THE IDENTIFICATION AND CHARACTERIZATION OF HIGHLY IMMUNOGENIC INTERNAL ANTIGENS OF AFRICAN TRYPANOSOMES

by

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ABSTRACT

Six monoclonal antibodies previously derived against internal *Trypanosoma brucei rhodesiense* ViTat 1.1 procyclic culture form antigens were characterized for species- and life cycle stage-specificity by immunoblotting. Five of the mAbs were specific for *T. brucei* spp. while the sixth (mAb #20) recognized an antigen in all trypanosome species and life cycle stages tested. Immunogold electron microscopy revealed that mAb #20 immunoreactivity localized in the mitochondrion. The antigen recognized by mAb #20 was partially purified and the N-terminal sequence showed significant identity with *T. cruzi* Hsp 60 and *E. coli* GroEL mitochondrial chaperones. Using a combination of expression library screening and polymerase chain reaction amplification, I isolated the cDNA encoding the trypanosome antigen. The translated sequence matched sequences of mitochondrial chaperones from several species. Taken together, the results indicate that the antigen recognized by mAb #20 is the *T. brucei* spp Hsp60 mitochondrial chaperone. The mAb #20 or the Hsp60 protein may be used in a simple immunodiagnostic test for African sleeping sickness.

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DEDICATION

I would like to dedicate this thesis to my parents, Sydney and Nora Tuckey for their continual support and encouragement throughout my years at UVIC. I would also like to dedicate this thesis to my grandfather, Charles Emery and to the memory of my grandmother, Mary Emery who encouraged me to always do my best.

The Identification and Characterization of Highly Immunogenic Internal Antigens of African Trypanosomes

Introduction

African trypanosomes are responsible for widespread disease in humans and domestic animals causing sleeping sickness in humans and Nagana in cattle. Sleeping sickness was first described in the 14th century by an Arab writer and physician, al Qualquashaudi (Hoeppli, 1959). It was not until 1880 that *Trypanosoma evansi* (the causative agent of surra in camels and horses) was discovered by Evans in India and the importance of trypanosomes in causing disease was recognized. David Bruce discovered *T. brucei brucei* in the blood of cattle suffering from Nagana in 1894 and subsequently reported transmission by tsetse flies (*Glossina*) in 1897. The causative agents of human sleeping sickness, *T. b. gambiense* and *T. b. rhodesiense* were found in 1902 and 1903 respectively (Historical information from Hoare, 1972).

Trypanosomiasis has killed hundreds of thousands of people in great epidemics in the past. The direct impact on human mortality has been reduced to approximately 25,000 new cases reported annually (Kuzoe, 1993) with this being an underestimate due to poor reporting, difficulty in diagnosis and the inaccessibility of affected areas (WHO, 1986). The disease is endemic in 36 countries in Africa, coinciding with the geographic range of the tsetse fly vector. Recently, the disease has been on the increase in many endemic areas due to political upheaval, breakdown in medical services and socio-economic factors and it is estimated that 50 million people are at risk of acquiring infection (Kuzoe, 1993).

The genus *Trypanosoma*

African trypanosomes belong to the order *Kinetoplastida* which are protozoa that possess a DNA-containing organelle, the kinetoplastid. Two genuses, *Trypanosoma* and *Leishmania* belong to this order and are important human pathogens. The genus *Trypanosoma* is composed of protozoa that have a single flagellum extending from the kinetoplastid and includes American and African trypanosomes. Members of this genus live alternately in the bloodstream and tissues of vertebrates and in the gut of leeches or arthropods. The genus is split into two divisions (*Stercoraria* and *Salivaria*) and classification is based upon the course of development in their vectors (Hoare, 1972). The division *Stercoraria* contains parasites that complete their development in the terminal gut of

their vector and are passed with vector feces. One such parasite is the American trypanosome *T. cruzi.* It causes Chagas Disease in humans, is found in South America and is transmitted via the Kissing bug (family *Reduviidae*) (Katz *et al.*, 1982a). African trypanosomes are salivarian trypanosomes and complete their development in the anterior part of the digestive tract of the tsetse fly (*Glossina* spp.). Mature parasites are then transmitted with vector saliva when the fly salivates while taking a blood meal (Katz *et al.*, 1982b).

The major pathogenic African trypanosomes fall into 3 subgenera; *Duttonella* (type species *Trypanosoma [Duttonella] vivax*); *Nannomonas* (type species *Trypanosoma [Nannomonas] congolense)* ; and *Trypanozoon* (type species *Trypanosoma [Trypanozoon] brucei*). Parasites of all species cause disease in domestic animals. The *T. brucei* species is further divided into 3 subspecies with different infectivities: *T. brucei brucei* infects cattle; *T. brucei gambiense and T. brucei rhodesiense* both cause the human forms of trypanosomiasis.

The Parasite and its Life Cycle

The life cycle of African trypanosomes is characterized by a succession of different forms adapted to the environments they encounter. The life cycle includes an alternation of rapidly dividing stages adapted to infection and nondividing stages specialized for transmission between the mammalian host and the vector. The parasites survive in their different environments by undergoing morphological and metabolic changes. Some of these changes include the expression of different surface molecules and the alteration of metabolic processes (Vickerman *et al.*, 1988).

The mammalian host of *T. brucei spp.* is infected by the bite of a tsetse fly carrying trypanosomes in its saliva. Metacyclic stage trypomastigote parasites are injected into the dermal connective tissue of the mammal and a local inflammatory reaction, the chancre, develops (Hoare, 1972). From the chancre, the parasites then gain access to the host bloodstream via the draining lymphatic vessels and differentiate into rapidly dividing long slender bloodstream forms (BSF) with a doubling time of 4 to 6 hours (Vickerman, 1971). This form of the parasite has a specialized metabolism that allows survival in the bloodstream of the mammalian host. The organism relies entirely on glycolysis for production of energy utilizing an organelle called the glycosome (Bowman and Flynn, 1976; Opperdoes, 1985 and 1987). Also, the mitochondrion appears to be non-functional and limited in structure (Vickerman, 1962). Survival in the mammalian bloodstream is also achieved by an immune system evasion tactic called antigenic variation (Tanner *et al.*,

1980). Each parasite is covered by a surface coat composed of a single form of the surface glycoprotein, the variant surface glycoprotein (VSG) (Cross, 1975). The host immune response is directed primarily against this immunodominant surface antigen (De Raadt, 1974). While most of the trypanosomes are eliminated from the circulation by this antibody response, a small subpopulation of parasites bear an antigenically different VSG thus allowing them to evade the host's immune response and form the next parasitemic wave (Vickerman, 1978). This allows the infecting trypanosomes to survive and proliferate as a population and results in a fluctuating parasitemia which is characteristic of systemic stage trypanosomiasis.

The bloodstream parasites rely on the abundant supply of host derived carbohydrates due to their relatively inefficient anaerobic metabolism. In order to survive in the midgut of the tsetse fly vector, they must become more metabolically efficient (Vickerman, 1971). As the host parasitemia declines, a small number of the surviving long slender parasites transform into non-dividing, morphologically distinct short stumpy BSF. As a pre-adaptation to further differentiation into fully metabolically active procyclic forms, these short stumpy forms possess a semi-developed mitochondrion (Vickerman, 1965). A similar transition to non-dividing parasites has been observed in *T. congolense* (Nantulya *et al.*, 1978) and in *T. vivax* (Shapiro *et al.*, 1984). Several mitochondrial enzymes are activated (Brown *et al.*, 1973) and the mitochondrion transforms from a simple tube into an elaborate network of cristae (Vickerman, 1962). This stage is thought to be an important intermediate for efficient transmission into the tsetse fly vector.

Trypanosome infected blood is ingested by the tsetse fly and it passes to the lumen of the midgut where the transition from bloodstream to procyclic forms takes place. Differentiation from BSF to procyclic forms involves several important morphological changes. These include loss of the VSG coat (Turner *et al.*, 1988) and appearance of the procyclin coat (Roditi *et al.*, 1989), cessation of endocytosis (Steiger, 1973), replication of the kinetoplastid DNA network (Hajduk *et al.*, 1984), a switch from the utilization of glucose to proline as an energy source (Bowman and Flynn, 1976; Bienen *et al.*, 1981), complete activation of the mitochondrion (Brown *et al.*, 1973) and a change in glycosome morphology (Hart *et al.*, 1984). This actively dividing form is not infective to mammals and maturation to metacyclics must occur before they become infective.

The procyclic trypanosomes migrate back up to the fly mouth parts and salivary glands where they differentiate into the epimastigote stage (Hoare, 1970). This occurs in two stages: first the procyclic trypanosomes cross the peritrophic membrane of the tsetse gut to reach the ectoperitrophic space (Evans and Ellis, 1983). The parasites migrate forward to the proventriculus, cease cell division and develop into elongate mesocyclic forms.

Secondly, the parasites again cross the peritrophic membrane and migrate to the tsetse's salivary glands. Mesocyclic trypanosomes then attach to the microvilli of the salivary gland cells by their flagella and divide as attached epimastigotes (Tetley and Vickerman, 1985).

Epimastigotes then transform to VSG-coated, free swimming metacyclic trypanosomes that reside in the salivary gland lumen (Tetley *et al.*, 1987). Transformation to infective metacyclics is marked by the repression of mitochondrial metabolic activity, reversion of glycosomes to form spherical organelles and the cessation of cell division (Vickerman *et al.*, 1988). When inoculated into the mammalian host as the tsetse takes a blood meal, the metacyclics transform into proliferating long slender BSF and establish the mammalian infection (Vickerman *et al.*, 1988).

Research into signals that trigger the developmental stage transformations in the trypanosome life cycle has identified factors involved in the transformation from bloodstream to procyclic forms. According to Czichos *et al.* (1986), in studies on the *in vitro* transformation of BSF to procyclic culture forms (PCF), a temperature drop (37°C to 26°C) and the addition of 3 mM cis-aconitate to minimal essential medium (with 15% heat inactivated horse serum) are sufficient to repress the synthesis of the VSG coat and to induce transformation. Recent studies by Bass and Wang, (1992) have indicated that the essential trigger for bloodstream to procyclic transformation *in vitro* is a general inhibition of protein synthesis in bloodstream forms caused by a temperature drop from 37°C to 26°C. TCA cycle intermediates such as cis-aconitate can shorten the initial lag phase of the differentiation *in vitro* (Bass and Wang, 1991).

Specific host-derived factors may also be directly involved in triggering life cycle stage changes in the vector and in the mammalian host. Lectins have been implicated in inducing developmental changes in the life cycle stages that parasitize the tsetse vector (Maudlin and Welburn, 1987, 1988). Evidence suggests that tsetse midgut lectin-trypanosome carbohydrate interactions influence parasite maturation in the tsetse fly (Maudlin and Wellburn, 1994). Experiments done *in vivo* examining the differentiation of procyclic *T. brucei* parasites to the metacyclic form show that inhibition of the midgut lectins by glucosamine or N-acetyl-D-glucosamine blocks maturation (Maudlin and Wellburn, 1994). Black, *et al.* (1985) suggested that the transformation of long slender BSF to short stumpy BSF may be controlled by a host molecule which inhibits parasite differentiation by promoting multiplication of the long slender forms. The discovery of an epidermal growth factor receptor homologue in *T. brucei* indicates that growth factor interactions are involved in regulation of cell growth in these parasites (Hide *et al.*, 1989). A *T. b. brucei* molecule has been isolated which triggers the production of interferon-γ (IFN-γ) by CD8+ T-cells in infected rats. IFN-γ has been found to play a crucial role in rats and humans by promoting

the proliferation of the infecting trypanosomes (Olsson *et al.*, 1991; Kristensson *et al.*, 1994: Bonfanti *et al.*, 1995).

Trypanosomes can complete their entire life cycle without genetic exchange in a sexual stage (Sternberg *et al.*, 1989). The existence of a sexual stage in the *T. brucei* life cycle has been implicated by studies of enzyme variation between natural parasite populations (Gibson *et al.*, 1980; Tait 1980; Tait *et al., 1985*), of content and polymorphism analysis of DNA (Borst *et al.*, 1980; 1982; Gibson *et al.*, 1985) and by studies of protein gene product polymorphisms (Anderson *et al.*, 1985; Pearson and Jenni, 1989). The first direct demonstration of genetic recombination after the co-transmission of two phenotypically distinct *T. brucei* clones was obtained by Jenni *et al.*, (1986). These experiments demonstrated that genetic exchange can occur in laboratory conditions using widely separated trypanosome isolates but not in naturally occuring populations (Degen *et al.*, 1995). Evidence that genetic recombination may occur between trypanosome populations transmitted within the same epidemic area has recently been published in further co-transmission experiments (Degen *et al.*, 1995). The importance of a sexual cycle in trypanosomes could be in the ability of the organism to adapt to many types of control measures. Such events would possibly reduce the efficacy of trypanocidal drugs.

Another important aspect of the trypanosome life cycle is the transmission between different mammalian hosts and the tsetse vector. African trypanosomes lose infectivity for mammals when they enter the tsetse fly gut and must complete their developmental cycle with differentiation to the metacyclic stage before infective parasites can be transmitted in tsetse saliva. This is called cyclical transmission. Mechanical transmission can occur when BSF parasites survive unchanged in the mouth parts of a fly long enough to be inoculated into the next animal which the fly feeds upon. This route of transmission is relatively rare and less efficient than cyclical transmission (Hoare, 1970). Cyclical transmission is subject to a variety of interacting factors. Trypanosome-infected humans, domestic animals and wild animals can serve as parasite reservoirs of both *T. b. gambiense* and *T. b. rhodesiense*. *T. b. rhodesiense* has been passed from wild game to humans and has been isolated from domestic cattle, sheep and goats. *T. b. gambiense* has been found in dogs, cattle, sheep and game animals in areas that are endemic for human sleeping sickness (Maurice and Pearce, 1987). Wild animals and some domestic animals are important carriers for human pathogenic trypanosomes because they are relatively trypanotolerant and do not develop clinical symptoms of the disease. Transmission cycles often involve man-fly-man, game-fly-man and domestic animal-fly-man cycles, further complicating the control of human African sleeping sickness.

Pathology and Immunology of African sleeping sickness

African trypanosomes cause disease in many species of mammals including humans. The disease in humans is fatal unless chemotherapy is administered (Hoare, 1970). There are two forms of African sleeping sickness based on the infecting subspecies, geography and the clinical course of the disease (Shapiro and Pearson, 1986). The chronic form is caused by *T. b. gambiense*, found mainly in West and Central Africa and may take years to kill an untreated patient. The acute disease is caused by *T. b. rhodesiense*, found mainly in East and South Africa and can cause death within several weeks. It is impossible to distinguish between *T. b. gambiense* and *T. b. rhodesiense* morphologically. Determination of the infecting species is usually performed based on geographic location and clinical course but in some cases more definitive tests such as isoenzyme determinations are required (Pépin and Milford, 1994).

The disease has three stages in humans which can be distinguished by the location of the parasites in the host and by the clinical manifestations. In the initial stage the parasites are localized at the site of the tsetse fly bite usually producing a chancre. The infecting trypanosomes then become distributed throughout the host's bloodstream and tissues characterizing the systemic stage. The host will exhibit fluctuating flu-like symptoms with recurrent parasitemia at this stage. In the advanced stages of the disease the parasites invade the central nervous system and other major organs of the host inducing irregularities in the nervous system, heart and other organs. The disease symptoms in all stages are caused both by the high parasite load and by the disruption of immunoregulation with the pathology of the disease largely due to the effect on the host's immune system (Shapiro and Pearson, 1986).

Five to fifteen days after the tsetse fly bite a chancre appears characterizing the initial stage of the infection (Basson *et al.*, 1977). The chancre is usually the first clinical manifestation of the disease and is a result of the host's immune response to the infecting parasites. The immune response results in the characteristic symptoms of oedema and the presence of many effector cells. The chancre subsides approximately 2 weeks after its appearance when parasites escape into the bloodstream via the local draining lymph node and the systemic process of parasite replication starts (Ssenyonga and Adam, 1975).

The systemic stage is characterized by a fluctuating parasitemia with symptoms such as fever, headache, joint pain, splenomegaly and lymphadenopathy (Apted, 1970; Greenwood and Whittle, 1980). The trypanosomes proliferate rapidly in the bloodstream and are found widely distributed throughout the animal. The fluctuating parasitemia is a result of antigenic variation which is used by the parasites as a population to evade the host's

immune system. Infection symptoms coincide with the increase in numbers of parasites in the circulation (Molyneux *et al.*, 1984).

In the advanced stages of the disease the parasites enter the central nervous system and the interstitial spaces of many organs (Wéry *et al.*, 1982). Dysfunction of the heart, liver, lungs, kidneys and brain are observed (Apted, 1970). This invasion causes lesions in organs which are associated with granular immunoglobulin deposits and with a marked cellular infiltration (Poltera and Cox, 1977). Patients can become irritated, psychotic and may go into a coma, giving the disease its name. Death is inevitable once the trypanosomes reach the central nervous system unless the patient receives chemotherapy. Unfortunately, drug treatment is not always effective at this late stage in the infection (Shapiro and Pearson, 1986).

Profound immunological perturbations occur in patients infected with African trypanosomes. The immune system plays an important role in controlling the parasite and in the pathology of the disease. As mentioned previously, the chancre, appearing early in infection, is a result of the host's immune response to the infecting parasites. Polymorphonuclear leukocytes and small lymphocytes initially infiltrate the chancre and later lymphoblasts, macrophages and plasma cells appear (Shapiro and Pearson, 1986). In order to establish the systemic infection, parasites must escape this immune response and reach the host's bloodstream. During the systemic phase of the disease an immunoproliferative reaction occurs producing much of the pathology seen. The systemic stage of the disease is characterized by a fluctuating parasitemia in which trypanosomes of one or a few antigenic types give rise to a peak of parasitemia in the bloodstream. The fluctuating parasitemia is the result of parasite clearance by anti-VSG antibodies directed against the major antigenic variant(s) in each parasitemic peak (Mansfield, 1990). Antibodies of the IgM isotype against VSG are responsible for the clearance of parasites from the bloodstream via complement-mediated lysis (Mansfield, 1990) and antibody-dependent phagocytosis (Greenblatt *et al.*, 1983; Ngaira *et al.*, 1983). These antibodies are effective in clearing most of the parasites and the subsequent parasitemic peak is the result of the rapid proliferation of a distinct antigenic type initially present in low number and to which no anti-VSG antibodies have yet been directed.

An intense immunoproliferative reaction of B- and T-cells, null cells and macrophages occurs in most lymphoid organs. This results in highly elevated serum IgM levels in the patient, with antibodies directed against the parasite, the host and non-specific antigens (Clarkson, 1976). The polyclonal activation of B-lymphocytes is thought to be mediated by the induction of a mitogen produced by the trypanosomes (Greenwood, 1974). This mitogen is believed to act on macrophages which stimulate the B- and T-lymphocyte

proliferation and cause the immunosuppression observed in later stages of infection (Sacks *et al.*, 1982; Askonas, 1985; Mansfield 1990). In a recent study using mice infected with *T. b. brucei* the polyclonal activation of B cells was investigated by determining the cell cycle position and the expression of activation antigens (Sacco *et al.*, 1994). It was found that B cells were activated during infection but the activation was aberrant in that the cells were cell cycle arrested and did not proliferate. This research may help elucidate the mechanisms by which immune dysfunction occurs in African trypanosomiasis.

Some aspects of the pathology in trypanosomiasis are caused by both the specific antibodies and other antibodies released after polyclonal activation, circulating in the host. Symptoms such as immune-complex dysfunction and autoimmunity are observed in late-systemic and advanced stage infections (Mansfield and Kreier, 1972; Lambert *et al.*, 1981). Antibodies are also produced against the trypanosome invariant plasma membrane components, nuclear and cytoplasmic cellular constituents. Autoantibodies against host antigens such as complement C3, fibrinogen-fibrin degradation products (Boreham and Facer, 1974) and tissue antigens such as liver, thymus and brain (Mansfield and Kreier, 1972; Poltera *et al.*, 1980) are also generated during the polyclonal B-cell activation. In human trypanosomiasis, anti-erythrocyte and anti-DNA antibodies have been reported (Parratt and Herbert, 1976). These autoantibodies play some role in the pathology of the disease and are implicated in hemolytic anemia and impairment of coagulation (Parratt and Herbert, 1976).

In the advanced stages of the disease, elevated IgM levels, lymphocytes and immune complexes formed by the parasite variant antigens and antibodies against them are found in the cerebrospinal fluid concurrent with CNS infection (Lambert *et al.*, 1981). Cerebral manifestations of the disease may depend on the inflammation sites, the interactions between parasites, antibodies and astrocytes and on the levels and types of mediator release at these sites (Pentreath, 1989). Immune complexes can damage capillaries and lead to localized oedema in the brain (Lambert *et al.*, 1981). Neuronal damage is also thought to be caused by astrocytes found in the CNS which are closely associated with the CNS tissue/blood system interface (McCarron *et al.*, 1985). Expression of astrocyte surface-exposed MHC class II antigens is induced by activated T-cells that cross the blood/brain barrier during the infection (Wekerle *et al.*, 1987) and these induced astrocytes can serve as antigen-presenting cells (Pentreath, 1989). Once the trypanosomes invade the CNS, astrocytes may interact with the activated T-cells to produce cytokines (Hunter *et al.*, 1991; 1992) and these cytokines may then induce proliferation of lymphocytes and astrocytes and provoke the B-cell response and antibody secretion (Pentreath *et al.*, 1994).

The ability to mount effective, specific antibody responses to trypanosomes decreases with time (Oka *et al.*, 1984) in the advanced stages of the infection. Various mechanisms to explain this immunosuppression have been proposed. Evidence for the involvement of suppressor cells (Pearson *et al.*, 1978; Yamamoto, *et al.*, 1985), soluble suppressor substances (Tizard *et al.*, 1978), differential macrophage activity (Paulnock *et al.*, 1988) and decreased IL-2 receptor expression (Kierszenbaum *et al.*, 1991) have been presented. The above are now believed to result from a parasite-induced effect on macrophages which then actively suppress immune responses or fail to process or present antigen properly (Askonas, 1985; Paulnock *et al.*, 1988).). The host will often die of secondary infections such as pneumonia due to the weakening of the immune system (Shapiro and Pearson, 1986). Drug cure of an infected immunosuppressed host does lead to rapid recovery of immune responsiveness and immunosuppression can be reversed within a few days of treatment (Askonas, 1985

Control of African sleeping sickness

The control of African sleeping sickness is important in at least 36 African countries where the disease is endemic. Control involves mobilization of trained personnel, drugs and hospital beds, and may involve vector control. Due to economic restrictions, political upheavals and competing health threats several measures aimed at controlling African sleeping sickness have not been met satisfactorily. The primary goal in combating a vector-borne disease is to reduce the reservoir of parasites available for transmission (Kuzoe, 1993). This is achieved by surveillance and treatment of infected individuals and by limiting vector populations.

Control of the tsetse fly vector has been attempted mainly through the ground and aerial spraying of insecticides such as DDT, Dieldrin and Endosulfan. The number of flies in the tsetse belt was significantly reduced until the appearance of insecticide-resistant flies, negative effects on other organisms and the high cost of this method proved it to be ineffective (WHO, 1986). The optimal way to avoid tsetse flies is to stay out of tsetse-infested areas and use fly traps in residential areas (WHO, 1986). In Uganda the control of tsetse flies with traps and community involvement has been shown to be effective in preventing an epidemic (Lucein, 1991).

