Human serum resistance in *Trypanosoma brucei*

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by

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Declaration

I, the undersigned, hereby declare that the contents of this thesis have been composed by myself, and that the work described herein is entirely my own except where stated otherwise. No part of it has been previously submitted for a degree at any university.

Gabriella Lindergård

10th February 1999

Abstract

T.b.brucei, *T.b.rhodesiense* and *T.b.gambiense* are all morphological identical and cannot be distinguished by microscopical observations. The original definition of these three subspecies was presented by Hoare (1970) taking into account their geographical distribution, their host specificity, their ability to infect humans and the pattern of disease they cause. By these criteria, *T.b.brucei* is defined as non-human infective and *T.b.gambiense* and *T.b.rhodesiense* as human infective since they have the ability to withstand lysis by human serum. The underlying mechanisms for human serum sensitivity/resistance are not yet fully understood and more than one factor in human serum may be involved. The overall aim of this thesis was to investigate the inheritance and molecular basis for human serum resistance/sensitivity. Inheritance studies were made possible because of the availability of recombinant progeny from a cross between a human serum resistant and a human serum sensitive trypanosome strain. A molecular approach based on differential display RT-PCR was also used to complement the more biochemical approaches adopted by others.

A range of variables were examined and optimised to develop a reliable *in vitro* bloodstream form human serum sensitivity assay but it was clear that cell viability could not be estimated from morphological appearance alone. A statistically significant difference in mean percentage lysis was observed between stocks that correlated with human serum sensitivity but the data varied too much between individual assays for the assay to provide unambiguous results. Instead, a reliable *in vivo* human serum resistance assay, which involved incubating bloodstream trypanosomes with human serum, injecting them into mice and screening of the mice for the development of parasitaemia, was optimised and adopted.

Genetic analysis and the heritability of human serum resistance/sensitivity was investigated with the aim of revealing the number of loci and alleles involved and determining the dominance relationships between alleles. From the pattern of inheritance a model for the inheritance of human serum resistance is suggested and a

number of models for inheritance which are incompatible with the results are refuted. As a result of detecting F_1 progeny clones of intermediate resistance, the simplest possible model is based on 3 alleles; codominant sensitive and resistant alleles and a recessive sensitive wild type allele, all at a single locus.

A candidate gene determining resistance to human serum has been previously described; the serum resistance associated (SRA) gene (De Greef & Hamers, 1994). To determine whether SRA expression was causing resistance in the present study, the presence of a genomic copy of the SRA gene was initially investigated by PCR in the cloned stocks STIB 386, STIB 247 and TREU 927. PCR products of the expected size were obtained from STIB 247 and TREU 927 but not from STIB 386. No PCR product could be amplified from STIB 386 whatever primer combination and PCR conditions were used implying that the SRA gene may be absent from this isolate. To further investigate the presence of a sequence related to SRA in STIB 386 and TREU 927, Southern blots were performed. Hybridisation with fragments of similar, but not identical, sizes were noted in all of the clones indicating that sequences with homology are present but that sequence variation occurs within the SRA gene. The expression of the SRA gene and its correlation with a HSR phenotype was further examined but its expression does not coincide with HSR in the stocks used in this study.

A study was initiated to identify candidate genes determining resistance or sensitivity to human serum. To do this differential display RT-PCR was used in order to identify cDNA fragments expressed in either of the serum sensitive or serum resistant trypanosome lines. Nine differentially amplified products were identified, reamplified, cloned and sequenced and 16 clones obtained. Four of these clones showed expression patterns associated with human serum resistance or sensitivity, three were specifically expressed in human serum sensitive trypanosomes and one was specifically expressed in human serum resistant trypanosomes. Two of the sensitive specific clones were related to EST sequences from a cDNA library of a *T.brucei* bloodstream clone which have not been further characterised. The other sensitive specific clone was part of a gene for ribosomal RNA while the resistance

specific clone was unrelated to any other sequences in the database. These data, taken together, suggest that human serum sensitivity in trypanosomes is determined by both a lack of resistance *and* a specific expression of gene product(s) conferring sensitivity.

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Abbreviations

BSA	Bovine serum albumin
BLAST	Basic Local Alignment Search Tool
CBSS	Carter's balanced salt solution
CFLP	Outbred strain of mice
CNS	Central nervous system
CSF	Cerebral spinal fluid
DAPI	4,6,-diamino-2-phenylindole
DDRT-PCR	Differential display reverse transcription PCR
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulphoxide
DFMO	α-difluoromethylornithine
DNA	Deoxyribonucleic acid
EDTA	Ethylene diaminetetraacetic acid
ETat	Edinburgh *Trypanozoon* antigenic type
EATRO	East African Trypanosomiasis Research Organisation
ES	Expression site
ESAG	Expression site associated gene
F1	First filial generation
FITC	Fluorescein isothiocyanate
g	Acceleration due to gravity
GPS	Guinea pig serum
GUP	Glasgow University Protozoology
GUT at	Glasgow University Trypanozoon antigen type
HBSS	Hank's balanced salt solution
HDL	High density lipoprotein
HSR	Human serum resistant
HSS	Human serum sensitive
hpr	Haptoglobin related protein
ISG	Invariant surface glycoprotein
i.p.	Intraperitoneal
ILT at	ILRAD Trypanozoon antigen type
IPTG	Isopropylthio-β-D-galactoside
kb	Kilobase
kDa	Kilo dalton
kDNA	Kinetoplast DNA
LB	Luria-Bertani medium
LDL	Low density lipoprotein
LMP	Low melting point agarose
μCi	Microcurie
MEM	Minimum essential medium
NaCl	Sodium chloride
Na_2HPO_4	Di-sodium hydrogen phosphate
NaOH	Sodium hydroxide
NHS	Normal human serum
PARP	Procyclic acidic repetitive protein

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PSG	Phosphate-buffered saline-glucose
RACE	Rapid amplification of cDNA ends
RADES	Random amplified differentially expressed sequences
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RFLP	Restriction fragment length poymorphism
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SE	Standard error
STIB	Swiss Tropical Institute, Basel
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TBS	Tris buffered saline
TLF	Trypanosome lytic factor
TREU	Trypanosomiasis Research, Edinburgh University
UTR	Untranslated region
VAT	Variable antigen type
VLDL	Very low density lipoprotein
VSG	Variable surface glycoprotein
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Chapter 1

General Introduction

1.1 GENERAL FEATURES OF AFRICAN TRYPANOSOMES

1.1.1 Life cycle

The *Trypanosoma brucei* group are protozoan parasites belonging to the subphylum Mastigophora, order Kinetoplastida and subgenus *Trypanozoon*. These trypanosomes are actively motile haemoflagellates that can parasitise the blood of a variety of mammals. They do not invade or live within cells but inhabit connective tissue spaces within organs and the reticular tissue spaces of the spleen and lymph nodes. They are especially abundant in the lymph vessels and, in later stages of infection, the intercellular spaces in the brain. Trypanosomes of the *T.brucei* group are all morphologically identical but have traditionally been divided into three subspecies depending on their infectivity to humans; *T.b.brucei* is not infective to humans while *T.b.rhodesiense* and *T.b.gambiense* are. The latter two are also distinguished on the clinical symptoms they can give rise to (Minter, 1991). *T.brucei* is transmitted by an infected bite of the insect tsetse fly vector of the genus *Glossina* and this requirement restricts the disease to sub-Saharan Africa. African trypanosomiasis and its cattle equivalent, nagana, are significant diseases in much of Africa and cause considerable suffering and economic loss (Schmidt & Roberts, 1989).

