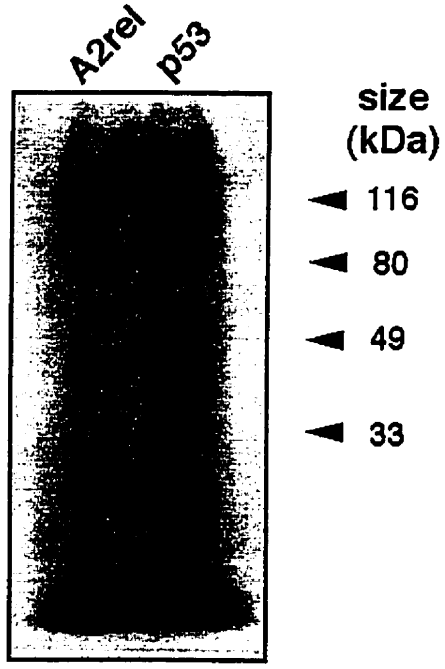

Figure 3. *In vitro* production of the A2rel protein. A 1.5 kb sequence, containing the A2rel deduced open reading frame, was amplified by PCR from the genomic clone GECO90 and subcloned *Bam*HI/ *Eco*RI into a pBluescript KS plasmid. This plasmid contains the T7 promoter and it was used in an *in vitro* transcription translation reaction (TNT system, Promega) to synthesize a S^{35}-Methionine-labelled A2rel protein. The p53 protein was used as a positive control for the *in vitro* assay. Proteins were separated on a 10% SDS-polyacrylamide gel and exposed to an autoradiograph film.

GENERAL DISCUSSION

In its transformation from a promastigote to an amastigote, *Leishmania* has developed means of adapting to different environments. This implies that there is differential expression of genes, since the control of stage-specific gene expression is one of the keys to successful adaptation. Our laboratory has focused on identifying *Leishmania* genes developmentally expressed during the amastigote stage, the rationale being that these genes would be essential for the survival of the parasite in its mammalian host. This research led to the characterization of one of the first amastigote-stage-specific gene families analyzed to date in *Leishmania*: the A2 gene family (Charest and Matlashewski, 1994; Charest *et al.*, 1996; Zhang *et al.*, 1996) shown to be a virulence factor in *L. donovani* (Zhang and Matlashewski, 1997).

In this thesis, I have presented follow-up research using the A2 gene family, and further characterization of the A2 locus by sequencing the gene which is associated with A2 in the genome of *L. donovani*, A2rel (Manuscript III). We have studied the applications of A2 by demonstrating, in Manuscript I, the potential of the A2 antigen in the serodiagnosis of visceral leishmaniasis. In Manuscript II, we have explored and demonstrated the feasibility of using the A2 gene regulatory system for the developmental expression of toxic products, in order to generate live attenuated *L. donovani* strains.

Diagnosis of visceral leishmaniasis using A2

The diagnosis of visceral leishmaniasis is often hampered by the lack of specificity of antigens due to antigenic relatedness between *Leishmania* species and other micro-organisms. This is particularly a problem when these pathogens co-exist. Through immunoblotting and ELISA, I have demonstrated that the anti-A2 antibody response was specific for patients with VL and with *L. mexicana* infections, and as such A2 holds great potential as a diagnostic antigen. However, because of cross-reactivity with *L. mexicana*, there may be a problem using A2 alone in regions where *L. donovani chagasi* and *L. mexicana amazonensis* are co-endemic, such as is the case in Brazil. Reed *et al.* (1987) have suggested the potential use of the 32- to 36-kDa region of total proteins subjected to SDS-PAGE for serologic distinction between these two types of infections. However, Tebourski *et al.* (1994) have observed that 58% of patients with Chagas' disease also reacted with the 32 kDa antigen.