Until recently, three main drugs were used to treat human sleeping sickness (Kuzoe, 1993). Pentamidine (an aromatic diamidine) inhibits S-adenosyl-L-methionine decarboxylase thereby reducing the synthesis of polyamines (Kuzoe, 1993) and is effective against early stage *T. b. gambiense* infections. Suramin (sulfated naphthylamine) inhibits

many enzymes including L-α-glycerophosphate oxidase and RNA polymerase and is effective against early stage *T. b. gambiense* and *T. b. rhodesiense* infections. Melarsoprol, a powerful inhibitor of pyruvate kinase, disrupts energy production via glycolysis in BSF, and thus, is effective against late stage *T. b. gambiense* and *T. b. rhodesiense* infections. All of these drugs have serious side effects with Melarsoprol causing reactive encephalopathy in 5-10% of patients with 1-5% fatality (Pépin and Milford, 1994). Also, trypanosomes can become resistant to Pentamidine and Melarsoprol (Kuzoe, 1993).

Eflornithine (difluoromethylornithine; DFMO) is a potent inhibitor of polyamine synthesis. Although this drug is relatively safe it is required in doses of 400 mg/kg/day split into four equal doses 6 hours apart and must be administered intravenously in a hospital (Pépin and Milford, 1994). This makes DFMO difficult to use in the field. This drug is also expensive and only effective against *T. b. gambiense* infection. Most chemotherapy regimes today involve the use of different combinations of these drugs depending on the infecting subspecies and stage of infection (Pépin and Milford, 1994). Therefore, current chemotherapy of sleeping sickness remains unsatisfactory. The ideal chemotheraputic agent should be safe, effective, affordable and easily administered in the field.

Drug treatment is most effective when administered early in infection. This makes early diagnosis very important in treatment. The principle of control and prevention of sleeping sickness relies on an integrated strategy of continuous surveillance involving diagnosis and treatment of the population at risk coupled with vector control (De Raadt, 1986).

Trypanosomal Antigens

African trypanosomes have a complex life cycle characterized by a number of different forms adapted to the various environments they encounter. Although many cellular constituents are present in all life cycle stages, some are differentially expressed at specific life cycle stages. The presence of differentially expressed cellular constituents is reflected in a varying antigenic profile found in trypanosomes as they differentiate between their life cycle stages (Anderson *et al.*, 1985). Much effort has been made to identify trypanosomal antigens both as tools for diagnosis and as potential targets for chemotherapy and vaccine development. Important stage specific surface molecules such as the bloodstream form variant surface glycoprotein (VSG) (Turner, 1982) and the procyclic form procyclins (Richardson *et al.*, 1988) have been relatively well characterized. These antigens are immunodominant and abundant on the surface of the trypanosomes in their respective life

cycle stages. Research to find other surface molecules on trypanosomes has lead to the discovery of a number of molecules such as kinetoplastid membrane protein-11 (KMP-11) (Stebeck *et al.*, 1995). Attempts to find other antigens of trypanosomes have lead to the discovery of molecules potentially useful as diagnostics and as chemotherapeutic targets.

The most studied antigens of trypanosomes are the variant surface glycoproteins (VSGs) found on the surface of BSF and metacyclic form parasites. *T. brucei* spp. survive in the bloodstream of their mammalian hosts as extracellular parasites under constant attack by the immune system. The Variable Antigenic Type (VAT) refers to the identity of a distinct BSF trypanosome as determined by its VSG. A cloned trypanosome population has the ability to produce a large number of VATs which constitute its VAT repertoire (WHO, 1986).

The BSF parasite surface is covered with a dense coat of approximately 10^7 VSG molecules attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (Ferguson *et al.*, 1985; 1988). This VSG coat is 12-15 nm thick and present as a tightly packed layer, one molecule deep (Vickerman and Luckins, 1969). The VSG polypeptide is approximately 450 to 500 amino acids long with a molecular mass of 55 to 65 kDa (Turner, 1985) and a carbohydrate portion that is 7 to 10% of the molecule by weight (Johnson and Cross, 1977).

Antigenically distinct VSG clones differ in the primary sequence in the N-terminal 350 residues (Cross, 1975). The C-terminal 50-100 residues share sequence homologies that allow VSGs to be classified into two or three groups (Donelson and Rice-Ficht, 1985). Carbohydrates attached to the VSG polypeptide consist of an N-linked oligosaccharide (McConnell *et al.*, 1983) and a glycan that is covalently attached to the C-terminal of the protein via an ethanolamine residue (Holder, 1983). The VSG molecule is anchored to the plasma membrane via this glycan and a GPI anchor (Ferguson *et al.*, 1985; 1988). A glycosylphosphatidylinositol-specific phospholipase C is able to cleave the membrane form VSG at the diacyl glycerol of this glycan which results in the release of the soluble form VSG (Hereld *et al.*, 1986). This also causes the exposure of an epitope at the C-terminal conserved carbohydrate portion called the cross-reacting determinant (CRD) that is responsible for the immunological cross-reactivity observed between most VSGs (Holder and Cross, 1981). Only a small N-terminal portion of the VSG is exposed to the host in the living parasite which leaves a small number of epitopes exposed on the surface of the trypanosome (Pearson *et al.*, 1980; Hall and Esser, 1984; Miller *et al.*, 1984a; 1984b; Clarke *et al.*, 1987). The antigenic cross-reactivity between all VSGs at the C-terminal carbohydrate does not appear to compromise the parasite because that epitope is not exposed on the surface of the living parasite (Holder, 1983).

The trypanosome genome has more than 1,000 VSG genes and 10 to 20 telomeric expression sites with a single gene expressed at any given time (Van der Ploeg *et al.*, 1982). Variation of the VSG surface coat occurs spontaneously at a rate of up to 10^{-2} per cell and per generation (Turner and Barry, 1989). The genes encoding the VSGs are located throughout the trypanosome genome at both intrachromosomal (Van der Ploeg *et al.*, 1982), and telomeric sites (DeLange and Borst, 1982). Only one VSG gene is transcribed at any given time and this expressed VSG gene is usually located at the end of one of the telomeric expression sites (Donelson and Rice-Ficht, 1985). Antigenic variation occurs by gene replacement in the active expression site and by alternative transcriptional activation of different expression sites. Gene replacement is achieved by recombinational events changing all or part of expressed VSG in the active site (Vanhamme and Pays, 1995). In the BSF, all but one of the VSG expression sites are repressed. The mechanism by which alternation between different expression sites occurs has not been fully elucidated but is thought to involve distant DNA rearrangements (Gottesdiener *et al.*, 1991; Zomerdijk *et al.*, 1991).

The trypanosome procyclic life cycle stage expresses an immunodominant species-specific glycoprotein that forms a surface coat in place of the VSG coat which is lost upon differentiation to this life cycle stage (Richardson *et al.*, 1986; 1988; Roditi *et al.*, 1989). The structure of these molecules in *T. brucei* is unusual in that approximately 40% of the polypeptide is a glutamic acid-proline dipeptide repeat differing in length between individual clones (Richardson *et al.*, 1988). These molecules have independently been named procyclin by Richardson *et al.* (1988) due to their stage-specific expression and high proline content and procyclic acidic repetitive protein (PARP) by Mowatt and Clayton (1987). An immunodominant, acidic surface glycoprotein also forms a coat on *T. congolense* procyclic forms. The primary sequence of these molecules shows no similarity to the procyclins found in *T. brucei* species but are thought to be analogs (Beecroft *et al.*, 1993). The *T. congolense* molecules were named glutamic acid/alanine rich protein (GARP). Collectively these molecules are referred to as procyclins (Stebeck and Pearson, 1994).

In both *T. brucei* and *T. congolense* species these molecules are lipid anchored and glycosylated and probably have an extended polyanionic structure (Stebeck and Pearson, 1994). The procyclin molecules are present at approximately 6 million copies per parasite and are highly negatively charged (Clayton and Mowatt, 1989). Each molecule is anchored to the parasite via a glycolipid membrane anchor that is distinct from the VSG anchor (Field *et al.*, 1991). The *T. brucei* procyclins possess an N-linked glycosylation site in a 31 amino acid N-terminal domain (Richardson *et al.*, 1988) and are also glycosylated at the

membrane anchor where a highly sialated structure exists (Ferguson *et al.*, 1993). The *T. congolense* analog contains no N-linked glycosylation site but has several sites for O-glycosylations and is heavily glycosylated (Beecroft *et al.*, 1993). The primary sequence of the *T. brucei* procyclin molecules is predicted to form extended and rigid tertiary structures with the $(Glu\text{-}Pro)_{22\text{-}29}$ repeats forming a cylindrical shape 14-18 nm in length and 0.9 nm in diameter in (Roditi *et al.*, 1989). The predicted molecular mass of this glycoprotein is 11-15 kDa (Mowatt and Clayton, 1987).

Both GARP and PARP are expressed in procyclic and epimastigote forms but not on metacyclic or BSF life-cycle stages (Stebeck and Pearson, 1994). Procyclin genes in *T. brucei* are arranged in two diploid, nontelomeric and unlinked loci, each containing two genes in direct repeat (Vanhamme and Pays, 1995). Both loci are expressed and transcription is mediated by a polymerase resistant to α-amanitin (Roditi *et al.*, 1989).

Using color flow cytometry, it has been shown that during transformation from BSF to PCF, loss of the VSG coat is followed by the gradual appearance of procyclin at the cell surface (Roditi *et al.*, 1989). When short stumpy BSF trypanosomes are placed under conditions that allow differentiation from BSF to procyclics, the expression of VSG is terminated. VSG mRNA is preferentially degraded and VSG is massively released from the cell surface between 4 and 6 hours after initiation of differentiation (Ziegelbauer *et al.*, 1993). Within 2 hours of triggering differentiation an increase in procyclin mRNA occurs (Roditi *et al.*, 1989) with the glycoprotein appearing on the surface of the parasite 8 to 24 hours later. Expression of VSG and procyclin on the surface membrane occurs in a gradual reciprocal fashion and this allows the parasites to be covered with a surface coat at all times (Stebeck and Pearson, 1994).

The biological function of procyclin has been postulated to involve more than a surface coat of the parasite. As a surface coat these molecules may protect the parasites from proteases in the tsetse midgut (Stebeck and Pearson, 1994). The structure of the molecule indicates that it would be resistant to most proteases due to the unusual primary sequence and the carbohydrates attached (Ferguson *et al.*, 1993). Other proposed biological functions of the procyclins include migration of the parasite to the correct place in the tsetse fly for maturation and completion of the life cycle (Stebeck and Pearson, 1994).

Much effort has been expended in finding nonvariant surface antigens in trypanosomes. Such antigens may be useful for development as potential vaccine candidates, as diagnostically useful cellular constituents or as targets for drug therapy. It has been determined that infected animals produce antibodies against VSG and other nonvariant parasite antigens (DeRaadt, 1974). Beneath the VSG coat, trypanosomes have a plasma membrane bilayer which probably contains potentially antigenic molecules. Some of these

molecules may be common between trypanosomes of different VAT types i.e. species-specific or common to all trypanosomes. It is believed that these underlying parasite membrane antigens are probably inaccessible to antibodies in living bloodstream forms (Shapiro, 1989) as the VSG coat would shield such molecules. The presence of VSG precludes antibody attack on invariant antigens, thus leading many to believe that it will not be possible to develop any vaccine against African trypanosomiasis using surface antigens (Shapiro, 1989).

To investigate invariant surface antigens, Beat *et al.* (1984), hyperimmunized rabbits with *T. brucei* homogenates and used the sera to identify an antigen common to BSF parasites having different VATs. Indirect immunofluorescence on living bloodstream forms revealed that an antigen may be diffusely distributed over the parasite surface although this antigen was not identified further (Beat *et al.*, 1984). A 31 kDa protein common to both BSF and PCF from *T. congolense* was found using mAbs against whole irradiated parasites (Parish *et al.*, 1985). This was not a surface antigen, however. In another study, mAbs prepared using mice exposed to living BSF trypanosomes were found to react against trypanosomes of different VATs. The antigen appeared to be on the surface of the parasite by immunofluorescence and when examined in immunoblot experiments was found to be a doublet of approximately 22 kDa (Burgess and Jerrels, 1985). Recently, an abundant 11-kDa molecule found on the surface of a wide variety of kinetoplastids (KMP-11) was found using a mAb generated against lipophosphoglycan-associated protein (LPGAP) of *Leishmania donovani* (Tolson *et al.*, 1994). The abundant expression of this molecule on a wide variety of kinetoplastids suggests it may be important for parasite survival (Stebeck *et al.*, 1995).

The identification of several trypanosome surface antigens has been accomplished using a variety of methods. The specific labeling of membrane proteins via iodination led to the identification of surface membrane antigens on procyclic *T. b. rhodesiense* (Gardiner *et al.*, 1983). Biotinylation of BSF *T. brucei* parasites has lead to the identification and characterization of invariant surface membrane proteins (ISG65 and ISG75) (Ziegelbauer and Overath, 1992a; 1992b; Zeigelbauer et al., 1995). These proteins, whose function has yet to be determined, belong to a group of minor surface proteins which are found on parasites expressing different VSGs.

Coated vesicle endocytosis occurs in trypanosome BSF and is the mechanism by which several materials are taken into the cells (Langreth and Balber 1975). Selected extracellular components are bound to specific surface receptors and then taken in by invagination (Goldstein *et al.*, 1979). Coated vesicles are formed only in the flagellar pocket (Langreth and Balber 1975) and evidence supports the hypothesis that antibody-accessible

endocytotic vesicle receptors exist on the surface of trypanosomes. Attempts to use flagellar pocket membrane fractions or proteins in vaccinations has shown some promise in mice (Olenick et al., 1988) and cattle (Mkunza *et al.*, 1995) indicating the presence of antibody-accessible invariant surface antigens.

Internal antigens in trypanosomes have also been identified. These antigens may be particularly useful in diagnosis and perhaps in identifying cellular constituents for drug therapy or for vaccine targets. Powell (1976; 1978) tried to find protective antigens by immunizing rabbits with crude mitochondrial fractions and claimed he achieved protection against heterologous strains of African trypanosomes. The relevant immunogen was never identified.

In an attempt to identify the common antigens that elicit the host's immune response, mouse infection and cure regimens have been used (Campbell *et al.*, 1981). Most of the monoclonal antibodies so obtained were found to be specific for VSG (Pearson *et al.*, 1980; Clarke *et al.*, 1987). Some monoclonal antibodies were found to be specific for non-VSG internal antigens but they were not characterized further (Campbell *et al.*, 1981). In another study, sera from cattle actively infected with *T. brucei* and sera from drug-cured cattle were used to identify antigens recognized during the clinical course of the disease (Shapiro and Murray, 1982). Antibodies from infected cattle bound eight different protein antigens in experiments using immunoprecipitation and SDS-PAGE, three of which were recognized by all recovered cattle (110, 150 and 180 kDa). The identity and the role of these antigens are not known.

Monoclonal antibodies produced against purified trypanosome enzymes or cellular constituents may lead to the identification of targets for chemotherapies against trypanosomes. Many monoclonal antibodies have been produced against the cytoskeletal components of *T. brucei*. These constituents include a 55 kDa tubulin (Gallo and Anderson, 1983; Gallo *et al.*, 1988), 320 kDa and 41 kDa microtubule associated proteins (Schneider *et al.*, 1988a; 1988b; Müller *et al.*, 1992), spectrin-like proteins (Schneider *et al.*, 1988c), 68 and 72 kDa paraflagellar rod structural proteins (Gallo and Schrevel, 1985), and a 60 kDa protein that interacts with microtubules and membranes (Stieger and Seebeck, 1986; Seebeck *et al.*, 1988; Müller *et al.*, 1993). An immunogenic protein with a repeating epitope of 24 amino acids has been shown to have similarities with cytoskeletal and intermediate filament proteins in other organisms (Duncan *et al.*, 1991). Two antigens from the flagellar cytoskeleton have proven to be potential candidates for the development of diagnostic tools for African trypanosomiasis (Imboden *et al.*, 1995).

Enzymes that have been purified and used in immunizations include trypanosome hexokinase (Risby and Seed, 1969a), aldolase (Risby and Seed, 1970) and phosphohexose

isomerase (Risby and Seed, 1969b). These enzymes failed to protect mice from challenge infections and may be inaccessible to host antibodies (Seed, 1974). Knowles *et al.* (1987) identified a trypanosome peptidase in the plasma of mice infected with *T. brucei brucei* and in the plasma of heifers infected with *T. congolense* (Knowles *et al.*, 1989) which may have a role in the pathology of the disease. Recently, an immunodominant antigen from *Trypanosoma congolense* that shares identity to a heavy chain binding protein (BiP) has been identified and characterized (Boulangé and Authié, 1994). A number of roles for this molecule in the pathogenesis of trypanosomiasis have been postulated by these authors.

Diagnostic Principles and Theory

The diagnosis of infectious disease includes determining the causative agent by isolating and identifying the infecting agent and/or and demonstrating the presence of immune reactivity in patients (such as an antibody-induced agglutination or skin reaction). Clinical symptoms are important in leading to a correct diagnosis but usually require a more definitive identification of the infectious agent by direct isolation of the pathogens or derived products from the patient (Gibbons *et al.*, 1985). In order to accurately identify an infectious agent, microscopical examination of samples or cultures from the patient are required, followed by further biochemical characterization of the isolated micro-organism (Duerden *et al.*, 1987).

Serological tests based on antibody-antigen interaction are useful in diagnosing disease because they can detect either microbe-specific antibodies or microbial antigens. The demonstration of pathogen-directed antibodies indicates exposure (by infection or vaccination) in the past to the agent of interest but does not indicate if the patient has an active infection (Jawetz *et al.*, 1987). The detection of microbe-specific antigens is more indicative of an active infection and requires the use of specific antibodies. The sensitivity and reliability of such assays directly depends on the antibodies used. The advent of monoclonal antibody technology (Köhler and Millstein, 1975) has allowed the production of specific biochemically homogeneous antibodies with known activity and offer the potential for reliable, sensitive and specific antigen detection assays. Many serodiagnostic formats have been developed for infectious agents, including immunofluorescence (Coons *et al.*, 1942; Collins, 1988), enzyme-linked immunosorbent assays (ELISAs) (Voller *et al.*, 1979; McLaren *et al.*, 1981), agglutination tests (Christensen *et al.*, 1973; McCarthy, 1985), and immunoprecipitation tests (Oudin, 1946; Ouchterlony, 1949; Asahi *et al.*, 1977). These techniques have all been applied to the diagnosis of bacterial, viral and parasitic infections (Jawetz *et al.*, 1987; Duerden, *et al.*, 1987). Newly developed

technologies involving DNA hybridization and PCR are currently used to identify infectious agents (Mullis and Faloona, 1987; Chou *et al.*, 1992). These techniques have proven to be very sensitive and accurate (Nantulya, 1991; Weiss, 1995).

Diseases caused by tropical parasites are often difficult to diagnose. Parasites often have complicated life cycles with different life cycle stages. In many diseases that are chronic immunosuppression can occur which can lead to secondary infections. Diagnosis is traditionally done by direct observation of the parasites in a sample or biopsy from the patient (Voller and de Savigny, 1981). Parasites that live in low numbers in the blood and tissues are difficult to observe directly. To improve on this situation, a large number of immunodiagnostic tests have been developed for tropical parasitic diseases, yet few are 100% reliable due to immunological complexities of the host-parasite interaction (Voller and de Savigny, 1981). In addition, many parasite antigens cross-react with antigens of other parasites. In areas where a large number of endemic tropical diseases exist there is a need for specific tests because of the possibility of multiple parasitic infections (Houba, 1980).

In vitro culture of parasites has allowed the large scale production of parasite antigens for use in diagnostic tests (Terry, 1985) and monoclonal antibodies have improved the specificity and sensitivity of several tests for parasite diseases (Bidwell and Voller, 1981; Gottstein *et al.*, 1985; 1987; Feldmeier *et al.*, 1985; Walls and Schantz, 1986; Lal *et al.*, 1987; Müller *et al.*, 1989). Recombinant DNA technology has allowed the mass production of specific antigens for vaccination trials (Miller *et al.*, 1986; Egan *et al.*, 1987; Patarroyo *et al.*, 1988) and for diagnostic purposes (Klinkert *et al.*, 1988; Affranchino *et al.*, 1989, Müller *et al.*, 1989). PCR can also be utilized for diagnosis of Chaga's disease (Sturm *et al.*, 1989), leishmaniasis (Barker *et al.*, 1986) and malaria (Delves, 1989).

Diagnosis of Human African sleeping sickness

Diagnosis of African sleeping sickness is difficult because of the similarities in symptoms between both forms of the human disease with each other and with other diseases. The fluctuating parasitemia during the systemic stage makes parasite detection unreliable and antigenic variation of BSF makes the development of sensitive serodiagnostic tests difficult. Currently, there are two main tests used for the diagnosis of sleeping sickness. Each has its own drawbacks (Turner, 1985).

Parasitological diagnosis is based on the detection of trypanosomes in blood or tissue samples by light microscopy. Demonstration of parasites in the chancre fluid, lymph fluid,

blood, bone marrow, or CSF is required to diagnose African sleeping sickness (Paris *et al.*, 1980). The number of parasites in the patient often falls below the detection limit of these tests due to the fluctuating parasitemia causing false negative diagnoses (Doyle, 1977). The detection limit of these tests ranges from 5 x 10^4 trypanosomes/ml blood for light microscopy to 5 x 10^3 trypanosomes/ml blood for DEAE-cellulose anion-exchange microconcentration technique (Paris *et al.*, 1982).

Serological methods used for the diagnosis of trypanosomiasis are based on the detection of anti-trypanosome antibodies in the patient's serum (Van Meirvenne and Le Ray, 1985). The Card Agglutination Trypanosomiasis Test (CATT) utilizes a formalin fixed and Coomassie blue stained bloodstream form of *T. b. gambiense* that expresses a particular VSG which is expressed fairly often in infections (Magnus *et. al.*, 1978). The suspension of trypanosomes is mixed with the test serum on plastic-coated cards and allowed to react. Sera containing antibodies to surface trypanosome antigens agglutinate the parasites as blue particles that are visible with the naked eye. This test has proven to be the most successfully applied screening technique for *T. b. gambiense* infections (WHO, 1986) with a 90% sensitivity. The expression of VSG on the surface of *T. b. rhodesiense* is much more variable when compared to *T. b. gambiense* rendering the CATT less effective at diagnosing *T. b. rhodesiense* infections since many false negatives are seen. In addition, with *T. b. gambiense*, approximately 5% false-positives can be expected (Van Meirvenne and Le Ray, 1985). An effective serodiagnostic test for the Rhodesian form of African sleeping sickness has not been achieved using VSG antigens.