T.brucei has a rather complex life cycle which involves morphological changes to its surface membrane and alterations in energy metabolism. During the bite of an infected tsetse fly, metacyclic stage trypomastigotes are injected into the dermal layer of the hosts' skin and an inflammatory reaction usually follows. The metacyclic forms transform into slender trypomastigotes and multiply extracellularly in the bloodstream and other body fluids. During the initial phase of rising parasitaemia these are the predominant forms but as the parasitaemia peaks and declines short, stumpy non-dividing forms come to be predominant. This morphological variation in

the bloodstream cells is called pleomorphism. If stumpy forms are ingested by a feeding tsetse fly, it is possible that the fly becomes infected but in most cases the trypanosomes are readily killed off by the tsetse flies' immune system and various digestive enzymes. If slender forms are ingested they will die or, sometimes, change into stumpy forms in the anterior midgut of the fly. Stumpy trypomastigotes that survive this hostile environment transform into procyclic forms in the fly midgut. From here trypanosomes penetrate the peritrophic membrane to colonise the ectoperitrophic space and migrate forward to the proventriculus where the peritrophic membrane is soft and allows the parasites to re-enter the lumen of the gut. They pass the oesophagus and move further up the labium before they finally reach the salivary glands where they transform into epimastigotes. Epimasigote forms are not free-swimming but are attached to the salivary gland endothelial cells by means of their flagellum. Epimastigotes are again a dividing stage which will mature into non-dividing infective metacyclic trypomastigotes, (Vickerman, 1985). It may take 18-34 days from the ingestion of stumpy trypomastigotes to the production of infective metacyclics when approximately 10^4 infective metacyclics are discharged during the feed of an infective fly (Service, 1986).

Apart from major changes in the gross morphology during the life cycle there are also alterations of the ultrastructure in the mitochondrion, activation of oxidize systems and the cytochrome electron transport chain as bloodstream trypanosomes transform into procyclics. Bloodstream trypomastigotes are covered by a densely packed coat consisting of single molecular species, the variable surface antigen. The surface coat protects against non-specific immune mechanisms and antibodies directed towards the cell and can also undergo antigenic variation. During the transformation of bloodstream forms to procyclic forms the surface coat is lost and the synthesis of variable surface glycoproteins (VSGs,) that constitute this coat, is discontinued (Barry, 1997). The loss of variable antigens occur faster in the posterior portion of the fly midgut than in the crop and anterior part. It is therefore concluded that the posterior midgut is the main site for transformation of bloodstream trypanosomes to procyclics and this transformation takes approximately 48 hours (Turner *et.al.*, 1988).

Tsetse flies are restricted to tropical Africa from between latitude $15°$ north and $20°$ south but extending to about $30°$ south along the eastern coastal area. Both male and female tsetse flies bite humans, a variety of domestic and wild animals, reptiles and birds. Most tsetse fly species show some host preference but none feed exclusively on one type of host. The flies take blood meals every 2-3 days but the interval may be longer in cooler, humid climates. The *Glossina* species can be divided into 3 groups; the Fusca group (forest flies), the Morsitans group (savannah flies) and the Palpalis group (riverine and forest flies). None of the fusca group are vectors of sleeping sickness. The Morsitans group contain the important vectors *G.morsitans* and *G.pallidipes* and the Palpalis group include *G.palpalis* and *G.fuscipes* which are important human sleeping sickness vectors (Service, 1986). Salivary gland infection rates are low, less than 1%, even in areas of endemic sleeping sickness (Nekpeni *et.al.*, 1991).

1.1.2 African Trypanosomiasis - spread and disease

Human sleeping sickness is endemic in a large area of Sub-Saharan Africa. It occurs in 36 African countries south of Sahara where at least 50 million people are at risk of infection (Kuzoe, 1993). Around 25 000 cases have been reported each year but since only 5-10% of the population is under surveillance it has been estimated that more than 300 000 people are carrying the disease (Smith *et.al.*, 1998). The spread follows the geographical distribution of the tsetse fly. African trypanosomes of the *T.brucei* species have traditionally been regarded as consisting of three sub-species: *T.b.brucei*, *T.b.rhodesiense* and *T.b.gambiense*. They have different host preferences and cause different disease patterns but are otherwise morphologically and antigenically indistinguishable (Hoare, 1970).

T.b.brucei is a parasite of African antelopes, other ruminants and lizards in tropical Africa but it also infects domestic animals such as cattle, horses, camels, pigs and sheep. Together with the related species *T.congolense* and *T.vivax*, which are the

primary pathogens of cattle, it causes a disease known as Nagana which involves severe anaemia and a significant reduced growth in domestic livestock. The clinical course of disease depends on the susceptibility of the host species. Horses, donkeys and dogs become acutely ill and usually survive only two weeks to four months. They suffer from anaemia, oedema, watery eyes and nose and fever, then become uncoordinated and paralysed and finally die. Cattle are usually more resistant to disease and can survive for several months after symptoms have been observed. Pigs are more refractory and may recover from the infection altogether (Schmidt & Roberts, 1989). *T.b.brucei* is common in most of the open country in Sub-Saharan Africa.

In a broad sense, *T.b.rhodesiense* and *T.b.gambiense* can be regarded as causing acute and chronic human sleeping sickness respectively, with the latter occurring in West and Central Africa and the former in East and Southern Africa. There is however, much complexity and diversity in these two sub-species which leads to variation and overlap in the disease pattern.