No single antigen appears to be sensitive and specific enough to be applied in all the areas where visceral leismaniasis is endemic. The best reported antigen so far is rK39, a recombinant protein part of LcKin which is related to the kinesin superfamily of motor proteins (Badarò *et al.*, 1996; Burns *et al.*, 1993). In a Fast-ELISA test, rK39 was found to have a sensitivity of 98% for detecting specific anti-*Leishmania* antibodies in human VL, and a specificity of 99%. This antigen was also evaluated in a different type of rapid test developed by Badarò *et al.* (1997) referred to as TRALd (Rapid test

Antibody *Leishmania donovani*). In this type of test, the rK39 antigen is spotted in the center of a nitrocellulose strip; one extremity of the strip contains a pad of lyophilized Protein A conjugated with colloidal gold, while the other end of the strip has an absorbent pad. A colored precipitate appears in the middle of the strip if anti-*Leishmania* antibodies are detected in the serum. The sensitivity of the TRALd was 94% and the specificity 95% for the detection of specific anti-K39 antibodies. The rK39 was tested by ELISA for the diagnosis of VL in HIV-infected patients. Once again rK39 proved to be highly sensitive (82%) as compared to other detection methods where sensitivity barely exceeded 50% (Gradoni, 1997).

We suggest that A2 may have the same diagnostic value as rK39 and that it could be used in combination with other antigens in the "dip-stick" method suggested by Badarò *et al* (1997). Similar to K39, A2 is expressed by amastigotes, while it is virtually absent in promastigotes, and it is composed of a repetitive amino acid sequence which codes for immunodominant epitopes. A2 was also demonstrated to be a virulence factor necessary for the replication of the organism in its mammalian host (Zhang and Matlashewski, 1997). Individuals without clinical signs of infection have a lower number of replicating amastigotes in their lymphoid tissues than patients with acute disease. This is the major reason why amastigote-specific antigens may have a much stronger diagnostic value, as was demonstrated with rK39.

Developmental expression of negative markers in the amastigote stage

A2 proteins are developmentally expressed in the parasite during promastigote to amastigote cytodifferentiation. Although not essential for the survival of the parasite in *in vitro* cultures, A2 genes are required for the amastigote survival in mice (Zhang and Matlashewski, 1997). Developmental expression of A2 involves A2 mRNA untranslated regions (UTRs). It was previously demonstrated in our laboratory that A2 UTRs can regulate expression of exogenous genes throughout the *Leishmania* life cycle (Zhang and Matlashewski, 1997; Charest *et al.*, 1996). The A2 gene regulatory system, easily inducible *in vitro* by simple passage of promastigotes to amastigotes, represents an excellent model for studies on stage-specific developmental expression of toxic genes. By targeting *neo* as a positive selection gene, combined with a negative marker regulated by the 3'UTR, we created a strain of infective *Leishmania* that were able to multiply as promastigotes in axenic conditions but that were hindered in their survival as amastigotes.

Such a targeted strain may have a role to play as a component in the development of safer attenuated vaccines. Recovery from leishmaniasis leads to solid immunity against subsequent reinfections, and vaccination using attenuated *Leishmania* strains is a particularly interesting approach for inducing long term protection while lowering the inherent risk associated with the use of live parasites. The production of live attenuated strains that contain

different modifications, such as the knockout of essential genes (Titus *et al.*, 1995), the blocking of essential proteins (Zhang and Matlashewski, 1997), and the expression of suicide genes, provide a safeguard against virulent revertants.

Live strains that become developmentally attenuated, due to the regulation of toxic product expression, can invade macrophages and persist for several days, mimicking a natural infection without long-term persistence of the organism. During this process, host responses to the infection can be monitored, whereby providing information on T cell proliferation and cytokine production. This may in turn help answer questions related to the mechanisms of differential induction of defined T cell subsets involved in *L. donovani* infection and in the subsequent clearing of the parasite. The study of parasite-specific T cell responses may lead to the development of more effective vaccines and/or immunotherapies.

The expression of toxic gene products in trypanosomatid systems is an experimental approach that may also provide important information on the biology of an organism. This has been previously demonstrated for mammalian cells, where the expression of toxic gene products in specific cell lineages was used in order to study cell-lineage relationships and to analyze cellular interactions during development (Borrelli *et al.*, 1988; Palmiter *et al*, 1987), as well as for the creation of transgenic mice with specific immune deficiencies (Heyman *et al.*, 1989). The approach used may result in the overexpression of a gene product that is necessary in low quantities

but toxic at higher levels. It may also result in the expression of a product at an unusual site for the cell, such as was shown by Clayton and Michels (1996) by the inaproppriate targeting of phosphoglycerate kinase to the cytoplasm of trypanosome cells. The expression of a negative selectable marker may also be used as a tool for the study of other processes: Valdès *et al.* (1996) have used TK, which is readily inactivated by mutation, to estimate the minimal rate of mutation in African trypanosomes. The cell-specific and inducible expression of viral *tk* as a suicide gene is an approach that was also used in the development of gene therapy strategies for cancer treatment (Ishii-Morita *et al.*, 1997; Link *et al.*, 1997).