The procyclic agglutination trypanosomiasis test (PATT) was developed to improve upon the CATT diagnostic test for *T. b. gambiense* infections and to expand it's utility to *T. b. rhodesiense* infections (Pearson *et al.*, 1986). This test is based on the detection of anti-procyclic antibodies in patients' sera using living PCF parasites in an agglutination format. Antibodies produced against procyclic forms of *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense* are detected in this test (Liu *et al.*, 1989) and are presumed to be induced in the patient by cross-reacting antigens released by lysed BSF trypanosomes during infection. The PATT has been proven to be effective in detecting anti-trypanosome antibodies in the sera of *T. b. rhodesiense* infected Vervet monkeys (Pearson, *et al.*, 1986), in *T. b. gambiense* infected humans (Liu *et al.*, 1989; 1990a) and in *T. b. rhodesiense* infected humans (Liu *et al.*, 1990b).

Serological testing based on the detection of anti-trypanosome antibodies in patients' sera cannot distinguish between active and past infections due to anti-trypanosome antibodies remaining in some patients including those that have been drug-cured (Liu and Pearson, 1987). These tests are therefore not ideal for the detection of active trypanosome

infections and the detection of circulating trypanosome antigens may be more effective. A double antibody sandwich ELISA was therefore developed and has proven to be effective in detecting antigen in the sera of trypanosome-infected mice (Liu and Pearson, 1987), Vervet monkeys (Liu *et al.*, 1988) and humans from both west and east Africa (Liu *et al.*, 1989; 1990a; 1990b). The detection of antigen gave results more indicative of active infection status than the antibody detection assays (CATT and PATT) and in several cases predicted when patients were about to relapse after drug treatment (Liu *et al.*, 1990b).

The CATT has been used extensively in the field, is inexpensive and simple to use. It is not effective in diagnosing *T. b. rhodesiense* infections. In contrast, the PATT and antigen capture assays are not restricted to detection of *T. b. gambiense* but are currently only lab-based assays which require adaptation to simple formats for use in the field (Ngaira *et al.*, 1992).

Although several lab-based and field assays for detection of human sleeping sickness have been developed there is a need for a more robust diagnostic test that can be inexpensively and effectively applied in the field, in the often adverse conditions in Africa.

Thesis objective and approach

The objective of my thesis research was to characterize highly immunogenic internal antigens of African trypanosomes that may be used to develop diagnostic tests for human African sleeping sickness. To do this I analyzed, in some detail, the specificity of six different monoclonal antibodies previously raised (by Margaret Liu) against internal antigens of African trypanosomes. Using the information on life cycle stage and species specificity, I chose one monoclonal antibody (#20) for further investigation. I used a combination of protein chemistry and recombinant DNA technology to obtain the DNA sequence and translated protein sequence of the protein recognized by monoclonal antibody #20 as a prelude to testing its utility as a diagnostically useful antigen.

Contributors to work presented in this thesis

The following individuals contributed to work presented in this thesis. In addition to this general description of work contributed by the respective scientists, specific descriptions are provided throughout the thesis.

a) Robert Beecroft (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) aided in the RNA-PCR and DNA sequencing for the 5'-portion of Hsp60 (antigen #20) and in the Southern and Northern blot analyses.

b) Michael Bridge (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) aided in characterizing the Hsp60 clones in the P1 cosmid library in preparation for knockout mutagenesis.

c) Dr. Ute Frevert (Department of Medical and Molecular Parasitology, New York University Medical Center, New York, New York, USA) performed the immunogold electron microscopy for localization of the antigen recognized by mAb #20.

d) Dr. Margaret Liu (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) generated the monoclonal antibodies against internal antigens in *T. b.rhodesiense* ViTat 1.1 PCF and performed the initial charaterization of the mAbs.

e) Dr. Caroline Stebeck (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) aided in the RT-PCR and DNA sequencing for the 5'-portion of Hsp60 (antigen #20), the Southern and Northern blot analyses and the P1 cosmid library screening.

Chapter 1: Derivation and characterization of trypanosome-specific mAbs

Introduction

The current methods available for diagnosis of African trypanosomiasis have not been completely reliable (Ngaira *et al.*, 1992). The need for an accurate and simple field test effective for both the Gambian and especially the Rhodesian forms of the disease still exists. The VSG coat present on BSF parasites is an immunodominant component of the trypanosome (Turner, 1982) and diagnosis based on the detection of this glycoprotein has not been accurate due to its variance. The mammalian host has been shown to immunologically react against other surface molecules and internal components despite the immunodominance of VSG (DeRaadt, 1974). Based on this observation, six mAbs were derived against soluble trypanosome antigens with the hope of finding a diagnostically useful nonvariant cellular constituent (Liu, 1990). An abundant, internal and nonvariant antigen may be diagnostically useful in an agglutination format or antigen trapping ELISA.

Previous studies by Liu, (1990) include the derivation and species- and life cycle stage-specificity determination of the mAbs. The epitope nature, cellular location and apparent molecular weight of the antigens recognized was also investigated. The species- and life cycle stage-specificity of the mAbs was found to be *T. brucei* specific for all but one mAb which was found to be *Trypanosoma* specific (Liu, 1990). The epitopes recognized by the mAbs appeared to be protein in nature and not carbohydrate for all six mAbs. The antigens recognized were determined to probably exist in the cytoplasm and be non-membrane associated with the exception of one which may be membrane associated.

A mixture containing the six mAbs was used in an antigen trapping double antibody sandwich ELISA to test for detection of antigen in sera using an experimental murine infection as well as human sera (Liu, 1990). It was found that these mAbs are potentially useful in diagnosing active infections with *T. brucei* spp. by detecting antigen in the serum and urine of infected mice and in the sera of humans. This chapter describes a further investigation into the species- and life cycle stage-specificity of the six mAbs.

Materials and Methods

Parasites. Bloodstream forms (BSF) of *T. b. brucei* 427 (Cross *et al.*, 1973), *T. b. brucei* GARP 16 (Hehl *et al.*, 1995), *T. b. rhodesiense* EATRO 1895 (Hill *et al.*, 1978), *T. b. gambiense* TH-1 (Gray, 1972), *T. congolense* K45/1, and *T. simiae* CP11 (Zweygarth and Rötcher, 1987) were obtained from Dr. R. Brun, Swiss Tropical Institute, Basel, Switzerland, Dr. I. Roditi, Institut für Allgemeine Mikrobiologie, Universitat Bern, Bern, Switzerland, Dr. G. Hill, Meharry Medical School, Nashville, Tennessee, USA, Dr. J. Doyle, International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya, Dr. R. Brun, Swiss Tropical Institute, Basel, Switzerland, and Dr. E. Zweygarth, Veterinary Laboratories, Kabete, Kenya, respectively. *T. b. rhodesiense* ViTat 1.1 was cloned by micromanipulation from the EATRO 1989 stock (Richardson *et al.*, 1986). With the exception of *T. simiae* CP11, BSF parasites were harvested aseptically from cyclophosphamide treated (Smith *et al.*, 1982) infected rats by Percoll™ isopycnic centrifugation (Grab and Bwayo, 1983) of whole heparinized blood followed by either lysis of contaminating erythrocytes with 0.83% NH_4Cl_2 in 100 mM Tris pH 7.4 or by purification over a DEAE-cellulose column (Lanham and Godfrey, 1970). *T. simiae* CP11 BSF were obtained by preparing buffy coats from the heparinized blood of an infected pig (Zweygarth and Rötcher, 1987). Procyclic culture forms (PCF) were established from all of the cloned bloodstream populations (Brun and Schönberger, 1979) and were maintained in culture at 26°C in minimal essential medium (MEM) containing Earle's salts (Gibco BRL, Gaithersburg, MD, USA), 25 mM HEPES (N-[2 hydroxyethyl]piperazine -N'-[2-ethanesulfonic acid]; Sigma, St. Louis, MO, USA), 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah), 1% non-essential amino acids (Gibco BRL), 2 mM glutamine (Gibco BRL), 60 mM proline (Sigma), 200 mM hypoxanthine (Sigma) and 50 mg/ml gentamycin (Gibco BRL) (procyclic culture medium). *T. vivax* IL-1392 (Barry and Gathuo, 1984) epimastigotes were obtained from Dr. I. Gumm (ILRAD, Nairobi, Kenya). Promastigotes of *Leishmania major* MHOM/SU/73/5-ASKH, obtained from the WHO reference collection at the Hebrew University-Hadassah Medical School, Jerusalem, Israel, were cultured in SM medium (Cunningham, 1973) containing 10% heat inactivated FBS as previously described (Tolson *et al.*, 1994).

Antigen preparation[1] . Proteins of *T. b. rhodesiense* ViTat 1.1 PCF whole cell lysate were separated according to Laemmli (1970) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gels) under reducing conditions. Prestained high molecular weight standards (Gibco BRL, Burlington, Ontario) were used as references. Fractions #1, 2 and 3, respectively, corresponding to approximate molecular masses of >62 kDa, 41-62 kDa and 28-41 kDa, were excised from gels using a scalpel. Proteins from each fraction were eluted by incubating the minced gel slices (3 x 5 mm) with 10 volumes of distilled water followed by dialysis against 4 liters of distilled water overnight at 4°C. Samples were concentrated to their original volumes by lyophilization. The extracted samples were analyzed by Coomassie blue staining and silver staining of 10% SDS-PAGE gels. The protein concentration of each fraction was determined by the method of Bradford (1976).

Immunization protocol[1]. Two female BALB/c mice (Charles River Laboratories, St. Constant, Quebec, Canada) were immunized with material from each gel fraction. Mice were injected three times at monthly intervals with approximately 30 μg of protein from each fraction. Each mouse received a primary intraperitoneal inoculation (0.1 ml in 0.01 M phosphate buffered saline (PBS), pH 7.4, emulsified in 0.25 ml of Freund's complete adjuvant) and two subsequent boosts in Freund's incomplete adjuvant. Mice were tail-bled after the first and second boost and the antibody titers in sera were determined by indirect ELISA (see below) using *T. b. rhodesiense* ViTat 1.1 detergent and water lysates as antigen (see below). The mice were injected in the tail veins with 10 mg of antigen preparation in 200 ml of 0.01 M PBS (pH 7.4) three days prior to removing the spleen and fusion of splenocytes with Sp2/0 parental myeloma cells (Schulman *et al.*, 1978) (see below). Mice injected with gel fraction #3 died approximately 14 days after the primary inoculation, or after the first boost, despite modifications to the immunization procedures, i.e. intramuscular injection, lower doses or injection without Freund's adjuvant. A total of six mice were used and all six died. As a result, cell fusion was possible only with splenocytes from mice injected with gel fractions #1 and #2.

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[1] This work was performed by Margaret Liu, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

Cell fusion and selection of hybridomas[1]. Splenocytes (10^8) from immunized mice were fused with Sp2/0 myelomas (10^7) in the presence of 30% polyethylene glycol (PEG, MW 1450) (Sigma Chemical Co., St. Louis, MO) according to standard procedures (Galfre and Milstein, 1981). Fused cells were incubated overnight at 37°C in an atmosphere of 5% CO_2 in air, 98% humidity, in 100 ml of fusion medium (RPMI 1640, 20% preselected, heat inactivated bovine calf serum, 1X Hypoxanthine/Aminopterin/Thymidine [HAT], 1 mM sodium pyruvate, 5 x 10^{-5} M 2-mercaptoethanol, 50 mg/ml gentamycin, 50 units/ml streptomycin, penicillin, 24 units/ml mycostatin and 10^7 syngeneic thymocytes). Cells were dispensed in 200 ml volumes into wells of 96 well plates and were maintained in fusion medium (1X HAT) at 37°C, in 5% CO_2 and 98% humidity, until they were ready for screening (10-14 days). The remaining 25 ml of cells were plated in petri dishes containing 2.2% methylcellulose (StemCell Technologies Inc., Vancouver, B. C.) in fusion medium according to the procedure of Davis *et al.* (1982). When hybridoma colonies had grown to 0.5 mm in diameter (10-14 days after plating), they were picked and transferred individually to 96 well plates in 200 µl of HT medium (fusion medium without aminopterin). In general, hybridomas were grown in fusion medium for 2 weeks, followed by 2 weeks incubation in HT medium and then were maintained indefinitely in growth medium (fusion medium minus HAT, thymocytes, and mycostatin).

Hybridoma supernatants were screened initially using indirect ELISA with detergent lysates and water lysates of *T. b. rhodesiense* ViTat 1.1 BSF and PCF as antigens. Positive hybridoma clones were transferred to 24 well plates and supernatants tested for antibody binding to detergent lysates and water lysates of other species of *Trypanosoma* (BSF of *T. b. gambiense*, *T. b. brucei* and *T. congolense*) and promastigotes of *Leishmania* (*L. major* and *L. donovani*). Human transferrin (5 µg/well) was used as antigen to identify "sticky" antibodies. Dilution cloning was performed with hybridomas that secreted antibodies binding specifically to *Trypanosoma* spp., or to both *Trypanosoma* and *Leishmania* spp. Stable clones were established immediately from their respective hybridoma cells after three dilution cloning steps and testing in indirect ELISA using various parasite lysates during each interval. Samples of these clones were cryopreserved in liquid nitrogen at 10^7 cells/ml of freezing medium (90% FBS, 10% dimethylsulfoxide).

Soluble antigens for ELISA[1]. Trypanosome PCF were harvested and washed twice by centrifugation (800 x g, 15 min.) in 0.01 M sodium phosphate/0.15 M NaCl containing 1% glucose (PSG). Lysates were then made as described by Richardson *et al.* (1986). Briefly, the pellet containing the parasites was resuspended at 1 x 10^8 parasites/ml in ice

cold distilled water containing 1% 3-[(3-cholamidopropyl) dimethylammonio}-1-propanesulfonate (CHAPS) (Sigma), 5 mM HEPES, and trypanosome protease inhibitor cocktail [1 mM N-tosyl-lysine chloromethyl ketone (TLCK) (Sigma), 1 mM N-tosyl-phenyl-alanine chloromethyl ketone (TPCK) (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), 1 mM L-trans-epoxysuccinyl leucyl-amidol-4-guanidino butane (E-64) (Sigma) and 5 mM Ethylenediamine-tetraacetic acid (EDTA) (Sigma)]. The resuspended cells were mixed using a vortex mixer, incubated at 37°C for 15 minutes, frozen in liquid N_2 , incubated at 37°C for 15 minutes and mixed again to solubilize the parasite proteins. Aggregates were removed by centrifugation at 14,000g for 5 min. at 4°C. The supernatant was aliquotted and frozen at -20°C. Lysates were used at a 1/20 dilution in dH_2O or phosphate buffered saline (PBS) to coat wells for indirect ELISA. Water lysates for ELISA were made as above without CHAPS in the lysis buffer.

Indirect ELISA[1]. Solid-phase ELISA was used to determine the binding specificity's of monoclonal antibodies to antigens in different aqueous and detergent lysates using the methodology of Parish *et al.* (1985). ELISA plate wells (Costar, Cambridge, MA) were coated with lysates prepared as above (ELISA lysates). One hundred microliters of 1/20 dilution of lysates in PBS (approximately 10 mg/ml) were coated onto ELISA plates. Antigen was dried onto the wells by incubating the microplates overnight at 37°C. Undiluted hybridoma tissue culture supernatants or 1/800 dilutions of ascites fluid were tested. An alkaline phosphatase labeled goat anti-mouse IgG/IgM (Caltag, South San Francisco, CA, USA) was used as second antibody at a dilution of 1/2000 with the substrate p-nitrophenol phosphate (Sigma).

Isotyping[1]. The isotype of monoclonal antibodies was determined using an antigen-capture-ELISA isotyping kit (American Qualex International, La Marida, CA, USA). The procedure was carried out as described by the manufacturer. Undiluted hybridoma tissue culture supernatants were used for isotyping to avoid detection of other mouse antibodies that are present in ascites fluids.

Lysates for gel electrophoresis. Trypanosomes from various species and life cycle stages and *Leishmania* promastigotes were counted, harvested and washed twice by centrifugation (800g, 15 min.) in PBS. The parasites were then solubilized at 1 x 10^6/ml in Laemmli (1970) sample buffer containing 0.0625 M TRIS (Tris-(hydroxymethyl) Aminomethane), 10% glycerol, 0.025 M DL-dithiothreitol, 2.3% SDS and bromophenol blue dye and

stored at -20°C. Lysates were used on gels at 1 x 10^7 cell equivalents per lane for stained gels and 2 x 10^6/lane for immunoblots.

Polyacrylamide gel electrophoresis (analytical). SDS-PAGE was performed according to Laemmli, (1970) using a minigel apparatus (Mini protean II, Biorad, Richmond, CA). Proteins were stacked using a 3% gel and were separated on a 10% gel at 10 mA. Rainbow™ high or low range molecular weight markers (Amersham, Oakville, Ontario) or Prestained Molecular Weight Markers (Gibco BRL, Burlington, Ontario) were run on each gel. Molecules were stained with Coomassie blue R-250 (BDH, Vancouver, B. C.), silver stained (Merrill, 1984) or were detected by immunoblotting (see below).

Immunoblots. Electrophoretic transfer of proteins from reducing SDS-PAGE gels onto Immobilon-P™ (Millipore Corporation, Bedford MA, USA) and subsequent antigen detection were performed by the procedure of Towbin *et al.* (1970), with modifications to reduce background (Birk and Koepsell, 1987; Bestagno *et al.* , 1987). For details see Beecroft *et al*, (1993). Undiluted hybridoma tissue culture supernatant or ascites fluids (1/1000 dilutions) were used as first antibody. Bound antibody was detected using horseradish peroxidase-conjugated goat anti-mouse IgG/IgM (Caltag, South San Francisco, CA, USA) at a 1/2000 dilution. Enhanced luminol chemiluminescence reagent (Renaissance™ DuPont NEN, Boston, MA, USA) was used as the substrate for the enzyme conjugate according to the instructions of the manufacturer.

Results

Derivation and characterization of mAbs. MAbs were derived and partially characterized by Margaret Liu as part of her Ph.D. thesis research in Dr. Pearson's laboratory. The mAbs were characterized further in the current thesis. Gel fractions of *T. b. rhodesiense* ViTat 1.1 PCF were excised from gels using prestained high molecular weight standards as references. Silver stained gels were used to examine the eluted gel fractions (Fig. 1). Gel fractions #s 1, 2 and 3 were found to correspond to molecular masses of >48 kDa, 52-37 kDa and 37-22 kDa, respectively (Fig. 1). A total of 26 mAbs were produced against *T. b. rhodesiense* ViTat 1.1 PCF gel fraction # 1 and 10 mAbs were raised against gel fraction # 2. Mice injected with proteins from gel fraction #3 died (see immunization protocols in Materials and Methods).

Indirect ELISA using water and detergent lysates of *Trypanosoma* and *Leishmania* species as antigen was employed to determine the binding specificities of the mAbs. Only 14 mAbs showed binding to trypanosome lysates, six of which were made against gel fraction #1 and the remaining eight against gel fraction #2 (data not shown). The 8 mAbs made against gel fraction #2 bound to the procyclin molecule of *T. brucei* spp. (Richardson *et al.*, 1986) as determined by ELISA on purified procyclin. The binding of the remaining six mAbs is summarized in Table 1. All six mAbs bound only to the *Trypanosoma* water or detergent lysates and not to *Leishmania* water- or detergent-lysates. Five of the anti-PCF mAbs (#65, 91, 148, 236 and 401) were *T. brucei* species-specific while mAb #20 bound to procyclic lysates of both *T. brucei* spp. and *T. congolense* . All six mAbs bound to BSF lysates of *T. b. rhodesiense.* The isotypes of these mAbs are also shown in Table 1. Three mAbs (#20, #148 and #236) were IgG while the remaining antibodies (#65, #91 and #401) were IgM.

Further characterization of mAbs. The species- and life-cycle stage specificities of the mAbs were further investigated by immunoblot analysis. The antigen recognized by mAb #20 was present in all species and life cycle stages tested (Figure 2). The molecular mass of this antigen was approximately 65 kDa and it was present in both the PCF and BSF of trypanosomes but not in *Leishmania* promastigotes. The molecular mass of this antigen appeared to be slightly lower in BSF (Lane B) than in PCF (Lanes A and C-G). Monoclonal antibody #148 recognized an antigen doublet of approximately 68 kDa and was specific to *T. brucei* spp. (Figure 3). Monoclonal antibody #148 was also tested in an

using mAb #65 is shown in Figure 4. This mAb recognized two bands (at 100 and 120 kDa) which appear to be *T. brucei* spp. specific (Lanes A-C). *T. b. rhodesiense* ViTat 1.1 BSF were also tested in an immunoblot with mAb #65 (data not shown) and found to be negative. Monoclonal antibody #91 recognized an antigen at 100 kDa (Figure 5, lanes A, C and D) only in lysates made from PCF. Monoclonal antibody #236 recognized high molecular weight bands in *T. brucei* spp. PCF at approximately 120, 200 and 300 kDa (Figure 6, Lanes A, C and D). Figure 7 illustrates that mAb #401 had an immunoblotting pattern similar to that of mAb #91, recognizing a 90 kDa antigen in *T. brucei* spp. PCF only. (Lanes A, C and D).

Table 2 summarizes the species and life-cycle stage specificities of each mAb. Monoclonal antibody #20 recognized an antigen that was found in all trypanosome species tested while the remaining 5 mAbs were *T. brucei* species specific. The mAbs #20 and 148 recognized antigens in BSF as well as PCF while 65, 91, 236 and 401 were PCF specific.

Figure 1

Silver stained SDS-PAGE gel patterns of extracted gel fractions used for immunization of BALB/c mice. Lane A, fraction # 1; Lane B, fraction #2; Lane C, fraction #3; Lane D, *T. b. rhodesiense* ViTat 1.1 PCF whole lysate (Reproduced from Liu, 1990).

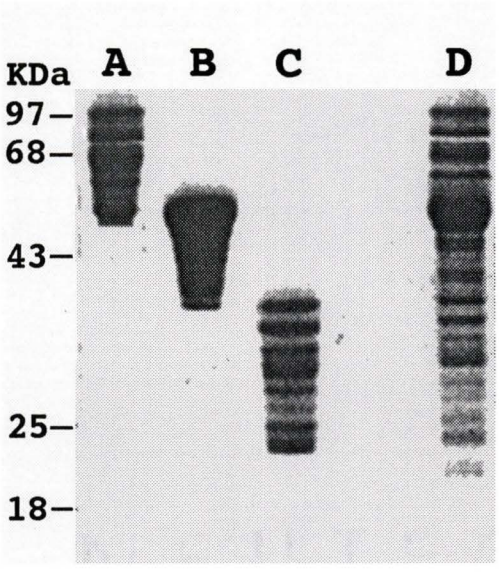

Table 1

Binding of selected monoclonal antibodies to detergent lysates[a] of *Trypanosoma* and *Leishmania* species in indirect ELISA.

Antibody[b]	Isotype[c]	*T. b. rhodesiense* Vitat 1.1 bloodstream forms	*T. b. rhodesiense* ViTat 1.1 PCF	*T. b. gambiense* U2 PCF	*T. b. brucei* 427.01 PCF	*T. b. congolense* 45/1 PCF	*L. donovani* IS2D	*L. major* A2	human transferrin[d]
20	IgG_{2b}	+	+	+	+	+	-	-	-
65	IgM	+	+	+	+	-	-	-	-
91	IgM	+	+	+	+	-	-	-	-
148	IgG_1	+	+	+	+	-	-	-	-
236	IgG_1	+	+	+	+	-	-	-	-
401	IgM	+	+	+	+	-	-	-	-
anti-gonococcus mAb	IgM	-	-	-	-	-	-	-	-
anti-urokinase mAb	IgG_1	-	-	-	-	-	-	-	-

[a]Parasites were lysed at 10^8/ml in 0.1% SDS/0.5% NP-40/10 mM Tris-saline (pH 7.4). Extracts from approximately $5x10^5$ parasites were used to coat ELISA wells.