At the site of a bite of an infected tsetse fly, a small sore - the chancre, often develops. This disappears after 1-2 weeks as the parasites enter the blood and lymph and start to reproduce rapidly. The lymph nodes become swollen, especially in the neck (Winterbottom's sign), groin and legs. This is accompanied by intermittent periods of fever, generalised pain, headache, mood changes and altered electroencephalograms (EEG), which all correlate with meningeal inflammation (Pentreath, 1995). The 'gambiense' form of the disease may go on for years with extensive central nervous system involvement while the interval between the start of the infection and the encephalitic stage in *T.b.rhodesiense* is much shorter, usually a few months, and may lead to death before central nervous system symptoms develop. The heart is particularly susceptible to the general increase in perivascular cellularity, haemorrhage and oedema and heart failure are often the cause of death in the 'rhodesiense' form (Manson-Bahr & Bell, 1991). CNS involvement progresses to mental deterioration which leads to reversal of sleep patterns and subsequently permanent sleep, coma and death. The neurological changes correlate with the progression from meningitis to encephalitis (Bentivoglio *et.al.*, 1994). Apart from the

causes mentioned, death may also occur as a consequence of malnutrition or pneumonia or accidents caused by disturbed co-ordination and balance (Schmidt & Roberts, 1986).

Sleeping sickness was relatively well controlled during the early 1950's but political and civil unrest as well as poverty have forced many African countries to prioritise other issues so that trypanosomiasis is still a major problem and epidemic outbreaks are frequently being reported (WHO, 1990; Pepin, 1997; Smith *et.al.*, 1998). Recrudescence of old foci and a spread into new areas have recently been reported in Southern Sudan, Angola, Uganda, Congo-Zaire and in the Republic of Central Africa (Smith *et.al.*, 1998). Other areas with increased incidence include Cameroon, Chad and the Kigoma region in Tanzania (Kuzoe, 1993). An increase in human sleeping sickness has also been seen in the Ivory Coast since 1976 and is now an important health problem (Duoa & Yapo, 1993). A contributing factor responsible for the outbreaks are large population movements and the subsequent increased contact between tsetse flies and newly settled people. Another big problem is sleeping sickness foci that extend across country borders where co-operation is hindered by poor relations between the countries (Pepin, 1997).

Since the presence of tsetse flies is a requirement for the spread of trypanosomiasis and nagana much effort has been put into tsetse fly control. Practised control methods include removal of brush, to prevent flies deposit their larvae, and clearing of land to remove resting places for the flies, bait catching and spraying insecticides by aircraft (Barrett, 1997). Unfortunately, this effort has not significantly reduced the incidence of disease in cattle or humans and vast areas of rich grassland can not be used for agricultural purposes or for keeping grazing cattle (Perry, 1988). Since many available treatments are toxic, parasite detection is an important part of clinical assessment and treatment.

1.1.3 Diagnosis of African sleeping sickness

Diagnosis of African trypanosomiasis is by detection of trypanosomes in the blood, lymph node aspirate or the CSF. The simplest and cheapest diagnostic technique is the examination of stained, thick blood films. The value of this technique was demonstrated by Truc *et.al.* (1994), who recommended it as a simple, effective diagnostic method. The quantitative buffy coat (QBC) is a development of the haematocrit centrifugation technique where acridine orange is used to stain the trypanosomes and this is now available as a commercial product (Bailey & Smith, 1992). The QBC© has the advantage that a lower concentration of trypanosomes can be detected. The most sensitive assay is the miniature anion-exchange centrifugation technique (Lumsden *et.al.* 1981), but unfortunately this is not an easy method to use in the field. Serological assays can be used to demonstrate the presence of antibodies against *T.b.gambiense* as the total IgM levels will be elevated during infection. The card agglutination test for human trypanosomiasis (CATT) is another commercial kit for detecting *T.b.gambiense* which has been used for initial screening surveys (Truc, *et.al.*, 1994). A development of the CATT is the card indirect agglutination trypanosomiasis test (*Tryp*Tect CIATT©) which can be used to detect both *T.b.gambiense* and *T.b.rhodesiense* and is rapid and sensitive for both patent and non-patent infections (Nantulya, 1997). Finally, the kit for *in vitro* identification (KIVI) can also be used for parasitological diagnosis of trypanosomiasis (Aerts, 1992). It is a very sensitive method, albeit time consuming, which has shown to detect infection missed by other direct techniques.

1.1.4 Treatment of African sleeping sickness

Treatment of late stage sleeping sickness is still mainly based on melarsoprol, a trivalent arsenical which was introduced in 1949 as Mel B Arsobal and, until 1990, was the only drug available for treatment of late stages of the disease. Melarsoprol was thought to be an inhibitor of pyruvate kinase (Flynn & Bowman, 1969) but

trypanothione was later proposed as the intracellular target (Fairlamb *et.al.*, 1989). These findings are now considered to be wrong and the target for melarsoprol remains unknown. Resistance to melarsoprol can be highly variable but has mainly remained at a low level probably because patients with melarsoprol resistant trypanosomes rarely have parasites in the bloodstream and so transmission is unlikely. Unfortunately, melarsoprol can also give rise to severe side effects, a danger which is often well known to inhabitants of endemic villages and can discourage people from participating in case detecting surveys (Pepin & Milord, 1994).

Another drug available is pentamidine, a diamidine which is effective against early stages of *T.b.gambiense*. It is a reversible inhibitor of S-adenosyl-L-methionine decarboxylase leading to reduction in the synthesis of polyamines but it may also have other modes of action (Sands *et.al.*, 1985). Treatment with pentamidine generally only gives rise to minor side effects, mainly affecting the digestive system, and a noticeable tenderness at the injection site. Resistance to pentamidine is less frequent than to melarsoprol and levels vary in different areas but has been comparatively stable for the last 30 years (Pepin & Milord, 1994). Pentamidine does not penetrate the blood-brain barrier and is therefore not effective against late stage sleeping sickness (Manson-Bahr & Bell, 1991).

A third drug, suramin, is effective against both *T.b.gambiense* and *T.b.rhodesiense* but is mainly used against *T.b.rhodesiense*. Its mode of action is poorly understood since it inhibits several enzymes of which many probably have nothing to do with the trypanocidal effect. Suramin has to be given intravenously because of its inflammatory effects on tissue. For early stage 'gambiense' infection, it is best given together with pentamidine since the two drugs will form a complex which seems to be less toxic and more effective than either drug given alone (Pepin & Milord, 1994). However, in early stage rhodesiense trypanosomiasis it is thought that suramin is more effective than pentamidine. It is not an appropriate treatment for late stage sleeping sickness because of its poor penetration of the CNS (Manson-Bahr & Bell, 1991).

For late stage *T.b.gambiense* infection there is a good and less toxic alternative to melarsoprol in eflornithine (α-difluoromethylornithine, DFMO) but unfortunately it is much more expensive than melarsoprol and ineffective against a number of *T.b.rhodesiense* strains. DFMO is an inhibitor of polyamine biosynthesis and has rather few side effects when administered intravenously (Doua & Yapo, 1993). A drawback, apart from the cost, is that it requires a very large amount administrated intravenously every six hours for fourteen days. All manufacture of DFMO has now been discontinued (C.M.R. Turner, pers.com.), due to its cost and the limited market.