Role of A2rel in *Leishmania*

In *Leishmania*, and trypanosomatid systems in general, genes that are clustered are transcribed polycistronically (Pays 1993; Clayton 1992). Post-transcriptional processing of the transcripts determines differential accumulation of clustered genes. This was demonstrated by Charest *et al* (1996) for A2 developmental expression, where A2rel transcript expression was constitutive in promastigotes when transferred into phagolysosomal conditions. In Manuscript III, we have demonstrated that this constitutive expression occurs throughout the parasite's life cycle by showing equal A2rel transcript levels in promastigotes and amastigotes, while A2 transcripts accumulate in the latter form. A2rel sequences were also shown to be conserved in all the *Leishmania* species tested, another

characteristic that differentiates it from the A2 gene. The deduced amino acid sequence of the A2rel encoded protein was compared with the sequences from the protein databank. The analyses show that A2rel is unique and does not have sequence similarity with any known leishmanial genes. While its putative function is as yet unknown, its transmembrane topology and its sequence homology to the herpes simplex virus glycoprotein I, suggests that A2rel may be targeted to the surface membrane. A cell attachment sequence, represented by the RGD tripeptide, was also found (amino acids 47-49) and suggests that A2rel may potentially play a role in cell adhesion. The fact that A2rel is constitutively expressed throughout the parasite life cycle, and that it is conserved across the *Leishmania* genus, suggests that it may have a more specialized function than A2.

Future directions

Future studies on A2rel entail the characterization of its protein and the determination of its role in the *Leishmania* life cycle. The latter may be achieved by generating A2rel-deficient organisms, as was performed with the blocking of the A2 protein using the antisense RNA approach (Zhang and Matlashewski, 1997). The A2rel gene alternates with copies of the A2 multigene family. The genes are arranged in repeated clustered arrays in the genome with short intergenic sequences between them, making it difficult to perform A2rel knockout studies without affecting the A2 genes.

Studies on A2 are being undertaken in the context of vaccine development and serodiagnosis of VL in Brazil. The WHO believes that A2 may have some potential as a vaccine candidate. The A2 protein is presently being included, along with other antigens, in the development of a vaccine cocktail that will be used to elicit protection against the leishmaniases (Greg Matlashewski, personal communication). The A2/GST fusion protein is presently being tested in ELISA field assays in Brazil for the diagnosis of visceral leishmaniasis, in a collaborative study with Dr. Ricardo Gazzinelli (Federal University of Minas Gerais, Belo Horizonte, Brazil).

For the effective diagnosis of visceral leishmaniasis, A2 should ideally be included in a more appropriate assay format, in order to simplify its use, reduce costs, and be applicable under rudimentary field conditions. Immunoblotting is a time-consuming technique that is not appropriate for mass epidemiological surveys, and the purification of large amounts of GST fusion protein is costly. We are currently working on the development of a synthetic A2 peptide, whose design is based on the sequence of the repeat region, to be used in ELISA or other assays. Synthetic peptides have proven to be valuable tools in the diagnosis of VL (Fargeas *et al.*, 1996) and of Chagas' disease (caused by *T. cruzi*) (Peralta *et al.*, 1994). We are also investigating the possibility of using the C9 monoclonal antibody raised, against A2, in simplified assays such as the dot-enzyme immunoassay (EIA) which was recently developed by Senaldi *et al.* (1996). In this assay, patient serum is spotted on nitrocellulose and

tested for the presence of a specific antigen using the monoclonal antibody.

In conclusion, our studies using A2-specific sequences have contributed to the characterization of a potentially powerful antigen for the serodiagnosis of visceral leishmaniasis, and to the elaboration of a methodology for the attenuation of *L. donovani.* This research, while contributing to our current knowledge on *Leishmania* systems, may lead to concrete applications using the A2 locus.

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