[b]Derived against extracted gel fraction #1 of *T. b. rhodesiense* ViTat 1.1 PCF.

[c]Determined by antigen-capture ELISA.

[d]Human transferrin was used as a negative control antigen and was assayed at 5 mg/well.

Reproduced from M. Liu (1990)

Figure 2

Detection of antigen in lysates of *Trypanosoma* and *Leishmania* species by immunoblotting using mAb #20. Lane A, *T. b. rhodesiense* ViTat 1.1 PCF; Lane B, *T. b. rhodesiense* ViTat 1.1 BSF; Lane C, *T. b. gambiense* TH-1 PCF; Lane D, *T. b. brucei* 427.01 PCF; Lane E, *T. simiae* CP11 PCF; Lane F, *T. vivax* IL-1392 trypomastigotes; Lane G, *T. congolense* K45/1 PCF; Lane H, *L. major* MHOM/SU/73/5-ASKH promastigotes.

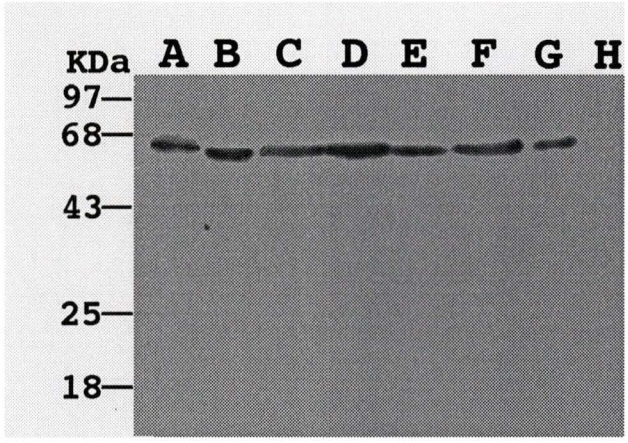

Figure 3

Detection of antigen in lysates of *Trypanosoma* and *Leishmania* species by immunoblotting using mAb #148. Lane A, *T. b. rhodesiense* ViTat 1.1 PCF; Lane B, *T. b. gambiense* TH-1 PCF; Lane C, *T. b. brucei* 427.01 PCF; Lane D, *T. simiae* CP11 PCF; Lane E, *T. vivax* IL-1392 trypomastigotes; Lane F, *T. congolense* K45/1 PCF; Lane G, *L. major* MHOM/SU/73/5-ASKH promastigotes.

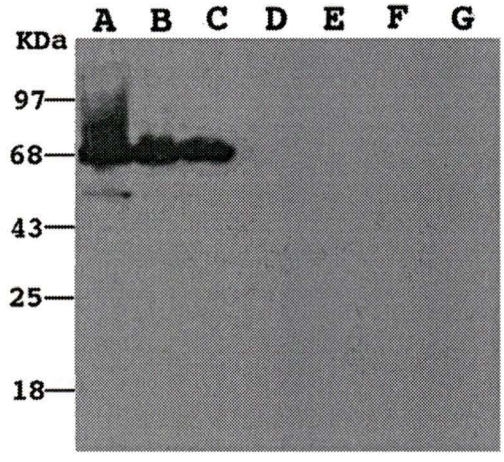

Figure 4

Detection of antigen in lysates of *Trypanosoma* and *Leishmania* species by immunoblotting using mAb #65. Lane A, *T. b. rhodesiense* ViTat 1.1 PCF; Lane B, *T. b. gambiense* TH-1 PCF; Lane C, *T. b. brucei* 427.01 PCF; Lane D, *T. simiae* CP11 PCF; Lane E, *T. vivax* IL-1392 trypomastigotes; Lane F, *T. congolense* K45/1 PCF; Lane G, *L. major* MHOM/SU/73/5-ASKH promastigotes.

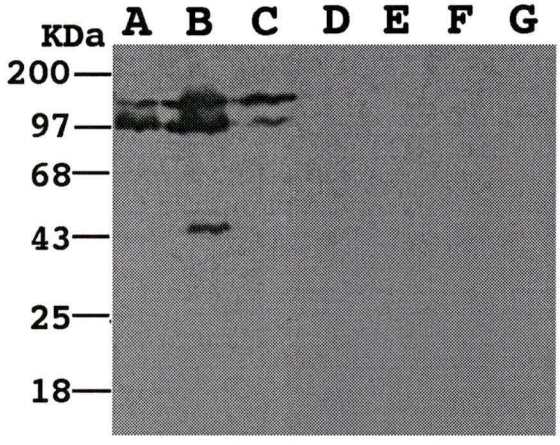

Figure 5

Detection of antigen in lysates of *Trypanosoma* and *Leishmania* species by immunoblotting using mAb #91. Lane A, *T. b. rhodesiense* ViTat 1.1 PCF; Lane B, *T. b. rhodesiense* ViTat 1.1 BSF; Lane C, *T. b. brucei* 427.01 PCF; Lane D, *T. b. gambiense* TH-1 PCF; Lane E, *T. congolense* K45/1 PCF; Lane F, *T. simiae* CP11 PCF; Lane G, *L. major* MHOM/SU/73/5-ASKH promastigotes.

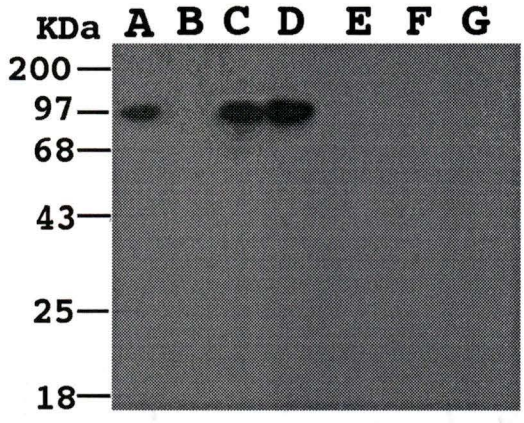

Figure 6

Detection of antigen in lysates of *Trypanosoma* and *Leishmania* species by immunoblotting using mAb #236. Lane A, *T. b. rhodesiense* ViTat 1.1 PCF; Lane B, *T. b. rhodesiense* ViTat 1.1 BSF; Lane C, *T. b. brucei* 427.01 PCF; Lane D, *T. b. gambiense* TH-1 PCF; Lane E, *T. congolense* K45/1 PCF; Lane F, *T. simiae* CP11 PCF; Lane G, *L. major* MHOM/SU/73/5-ASKH promastigotes.

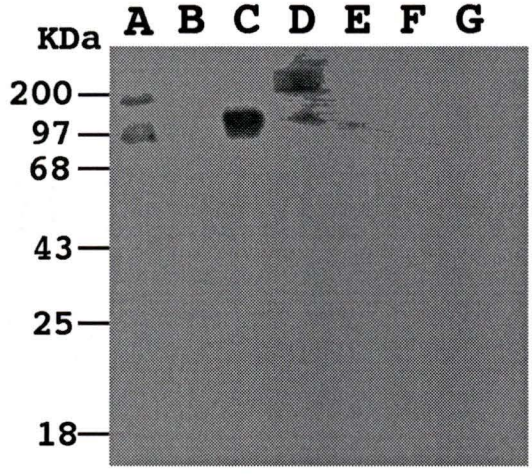

Figure 7

Detection of antigen in lysates of *Trypanosoma* and *Leishmania* species by immunoblotting using mAb #401. Lane A, *T. b. rhodesiense* ViTat 1.1 PCF; Lane B, *T. b. rhodesiense* ViTat 1.1 BSF; Lane C, *T. b. brucei* 427.01 PCF; Lane D, *T. b. gambiense* TH-1 PCF; Lane E, *T. congolense* K45/1 PCF; Lane F, *T. simiae* CP11 PCF; Lane G, *L. major* MHOM/SU/73/5-ASKH promastigotes.

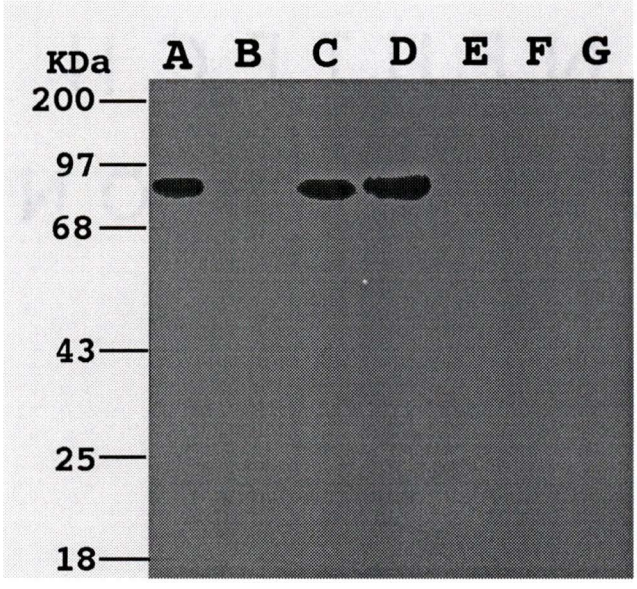

Table 2

Summary of characteristics of mAbs and their specific antigens as determined by ELISA (adapted from Liu, 1990) and immunoblot analyses.

	mAb #20	mAb #65	mAb #91	mAb #148	mAb #236	mAb#401
isotype	IgG_{2b}	IgM	IgM	IgG_1	IgG_1	IgM
specificity	*Trypano-soma* (PCF/BSF)	*T. brucei* (PCF)	*T. brucei* (PCF)	*T. brucei* (PCF/BSF)	*T. brucei* (PCF)	*T. brucei* (PCF)
molecular weight on immuno-blots	65 kDa	100 kDa 120 kDa	100 kDa	68 kDa	120 kDa 200 kDa 300 kDa	90 kDa

Discussion

The species- and life cycle stage-specificity of the six mAbs produced against *T. brucei* PCF (Liu, 1990) was investigated. One mAb (mAb #20) recognized all *Trypanosoma* species tested while the remaining mAbs were *T. brucei* spp. specific. No mAbs were found to react against the *Leishmania* parasites tested. Immunoblots were performed against whole lysates from various *Trypanosoma* species and life cycle stages using each mAb as the primary antibody.

The antigen recognized by mAb #20 was found in all *Trypanosoma* species and life cycle stages tested (Figure 2). This mAb appeared to recognize a band at apparent molecular mass of 65 kDa in all PCF tested as well as trypomastigotes of *T. vivax* (Figure 2). The apparent molecular mass of the antigen in *T. b. rhodesiense* BSF was slightly lower than in the PCF perhaps due to a change in conformation or post-translational modification. The species-specificity of this mAb was expanded from previous work to include *T. simiae* PCF and *T. vivax* trypomastigotes and was also found to be negative against another serotype of *L. major* (MHOM) promastigotes. The apparent molecular masses reported by Liu, (1990) are slightly different than reported here. This could be due to slight differences in SDS-PAGE running conditions or molecular mass standards.

The antigen recognized by mAb #148 was found only in *T. brucei* spp. (Figure 3). The antigen was found in both PCF and BSF tested and was a doublet with an apparent molecular mass of 68 kDa. Liu (1990) had reported different banding patterns on immunoblots for the *T. brucei* subspecies and thought this mAb may be useful in distinguishing between them (Liu, 1990). Different banding patterns were not observed on immunoblots performed here.

Monoclonal antibody #65 recognized two *T. brucei* spp. bands at 100 and 120 kDa (Figure 4). This mAb was also found to be PCF specific unlike Liu's (1990) findings which showed this antigen in BSF as well. Similar results were obtained for mAbs #91 and #401 (Figures 5 and 7). These mAbs recognized *T. brucei* PCF specific bands at 100 kDa and 90 kDa respectively and did not recognize BSF. This could be due to slightly different whole lysate preparations used for SDS-PAGE or low sensitivity of the chemiluminescent detection system used here compared to the I^{125} detection method used by Liu, (1990).

The antigen recognized by mAb #236 was found to be *T. brucei* PCF specific (Figure 6). Different banding patterns were observed for the *T. brucei* subspecies with *T. b. rhodesiense* having a doublet at 100 kDa and a third band at 200 kDa (Figure 6; lane A). *T.*

b. brucei showed a doublet at 100 kDa (Figure 6; lane C) while *T. b. gambiense* had a single band at 100 kDa and a doublet at 300 kDa (Figure 6; lane D). This mAb may therefore be used to distinguish between the different *T. brucei* PCF subspecies in immunoblots.

Although several of the mAbs recognized antigens that appear to be *T. brucei* spp.-specific, the antigen bound by mAb #20 was detected in all trypanosome species and subspecies tested. For this reason, this antigen was characterized further as it may have potential in a diagnostic test for human sleeping sickness. This characterization is described in Chapter 2.

Chapter 2: Further characterization of mAb #20 and identification and characterization of its specific antigen.

Introduction

The antigen recognized by mAb #20 was found in all *Trypanosoma* species and life cycle stages tested as described in chapter 1. This indicates that it may be an important antigen for diagnostic use. Diagnostically, the antigen will not distinguish between different trypanosome species but may be useful as a nonvariant antigen that is present in all African trypanosomes. Monoclonal antibody #20 was therefore further characterized for species and life cycle stage specificity. The identity of antigen #20 was also determined. As will be seen, antigen #20 was found to be a heat shock protein of the mitochondrial Hsp60 chaperone class.

Heat shock proteins (Hsps) or chaperones are found in all cells, function to protect the cell during stress and are also important in many normal cellular functions (Parsell and Lindquist, 1993). The stress response involves the activation of this set of conserved genes and the subsequent synthesis of the encoded proteins as a result of a change in environmental conditions normally experienced by a cell. The genes are activated by agents that stress the cell such as organic solvents, heavy metals, nutrient deprivation, oxidants and viral infection (Lindquist, 1986). The common signal for Hsp induction in a stress response appears to be the presence of "abnormal" or denatured proteins (Ananthan *et al.*, 1986). Many Hsps are present during normal cell growth conditions and play an important role as chaperones (Gething *et al.*, 1992). These proteins can be grouped into four families based on their molecular size (1) 15,000-30,000 MW, (2) 90,000-100,000 MW, (3) 70,000-80,000 MW and (4) 50,000-60,000 MW Hsp families. Within each family a high degree of conservation in protein structure and function exists between highly divergent species.

Heat shock proteins have been of great interest in parasites which infect two different hosts during their life cycle because the temperature shift and oxidant exposure they encounter when transmitted from insect vector to mammalian host induces a stress response. The nutrient deprivation occurring in the insect vectors also results in an increased expression of various heat shock proteins (Van der Ploeg *et al.*, 1985). These proteins may have important roles beyond their chaperoning functions such as helping the parasites to adapt to the higher temperatures and to the differentiation states of parasites in the various life cycle stages (Van der Ploeg *et al.*, 1985). In studies using *Leishmania*

parasites, heat shock proteins were linked to an increase in infectivity (Smejkal *et al.*, 1988), increased resistance to oxidants (Wilson *et al.*, 1994), and increased virulence (Salotra *et al.*, 1994).

When parasitic protozoa invade the human body the immediate heat shock response produces Hsps. Heat shock proteins are abundant, immunodominant proteins in parasites. The immune response to infection by many parasites elicits immunoglobulins and T-lymphocytes that recognize Hsps as the major foreign antigens (Kaufmann, 1990). Stress protein epitopes that do not cross-react with host stress proteins could be useful diagnostically and may be vaccine candidates (Kaufmann, 1990).

Heat shock proteins also exist in normal human cells, are present at low levels and confined to inside the cell. In abnormal, stressed or damaged cells, there are higher levels of these proteins and some may be present at the cell surface (Kaufmann, 1990). Antibodies or T-cells produced against Hsps of infecting pathogens can then recognize shared epitopes. This situation would lead to immune-mediated destruction of these cells through the system of immune surveillance. Individuals suffering from autoimmune disease possess antibodies and $\delta\gamma$-T-lymphocytes that recognize Hsps. This occurs in rheumatoid arthritis and diabetes mellitus (Jones *et al.*, 1993).

The Hsps of the 15,000-30,000 MW family are found in the cytosol of prokaryotes and eukaryotes. These proteins have 15-20% amino acid sequence identity and share a conserved hydrophobic region at their C-terminal end (Parsell and Lindquist, 1993). They are most abundant in plant cells and found in the chloroplasts as well as in the cytosol (Vierling, 1991). Cytosolic small Hsps form heat shock granules in both plants and mammals in response to extreme stress (Lindquist and Craig, 1988). The function of these granules is not clear but they can be highly organized in structure and may contain small RNAs (Lindquist and Craig, 1988). The small Hsps contain a domain that is closely related to a sequence in vertebrate lens α-crystallin in their C-terminal region and this is thought to be the region responsible for polymerization (Lindquist and Craig, 1988). Studies show these Hsps are phosphorylated in response to heat and other stresses and it has been suggested that they function in signal transduction pathways (Mendelsohn *et al.*, 1991). Research also shows that they display an ATP-independent chaperone function *in vitro* (Jakob *et al.*, 1993). Two mycobacterial surface antigens (de Jong *et al.*, 1993) and a major egg antigen of *Schistosoma mansoni* (Nene *et al.*, 1986) are members of this Hsp family. Members of the family have also been found in *Kinetoplastida* but have not been characterized. An immunogenic 22-kDa Hsp protein localized to the mitochondria was reported in *Leishmania mexicana amazonensis* in studies using two-dimensional gel electrophoresis (Pinelli and Shapira, 1990). Recently, a cDNA encoding a domain

homologous with these heat shock proteins has been identified in *T. cruzi* (Schoneck *et al.*, 1994).

Members of the Hsp90 family are found in the cytosolic and nuclear compartments of all eukaryotes and in the endoplasmic reticulum of higher eukaryotes (Parsell and Lindquist, 1993). The *E. coli* homologue HtpG shares 40% amino acid identity with the eukaryotic proteins, is strongly induced by heat and is not essential for cell survival (Bardwell and Craig, 1988). The eukaryotic Hsp90 proteins are essential for cell survival at normal to moderately high temperatures with the quantity required by the cell increasing as the temperature increases (Borkovich *et al.*, 1989). These Hsps function as ATP-dependent chaperones and have been shown to prevent denatured proteins from aggregating (Wiech *et al.*, 1993). At normal temperatures the Hsp90 proteins interact with many cellular proteins including casein kinase II (Miyata and Yahara, 1992), several steroid hormone receptors (Pratt *et al.*, 1992), oncogenic tyrosine kinases (Brugge, 1986), calmodulin (Minami *et al.*, 1993), actin (Nishida *et al.*, 1986) and tubulin (Fostinis *et al.*, 1992). The Hsp90s display a high specificity in associating with target proteins and these interactions are important in regulating the function of the target proteins (Parsell and Lindquist, 1993). The regulation of the target protein is accomplished by the Hsp90 functioning as a "match-maker" that brings molecules together. By binding to the protein Hsp90 helps them assume an activation-competent conformation but keeps the target inactive as long as it stays associated with the protein (Parsell and Lindquist, 1993).

Hsp90 proteins are essentially intracellular, although cell surface expression has been reported (Erkeller-Yüksel *et al.*, 1992). Immunogenic Hsp90 (Hsp83) proteins have been reported in *Plasmodium falciparum* (Jendoubi and Bonnefoy, 1988), *Leishmania mexicana amazonensis* (Shapira and Pinelli, 1989), *Crithidia fasciculata* and *Trypanosoma cruzi* (Nadeau *et al.*, 1992) and *Trypanosoma brucei* (Mottram *et al.*, 1989). High heat inducibility of the hsp83 gene appears to correlate with vertebrate infectivity and virulence (Van der Ploeg *et al.*, 1985; Shapira *et al.*, 1988). The presence of stress proteins prior to transmission may preadapt the parasite for additional stress in the mammalian host.

Members of the Hsp70 family are found in the ER, mitochondria, chloroplasts, nucleus, nucleolus and cytoplasm in eukaryotic cells. These heat shock proteins are constitutively expressed and are induced by stress in the cell. The DnaK protein of *E. coli* is 50% homologous to eukaryotic Hsp70 proteins. Hsp70 proteins have two domains, a N-terminal domain containing an ATP-binding site and a C-terminal domain responsible for binding proteins (Flaherty *et al.*, 1990). These heat shock proteins bind to hydrophobic surfaces, stabilizing target proteins in a fully or partially unfolded state (Pelham, 1986). Hsp70 chaperone activity is ATP-dependent and the transition of Hsp70 from an ADP-

bound to an ATP-bound form drives the protein release (Parsell and Lindquist, 1993). Hsp70 proteins function to protect the cell in heat shock by promoting growth at moderately high temperatures and protecting the organism from being killed at extreme temperatures. This is accomplished by the chaperoning of proteins damaged by stress. At normal temperatures Hsp70 proteins have important chaperone activities in the cell. These proteins bind to small peptides, nascent polypeptides, protein subunits and mutant proteins (Parsell and Lindquist, 1993). Activities include participation in protein folding, unfolding, assembly, disassembly and translocation processes.

Heat shock proteins belonging to the Hsp70 class have been identified as important immunogens and in parasite survival in the host. A few examples include *Schistosoma mansoni* (Hedstrom *et al.*, 1987), *Plasmodium falciparum* (Sharma, 1992), *Crithidia fasciculata* (Effron *et al.*, 1993), *Leishmania* spp. (Hanekamp and Langer, 1991), *Trypanosoma cruzi* (Requena *et al.*, 1988) and *T. congolense* (Boulangé and Authié, 1994) and *T. brucei* (Glass *et al.*, 1986). As immunogens, these proteins were immunologically recognized in the majority of patients tested in *Schistosoma mansoni* (Hedstrom *et al.*, 1987), *Plasmodium falciparum* (Bianco *et al.*, 1986), *Crithidia fasciculata* (Effron *et al.*, 1993), *Leishmania donovani* (MacFarlane *et al.*, 1990) and *T. cruzi* infections (Engman *et al.*, 1990; Tibbetts *et al.*, 1994) and in animals infected with *T. congolense* (Boulangé and Authié, 1994). Some Hsp70 proteins are found within the kinetoplast in *C. fasciculata* (Effron *et al.*, 1993), *Leishmania* (Searle *et al.*, 1993), *Trypanosoma cruzi* (Engman *et al.*, 1989; Olson *et al.*, 1994) and *T. brucei* (Klein *et al.*, 1995) and were postulated to not only chaperone proteins imported in to the mitochondrion but also to associate with the kinetoplastid DNA (kDNA). Results suggest that in all kinetoplastid species mitochondrial Hsp70 has a specific function in kDNA replication, editing and structure (Klein *et al.*, 1995). Cytoplasmic Hsp70 proteins are postulated to have a more classical chaperoning function in *Leishmania major* (Searle and Smith, 1993) and in *Trypanosoma cruzi* (Olson *et al.*, 1994).