In summary, there is no trypanocidal drug available that can claim to fulfil the criteria of being effective, non-toxic, cheap, well-absorbed orally and with good penetration of the CNS.

1.2 STOCKS AND SUB-SPECIES OF *T.BRUCEI*

T.b.brucei, *T.b.rhodesiense* and *T.b.gambiense* are all morphological identical and cannot be distinguished by microscopical observations. The original definition of these three subspecies was presented by Hoare (1970), taking into account their geographical distribution, their host specificity, their ability to infect humans and the pattern of disease they cause. Human infectivity was tested by the Blood Incubation Infectivity Test (BIIT), which measures resistance to human serum by exposing trypanosome isolates to human serum followed by infection of rodents to detect surviving parasites (Rickman & Robson, 1970a). By these criteria, *T.b.brucei* is defined as infecting animals but not humans and is present in all of the tsetse fly areas in Africa, *T.b.gambiense* is human-infective, localised to West and Central Africa and gives rise to chronic trypanosomiasis while *T.b.rhodesiense* is also human infective but is present in East Africa and causes acute sleeping sickness (**table 1.1**). Several studies have been published that are not consistent with these original criteria and there are still many practical problems when it comes to distinguishing the

subspecies. The question therefore arises as to whether it is acceptable to divide *T.brucei* into these three subspecies.

Trypanosome	Mammalian host	Disease	Distribution
T.b.brucei	wild and domestic animals	nagana	Tropical Africa
T.b.rhodesiense	humans, wild and domestic animals	acute human trypanosomiasis	East tropical Africa
T.b.gambiense	humans and domestic animals	chronic human trypanosomiasis	West and Central tropical Africa

Table 1.1. Distinguishing features of the *T.brucei* group of trypanosomes (after Hoare, 1970).

1.2.1 Methods for identifying Trypanosome stocks

There are various molecular and biochemical approaches that can be used to distinguish and characterise trypanosome species and subspecies. A method widely used for parasitic and non-parasitic protozoa is enzyme electrophoresis where differences in the electrophoretic mobility of isoenzymes are used to detect a specific banding pattern for stocks and also to determine allele frequencies in populations of stocks (Tait *et.al.*, 1980; Gibson *et.al.*, 1980). Several isoenzymes can be compared in separate populations of trypanosomes and the relatedness of stocks assessed. A trypanosome stock is defined as a population derived by serial passage *in vitro* and/or *in vivo* from a primary isolation and there is no implication of homogeneity or characterisation. Stocks which turn out to have identical patterns are termed zymodemes and stocks with a similar pattern are said to belong to the same strain group (WHO, 1978). The greater the number of isoenzymes used, the more reliable the assessment of relationships will be. Hide *et.al.* (1994) showed however, that stocks with identical isoenzyme patterns may differ when analysed using repetitive DNA probes. This means that using isoenzyme profiles to suggest that two isolates are genetically identical can be misleading.

There are various DNA analysis methods available which have been used to investigate the variation of DNA sequences and identify trypanosome stocks. These include restriction fragment length polymorphism (RFLP) analysis of kDNA maxicircles (Gibson *et.al.*,1985a) and RFLP analysis of repetitive DNA sequences (Hide *et.al.*, 1990). The latter can be undertaken by Southern blotting of DNA cleaved by a restriction endonuclease and using a repetitive DNA probe, which will detect variation in DNA sequence, to generate a ladder of bands which act as a DNA fingerprint. Conserved single copy gene sequences or satellite sequences can also be used to detect variation in restriction sites by hybridisation (Hide *et.al.*, 1997). Many other types of genes can also be targeted and Sogin *et.al.* (1986) used rapid sequencing techniques of small subunit ribosomal-RNA genes to analyse the evolutionary diversity within species of *Trypanosoma*. It is also possible, but laborious, to use direct DNA sequencing of appropriate genes.

Recently, the use of PCR with specific primers or with random primers referred to as RAPDs (Randomly Amplified Polymorphic DNA fragments) have proven very useful for distinguishing groups of isolates (Mathieu-Daude *et.al.*, 1995). PCR amplification of repetitive DNA sequences, which are specific for trypanosome species or subspecies, proved to be a valuable tool in field surveys when investigating the identity of infections in tsetse flies (Masiga *et.al.*, 1996). The prevalence of various mature trypanosome infections could be evaluated when satellite DNA was used as a target for primers specific for the *T.brucei* group allowing this group to be distinguished from *T.vivax* and *T.godfreyi*.

1.2.2 Animal reservoirs of sleeping sickness

It was long assumed that bushbucks and other wild animals were the main reservoirs of sleeping sickness, mainly because the first human infective trypanosome, isolated from a non-human source, came from a bushbuck in East Africa (Heisch *et.al.*, 1958). More recent studies suggest that at least a third of all *T.brucei* infected domestic animals carry trypanosomes that are also infective to

humans and includes both *T.b.rhodesiense* and *T.b.gambiense* (Hide *et.al.*, 1996; Hide *et.al.*, 1994). Of three trypanosome stocks isolated from domestic animals in Congo and Zaire and investigated for enzyme variation, one showed the typical pattern for *T.b.gambiense* (Tait *et.al.*, 1984) and it has been confirmed that *T.b.gambiense* can be found in domestic animals such as pigs, dogs and sheep (Paindavoine *et.al.*, 1986).

A number of studies have examined the prevalence of *T.b.rhodesiense* in both game and domestic animals using the BIIT test as a criteria for identification. For example when 95 game animals in the Serengeti Area in Tanzania were examined for the presence of trypanosomes, 22% was found to be infected with *T.brucei* subgroup. 10% of these were resistant to human serum according to a BIIT test (see next section) and therefore regarded as *T.b.rhodesiense*, 32.5% of them gave equivocal results and the remaining 57.5% were completely sensitive i.e. *T.b.brucei* according to the criteria of Hoare (1970) (Geigy *et.al.*, 1973). Another study by Bertram (1973), also in the Serengeti National Park, estimated that about 7.5% of the mammals were infected with *T.brucei* but only a small proportion of strains proved to be infective to humans by the BIIT test and this was regardless of the host species they were found in. Hide *et.al.* (1990) collected a large number of *T.brucei* isolates from humans, cattle, pigs and tsetse flies in south-east Uganda and showed that a number of stocks isolated from cattle were human infective *T.b.rhodesiense* isolates and that cattle were reservoirs for *T.b.rhodesiense*. Hide *et.al.* (1996) calculated that it is five times more likely that an infected tsetse fly has obtained an infection from cattle than from an infected human.

In summary, these analyses show that domestic animals act as reservoir hosts for *T.b.gambiense* and that both domestic and wild animals are reservoir hosts for *T.b.rhodesiense*. However, in the case of *T.b.gambiense* it is uncertain what role the animal reservoir plays as a source of human infection.