Members of the chaperone Hsp60 family are found in the matrix of the mitochondria accounting for 1% of the total mitochondrial matrix protein and in the stromal compartment of chloroplasts (Ellis and van der Vies, 1991). These proteins are the most highly conserved in structure among Hsps and share 60% amino acid identity along the length of the protein (Hemminsen *et al.*, 1988). Most consist of 14-mers of 60,000 MW subunit forming two seven-membered rings (Ellis and van der Vies, 1991). The mitochondrial protein forms a single seven-membered ring (Viitanen *et al.*, 1991) and is localized to the inner compartment of the mitochondria. Hsp60 has an ATPase activity that increases with temperature and the binding of ATP induces a major conformational change in the structure

of the oligomer (Saibil *et al.*, 1993). Another heat shock protein, Hsp10 (GroES in *E. coli*), associates with Hsp60 by complexing with one end of the oligomer and regulating its ATPase activity and substrate associations (Saibil *et al.*, 1993). This chaperone forms a single seven-membered ring (Chandresekhar *et al.*, 1986). The unfolded polypeptide appears to bind exclusively to the Hsp60 portion of the Hsp60/Hsp10 complex in the central channel of the cylinder (Wynn *et al.*, 1994).

The Hsp60 proteins are induced by heat and increase by 2-3 fold while the Hsp60 of *E. coli* (GroEL) increases to 10-15% of total cellular protein upon heat shock (Niedhardt *et al.*, 1984). During heat shock these chaperones protect the cell by preventing the aggregation of denatured proteins and by promoting refolding when cells are returned to lower temperatures (Martin *et al.*, 1992). At normal temperatures Hsp60 binds to unfolded proteins and promotes their folding. Hsp60 has a high affinity for denatured proteins and while bound to Hsp60 the substrate will acquire secondary structure (Martin *et al.*, 1991). In most cases Hsp10 and ATP are required for further folding and substrate release from Hsp60 (Martin *et al.*, 1991). Both Hsp60 and Hsp10 are required for growth at all temperatures by bacteria and eukaryotic cells (Fayet *et al.*, 1989). In the mitochondrion, Hsp60 is important in chaperoning proteins that have been unfolded for transport across the mitochondrial membrane. A member of the Hsp70 family unfolds the protein destined for the mitochondria and upon emerging into the lumen of the mitochondria, Hsp60 will participate in the refolding of the protein (Parsell and Lindquist, 1993).

Chaperones in the Hsp60 family have not been studied extensively in parasitic protozoa and their relevance in parasitic infection is just beginning to be investigated. Many principles that apply to Hsp70 chaperones also apply to Hsp60 proteins such as immunogenicity, parasite transmission and differentiation. To date Hsp60 genes have been found in *Leishmania donovani* (Rey-Ladino and Reiner, 1993), *Trypanosoma cruzi* (Giambiagi-de Marval *et al.*, 1993; Sullivan *et al.*, 1994) and recently in *T. b. brucei* (Bringaud *et al.*, 1995). These heat shock proteins have been studied in other pathogens and are implicated in autoimmunity and other disease pathologies (Macario, 1995).

Materials and Methods

Parasites. T. b. brucei 427, *T. b rhodesiense* ViTat 1.1 and *T. congolense* K45/1 were obtained and cultured as described in Chapter 1. Bloodstream forms of *T. congolense* IL-3000 (Fish *et al.*, 1989) were obtained from Dr. W. Fish, ILRAD, Nairobi, Kenya. *T. congolense* IL-3000 PCF, epimastigotes and metacyclics were grown *in vitro* using culture methods developed at ILRAD (Fish *et al.*, 1989 and Bienen *et al.*, 1991). *Trypanosoma cruzi* Y strain trypomastigotes and epimastigotes were obtained from Dr. V. Nussenzweig (NYU, New York, NY) and were grown in Vero cell cultures as described by Schenkman *et al.* (1991).

Transformation of BSF to PCF. BSF of *T. congolense* IL3000 were harvested aseptically from rat blood, adjusted to $2x10^7$ ml^{-1} in procyclic culture medium and placed into each of ten 50 ml tissue culture flasks. The flasks were incubated at 26°C and at each of ten intervals, parasites were harvested from one flask and cell lysates prepared for subsequent gel electrophoresis and immunoblotting experiments as described below.

Solubilization. T. b. rhodesiense ViTat 1.1 PCF (1×10^{11}) were harvested, washed once in MEM (without additives) and once in PSG at 1×10^9 trypanosomes/ml by centrifugation (2000g, 15 min. at 4°C). The parasites were then frozen in liquid N_2 as wet pellets. Lysis was performed by thawing the parasites at 37°C and resuspending them at 1×10^9/ml in ice cold HPLC grade dH_2O containing protease inhibitors (see under ELISA lysates) and sonicating for 8 cycles of 30 sec bursts at 45 watts (setting 5 in a Sonifier Cell Disrupter, model w185E, Heat System-Ultrasonics, Inc., Plainview, NY, USA) on ice. The resulting mixture was then centrifuged at 4000g for 10 min. at 4°C to remove insoluble cellular debris. The supernatant was then centrifuged at 105,000g for 60 minutes at 4°C and the pellet discarded. The 105,000g supernatant containing the antigen of interest was saved for further purification steps.

Ammonium sulfate precipitation. Ammonium sulfate (Fisher Chemical Co. Fairlawn, NJ) was added to the 105,000g supernatant to a final concentration of 50% saturation (Saturation is 4.1 M) at 4°C while stirring. The solution was stirred for 30 minutes and then centrifuged at 4000g for 30 minutes at 4°C to obtain the precipitated proteins. The pellet was then dialyzed, lyophilized and resuspended in 12.5 ml of sample buffer for preparative SDS-PAGE. The sample buffer contained 0.06 M Tris-HCl pH 6.8, 2% SDS,

5% beta-mercaptoethanol, 10% glycerol and 0.025% bromophenol blue. Samples were stored at -20°C.

Preparative SDS-PAGE. Proteins concentrated by ammonium sulfate precipitation were separated using a Model 491 Prep Cell preparative SDS-PAGE (PSP) electrophoresis unit (Biorad, Richmond, CA). An 8% acrylamide monomer solution was determined to give optimal separation of the proteins of interest. A 75 ml solution was used in a 37 mm ID gel tube with a 4% stacking gel. The gels were run according to the manufacturer's instructions with approximately 2 ml of above protein sample for each run at 4°C and 12 watts constant power. After the dye front reached the bottom of the tube gel, 150 fractions of 3 ml each were collected at a flow rate of 1 ml/min. using a fraction collector (Pharmacia model Frac-300, Uppsala, Sweden) and a peristaltic pump (Pharmacia pump model P-1). Fractions were analyzed for the presence of antigens recognized by mAb #20 by indirect ELISA essentially as described in Chapter 1. Briefly, 10 ml of every second fraction was added to an ELISA plate well with 90 ml of distilled H_2O (dH_20) and dried at 37°C overnight. Lysates made from *T. b. rhodesiense* ViTat 1.1 PCF were used as positive control antigens diluted at 1/20 in dH_2O. Ascites fluid containing mAb #20 was diluted to 1/1000 and used as the first antibody. Alkaline phosphatase-labeled goat anti-mouse IgG/IgM (Caltag, South San Francisco, CA) was used as detecting antibody at a dilution of 1/2000. Peak fractions containing the antigens were pooled, dialyzed against dH_2O at 4°C, lyophilized to dryness and resuspended in PSP sample buffer. Proteins in peaks from six separate preparative runs were assessed for their antigen purity by analytical SDS-PAGE followed by Coomassie blue, silver staining and immunoblot analysis, and the best ones (showing the appropriate antigen with fewest contaminating proteins) were pooled for further PSP separation. Separation was performed on the selected pooled peak proteins using a 7% monomer separating solution of 75 ml with a 37 mm ID gel tube and a 4% stacking gel for each antigen. Fractions were collected as above and analyzed by indirect ELISA. Positive peak fractions for each antigen were pooled, dialyzed and lyophilized as above after assessment for purity by analytical SDS-PAGE followed by Coomassie blue and silver staining and by immunoblot analysis. Protein microsequencing was performed on the 60 kDa Coomassie blue stained band on Immobilon™ blots.

Sample preparation for analytical SDS-PAGE. Trypanosomes from various species and life cycle stages were prepared for SDS-PAGE as described in Chapter 1. Lysates were used on SDS-PAGE gels at 1 x 10^7 cell equivalents per well for stained gels and 2 x

10^6/well for immunoblots. Samples from fractions in the purification procedure were prepared by adding 10 ml of sample to 10 ml of 2 X sample buffer for SDS-PAGE.

Polyacrylamide gel electrophoresis (analytical). SDS-PAGE was performed according to Laemmli, (1970) as described in Chapter 1. Molecules were stained with Coomassie blue R-250 (BDH, Vancouver, Canada), silver stained (Merrill, 1984) or were detected by immunoblotting (see below). For protein microsequencing and amino acid microanalysis, SDS-PAGE gels were run under special conditions using recrystallized SDS as described by Matsudaira, (1987).

Immunoblots. Electrophoretic transfer of proteins from reducing SDS-PAGE gels onto Immobilon-P™ (Millipore Corporation, Bedford MA, USA) and subsequent antigen detection were performed by the procedure of Towbin *et al.* (1970) as described in Chapter 1. Ascites fluid containing mAb #20 (at a 1/1000 dilution) was used as the first antibody. Bound antibody was detected using horseradish peroxidase-conjugated goat anti-mouse IgG/IgM (Caltag, San Francisco, CA, USA) at a 1/2000 dilution. Enhanced luminol chemiluminescence reagent (Renaissance™ DuPont NEN, Boston, MA, USA) was used as the substrate for the enzyme conjugate according to the instructions of the manufacturer. For protein microsequencing and amino acid microanalysis, protein or peptide bands were located by staining the membranes with Coomassie blue R-250 (Matsudaira, 1987).

Protein Microsequencing. Coomassie blue-stained bands of PSP purified proteins from Immobilon-P™ blots were placed directly into a gas phase sequencer (Model 470A, Applied Biosystems, Foster City, Ca, USA) and sequence analysis was performed in the University of Victoria Tripartite Microanalytical Center. Sequences obtained were searched against the SWISS-PROT protein database (Altschul *et al.*, 1990).

Two-dimensional gels. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was performed on samples purified by preparative SDS-PAGE or on *T. b. rhodesiense* ViTat 1.1 PCF SDS-lysates (2 x 10^6/ml). These were applied to isoelectric focusing gels in the ISO-DALT 2-D PAGE system (Anderson and Anderson, 1978a, b) and run for 20 hours at 800 V. Ampholines used in the first dimension tube gels were a mixture of 75% Pharmalyte pH 3-10 and 25% Pharmalyte pH 5-8 (Pharmacia, Uppsala, Sweden). Gradient gels (5-15% acrylamide) were used for the second dimension. The ampholine mixture and second dimension were optimized empirically to give good dispersion and resolution of protein spots. Gels were either stained with Coomassie blue or proteins were

analyzed by immunoblotting (see below) after transfer to Immobilon™ using mAb #20 as the detecting antibody at a 1/1000 dilution and HRPO-conjugated goat anti-mouse IgM/IgG (Caltag) at a 1/2000 dilution. The immunoblots were developed using enhanced chemiluminescence™ (ECL) (Amersham, Oakville, Ontario) according to the instructions of the manufacturer.

Comparison of 2-D PAGE patterns. Coomassie blue stained 2-D gels were compared to immunoblot film surfaces by placing the film over the corresponding stained gel on a light box. Superimposition of gel spot constellations allowed easy identification of antigen. This simple procedure is accurate and reliable because the ISO-DALT 2-D PAGE gel system allows 10 gels (poured from the same batch of reagents) to be run simultaneously, thus all samples can be electrophoresed in one run. Interpretations of gel spot constellations were performed using previously published criteria (Anderson *et al.*, 1979 and Pearson *et al.*, 1983).

Immunogold electron microscopy[2]. *T. b. brucei* BSF or PCF were processed for Lowicryl K4M embedding at -20°C as previously described (Frevert, 1992). Thin sections were cut with a RMC MT-7 ultramicrotome and sequentially labeled with mAb #20 ascites (1:1 dilution) and a 1:30 dilution of protein A gold (10 nm, Amersham, Arlington Heights, IL). Photographs were taken with a Zeiss EM 910 electron microscope.

Heat shock. PCF of *T. b. brucei* 427-01, *T. b. rhodesiense* ViTat 1.1 and *T. congolense* 45/1 were tested for expression of antigen #20 before and after exposure to 37°C heat shock *in vitro*. PCF (2×10^8) growing at 26°C in procyclic culture medium were pelleted (800g for 5 min.), resuspended at 1×10^7/ml in fresh procyclic culture medium, and incubated at either 26°C or 37°C for 1 hour. Trypanosomes were then immediately pelleted, washed once in PSG and resuspended to 5×10^5 per ml in SDS-PAGE sample buffer (SDS lysates). SDS lysates were then electrophoresed on 10% SDS-PAGE gels (1×10^6 trypanosome equivalents per lane) and immunoblotted. Relative differences between antigen #20 immunoreactive bands on autoradiograph film were compared visually.

Gelatin-agarose chromatography. Heat shock proteins from the 70 and 90 kDa families can be purified using gelatin-agarose beads (Nandan *et al.*, 1994). Proteins from *T. b.*

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[2] This work was performed by Ute Frevert, Department of Pathology, New York University, New York, NY, USA.

rhodesiense ViTat 1.1 PCF were solubilized in lysis buffer by resuspending 2 x 10^8 trypanosomes per ml in 20 mM tris-HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100 containing a cocktail of protease inhibitors. This cocktail consisted of 10 mM leupeptin, E64, and antipain, 0.5 mM PMSF, 0.5 M EDTA and 5 mg/ml TLCK. The lysate was centrifuged at 10,000g for 20 minutes at 4°C to remove insoluble materials. Supernatants were incubated with 1.0 ml of gelatin-agarose beads (Sigma Chemical Co., St. Louis, MO) by mixing for 2 hours at 4°C. The beads were collected by centrifugation and washed 3 x with lysis buffer adjusted to 0.5 M NaCl, and once with 5 mM HEPES, pH 7.5. Bound proteins were released by incubating the gelatin-agarose beads with 3 mM ATP, 1 mM magnesium chloride in 5 mM HEPES, pH 7.5 at room temperature. Released proteins and breakthrough proteins were lyophilized and analyzed by SDS-PAGE on 10% gels and immunoblotting as previously described.

cDNA Library screening: A unidirectional cDNA expression library was constructed using the Superscript™ Lambda system (Gibco BRL) by Dr. Isabel Roditi, Berne, Switzerland. *T. b. brucei* GARP 16 (Hehl *et al.*, 1995) mRNA was reversed transcribed into the λgt22A cloning vector (Han and Rutter, 1987). The library was kindly sent to us at UVIC by Dr. Roditi. For the primary library screen, 10,000 pfu/plate were used. The library host strain, *E. coli* Y1090 was grown at 37°C for 8 hours (shaking at 250 rpm) in 5 ml Luria-Bertani medium pH 7.0 (LB) containing 1% (w/v) trypticase peptone (Becton Dickinson, Cockeysville, MD, USA), 0.5% yeast extract (BDH), and 1% NaCl (Sambrook, *et al.*, 1989) and supplemented with 0.2% maltose and 0.1% $MgSO_4$. One µl of a 1/380 dilution of the library stock (titer 3.8 x 10^9 pfu/ml) was added to 100 µl of fresh Y1090 culture and incubated at 37°C. After 20 min, 8 ml of molten (48°C) H top agarose (1% trypticase peptone, 0.5% NaCl, and 0.75% agarose (w/v))was added, the mixture poured onto H agar plates (1% trypticase peptone, 0.5% NaCl, 1.5% bacto-agar; DIFCO Laboratories, Detroit, MI, USA) and the plates incubated at 42°C for 3.5 to 5 hours (until plaques were visible). At this point nitrocellulose filters (Schleicher and Schuell, Keene, NH, USA) soaked in 100 mM isopropyl-B-thiogalactopyranoside (IPTG) (Stratagene, La Jolla, CA, USA) and air dried were layered onto the plates and incubated at 37°C overnight (10 to 12 hours). Duplicate lifts were made. Before the filters were removed, several marks on the filter and agar were made with a needle containing india ink. The filters were then removed and placed in PBS (4°C). For secondary plaque screening, positive plaques were picked and stored in SM with $CHCl_3$ at 4°C. The phage suspension was plated by diluting 1:100 in fresh overnight Y1090 culture by adding 1 µl of diluted phage to 100 µl of Y1090.

Immunoscreening of the library. Filters were screened according to Amersham's tech tip #133 (R932148 for use with ECL™). Briefly, filters were blocked in PBS containing 10% milk powder for 10 minutes and then washed in 200 ml of PBST [PBS plus 0.1%(v/v) Tween-20 (Sigma)] for 3 x 30 seconds. Filters were incubated in ascites fluids containing mAb #20 (1/2000) diluted in PBS, for 30 minutes, washed 2 x 10 minutes in PBST and incubated in horseradish peroxidase-conjugated goat anti-mouse IgG/IgM diluted in PBS at 1/5000 for 30 minutes. Filters were washed 2 x 10 minutes in PBST and processed for chemiluminescence detection. All steps were performed at room temperature. ECL detection was performed according to the manufacturer's instructions (see immunoblotting ECL). Positive plaques were picked using a sterile Pasteur pipette, placed into 1 ml SM (Sambrook *et al.*, 1989) and left overnight at 4°C. A secondary screening was performed with positive plaques using 1 µl phage suspension in 100 µl of fresh Y1090 culture and 1 µl of a 1:100 dilution of phage suspension. Library filters and immunoscreening were performed as above and a well isolated plaque was then picked into 1 ml of SM as a plaque purified suspension of a positive phage.

High titer phage stocks. High titer λgt22A phage stocks were made essentially by the method of O'Toole and Foster (1988). Briefly, using a fresh plate of purified plaques as the source, a series of tubes containing 100 µl fresh overnight Y1090 culture were inoculated with 1, 2, 3 or 4 picked plaques and mixed on a vortex mixer. The tubes were incubated at 37°C for 20 minutes, 2.5 ml of molten H top agarose was added and the mixture poured onto H agar plates. The plates were incubated base downwards at 37°C and after 4 hours were monitored every 30 minutes for confluent lysis. The plates with the number of plaques that gave the desired amount of lysis (i.e. just confluent) by 6 hours were then used as the source of phage and harvested. Phage were harvested by adding 3 ml of SM to each plate, the top agarose was scraped off and placed into a polypropylene tube. One hundred microliters of $CHCl_3$ was added and the mixture vortexed to release phage particles. The tubes were then centrifuged at 4000g for 10 minutes at 4°C and the supernatant used as the high titer stock. The titer of the stocks was determined (Sambrook *et al.*, 1989).

Preparation of phage. Phage particles were prepared by the methods of O'Toole and Foster (1988) with some modifications. Briefly, using 0.5 ml of fresh overnight culture of *E. coli* Y1090, 4 flasks containing 300 ml of complete medium consisting of 1% casamino acids, 1 x M9 salts (Sambrook *et al.*, 1989), 2% glucose, 2% maltose, 5 mM $MgCl_2$ and 0.001

M $CaCl_2$ were inoculated and 100 µl of phage stock (1 x 10^9 pfu for a MOI of 10) were added. The flasks were incubated at 37°C for 5-9 hrs until lysis occurred. When lysis occurred, 5 ml of chloroform was added to each flask and the culture was shaken well and left to stand for 15 minutes. Debris was removed by centrifugation at 10,000g for 10 minutes at 4°C and the supernatant was decanted into an Erlenmeyer flask. NaCl was added to a final concentration of 0.5 M and polyethylene glycol 8000 to 10% (w/v). The polyethylene glycol was dissolved by shaking the flask well and then leaving to stand overnight at 4°C.

The phage particles were then pelleted by centrifuging at 4000g for 30 minutes, decanting the supernatant and centrifuging for 5 minutes at 4000g to compact the pellet. The phage pellet was resuspended in 7.5 ml of buffer containing 0.25 M NaCl, 10 mM Tris pH 7.5, and 5 mM $MgCl_2$. The phage particles were purified on a CsCl block gradient consisting of CsCl at density 1.7 on the bottom, 1.55 in the middle and 1.3 on the top layer in a Beckman polyallomer centrifuge tube (14 x 89 mm). The phage was layered on top of the CsCl 1.3 density layer and the tube centrifuged at 77,000g for 90 minutes at 4°C. The phage band was removed with a syringe (18 gauge needle) from between the 1.55 and 1.3 CsCl density layers and stored at 4°C.

Preparation of phage DNA. The phage DNA was released from the phage capsids by adding 1/10 volume 0.2 M EDTA in 2 M Tris-HCl (pH 8.5) and an equal volume of formamide to the CsCl band and mixing on a vortex mixer (O'Toole and Foster, 1988). The mixture was incubated overnight at room temperature. The phage DNA was then precipitated by adding 1 ml of 95% ethanol (-20°C) (Sambrook *et al.*, 1989).

DNA cloning. Phage clone DNA containing positive cDNA inserts was digested with *Not* I and *Sal* I restriction enzymes (New England Biolabs, Beverly, MA) at 37°C overnight and heated to 65°C for 5 minutes prior to being electrophoresed on an agarose gel (1%). Phage insert cDNA was purified from the agarose using Promega Wizard™ prep kit (Promega, Madison WI) and precipitated. The vector Bluescript SK+ was also digested, electrophoresed and purified as for phage DNA. The phage cDNA insert and vector DNA were ligated overnight at 16°C with T4 DNA ligase (New England Biolabs). The *E. coli* strains DH5α and XL-1 Blue were used for transformation experiments according to the method of Hanahan (1983) and standard gene cloning techniques were applied as described by Sambrook *et al.* (1989). Several subclones of phage cDNA inserts were made in order to gain more sequence information using various restriction enzymes.

DNA sequencing[3]: Single-stranded DNA was prepared using a modification of the protocol by Vieira and Messing (1987). Double-stranded DNA was prepared using Nucleobond™ AX cartridges (Machery-Nagel, Düren, Germany). Sequencing of both single-stranded and double-stranded sequencing was performed using the Sequenase™ Version 2.0 DNA sequencing kits (United States Biochemical, Cleveland, OH, USA) with either universal sequencing primers or internal primers designed from DNA sequences. The CircumVent™ Thermal Cycle Dideoxy Sequencing Kit (NEB, Beverly, MA) was also used according to the manufacturer's instructions. In some cases automated DNA sequencing using an Applied Biosystems DNA Sequencer Model 373 (Applied Biosystems Inc., Foster City, CA, USA) was performed with fluorescent dye primer sequencing reactions utilizing the fmol sequencing kit (Promega) according to the manufacturers instructions.

Polymerase chain reaction techniques[3]: *T. b. brucei* GARP16 PCF poly $(A)^+$ RNA was isolated directly from trypanosome lysates by the procedure described in Sambrook *et al.* (1989) except poly-U-Sepharose (Pharmacia) was used. Poly $(A)^+$ RNA (0.4 μg) was reversed transcribed using an oligo(dT) $_{16}$ primer and Superscriptase II™ (GIBCO/BRL) reverse transcriptase (42°C, 40 min; 99°C, 5 min; 4°C, 5 min). To amplify the upstream noncoding region and the remaining 5' cDNA sequence of antigen #20, a specific 5' (forward) primer [5'-CAGTTTCTGTACTATATTG-3'] designed from the mini-exon (Walder, et al., 1986) and a 3' internal (reverse) primer designed from the expression library clone cDNA sequence [5'-CAGACACAAGCACGAAC-3'] were used in PCR. The following PCR protocol was performed using a Thermolyne Temp-Tronic™ Thermal Cycler: 35°C annealing, 74°C extension, 4 cycles; 40°C annealing, 74°C extension, 30 cycles. In all amplifications "hot start" PCR (Chou *et al.*, 1992) was performed by adding 2.5 units of Taq DNA polymerase (Pharmacia) after the initial denaturation step.

Cloning of the PCR product[3]: The amplified PCR product was recovered from 1% agarose using Wizard™ PCR Preps (Promega, Madison, WI) and cloned into the pGEM-T™ vector (Promega) or into Litmus™ cloning vectors (New England Biolabs) to allow sequencing in both directions. DNA sequencing was performed on positive clones as described above.

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[3] This work was performed in part by Caroline Stebeck and R. Beecroft, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

Purification of genomic DNA. Genomic DNA from *T. b. rhodesiense* ViTat 1.1 and *T. b. brucei* GARP 16 PCF was purified essentially according to the procedure described in Sambrook *et al.*, (1989) with some modifications. Briefly, trypanosomes were harvested by centrifugation at 800g, 15 min., washed in PSG and resuspended in lysis buffer (0.2 M Tris-HCl: pH 8.0, 0.2 M EDTA) at 2 x 10^8 trypanosomes/ml. 10% SDS was added to a final concentration of 0.5% and the tube inverted several times to mix. The tube was incubated at 37°C for 10 minutes, RNAse (DNAse free Bovine pancreatic RNAse from Quiagen, Chatsworth, CA) was added to 100 mg/ml and incubated for 1-2 hours at 37°C. Proteinase K (Gibco BRL) at 10 mg/ml in lysis buffer pretreated by incubation at 56°C for 2 hours was added to the DNA preparation to a final concentration of 1.0 mg/ml and the tube was incubated overnight at 56°C with periodic mixing. The DNA preparation was then extracted with phenol: chloroform: isoamyl alcohol (25:24:1) twice and the aqueous phase was extracted with chloroform to remove any remaining phenol. The DNA was dialyzed overnight against 4 L dialysis buffer (10 mM Tris-HCl (pH 8.0), 100 mM NaCl and 1 mM EDTA), quantitated, precipitated and resuspended in TE buffer (pH 7.5) (10 mM Tris-HCl, 1 mM EDTA) to the desired concentration.

Southern and Northern blot analyses[3]. For Southern blot analysis, 8 µg of *T. b. rhodesiense* ViTat 1.1 PCF genomic DNA were digested overnight at 37°C with 100 units of each of *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Nde*I, *Sau*3AI and *Xho*I (New England Biolabs, Beverly, MA, USA), followed by a further 4 hour incubation at 37°C with an additional 100 units of enzyme to ensure complete digestion. The digests were electrophoresed for 11 hours at 15 V on a 0.6% agarose gel, using as size standards a lambda DNA-*Hind*III digest (New England Biolabs). For Northern blot analysis, 0.9 µg of *T. b. rhodesiense* ViTat 1.1 PCF poly(A)+ RNA was electrophoresed for 15 hours at 20 V on a 1% formaldehyde gel using established protocols (Sambrook *et al.*, 1989). A 0.24-9.5 Kb RNA ladder (Gibco BRL, Burlington, ON, Canada) was run simultaneously in order to accurately establish transcript size. The genomic DNA restriction digests and the poly(A)+ RNA were transferred via vacuum blotting (model 785, BioRad, Hercules, CA, USA) to Zeta-Probe® membrane (BioRad, Hercules, CA, USA) according to the manufacturer's instructions with slight modifications. Briefly, the agarose gel for Southern blot analysis was treated with 0.25 N HCl (1 x 15 min), 1.5 M NaCl/0.5 M NaOH (2 x 15 min) and 1.5 M NaCl/0.5 M Tris-HCl pH 7.5 (1 x 30 min) prior to transfer in 0.5 M NaOH/0.6 M NaCl. One microgram of a 500 base pair fragment of the 20-4 cDNA (*Sal* I and *Hind* III digested) was labeled with [α-^{32}P]dATP (specific activity 3000 Ci/mmol) using the Multiprime DNA labeling system (Amersham, Oakville, ON, Canada) according

to the manufacturer's instructions. The Southern and Northern blots were prehybridized 5 minutes at 50°C in 0.25 M Na_2HPO_4, 7% SDS and hybridized overnight at 50°C with mild agitation in the same buffer with the labeled homologous probe (1.3 $x10^{-6}$ c.p.m. μl^{-1}; specific radioactivity 1.8 x 10^9 c.p.m. μg^{-1}). The membranes were washed 2 times for 45 minutes each at 50°C with 200 ml of 20 mM Na_2HPO_4 pH 7.2, 5% SDS, followed by 2 washes for 20 minutes each at 50°C with 200 ml of 20 mM Na_2HPO_4 pH 7.2, 1% SDS. The membranes were then air-dried and autoradiographed.

Bacteriophage P1 library screening[4]. A bacteriophage P1 library high density filter (SM7 No 8) containing DNA from *L. donovani* strain 2903 and *T. brucei* strain 927 (Turner *et al.*, 1990) was kindly provided by Drs. Sara Melville and Vanessa Leech (Cambridge University, Department of Pathology, Laboratory for Parasite Genome Analysis, Cambridge, England). The library was constructed in the pAD10SacBII cloning vector as previously described (Pierce *et al.*, 1992). Briefly, *L. donovani* and *T. brucei* genomic DNA was partially digested with *Sau*3AI to generate inserts of approximately 60-70 kb which were subsequently cloned into the *Bam*HI site of the cosmid vector. The library was grown in 96-well microtitre plates and the colonies in each well were subsequently transferred to a high density filter (Hybond N, Amersham, England). The high density filter was probed using the α-^{32}P-labeled Hsp60 cDNA fragment generated for the Southern and Northern blot analyses (described above). Prehybridization, hybridization, filter washing and autoradiography were performed exactly as described for the Southern and Northern blot analyses.

Processing of bacteriophage P1 clones[5]. P1 clones demonstrating reactivity with the homologous probe were selected and DNA was subsequently isolated from these clones using conditions suggested by the Laboratory for Parasitic Genome Analysis (Birnboim and Doly, 1979). The DNA was denatured (95°C, 3 min) and subsequently dotted onto Zeta-Probe® membrane (BioRad) according to the manufacturer's instructions. Dot blots were rescreened to verify positivity using the homologous Hsp60 probe under conditions exactly as outlined for Southern and Northern blot analyses. In order to decrease the insert size of the P1 clone in preparation for obtaining flanking regions of the Hsp60 gene, DNA

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[4] This work was performed by Caroline Stebeck and R. Beecroft, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

[5] This work was performed by Caroline Stebeck and Michael Bridge, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

was isolated from one positive P1 clone from the second screen, digested with *Eco*RI and *Nde*I (New England Biolabs) and separated on a 0.6% agarose (Promega) gel. The DNA was transferred to Zeta-Probe® membrane (Bio-Rad) according to the manufacturer's instructions, probed with the homologous Hsp60 DNA as described for the Southern and Northern blot analyses and one hybridizing DNA fragment was selected.

Database searches, deduced amino acid sequence characterization and sequence alignments. Database searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al*, 1990) and either a non-redundant DNA database (includes GenBank, GenBank updates, EMBL, and EMBL updates) or a non-redundant protein database (includes SWISS-PROT, PIR, GenPept, and GenPept updates). The nucleotide sequences were translated and analyzed using the SeqApp™ software (Gilbert, 1992). The molecular mass and isoelectric point (pI) of the translated product were calculated using the MacProMass™ v1.05 software (Beckman Research Institute, City of Hope, Duarte, CA). The net charge was calculated manually assuming physiological pH. Alignment of the amino acid sequences (of antigen #20 with heat shock proteins and chaperones from various organisms) was carried out using the Clustal V™ multiple alignment program (Higgins *et al*, 1992). The percentage of positional identity between sequences was calculated from the number of identical residues between aligned sequences; insertions and deletions were not counted.

Results

A. Purification of antigen recognized by mAb #20

Solubilization of Antigen #20. A total of 1 x 10^{11} *T. b. rhodesiense* ViTat 1.1 PCF were thawed at 37°C and lysed by resuspending at 1 x 10^9/ml in ice cold HPLC grade dH_2O containing protease inhibitors and sonicating on ice. To obtain the soluble cellular fraction, the mixture was centrifuged at 4000g and then at 105,000g as described above. The supernatant and pellet were assayed for the presence of the antigen recognized by mAb #20 (Ag #20) using indirect ELISA (data not shown). Antigen #20 was found in the 105,000g supernatant and not in the pellet. A total of 100 ml of 105,000g supernatant was obtained.

Ammonium sulfate precipitation. Enrichment of Ag #20 was performed by ammonium sulfate precipitation of the 105,000g supernatant. Ammonium sulfate was added to 50% saturation at 4°C. The pellet and supernatant were analyzed for total protein and antigen #20 content by SDS-PAGE followed by silver staining and immunoblot analysis (data not shown). The 50% pellet was found to contain Ag #20 and approximately 80% of the total protein. Therefore, approximately 20% of the total protein remained soluble and was removed from the Ag #20 sample. The ammonium sulfate was dialysed from the pellet using dH_2O. The resulting protein sample was lyophilized to dryness and resuspended in 12.5 ml of sample buffer for preparative SDS-PAGE.

Preparative SDS-PAGE. The protein sample containing Ag #20 from above was subjected to preparative SDS-PAGE. The sample was split into 4 x 3 ml samples and each was electrophoresed on an 8% gel in four separate runs. This was done to reduce the total amount of protein added to the electrophoresis unit and to improve the separation of proteins. One hundred and fifty fractions of 3 ml each were collected during each run and analyzed for Ag #20 by ELISA. A similar peak containing Ag #20 was obtained from each of the four runs corresponding to an O.D. 405 nm of greater than 0.5 on ELISA. The peak profile from run 1 is shown in Figure 8. Data from the other runs are not shown. Peak fractions from each run were pooled, dialyzed against dH_2O and lyophilized.

Analysis of the total protein and Ag #20 content of each peak was performed by analytical SDS-PAGE with Coomassie blue staining, silver staining and immunoblotting using mAb #20. Figure 9 shows the Coomassie blue stained gel from each run. A heavily stained

band at approximately 60 kDa can be seen. Figure 10 shows an immunoblot corresponding to the Coomassie blue-stained gel of each run. A band at 60 kDa corresponds to the large band on the gel. Close inspection of the gels and immunoblots revealed that the band containing Ag #20 also contained other proteins of a similar apparent molecular mass. In order to separate Ag #20 from these proteins a second preparative SDS-PAGE run was performed.

In order to reduce the total protein load and therefore increase resolution, two separate secondary runs were performed (runs 2-1 and 2-2). For the secondary runs a 7% separating acrylamide gel was used and 150 x 3 ml fractions were collected as described. Analysis of the fractions for Ag #20 was done by ELISA as for the primary runs. Figure 11 shows the peak profiles of the secondary runs. A large peak containing Ag #20 was seen for each run. Peak fractions were analyzed for total protein content by analytical SDS-PAGE with silver stain (data not shown) and immunoblot analysis before pooling fractions. Figure 12 shows the immunoblot analysis of peak fractions from run 2-1. Fractions #58-74 for run 2-1 and #50-74 for run 2-2 were determined to be the cleanest. These fractions were pooled, dialyzed against dH_2O and lyophilized to dryness.

2-D gel electrophoresis. To determine if contaminating proteins were present in the sample containing Ag #20, 2-D gel electrophoresis was performed. Coomassie blue-stained gels of Ag #20 were compared to an immunoblot of Ag #20 to determine the purity of the sample. Figure 13 shows the Coomassie blue stained gel pattern of Ag #20 in panel A with the immunoblot pattern in panel B. It was determined that at least one contaminating protein was present with Ag #20. This protein migrated below the 60 kDa protein and was not detected by mAb #20 on the immunoblot.

Protein microsequencing. Gas phase microsequencing of the N-terminal amino acids of Ag #20 was performed by electrophoresing the sample from preparative SDS-PAGE onto Immobilon-P™. Sequencing of the appropriate Coomassie blue stained band was performed by Sandy Kielland in the University of Victoria Tripartite Microanalytical Centre using a gas-phase sequencer (model 470A, Applied Biosystems, Foster City, CA). A total of 11 amino acid residues were obtained and were found to match the N-terminal sequences of *T. brucei* and *T. cruzi* Hsp60 and to be similar to the *E. coli* GroEL N-terminus (Figure 14). The sequence of Ag #20 matched the predicted amino acid sequence from *T. brucei* and *T. cruzi* Hsp60 starting after the signal sequence that directs the protein to the mitochondrion (Bringaud *et al.*, 1995; Giambiagi-de Marval *et al.*, 1993). The N-terminal

sequence of Ag #20 was 100% identical to the *T. brucei* sequence (Figure 14) and was 99% identical and 100% homologous to the *T. cruzi* Hsp60 N-terminus. It was 18% identical and 45% homologous to the GroEL protein of *E. coli.* (Hemmingsen *et al.*, 1988).

B. Further characterization of mAb #20 and its specific antigen

Life-cycle stage and species determination. Monoclonal antibody #20 recognized an antigen present in all trypanosome species and life cycle stages examined as reported in Chapter 1. However, to further evaluate its potential as a diagnostic molecule, the presence of Ag #20 in different species and life cycle stages was examined further. A time course transformation from BSF to PCF was performed with *T. congolense* IL-3000 parasites in immunoblotting experiments. Each lane on the SDS-PAGE gels was loaded with equal amounts of lysate in order to determine relative amounts of protein expressed at each time point. The results are shown in Figure 15. The antigen recognized by mAb #20 appeared at all time points.

In another set of experiments, *T. congolense* IL-3000 parasites were harvested at specific life-cycle stages, lysates were prepared, electrophoresed on SDS-PAGE gels and immunoblotted with mAb #20. Again, the SDS-PAGE lanes were loaded with equal amounts of lysates. The results are shown in Figure 16. All life cycle stages except metacyclics expressed a similar amount of antigen #20.

The presence of antigen #20 was also tested in American trypanosomes (*T. cruzi* Y strain). Figure 17 shows an immunoblot of lysates prepared from *T. cruzi* trypomastigotes and epimastigotes. The antigen was detected in both life cycle stages and demonstrated that mAb #20 cross reacts with a *T. cruzi* antigen of 60 kDa.

Subcellular localization. The subcellular localization of antigen recognized by mAb #20 was determined by immunogold electron microscopy (Figure 18) (Ute Frevert, NYU). *T. b. brucei* 427.01 BSF parasites were used in this procedure and it can be seen that mAb #20 localized to the mitochondrion of the parasite.

Heat-shock induction and gelatin-binding characteristics. Experiments were performed with PCF of *T. brucei* and *T. congolense* to determine if the expression of Ag #20 increased upon heat shock in culture. Figure 19 shows an immunoblot of PCF lysates

comparing Ag #20 expression in heat shocked and non-heat shocked cultures. The same number of parasites was added to each lane in the gel. As can be seen, the expression of Ag #20 increased markedly in PCF subjected to 37°C heat shock (compare lanes A and B, C and D, E and F).

To test whether or not Ag #20 could bind to denatured proteins, a chromatography experiment using gelatin-agarose was performed. Gelatin-agarose is known to bind chaperonins of the HSP70 and 90 classes (Nandan *et al.*, 1995). Lysates of *T. b. rhodesiense* ViTat 1.1 PCF were passed over a gelatin-agarose column. Both the breakthrough (nonbinding) and the eluted (binding) fractions were tested for the presence of Ag #20 by immunoblotting (Figure 20). Antigen #20 was present in the breakthrough fraction and did not bind to the column, indicating that it did not bind to the gelatin-agarose.

C. Cloning and nucleotide sequencing of the cDNA encoding antigen #20 and analysis of the encoded protein.

Library screening. A *T. b. brucei* GARP 16 BSF λgt22A cDNA expression library was screened with mAb #20. Seven positive clones were identified in the primary screen (data not shown). The positive clones were picked and plated for secondary screening. Upon secondary screening, one clone remained positive. This was plaque purified, amplified and the phage prepared. Phage DNA was then purified, digested with *Not I/ Sal* I to release the cDNA insert and electrophoresed on a 1% agarose gel (Figure 21). The clone 20-4 cDNA insert was approximately 1450 base pairs in length. This insert was excised from the agarose gel, purified using the Wizard PCR preps kit and cloned into Bluescript SK^+ (Stratagene, La Jolla, CA) via the *Not I/ Sal* I restriction sites to prepare for double-stranded DNA sequencing. Figure 22 shows the cDNA sequence of clone 20-4. The cDNA is 1411 bp in length with a 387 amino acid open reading frame spanning from bp 1 to 1161. The *Sac*II sites at 85 and 1074 bp and the *Hind*III site at 596 bp were used to subclone and sequence the cDNA and are underlined. A data base search was performed using the BLAST algorithm (Altschul *et al.*, 1990). The cDNA sequence was found to match the sequence of a *T. b. brucei* Hsp60 mitochondrial chaperone (Genbank accession number L43797) and a *T. cruzi* mitochondrial Hsp60 (Genbank accession number 108791) and a variety of other heat shock proteins. This sequence corresponded to the C-terminal 387 amino acids of the mitochondrial Hsp60 cDNA. The remaining 175 residues as well as the upstream flanking region was not included in the cDNA.

RNA-PCR was used to obtain the 5' portion and upstream flanking regions of the Hsp60 gene. A 600 bp DNA fragment was obtained, cloned and sequenced. This included the 5' mini-exon, upstream flanking region and 5' portion of the Hsp60 gene. Figure 23 shows the entire coding region of the Hsp60 cDNA. The cDNA was found to be 1686 bp long with an open reading frame encoding the 562 amino acid mitochondrial chaperone. Figure 24 shows an alignment of the predicted amino acid sequence of antigen #20 with *T. b. brucei* Hsp60 (Bringaud *et al.*, 1995), *T. cruzi* Hsp60 (Giambiagi-de Marval *et al.*, 1993) and *E. coli* GroEL (Hemmingsen *et al.*, 1988). Antigen #20 was found to be 99.3% identical to the *T. b. brucei* Hsp60, 85.9% to *T. cruzi* Hsp60 and 49.8% to *E. coli* GroEL. The predicted molecular mass of antigen #20 Hsp60 was calculated to be 59.5 kDa with a pI of 5.34.

Southern and Northern blot analyses. These were performed using a fragment of the expression library cDNA clone as a probe. The probe used should hybridize with the gene at 525 bp to 1120 bp and is approximately 600 bp in length. The Northern blot shows a mRNA transcript of approximately 2.1 kilobases (Figure 25, Panel B). The Southern blot shows that the Hsp60 genes are present in more than one copy, probably three or more (Figure 25, Panel A). The *BamH*I digest (lane 7) gives one large band at 10,000+ bp and since there are no *BamH*I sites within the gene can only tell us that the probe hybridizes with a large fragment of DNA that could hold as many as five copies of the Hsp60 gene. This is also true for *Kpn*I (Lane 4). There are 10 *Sau3A*I restriction sites within the Hsp60 gene most of which produce fragments smaller than 150 bp. One band that overlaps with the probe spans from 764 bp to 1323 bp and is 559 bp long. This is most likely the band appearing in lane 2. The 2100 bp band in lane 1 produced from the *Xho*I digest is most likely due to digestion at the site within the Hsp60 gene at 1447 bp and is the same size of the mRNA transcript. This may also indicate that more than one gene exists because digestion of two or more genes would produce this band. The three bands produced by *EcoR*I digestion in lane 6 and by *Nde*I digestion in lane 3 indicate that there are two or more copies of the Hsp60 gene present. In both cases, the largest band could be a result of incomplete digestion. It is more likely that the digestion was complete and there are three or more copies of the Hsp60 gene present. Each band is large enough to contain more than one copy of the gene so three copies is a minimum estimate. The *Hind*III digest produced four bands (lane 5) and one *Hind*III restriction site at 1120 bp exists within the Hsp60 gene. Assuming the digestion was complete, at least four copies of the gene would be present within the *T. brucei* genome.

A bacteriophage P1 library screen was performed in order to obtain the entire Hsp60 gene with flanking regions for use in future gene knockout experiments (data not shown). Five clones that hybridized with the probe were selected for further study. DNA isolated from each was rescreened and one positive clone was selected. In order to reduce the size of the fragment carrying the Hsp60 gene, the DNA from this clone was isolated and digested with *Eco*RI and *Nde*I. When the DNA was reprobed by Southern blotting with the original Hsp60 probe one hybridizing fragment of 6,500 bp for the *Eco*RI digest and two at 4,000 and 6,500 bp for the NdeI digest were identified.

At this point the work for this thesis was considered completed as it is beyond the scope of the present project to derive knockout mutants.

Figure 8

Enzyme-linked immunosorbent assay (ELISA) of fractions from T. *b. rhodesiense* ViTat 1.1 PCF after preparative SDS-PAGE. Run 1 using mAb #20 as the first antibody.

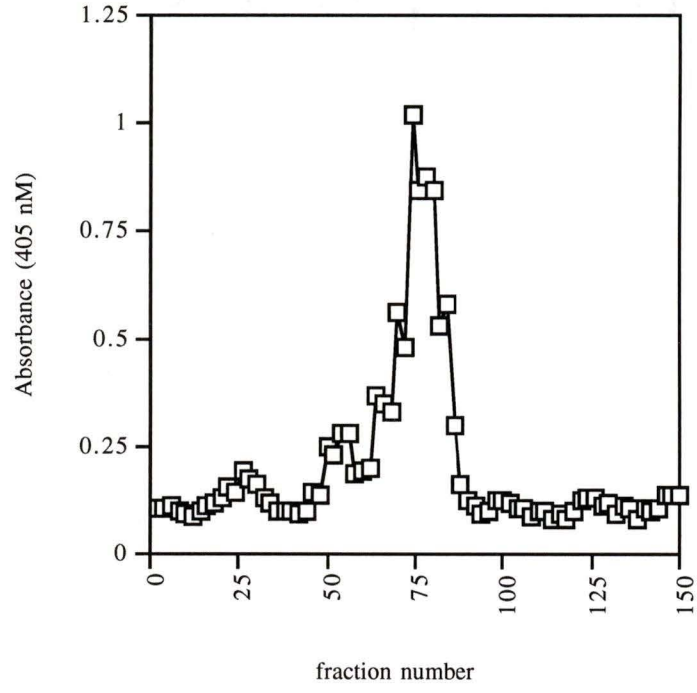

Figure 9

Coomassie blue stained SDS-PAGE gel of peaks from preparative SDS-PAGE containing antigen #20. Lane A, Run 1 peak; Lane B, Run 2 peak; Lane C, Run 3 peak; Lane D, Run 4 peak.