1.2.3 Distinguishing *T.b.gambiense* from *T.b.rhodesiense* and *T.b.brucei*

One of the first attempts to measure genetic differences between *T.b.gambiense*, *T.b.rhodesiense* and *T.b.brucei* was carried out by the use of restriction enzyme analysis of kinetoplast DNA (Borst *et.al.*, 1981). The results showed strain specificity but without a common pattern for the *T.b.rhodesiense* or *T.b.gambiense* kDNA. The three subspecies were apparently so closely related that they could not be distinguished by restriction enzyme analysis alone. Indeed, the differences found between trypanosome stocks from different areas in Africa are similar to that found in a single subspecies of mice from different areas of North America (Avise *et.al.*, 1994). If isoenzyme electrophoresis is used instead of restriction site analysis however, *T.b.gambiense* can be differentiated from the other variants as shown by Tait *et.al.* (1984), who used electrophoretic enzyme variation to compare the degree of divergence between the three *T.brucei* species. The stocks used had previously been isolated and identified on the basis of their geographical and host origin as well as their sensitivity to human serum. From 20 screened enzymes, one, peptidase C, could differentiate all the *T.b.gambiense* stocks from the other stocks and together with another 5 enzymes could be used to differentiate all stocks. It was also clear that *T.b.gambiense* was more different from *T.b.brucei* and *T.b.rhodesiense* than these two are from each other. The basis for this argument was the measurement of genetic identity and genetic distance.

The combined use of restriction enzyme digestion and molecular hybridisation in order to determine relationships between *T.brucei* stocks was also used by Paindavoine *et.al.*, (1986). The overall conclusions were similar to those obtained from isoenzyme analysis, i.e. all *T.b.gambiense* isolates have a conserved and specific DNA band pattern in common which was different from *T.b.brucei* and *T.b.rhodesiense* isolates. More recently Hide *et.al.* (1994; 1998) compared *T.brucei* stocks from various locations by RFLP analysis and showed that *T.b.gambiense* could again be specifically identified as a distinct group.

1.2.4 *T.b.gambiense* type II

The main criteria to distinguish *T.b.gambiense* in the *T.brucei* group is its infectivity to humans and its origin in West Africa. It is not clear however, if *T.b.gambiense* is a true subspecies of *T.brucei* or if there is another subset of human infective *T.brucei* in West Africa as suggested by Gibson (1986). She examined the isoenzyme profiles from several separate studies involving *T.b.gambiense* (Godfrey *et.al.*, 1976; Tait *et.al.*, 1984; Gibson *et.al.*, 1980; Mehlitz *et.al.*, 1982; Zillmann *et.al.*, 1984) and found that *T.b.gambiense* could be divided into two groups, one group with limited isoenzyme variability which included the majority of isolates and one, less defined, group with greater isoenzyme variation. The latter group, which was termed Group 2, also showed variable results in blood incubation infectivity tests (Mehlitz *et.al.*, 1982; Zillmann *et.al.*, 1984) whereas the Group 1 stocks were highly resistant to human serum. When first identified as a species, the original *T.b.gambiense* was characterised by its low virulence to rodents but the defined Group 2 caused a more rapid onset and severe infection in experimental rodents (Mehlitz *et.al.*, 1982). As yet, the Group 2 *T.b.gambiense* has only been found in Ivory Coast and Burkina Faso (Gibson, 1986) but RFLP analysis by Hide *et.al.* (1994) also identified two groups of *T.b.gambiense*, one of which showed a close relationship to West African *T.b.brucei*. A further study by Godfrey *et.al.* (1987) found that some isolates collected from patients in gambian sleeping sickness areas in Ivory Coast and Burkina Faso grew quickly in laboratory animals and that the isoenzyme pattern of these isolates differed from a defined *T.b.gambiense* enzymic pattern. These studies suggest that a sub-set of *T.b.gambiense* isolates have the characteristics of *T.b.rhodesiense*.

1.2.5 Distinguishing *T.b.rhodesiense* from *T.b.brucei*

T.b.rhodesiense was first identified and described by Stephens & Fantham, (1910), but its identity as a distinct species has since been questioned initially, by Rickman & Kolala, (1980). They carried out sequential BIIT tests on successive clones produced

in vivo from 3 different *T.b.brucei* isolates, serially syringe-passaged in rats at 10-days intervals. After being initially BIIT negative, all three stocks eventually gave rise to human serum resistant clones typical of *T.b.rhodesiense*. There were also changes in VAT expression. In a follow up study using enzyme electrophoresis no enzyme variants or alleles specific for *T.b.rhodesiense* could be found and it was shown that *T.b.rhodesiense* is a more homogeneous group than *T.b.brucei* (Rickman et.al., 1984).

T.b.rhodesiense typically occurs in East Africa as a disease of low prevalence but with epidemics occurring periodically. The reasons for these epidemics are not fully understood and various factors have been considered including the occurrence of new strains, animal reservoirs, movement of human populations and the development of more virulent strains in the same region. Studies by isoenzyme electrophoresis of *T.b.rhodesiense* stocks from Botswana and Zambia have shown that they are clearly different from strains causing disease in the northern Uganda/Kenya region and there are also differences in disease patterns (Godfrey et.al., 1990; Stevens et.al., 1992). Many hundred stocks of *T.brucei* from several countries in Africa were characterised by isoenzyme electrophoresis and the data obtained indicated that specific strains have a rather limited geographical distribution and little genetic exchange takes place between stocks from different areas (Godfrey et.al., 1990). *T.brucei* zymodemes were only similar within a restricted area and many differences were found within its geographical distribution suggesting that the parasites may be adapted to different specific environments. Hide et.al. (1994), showed that *T.b.rhodesiense* populations collected in Tororo district of Uganda formed a distinct group but that this group was very different from another distinct group of *T.b.rhodesiense* collected in Zambia. From these observations it was subsequently suggested that *T.b.rhodesiense* strains from Zambia and Kenya/Uganda have different and independent origins. (Hide et.al., 1991; 1994). In order to understand the geographical spread, Komba et.al. (1997), compared Tanzanian *T.b.rhodesiense* isolates with stocks from Uganda, Kenya and Zambia and showed that there was a considerable homogeneity within the Tanzanian stocks which supports the suggestion that there are different genotypes of *T.b.rhodesiense* in each endemic region of East Africa.