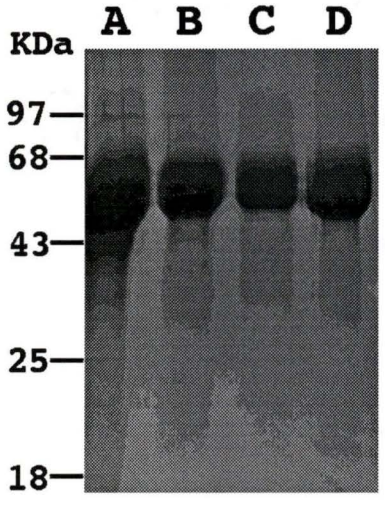

Figure 10

Immunoblot of primary preparative SDS-PAGE peaks using mAb #20. Lane A, Run 1; Lane B, Run 2; Lane C, Run 3; Lane D, Run 4.

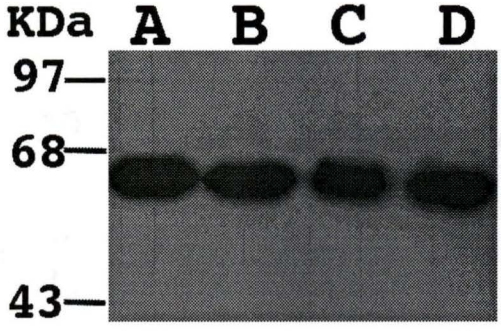

Figure 11

Enzyme-linked immunosorbent assay (ELISA) of fractions after a second preparative SDS-PAGE run. Monoclonal antibody #20 was used as the first antibody.

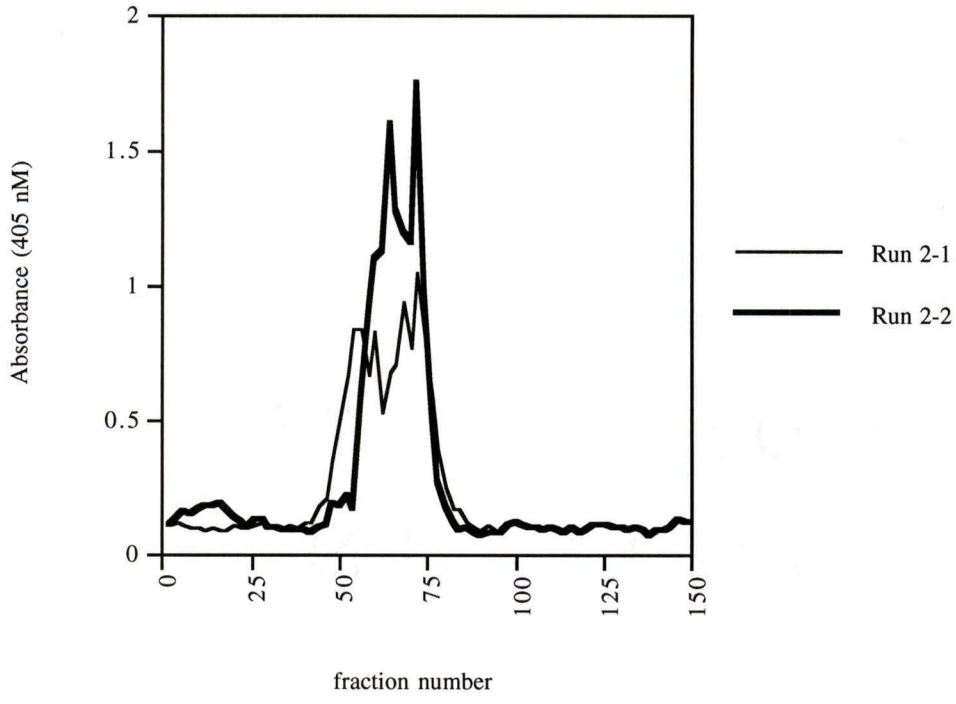

Figure 12

Immunoblot of fractions after a second preparative SDS-PAGE run. Fractions from run 2-1 were tested using mAb #20. Lanes A-Q are from fractions 58-74 (run 2-1) in Figure 11.

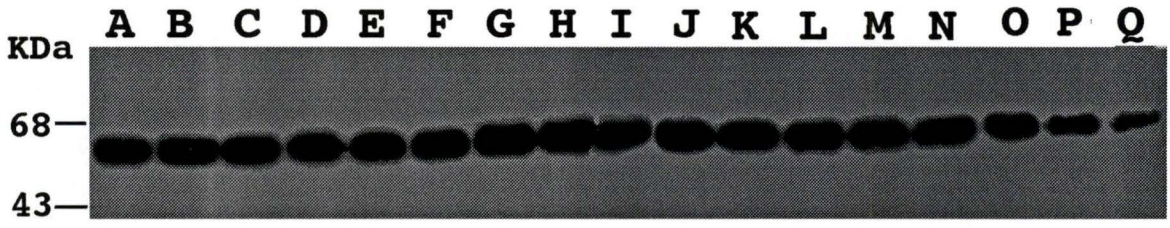

Figure 13

2-D gel analysis of Ag #20 after preparative SDS-PAGE purification. Panel A, Coomassie stained 2-D gel of Ag #20; Panel B, Corresponding immunoblot using mAb #20. Arrow in panel A shows Ag #20 pattern.

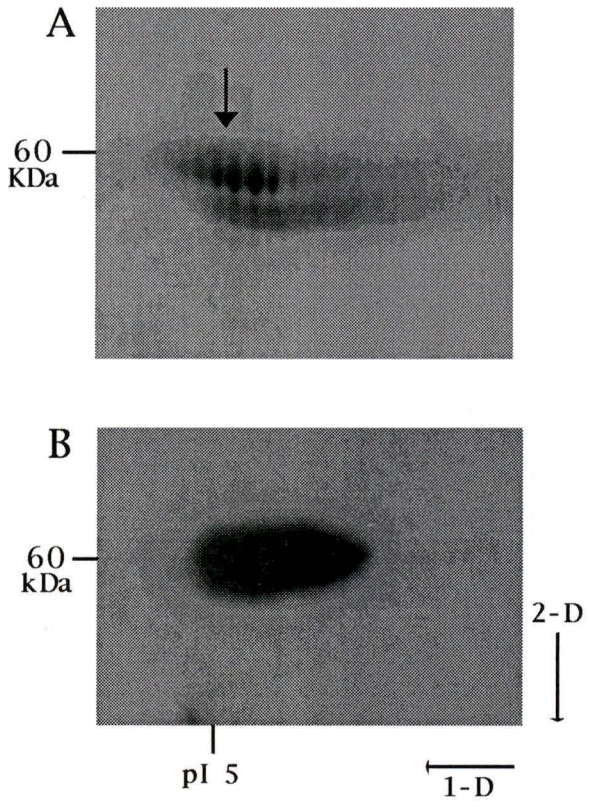

Figure 14

N-terminal sequence of Ag #20 and alignment with *T. brucei* Hsp60, *T. cruzi* Hsp60 and GroEL N-terminal sequences. Sequence A, Antigen #20; sequence B, *T. brucei* Hsp60 sequence C, *T. cruzi* Hsp60; sequence D, *E. coli* GroEL.

(A)	-	-	-	-	-	-	-	-	-	-	A	K	D	I	R	F	G	T	E	A	R	-	-
(B)	M	F	R	C	V	V	R	F	G	A	K	D	I	R	F	G	T	E	A	R	Q	S	
(C)	M	F	R	S	A	A	R	F	A	G	K	E	I	R	F	G	T	E	A	R	Q	S	
(D)	M	-	-	-	-	-	-	-	A	A	K	D	V	K	F	G	N	D	A	R	V	K	

Figure 15

Detection of antigen in immunoblots of *T. congolense* IL-3000 parasites during transformation from BSF to PCF *in vitro* . Time points represent hours from start of differentiation. Lane A, 0; Lane B, 2; Lane C, 4; Lane D, 8; Lane E, 12; Lane F, 24; Lane G, 48; Lane H, 72; Lane I, 96 hours. Monoclonal antibody #20 was used as the first antibody throughout.

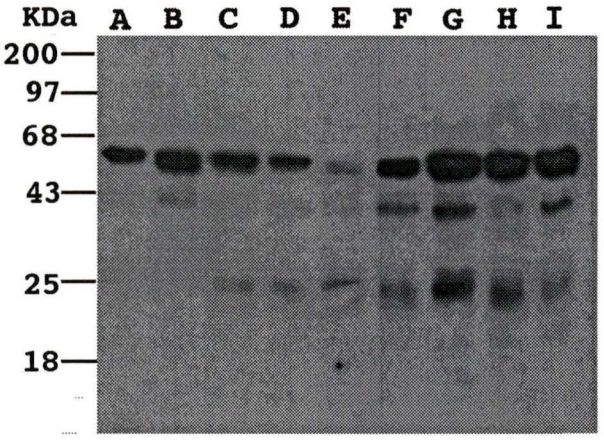

Figure 16

Detection of antigen #20 by immunoblot of various life cycle stages of *T. congolense* IL-3000. Lane A, PCF; Lane B, epimastigotes; Lane C, metacyclics; Lane D, BSF. Monoclonal antibody #20 was the primary antibody.

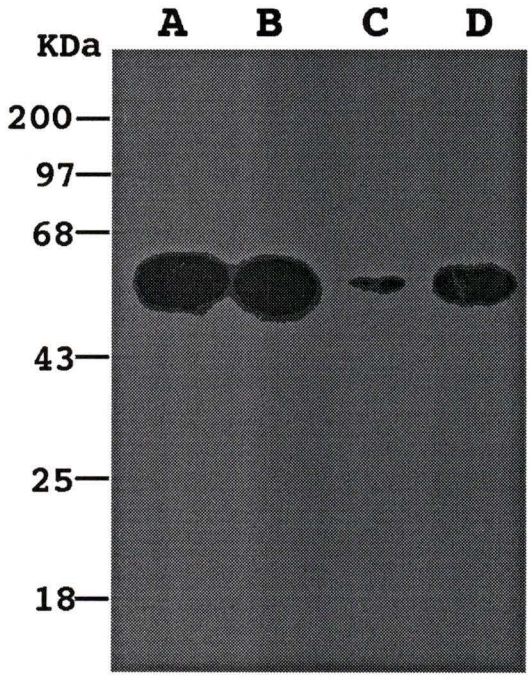

Figure 17

Detection of antigen #20 by immunoblotting of various life cycle stages of *Trypanosoma cruzi* parasites. Lane A, *T. b. rhodesiense* ViTat 1.1 PCF; Lane B, *T. cruzi* Y strain trypomastigotes; Lane C, *T. cruzi* Y strain epimastigotes. Monoclonal antibody #20 was used as the primary antibody.

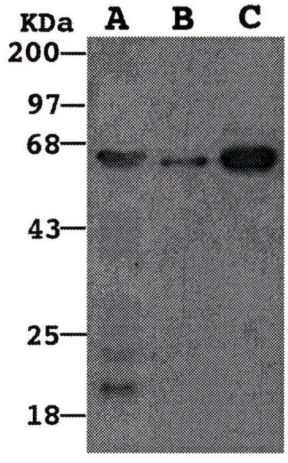

Figure 18

Immunogold electron microscopic localization of antigen in *T. b. brucei* 427.01 BSF parasites using mAb #20 as the primary antibody.

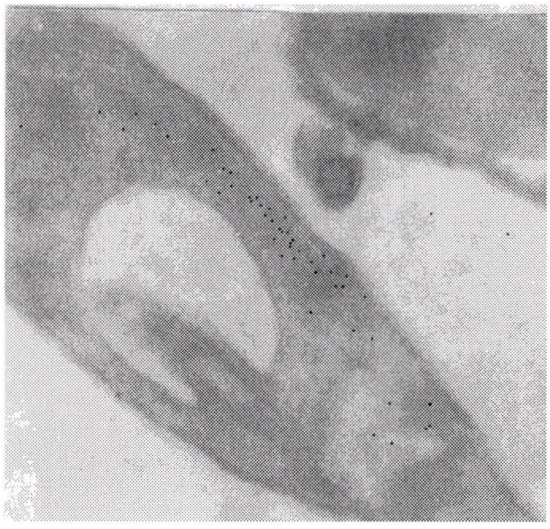

Figure 19

Immunoblot of cell lysates of heat shocked and non-heat shocked PCF Trypanosomes of different species. Lane A, *T. b. rhodesiense* ViTat 1.1 PCF non-heat shocked; Lane B, *T. b. rhodesiense* ViTat 1.1 PCF heat shocked; Lane C, *T. b. brucei* 427-01 PCF non-heat shocked; Lane D, *T. b. brucei* 427-01 PCF heat shocked; Lane E, *T. congolense* 45/1 PCF non-heat shocked; Lane F, *T. congolense* 45/1 PCF heat shocked. Monoclonal antibody #20 was used as the first antibody.

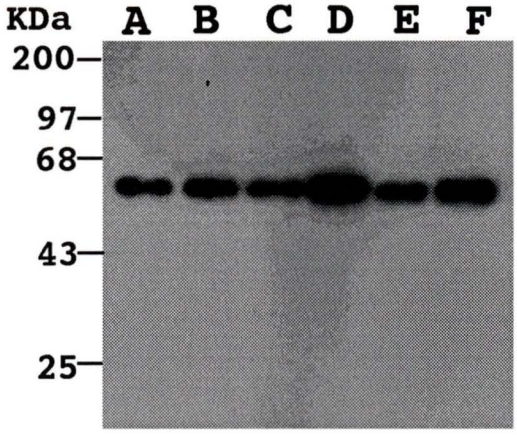

Figure 20

Immunoblot analysis of antigen #20 in *T. b. rhodesiense* ViTat 1.1 PCF fractions eluted from a gelatin-agarose column. Column breakthrough and eluted fractions were electrophoresed on SDS-PAGE gels and immunoblotted using mAb #20. Lane A, *T. b. rhodesiense* ViTat 1.1 PCF whole lysate; Lane B, gelatin-agarose eluted fraction; Lane C, gelatin-agarose breakthrough fraction.

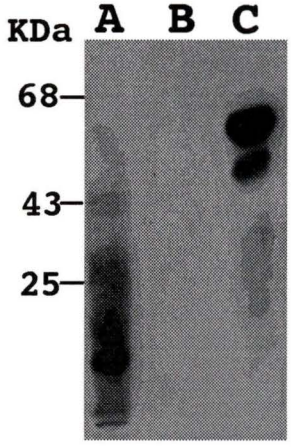

Figure 21

Agarose gel of *Not* I/*Sal* I digested phage DNA from plaque purified λgt22A expression library clone 20-4. Phage DNA was digested to excise insert cDNA and electrophoresed on a 1% agarose gel. Lane A, clone 20-4.

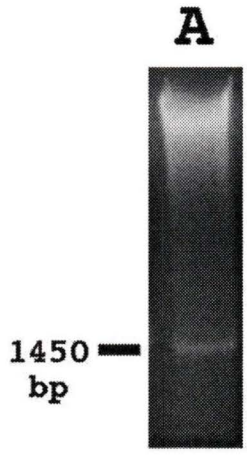

Figure 22

Clone 20-4 cDNA sequence. The cDNA sequence length is 1411 bp. Underlined sequences show restriction sites: *Sac* II at 85 and 1074 bp, *Hind* III at 596 bp. (Open reading frame from 1 to 1161 bp).

```
   1 GGTGGGGAAG GATGGCGTGA TCACAACACA AGATGGGAAG ACATTGACGA
  51 CTGAGCTGGA AGTTGTGGAA GGCATGAGTG TGGACCGCGG TTACATCAGC
 101 CCGTACTTCG TAACTGACGC GAAGACTCAG AAGGCCGAGC TTGAAGATGC
 151 GTTCGTGCTT GTGTCTGCAA AGAAGTTGAA CAACATTCAT ACGATCTTAC
 201 CGGTGTTGAA TCACGTGGTG CGCAGTGGGC GACCATTGCT GATCATTGCG
 251 GATGATGTGG AGAGTGAGGC CCTGACGACG ATGATTTTCA ATAAACTTCA
 301 AGGAAAGCTG AAGATTGCGT GCGTGAAGGC TCCAGGTTTC GGGGACAACA
 351 AGGCTGCGAT GCTGCAAGAC ATCGCCATTT TCAGTGGTGC CTGCGTTGTT
 401 GGTGAGGAAG GCAGTGGTGT GGAACTTGAC GCTGAGAAAT TCGACGCCAG
 451 CATCTTGGGG AGTGTGAAGA AGGCAACAAT CACGAAGGAC GATACAGTAC
 501 TGTTGAACGG TGGTGGCGAC GTTGCGATGA TGAAAGAACG CGTGGACCTG
 551 CTGCGCGGGC TCATTGAGCG CGAGACGAGT GACTATAACC GCGAGAAGCT
 601 TCAAGAACGT CTTGCAAAAC TGAGTGGTGG CTGTGCCGTA ATCCGCGTTG
 651 GTGGTCCTTC TGAGGTGGAG GTGAACGAGA AGAAGGACCG CATCACAGAT
 701 GCCCTGTGCT CGACCCGCGC TGCGGTGCAG GAAGGCATTG TCCCTGGTGG
 751 TGGCGCTGCG TTGCTGCGTG CGAGCAAAGC ATTGGACGGA TTACTACAGG
 801 ATCAGTCACT CACCGCTGAT CAACGGACTG GCGTGCAGAT CATCCGTAAC
 851 GCGGTGCGGT TGCCCGCCCA CCGCATTGTT GCCAATGCTG GAAGGGAAGG
 901 TGCTGTTGTT GTTGAGAAGG TGCTCGAGAA CACTGATGCC GCTGTTGGTT
 951 ACGATGCGAC GCTTGATCGC TACGTGAACA TGTTTGAAGC CGGAATAATC
1001 GACCCCGCGC GTGTGGTTCG TGTTGCGCTT ACTGACGCTG CGTCTGTTGC
1051 CAGCCTCATG ATGACGGCAG AGGCCGCGGT TGTGGATTTA CCGAAGGATG
1101 ATGCACCTGC TGCAGGTGGT ATGGGAGGCA TGGGTGGTAT GGGAGGTATG
1151 GACGGCATGT ATTGATGCTG AGGCAACCTG AGAGCATTGA GCAGGTGTTG
1201 TCCTGGTCAT AATGTTCCGC ACAGAATTGA ACGTCATCAT TATTTATTTA
1251 TTTATTTATT TATTTATTTA TTATTATTAT TCAGCTCGTG TTTTAAATTA
1301 ATTATTATCA TTATTATTGT CGCTTCAGTA GGGTTCTAAT TCCAGGTTAT
1351 TCGAGTTGTC GAGTCTGTGG TTCGCGGGAG GGGAGACTCT TGGAGGGAGG
1401 TACTGTGTTT G
```

Figure 23

T. b. brucei mitochondrial Hsp60 cDNA encoding Antigen #20. The coding region starts at bp 1 and ends with a stop codon at bp 1687 (underlined).

```
   1 ATGTTCCGCT GTGTCGTCCG TTTTGGTGCC AAAGACATCC GTTTTGGCAC
  51 GGAAGCACGT CAATCTATGC TGAAGGGCGT ACAACGCGCT GTGGAGGCTG
 101 TTGCAACGAC CCTTGGGCCT AAGGGACGTA ACGTGATTAT CGAGCAATCG
 151 TACGGTGCTC CGAAGATCAC GAAGGATGGT GTAACCGTTG CGAAGTCGAT
 201 CGAGTTCAAG GACCCGTTTG AGAACATGGG TGCGCAGCTC GTGCGGCAGG
 251 TATGCAATAA GACAAATGAC CTCGCGGGTG ATGGAACGAC GACATCGGCT
 301 GTCCTCGTTG CAAGCATCTT TAGCGAGGGT ATCAAATCGA TTGCAACCGG
 351 GACGAATCCC ATTGACATGA AGCGTGGTAT GGACCGCGCC GTGGAGGTGA
 401 TCCTGAAGAA CATCGAATCT CAGAGCCGAA CGGTAACAAA TACGGAGAAC
 451 GTTGTGCAGG TTGCGACGAT TTCCGCGAAC GGTGATGTTG AACTCGGCAA
 501 GCTGATTGGG GAGGCGATGG AGAAGGTGGG GAAGGATGGC GTGATCACAA
 551 CACAAGATGG GAAGACATTG ACGACTGAGC TGGAAGTTGT GGAAGGCATG
 601 AGTGTGGACC GCGGTTACAT CAGCCCGTAC TTCGTAACTG ACGCGAAGAC
 651 TCAGAAGGCC GAGCTTGAAG ATGCGTTCGT GCTTGTGTCT GCAAAGAAGT
 701 TGAACAACAT TCATACGATC TTACCGGTGT TGAATCACGT GGTGCGCAGT
 751 GGGCGACCAT TGCTGATCAT TGCGGATGAT GTGGAGAGTG AGGCCCTGAC
 801 GACGATGATT TTCAATAAAC TTCAAGGAAA GCTGAAGATT GCGTGCGTGA
 851 AGGCTCCAGG TTTCGGGGAC AACAAGGCTG CGATGCTGCA AGACATCGCC
 901 ATTTTCAGTG GTGCCTGCGT TGTTGGTGAG GAAGGCAGTG GTGTGGAACT
 951 TGACGCTGAG AAATTCGACG CCAGCATCTT GGGGAGTGTG AAGAAGGCAA
1001 CAATCACGAA GGACGATACA GTACTGTTGA ACGGTGGTGG CGACGTTGCG
1051 ATGATGAAAG AACGCGTGGA CCTGCTGCGC GGGCTCATTG AGCGCGAGAC
1101 GAGTGACTAT AACCGCGAGA AGCTTCAAGA ACGTCTTGCA AAACTGAGTG
1151 GTGGCGTTGC CGTAATCCGC GTTGGTGGTC CTTCTGAGGT GGAGGTGAAC
1201 GAGAAGAAGG ACCGCATCAC AGATGCCCTG TGCTCGACCC GCGCTGCGGT
1251 GCAGGAAGGC ATTGTCCCTG GTGGTGGCGC TGCGTTGCTG CGTGCGAGCA
1301 AAGCATTGGA CGGATTACTA CAGGATCAGT CACTCACCGC TGATCAACGG
1351 ACTGGCGTGC AGATCATCCG TAACGCGGTG CGGTTGCCCG CCCACCGCAT
1401 TGTTGCCAAT GCTGGAAGGG AAGGTGCTGT TGTTGTTGAG AAGGTGCTCG
1451 AGAACACTGA TGCCGCTGTT GGTTACGATG CGCAGCTTGA TCGCTACGTG
1501 AACATGTTTG AAGCCGGAAT AATCGACCCC GCGCGTGTGG TTCGTGTTGC
1551 GCTTACTGAC GCTGCGTCTG TTGCCAGCCT CATGATGACG GCAGAGGCCG
1601 CGGTTGTGGA TTTACCGAAG GATGATGCAC CTGCTGCAGG TGGTATGGGA
1651 GGCATGGGTG GTATGGGAGG TATGGACGGC ATGTATTGA
```

Figure 24 Comparison of the predicted amino acid sequence of Antigen #20 with *Trypanosoma* Hsp60 and *E. coli* GroEL (•=identity; |=similarity).