An epidemic of human sleeping sickness occurred in the Tororo district of south-east Uganda in the late 1980's. After an epidemic in the 1920's, no cases of human sleeping sickness were detected until 1984 but many hundred were reported each year until 1990. Large numbers of *T.brucei* isolates were collected from infected humans, pigs, cattle and tsetse flies during these years (Maudlin *et.al.*, 1990) and analysed for variation in isoenzyme patterns and for RFLPs (Hide *et.al.*, 1990, 1991, 1994, 1996). Repetitive DNA sequences, derived from ribosomal RNA genes, were used as probes and the resulting restriction enzyme patterns were used to construct a dendrogram to define the similarity between stocks (Hide *et.al.*, 1991). The isolates were also analysed for their resistance or sensitivity to human serum. Trypanosomes collected from humans, tsetse flies and various animals from both West and East Africa were included in this dendrogram. In summary, it was shown that all isolates collected were very different from a group of *T.b.gambiense* isolated in West Africa but that the human non-infective trypanosomes, assumed to be *T.b.brucei*, were a diverse set of stocks which were distributed among several different similarity groups. Human infective and human non-infective trypanosomes were distinguishable from each other so that human serum resistant stocks all fell into one group and formed a defined separate sub-population compared with other stocks isolated from animals in Kenya and Uganda. The human serum resistant and human serum sensitive stocks were regarded as *T.b.rhodesiense* and *T.b.brucei* respectively. There was however, one exception with a stock from a human in West Africa which was indistinguishable from East African *T.b.brucei*. The human serum resistant stocks had a repetitive DNA fingerprint that was similar to that from other human serum resistant stocks from human sleeping sickness foci in Kenya and Uganda. Hide *et.al.* (1994) are the only group that claims to be able to distinguish *T.b.rhodesiense* isolates from *T.b.brucei* isolates and it has yet to be investigated whether such differences exist in other areas apart from south-east Uganda. Mihok *et.al.* (1990) analysed a series of isolates of *T.b.rhodesiense* collected from the Lambwe valley, Kenya, over a long time period and, based on isoenzyme markers, concluded that the human infective parasites were different from human non-infective trypanosomes. They suggested that the markers used were linked to genes with direct importance to

human infectivity. This was, however, only a speculation since the isoenzyme markers identified differed from those found in human isolates in Uganda by Gibson & Gashumba, (1983).

In conclusion, *T.b.rhodesiense* may be more related to *T.b.brucei* stocks in the same areas than to other *T.b.rhodesiense* stocks in different areas and *T.b.rhodesiense* is likely to be a subset of *T.b.brucei* rather than a genetically distinct type. This would explain the difficulties in finding specific isoenzyme markers that can distinguish between *T.b.brucei* and *T.b.rhodesiense*.

1.2.6 The origin of human infective trypanosomes

It can be postulated that human serum resistant trypanosomes arose from non-human infective strains, possibly through selective pressure. This can certainly occur in laboratory strains when human serum sensitive strains are made human infective by exposing them to human serum as demonstrated by Rifkin *et.al.* (1994) and Seed *et.al.* (1993). In the field this may be initiated by contact of trypanosomes with human serum in tsetse flies feeding on humans and non-human infective trypanosomes may then evolve to human serum resistant variants. However, the low prevalence of trypanosomes in humans and the epidemic nature of the disease makes it difficult to see how sufficient selective pressure could occur for human infective trypanosomes to develop. Another possibility is that genetic exchange between *T.b.brucei*, *T.b.rhodesiense* and *T.b.gambiense* could lead to the inheritance of human serum resistance into populations with minimal contact with humans. The implications of genetic exchange will be discussed in detail in section 1.5.

1.3 ANTIGENIC VARIATION IN *T.BRUCEI*

The mammal infective stages of African trypanosomes are completely enwrapped in a monolayer of a single species of glycoprotein, the variant surface glycoprotein

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(VSG). The VSG protects the parasite from components of non-specific immune responses but is itself immunogenic and stimulates a strong antibody-mediated immune response. To avoid killing by this specific immunity, trypanosomes periodically switch from expression of one VSG to that of another. Each VSG determines a different variable antigen type (VAT) and this switching process is thus termed antigenic variation (Cross, 1975). When bloodstream stumpy forms are ingested by the tsetse fly their surface-coat is rapidly lost in the midgut of the fly. This is correlated with the loss of infectivity and occurs within 48 hours (Barry & Vickerman, 1978). The surface coat is again acquired at the metacyclic stage, as a pre-adaptation for survival in the mammalian host (Vickerman, 1985).

VSGs have molecular weights of 53-63 kD and each VSG consists of an N-terminal domain of 350-400 residues and a C-terminal domain of 50-100 residues. The C-terminus is anchored to the cell surface through the covalent attachment to a glycosylphosphatidylinositol (GPI) anchor in the cell membrane (Ferguson *et.al.*, 1988). The N-terminal domain comprises two antiparallel α-helices with several smaller elements, together forming an elongated structure (Blum *et.al.*, 1993). More than 20 VSG genes have been sequenced to date and they share very little sequence homology and no common biochemical activity has been found. The only common requirement known is their ability to form a protective surface coat. The N-terminal domains share the lowest sequence homology and antigenic variation occurs through sequence variation throughout the whole of the N-terminal domain while the C-terminal domain is the more conserved region. There are, however, conserved regions both in the N- and C-terminal domains and these have been grouped into 3 classes: A, B and C for the N-terminus and, by sequence homology, into 4 classes, I, II, III and IV for the C-terminus (Carrington *et.al.*, 1991). When the variable domains of two VSGs were compared by X-ray crystallography it was shown that the tertiary structures were highly similar even though the overall sequence similarity was very low (Blum *et.al.*, 1993). It is remarkable for a family of proteins, with a similar function, to have such low homology with each other and yet form a very similar structure. The switch from one VSG to another is a gradual process so that during switching the trypanosome will initially expresses a mixed surface coat. If different

VSGs had different tertiary structures they would not fit in properly thus compromising the integrity of the surface coat. In summary, antigenic variation is most likely caused by variation in sequences and not by variation in structures.

1.3.1 Expression of VSG genes

Each VSG is transcribed from a separate VSG gene and there are several hundred VSG genes in the genome but only one VSG is expressed per cell. Expression is controlled at the transcriptional level and VSG genes can only be expressed from one of the approximately 20 VSG expression sites (ES). Only one of the ESs is active at any given time (Borst & Rudenko, 1994). The most common mechanism for switching between VSGs is for a non-expressed VSG gene to be copied into an expression site and replace the previously expressed VSG gene. There are also VSG genes which normally reside in expression sites and can be activated where they are by stopping transcription from one ES and simultaneously starting transcription from another. Alternatively, VSG genes can be transposed between ESs. The activation of one site and inactivation of another appear to be independent, spontaneous processes but the mechanisms of activation/inactivation remain unknown (Borst *et.al.*, 1997). In an ES, a VSG gene is associated with, at least, eight other genes referred to as expression site associated genes (ESAGs) that are co-transcribed as a single polycistronic transcription unit (Pays *et.al.*, 1989; Cully *et.al.*, 1985).