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                 •       ||•|||•• |••  • |••|    ••  •••••••••||||•|••• ••••••|••| ••| • ••••••••|•||
Antigen #20      MFRCVVRFGAKDIRFGTEARQSMLKGVQRAVEAVATTLGPKGRNVIIEQSYGAPKITKDGVTVAKSIEFKDPFENMGAQLVRQ
T. brucei Hsp60  MFRCVVRFGAKDIRFGTEARQSMLKGVQRAVEAVATTLGPKGRNVIIEQSYGAPKITKDGVTVAKSIEFKDPFENMGAQLVRQ
T. cruzi Hsp60   MFRSAARFAGKEIRFGTEARQSMQKGVQRAVSAVATTLGPKGRNVIIEQSYGAPKITKDGVTVAKAIEFKDPFENMGAQLVRQ
E. coli GroEL    M-------AAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGAPTITKDGVSVAREIELEDKFENMGAQMVKE

                 • |•|•• •••••••||••  ||||•||| |•|• ••|•|•••|•|••   | ||   |   |||  | ••|•••••|•  |•
Antigen #20      VCNKTNDLAGDGTTTSAVLVASIFSEGIKSIATGTNPIDMKRGMDRAVEVILKNIESQSRTVTNTENVVQVATISANGDVELG
T. brucei Hsp60  VCNKTNDLAGDGTTTSAVLVASIFSEGIKSIATGTNPIDMKRGMDRAVEVILKNIESQSRTVTNTENVVQVATISANGDVELG
T. cruzi Hsp60   VCNKTNDLAGDGTTTSAVLVASVFSESLRCIATGTNPIDMKRGMDRAVGVILQSVAEQNRKVTSTENIVQVATISANGDEELG
E. coli GroEL    VASKANDAAGDGTTTATVLAQAIITEGLKAVAAGMNPMDLKRGIDKAVTAAVEELKALSVPCSDSKAIAQVGTISANSDETVG

                 |••||••|••••|••••  |••  |  ••|•••••  ••••|••••|  | |   ••• |•|•|| ••|||•|  |•• •| •
Antigen #20      KLIGEAMEKVGKDGVITTQDGKTLTTELEVVEGMSVDRGYISPYFVTDAKTQKAELEDAFVLVSAKKLNNIHTILPVLNHVVR
T. brucei Hsp60  KLIGEAMEKVGKDGVITTQDGKTLTTELEVVEGMSVDRGYISPYFVTDAKTQKAELEDAFVLVSAKKLNNIHTILPVLNHVVR
T. cruzi Hsp60   RLIGQAMEKVGKDGVITTQDGKTMTTELEVVEGMSIDRGYISPYFVTDAKAQKAELEDAFVLVSAKKVSSIHTILPALNHVVG
E. coli GroEL    KLIAEAMDKVGKEGVITVEDGTGLQDELDVVEGMQFDRGYLSPYFINKPETGAVELESPFILLADKKISNIREMLPVLEAVAK

                 |•|••••••|•••|•••|•|| • ||• |•|• ••••••••  | ••|•••• ||•|  |||••  •|••|    ||   ••  •|
Antigen #20      SGRPLLIIADDVESEALTTMIFNKLQGKLKIACVKAPGFGDNKAAMLQDIAIFSGACVVGEEGSGVELDAEKFDASILGSVKK
T. brucei Hsp60  SGRPLLIIADDVESEALTTMIFNKLQGKLKIACVKAPGFGDNKAAMLQDIAIFSGACVVGEEGSGVELDAEKFEASILGSVKK
T. cruzi Hsp60   TGRPLLIIADDVESEALTTMIFNKLQGKLKIACVKAPGFGDNKTAMMQDIAIFAGARLVGEEGSGLELDAENFDPAILGTVKK
E. coli GroEL    AGKPLLIIAEDVEGEALATLVVNTMRGIVKVAAVKAPGFGDRRKAMLQDIATLTGGTVISEE-IGMELEKATLED--LGQAKR

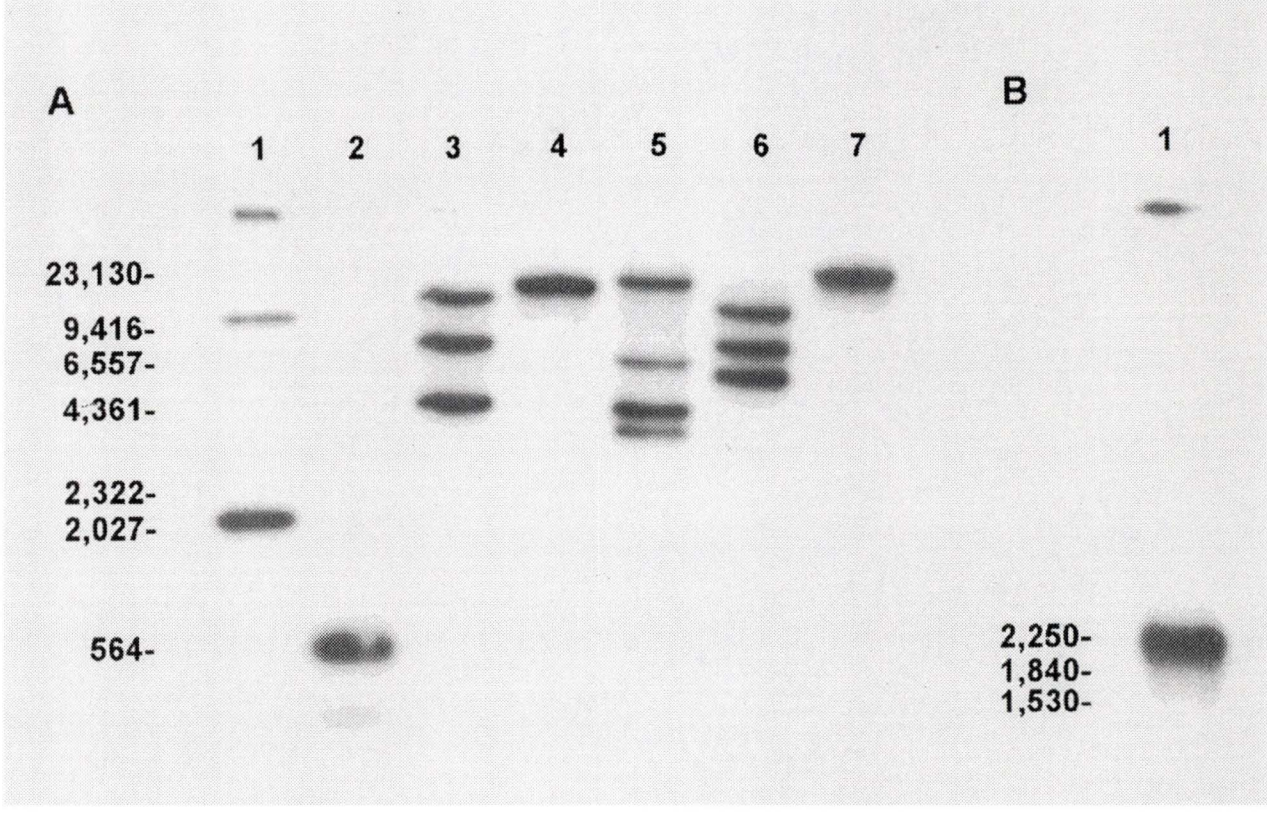
                 • •• • |||• •|  | || ••  |•  •|  ••••|••••••••|•••|••••••|••|||•••||••• •| ••• |•••
Antigen #20      ATITKDDTVLLNGGGDVAMMKERVDLLRGLIERETSDYNREKLQERLAKLSGGVAVIRVGGPSEVEVNEKKDRITDALCSTRA
T. brucei Hsp60  ATITKDDTVLLNGGGDVAMMKERVDLVRGLIERETSDYNREKLQERLAKLSGGVAVIRVGGASEVEVNEKKDRITDALCSTRA
T. cruzi Hsp60   ATITKDDTVLLNGGGESSMVKERVELLRGLIDGETSDYNREKLQERLAKLSGGVAVIKVGGGSEVEVNEKKDRITDALCSTRA
E. coli GroEL    VVINKDTTTIIDGVGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKARVEDALHATRA

                 ••|••|•|••• ••|• |  • |•  |     ••  •|||  •|  • | •• • • • |•• | •   •  ••|•  |
Antigen #20      AVQEGIVPGGGAALLRASKALDGLLQDQSLTADQRTGVQIIRNAVRLPAHRIVANAGREGAVVVEKVLENTDAAVGYDAQLDR
T. brucei Hsp60  AVQEGIVPGGGPALLRASKALDGLLQDQSLTADQRTGVQIIRNAVRLPAHRIVANAGREGAVVVEKVLENTDAAVGYDAQLDR
T. cruzi Hsp60   AVQEGIVPGGGVALLRASKALDSLLGDSSLTADQRTGVQIIPNAVRLPAHTIVLNAGKEGAVVVEKVLENNDVTVGYDAQRDR
E. coli GroEL    AVEEGVVAGGGVALIRVASKLADLRGQNE---DQNVGIKVALRAMEAPLRQIVLNCGEEPSVVANTV-KGGDGNYGYNAATEE

                 • ••|| ••|••||• • •|  | •••|••|•|•  | •|••|||| •| •••••••••||•
Antigen #20      YVNMFEAGIIDPARVVRVALTDAASVASLMMTAEAAVVDLPKDDAPAAGGMGGMGGMGGMDGMY
T. brucei Hsp60  YVNMFEAGIIDPARVVRVALTDAASVASLMMTAEAAVVDLPKDDAPAAGGMGGMGGMGGMDGMY
T. cruzi Hsp60   YVNMFEAGIIDPARVVRVAITDPVSVASLMMTTEASIVDFPKEETPAAGGMGGMGGMGGMDMY
E. coli GroEL    YGNMIDMGILDPTKVTRSALQYAASVAGLMITTECMVTDLPKNDAADLGAAGGMGGMGGMGMM
```

Figure 25

Southern and Northern blots of *T. b. rhodesiense* ViTat 1.1 PCF DNA and RNA probed with 20-4 cDNA. Panel A: Southern blot of genomic DNA digests. Panel B: Northern blot of poly(A)$^+$ RNA. Lane 1, *Xho*I; Lane 2, *Sau3A*I; Lane 3, *Nde*I; Lane 4, *Kpn*I; Lane 5, *Hind*III; Lane 6, *Eco*RI; Lane 7, *Bam*HI digest.



Discussion

The antigen recognized by mAb #20 was found to be a chaperone of the Hsp60 family. Initial evidence consisting of a short N-terminal sequence from the partially purified antigen was found to match a *T. b. brucei* Hsp60 mitochondrial chaperone (Bringaud *et al.*, 1995). A λgt22 expression library was screened with mAb #20 and a 1400 bp cDNA fragment partially encoding the Hsp60 was isolated, cloned and sequenced. This cDNA fragment contained an open reading frame which corresponded to the C-terminal 387 of 562 residues of the Hsp60 protein. RT-PCR was performed to obtain the 5' end of the cDNA. The full length cDNA was found to be 1689 bp in length with an open reading frame of 562 amino acids. The predicted amino acid sequence of the cDNA is 99% identical to the *T. brucei* Hsp60 reported after this thesis research was completed (Bringaud *et al.*, 1995) and 85.9% identical with the *T. cruzi* Hsp60 (Giambiagi-de Marval *et al.*, 1993). These findings show that antigen #20 is a mitochondrial chaperone of the Hsp60 family.

Antigen #20 (henceforth referred to as Hsp60) was found to be present in all *Trypanosoma* species and life cycle stages tested by immunoblotting. The mAb #20 was found to be *Trypanosoma* specific as a cross reacting protein was not detected in *Leishmania* species. The monoclonal antibody (H95) produced by Bringaud *et al.*, (1995) was also used by them in immunoblots to determine species- and life cycle stage-expression of Hsp60. This antibody detected the Hsp60 in all kinetoplastid species including *Leishmania* which indicates that the epitope recognized by this mAb is different from the one recognized by mAb #20.

The Hsp60 appears to be present in slightly lower quantities in BSF stages when compared to PCF (Figures 15 and 16). The mitochondrion is fully active in the PCF reflecting different metabolic processes in various life cycle stages. This chaperone may help the parasite survive in the tsetse vector by supporting mitochondrial function. A lesser amount of Hsp60 would be required in the BSF since the mitochondrion is inactive. There appears to be a markedly lower amount of Hsp60 in the metacyclic stage of the parasite. This probably reflects the morphological changes occurring while the parasite shuts down its mitochondrial function in preparation for survival in the vertebrate host. Expression could increase again in bloodstream forms due to a general heat shock response when the parasite passes from the tsetse fly (26°C) to the vertebrate host (37°C). Studies by Bass and Wang, (1992) have indicated that the essential trigger for bloodstream to procyclic transformation *in vitro* is a general inhibition of protein synthesis in BSF caused by a temperature drop from 37°C to 26°C which could be part of a general stress response.

Subcellular localization of this antigen using mAb #20 on BSF parasites in immunogold EM (Figure 18) showed it is localized in the mitochondrion of the parasite, further confirming its identity as a mitochondrial chaperone. In immunofluorescence studies using Hsp60-specific human sera with *T. cruzi* epimastigotes, Sullivan *et al.*, (1994) showed that Hsp60 homologue was also localized in the mitochondrial matrix. Bringaud *et al.*, (1995) were not able to directly demonstrate the localization of Hsp60 to the mitochondrion using immunofluorescence. Indirect evidence showed that the mAb H95 cross reacted with a *Leishmania tarentolae* mitochondrial fraction in immunoblots. The N-terminal sequence (Figure 14) obtained by the partially purified Hsp60 is missing 9 residues that are present in the predicted amino acid sequence (Figure 24). These residues are all aromatic, aliphatic and basic agreeing with the observation that most mitochondrial proteins are synthesized as precursors with positively charged amino-terminal targeting sequences (Höhfeld and Hartl, 1994). This may be an import signal that is cleaved upon transport to the mitochondria.

It was also shown that expression of the Hsp60 was slightly induced by heat shock from 26°C to 37°C of PCF *in vitro* (Figure 19). In experiments using *T. cruzi* epimastigotes that were subjected to incubation at 26°C, 37°C and 42°C *in vitro* by Sullivan *et al.*, (1994) a 6 fold increase in Hsp60 mRNA expression but no concurrent increase in Hsp60 levels after incubation at 37°C was seen. Previous studies by Olson *et al.*, (1994) and Tibbetts *et al.*, (1994) showed that *T. cruzi* parasites do not show a dramatic increase in heat shock protein expression upon heat shock as observed in many other organisms. They surmise that experiments performed *in vitro* using trypanosomes cultured in serum rich medium may be responsible for this phenomenon. Serum is a known inducer of heat shock protein expression (Wu and Morimito, 1985) and parasites grown in media containing serum may have already artificially high levels of heat shock protein.

Hsp70 proteins are also found in the mitochondrion and are known to bind to denatured proteins (Nandan *et al.*, 1994). Heat shock proteins of the 70 and 90 kDa families can be purified using a gelatin-agarose chromatography technique developed by Nandan *et al.*, (1994). Hsp60 proteins do not bind gelatin-agarose (personal communication with D. Nandan, 1995). In order to confirm that the antigen recognized by mAb #20 was member of the Hsp60 class and not the Hsp70 or 90 families, a gelatin-agarose binding experiment was performed (Figure 20). Antigen #20 was detected in the column flowthrough and therefore did not bind to the column.

The cDNA encoded by the λgt22A expression library clone contained a region located downstream from the 3' end of the Hsp60 translated region (Figure 22). This region contains a repeated motif consisting of (TTA) and (TTTA) which is known to occur in *Trypanosoma* heat shock genes (Dragon *et al.*, 1987; Engman *et al.*, 1989) and has been

suggested to play a role in their expression (Engman *et al.*, 1992). Also, the predicted amino acid sequence in Figure 24 shows another motif at the C-terminus of the protein. Five repeats of (GGM) residues are present in all *Trypanosoma* Hsp60 genes shown. This motif has also been found at the carboxyl terminus of a cytosolic Hsp70 from *T. brucei* (Glass *et al.*, 1986) and *Leishmania major* (Lee *et al.*, 1988). The role of this motif is unknown.

Studies on the expression and genomic organization of the Hsp60 genes in *T. b. brucei* revealed a 2.1 kb mRNA transcript (Figure 25, Panel B). Homologous Hsp60 genes in *T. cruzi* have been shown to exist in two allelic, multicopy gene clusters arranged in simple direct tandem arrays (Giambiagi-de Marval *et al.*, 1993; Sullivan *et al.*, 1994). These genes are found in the nuclear DNA and not encoded in the mitochondrial genome (Sullivan *et al.*, 1994). The Hsp60 genes exist in different isoforms in *T. cruzi* and it is known that there are at least five (Sullivan *et al.*, 1994). Southern blotting performed on *T. b. brucei* (Figure 25, Panel A) showed that there is more than one Hsp60 gene copy present in *T. b. brucei* and probably three or more. More definitive tests will have be done to ascertain the exact number of genes and their organization.

The identification of Hsp60 protein and encoding genes in African trypanosomes will allow studies on the role these stress proteins play in mitochondrial biogenesis and parasite survival. The role this protein plays as an antigen in African sleeping sickness can also be studied. This antigen (or mAb #20) may be useful as a diagnostic tool for African (and American) sleeping sickness.

General Discussion

Six monoclonal antibodies were previously generated against *T. brucei rhodesiense* ViTat 1.1 PCF internal antigens. Five of the six mAbs were found to be *T. brucei* spp. specific and may be useful as a mixture in a diagnostic test for parasite antigens as discussed in Chapter 1. The remaining monoclonal antibody (#20) was found to recognize all *Trypanosoma* species and life-cycle stages tested and the antigen was identified in this thesis work as an Hsp60 chaperone. This antigen may be useful in the diagnosis of African sleeping sickness. Heat shock proteins are well known to be immunogenic proteins and have potential utility as vaccine targets and diagnostic markers for many pathogens.

Proteins from the 60 kDa and 70 kDa Hsp families are well characterized as major targets for both antibody and T-cell responses in infections involving helminths, protozoa and bacteria (Macario, 1995). Antibody responses to Hsp70 proteins have been described for the parasites *Plasmodium falciparum* (Kumar *et al.*, 1991), *T. cruzi* (Glass *et al.*, 1986), *L. donovani* (Louzir *et al.*, 1994), *Schistosoma spp.* (Hedstrom *et al.*, 1988) and *Brugia malayia* (Selkirk *et al.*, 1989). To date Hsp60 family members have been described as the major antigens of bacterial pathogens such as *Mycobacterium spp.*, *Coxiella burneti*, *Treponema pallidium*, *Legionella pneumophilia*, *Borrelia burgdorferi* and *Chlamydia trachomatis* (Macario, 1995). These antigens are recognized by the humoral and cellular arms of the immune system and in the case of T-cell reactivity, γδ-T-cells have been identified as important mediators of the immune response (Born *et al.*, 1990). Stress proteins from the 60 kDa family have been described as major antigens in *Leishmania donovani* (Rey-Ladino and Reiner, 1993), *Trypanosoma cruzi* (Giambiagi-de Marval *et al.*, 1993; Sullivan *et al.*, 1994) and *T. b. brucei* (Bringaud *et al.*, 1995).

In vitro culture of parasites has allowed the large scale production of parasite antigens for use in diagnostic tests (Terry, 1985). Monoclonal antibodies have improved the specificity and sensitivity of several tests for parasite diseases (Bidwell and Voller, 1981; Gottstein *et al.*, 1985; 1987; Feldmeier *et al.*, 1985; Walls and Schantz, 1986; Lal *et al.*, 1987; Müller *et al.*, 1989). The use of a purified antigen (such as the Hsp60 mitochondrial chaperone described in this thesis) in an ELISA could be highly specific and useful for both diagnostic and epidemiological applications (Voller and De Savigny, 1981).

Recombinant DNA technology has allowed the mass production of specific antigens for vaccination trials (Miller *et al.*, 1986; Egan *et al.*, 1987; Patarroyo *et al.*, 1988) and for diagnostic purposes (Klinkert *et al.*, 1988; Affranchino *et al.*, 1989, Müller, 1989). If a

particular antigen can be partially or fully cloned and expressed, it can then be produced in large quantities and used to detect antibody or even antigen in patients. Large amounts of diagnostically useful antigens could be used in many serological tests including antigen-coated latex beads on agglutination cards for serodiagnosis. The use of Hsp60 in this type of diagnostic assay has potential if *Trypanosoma*-specific epitopes can be found and/or cross reactivity does not occur with host or other parasite Hsp60 proteins. Due to the high conservation among these proteins, a *Trypanosoma*-specific epitope would probably be more useful. A synthetic peptide corresponding to this epitope coupled to latex beads or a carrier protein could then be used for the detection of antibodies specific for trypanosomes. Evidence that a trypanosome-specific epitope exists was obtained in this thesis since mAb #20 was trypanosome-specific.

A kinesin-related antigen from *Leishmania chagasi* was recently cloned and used to detect antibody in patients infected with Leishmania (Burns *et al.*, 1993). This cloned antigen was specific for visceral leishmaniasis and could replace crude parasite antigen preparations as a basis for more specific serological diagnosis of visceral leishmaniasis.

Assays based on the detection of antibodies have two important disadvantages. First, the delay between infection and the appearance of detectable levels of circulating antibody leave a window where false negatives can occur. Secondly, antibody levels may persist after the infecting organism has been cleared, yielding false positive results in diagnostic tests. The detection of antigen in the host may eliminate these errors and may give a more accurate diagnosis of current infection status (Voller and De Savigny, 1981).

Through the use of monoclonal antibodies it is possible to design highly specific and sensitive assays for parasite antigen detection in tissue fluids and in the excreta of infected hosts as a means of diagnosis (Liu and Pearson, 1987; Nantulya, 1991). Monoclonal antibodies produced against antigen found circulating in the sera of infected patients can be used in an indirect ELISA to detect circulating parasite antigens.

DNA hybridization techniques offer an approach to the identification of parasites in tissue specimens of infected hosts (Nantulya, 1991). In addition, the advent of PCR techniques has made it possible to increase the sensivity of diagnostic techniques through amplification of parasite specific DNA sequences in patient test material. DNA probe and PCR based assays used to identify and detect parasites are highly sensitive and can directly detect organisms independent of the immunocompetence or previous clinical history of the patient (Nantulya, 1991). These techniques can often distinguish between organisms that are morphologically similar. PCR techniques can be utilized for diagnosis of Chaga's disease (Sturm *et al.*, 1989), leishmaniasis (Barker *et al.*, 1986) and malaria (Delves, 1989). The use of PCR in a simplified procedure for the specific detection of *T. cruzi* in

human blood specimens using primers specific for kinetoplast DNA suggests that this technique may be a powerful way to detect parasites in patients with a chronic infection (Wincker *et al.*, 1994). Probes based on kinetoplastid DNA sequences have also been used to develop *in situ* hybridization tests for the diagnosis of leishmaniasis (Barker *et al.*, 1986). The sequence of the trypanosome Hsp60 antigen gene can provide information to design specific DNA probes for *in situ* hybridization or PCR in diagnosis of African trypanosomiasis. Despite the sensitivity and discriminatory powers of PCR techniques it is unlikely that they will be adapted to field use in the near future.

Does the trypanosome Hsp60 have vaccine potential? To serve as vaccines, antigens must be recognized by the immune system and be capable of inducing the appropriate cellular and/or humoral responses that are effective against the pathogens. Due to the extensive identity found between heat shock proteins of different species there is much immunological cross reactivity between these proteins. The cross-reactive immunity to Hsps of different microbes makes them less suitable for vaccines. Specific epitopes of heat shock proteins such as the one recognized by my mAb #20 described in this thesis would be potentially more useful as candidates for vaccine development.

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