VSGs are not expressed at random but in a semi-predictable order in an infection. The order of VSG gene expression in *T.brucei* appears to correlate to the ease with which VSGs are switched on so that telomeric genes in expression sites that are more easily activated tend to appear first in an infection. Other telomeric genes are then expressed because they show good homology with the active expression site and these are followed by expression of non-telomeric genes with good homology at both 3' and 5' ends and finally genes with poor homology in flanking sequence with the expression site will be expressed (Borst, 1991).

Metacyclic trypanosomes also have a VSG coat which protects them from lysis by host serum factors. VSG genes are activated and expressed differently in the metacyclic and bloodstream stages. The expression of VATs by metacyclic trypanosomes shows much more predictability than VATs by bloodstream trypanosomes. There are no more than 27 metacyclic VATs (M-VATs) in *T.b.rhodesiense* and there is no switching between different VSGs in metacyclic forms. Instead of antigenic variation, there is a mixture of VATs present in the metacyclic population before they enter a mammal (Barry *et.al.*, 1983). This occurs by simultaneous and random activation of individual telomeres containing the M-VSG repertoire and the VSG genes in each M-expression site are conserved so that the M-VSG repertoire is predictable (Tetley *et.al.*, 1987). M-VSG genes all occupy the telomeres of the largest chromosomes (Barry *et.al.*, 1990). The advantage of having a mixture of VSGs is thought to derive from the improved probability of establishing infection in semi-immune hosts.

Procyclic trypanosomes do not express VSG genes. Instead they produce an abundant invariant surface protein called the procyclic acidic repetitive protein (PARP) or procyclin (Roditi & Pearson, 1990).

1.3.2 VSG switching during trypanosome infection

A trypanosome infection can be induced by a single trypanosome after which a parasitaemia takes the form of waves peaking at irregular intervals. The profile of the parasitaemia can take a variety of shapes including small sharp peaks arising from a background of more or less permanent parasitaemia or regular or irregular peaks of parasitaemia. These differences in parasitaemia patterns are reproducible and only partly determined by the host's immune response (Barry & Turner, 1991). For antigenic variation to be successful in evading the immune system, the different VATs must appear consecutively and this takes place in a semipredictable way, in a hierarchical but ill-defined sequence of expression. The switching rate is approximately 100 switches per generation (Turner & Barry, 1989). That is, at least one in every 100 trypanosomes switches its VAT expression every generation. Some

VATs have a high tendency to appear early in infection whereas others are more common later in infections even though this relationship is not totally fixed. VATs appearing early in infection continue to be produced throughout infection but these trypanosomes are killed off by the immune response. Factors involved in determining the pattern of parasitaemia involve the growth rate and trypanosome population size in a particular host species, the overall number of different antigenic variants and the hosts' immune response. However, no adequate model of how the hierarchy of switching occurs has been proposed.

Switching is not induced by antibody since switching in VSG expression also occurs in immuno-compromised animals and in *in vitro* cultures (Doyle *et.al.*, 1980). The presence of other bloodstream populations in lymphatics or extravascular sites does not influence the rate of switching and it is thought that selective pressure from the hosts' immune response is important for the generation and maintenance of the high rate of switching (Turner & Barry, 1989).

1.3.3 Surface receptors of *T.brucei*

The entire external surface of *T.brucei* is covered by VSG molecules with the exception of the flagellar pocket. The flagellar pocket is an invagination in the surface at the site of flagellar attachment and forms 1-3 % of the total cell surface. The layer of VSG molecules is much less dense in the flagellar pocket and receptors for the uptake of host macromolecules such as transferrin and low density lipoproteins (LDL) are located in the pocket. The presence of surface receptors for these large molecules has been demonstrated by showing that uptake of transferrin and LDL occurs much faster than fluid phase endocytosis and that uptake is saturable (Coppens *et.al.*, 1987). LDL and transferrin are required by the bloodstream stages of *T.brucei* for their growth (Coppens *et.al.*, 1988; Schell *et.al.*, 1991). All the available iron in the blood of mammals is bound by transferrin and the concentration of free iron is reduced to a level which cannot support growth of micro-organisms so the

specific uptake of transferrin is a method for trypanosomes to overcome this limitation in iron availability (Coppens *et.al.*, 1987).

The structure of LDL and transferrin molecules may vary between mammals but they are invariant within a specific host and the receptor binding site must therefore also be relatively invariant. The receptors for these molecules may extend out beyond the VSG coat but it has been suggested that in order not to be recognised by the immune system they may be capable of some variation (Borst, 1991). ESAG 6 and 7 together form a transferrin binding protein complex so that the transferrin receptor of *T.brucei* is encoded by ESAG 6 and 7. This means that the parasite could modify the receptor by switching to another ES as different members of the ESAG 6/7 gene family vary in gene sequence. Bitter *et.al.* (1998), have shown that the small differences between transferrin receptors can be very important and allow *T.brucei* to adjust to the large sequence diversity in transferrin from different hosts.

The most abundant surface protein, after the VSGs, are the invariant surface glycoproteins (ISGs). The functions of the ISGs are not yet known and the sequences of the genes for ISG-65 and ISG-75 do not show homology to any known proteins (Ziegelbauer *et.al.*, 1992). They are distributed over the whole surface of the cell and the flagellum but they are not readily accessible to antibodies in live cells because they are 'shorter' than the VSGs so that binding is prevented (Overath *et.al.*, 1994). Invariant surface proteins are expressed at a level 100-10 000 fold less than VSGs (Overath *et.al.*, 1994). Several other non-VSG proteins are also thought to have the same kind of dimensions as a VSG N-terminal domain and examples of these non-VSG surface molecules with the same tertiary structure, apart from the ISGs, are the polypeptides produced by ESAG 6 and 7 and the procyclin associated gene 1 (PAG1) product (Carrington & Boothroyd, 1996).

1.4 HUMAN INFECTIVITY

1.4.1 Sensitivity of African trypanosomes to human serum

Identification of the *T.brucei* subspecies pathogenic to humans was originally dependent on human volunteers. The first demonstration that animals can be reservoirs of *T.b.rhodesiense* was made by the use of human volunteers (Heisch *et.al.*, 1958) and human volunteers were also used to show that *T.b.gambiense* and *T.b.rhodesiense* can retain their infectivity even after long periods of cyclical transmission by tsetse flies (Willett & Fairbairn, 1955). These unethical infectivity assays have now been replaced with new methods and several trypanosome species have been tested for their sensitivity/resistance to normal human serum using both *in vitro* and *in vivo* assays. Isolates of the cattle trypanosome *T.b.brucei*, the horse species *T.equinum* and *T.equiperdum* (Verducci *et.al.*, 1989) and isolates of the camel parasite *T.evansi* are all lysed by the presence of human serum while the human infective subspecies *T.b.gambiense* and *T.b.rhodesiense* are resistant and can withstand lysis by normal human serum (Seed *et.al.*, 1990; Paindavoine *et.al.*, 1986; Van Meirvenne *et.al.*, 1976). Most isolates, but not all, of the cattle trypanosome, *T.congolense*, are also lysed when incubated with human serum (Hajduk *et.al.*, 1994).

When discussing the susceptibility or resistance to trypanosome infections it is important to separate the effect of the host's immune response, both specific and non-specific, and the non-immune, innate resistance that results in host selectivity. In addition to human serum, serum from other primates can have a killing effect upon *T.b.brucei* and a survey by Seed *et.al.* (1990) showed that baboon serum had the highest trypanocidal activity followed by serum from human, mandrill and gorilla. Many other host species show some degree of tolerance to trypanosome infection. Parasitaemia can be controlled in the animal by specific and non-specific antibodies and by cell mediated responses although naturally evolved, non-immune serum factors may also play an important role (Mulla & Rickman, 1988; Muranjan *et.al.*, 1997).

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The stability of the human serum resistant (HSR) or human serum sensitive (HSS) phenotypes appears to vary between isolates. Most isolates of *T.b.gambiense* appear to be stable for the HSR phenotype as demonstrated by the fact that isolates maintained in laboratory animals for long periods are still capable of infecting humans. The HSR phenotype of many *T.b.rhodesiense* isolates can, on the other hand, be lost if passaged in laboratory rodents for prolonged periods (Yorke *et.al.*, 1930; Rifkin *et.al.*, 1994; Rickman *et.al.*, 1982). Some *T.b.brucei* isolates have been made resistant to human serum by repeatedly injecting infected animals with human or baboon serum in sub-curative doses but others appear to have a stable HSS phenotype (Rickman & Kolala, 1980). It is however, unclear whether the HSS isolates which have been made serum resistant can infect humans. Old data implies that human volunteers did not develop a parasitaemia after infection with such strains (Yorke, *et.al.*, 1930). In these isolates, which can be changed from resistance to sensitivity, or from sensitivity to resistance, extensive passaging in mice or rats is required perhaps implying that this is not a single step process. Ortiz *et.al.* (1994) reported that it took at least one year to convert *T.b.gambiense* to a sensitive state (24-36 mouse passages). It always took longer for clones to acquire resistance than it took for clones to revert to sensitivity. Studies on *T.b.rhodesiense* and *T.b.brucei* report a similar time scale and number of passages for change in sensitivity/resistance (Hawking, 1976; Rifkin *et.al.* 1994). At least one *T.b.rhodesiense* has however, been passed through sheep and other animals for 23 years without contact with human serum but was still human infective at the end of the experiment (Ashcroft, 1959).

A number of studies have indicated a change in HSS or HSR phenotype is associated with a change in VAT expression (Hawking, 1977; Rickman & Kolala, 1980; Rickman *et.al.*, 1981; Gibson, 1989; Van Meirvenne *et.al.*, 1976) but no in-depth studies were completed until Rifkin (1994) analysed 3 pairs of HSS and HSR *T.b.rhodesiense*. No correlation between changes in resistance and VAT expression was observed. In a parallel study, using *T.b.gambiense*, a change in VAT was always observed when there was a change in human serum susceptibility (Ortiz *et.al.*, 1994).

Each time a HSR clone was selected from a HSS clone, by gradually increasing the amount of normal human serum (NHS) injected into an infected mouse, they seemed to change their VAT. The same VAT was therefore never observed in both resistant and sensitive clones. It may well be that there is great variation in stability between different stocks for reasons that will be discussed below.

Fresh human serum can have a trypanocidal effect upon both procyclic *T.b.brucei*, which otherwise are resistant to human serum, and upon bloodstream *T.b.rhodesiense* (Rifkin, 1978). This is probably due to non-specific activation of the complement system. When the serum is heat-treated at 56° C for 30 minutes to inactivate complement, this sensitivity disappears. Non-heat inactivated fresh sera from rat, guinea pig, mouse and rabbit, on the other hand, have been reported to have no effect upon trypanosomes and are therefore often used as control sera in sensitivity/resistance assays (Rifkin, 1978). Early studies showed that a trypanosome lytic factor was present in human serum which was non-immune and very unlikely to involve the host's complement pathway. The basis for this conclusion was mainly that heat-inactivation of both the classical and alternate pathways of complement has little or no effect on the trypanocidal activity (Rifkin, 1978). Furthermore, human serum did not cause the *T.brucei* group trypanosomes to agglutinate and no decrease in trypanosome killing could be noted when the IgG fraction of an anti-IgM serum was used to absorb out human IgM (Rifkin, 1978).

Apart from certain stocks being resistant or sensitive to human serum there are also differences in susceptibility between the various developmental stages in the life cycle. All of the bloodstream stages of *T.b.brucei* are susceptible to human serum even though the dividing slender forms are more sensitive than the non-dividing stumpy forms, probably due to the slender forms having a higher rate of endocytosis (McLintock *et.al.*, 1993). Procyclic stages are all sensitive to complement mediated lysis but they are resistant to killing by heat-inactivated human serum. This is probably because procyclics take up macromolecules by a mechanism different from that of the bloodstream and metacyclic forms and some studies have shown that procyclics do not endocytose macromolecules at all and therefore will avoid any

cytotoxic factors in the NHS (Vickerman, 1985). If bloodstream *T.b.brucei* are incubated under conditions that promote the differentiation into procyclics they will develop resistance to NHS (Moore *et.al.* 1995). Long slender forms, that cannot differentiate into procyclics, did not develop any resistance when they were kept under the same conditions. Differentiating stumpy forms further showed a decrease in protein synthesis and it was suggested that a protein required for binding and endocytosis of a cytotoxic factor is lost during the differentiation from bloodstream forms into procyclics (Moore *et.al.*, 1995).

1.4.2 Methods for measuring HSS/HSR

Originally, the only way to test a particular strain of trypanosomes for its infectivity to humans was to inject it into human volunteers (Heisch *et.al.*, 1958; Willett & Fairbairn, 1955). For obvious ethical reasons, there was a need for a reliable method for measuring HSS/HSR to replace these volunteer studies. To this end, Rickman & Robson (1970) developed an assay, involving the use of rodents instead of humans, known as the blood incubation infectivity test (BIIT). In this assay trypanosomes were isolated and incubated in the presence of human blood for 5 hours at 37°C and then injected into mice or rats. Next, the animals are screened for parasitaemia and, if an infection developed, the initial trypanosomes are scored as resistant to human serum (HSR) and if not, they are referred to as human serum sensitive (HSS). The BIIT proved to be a valid and reliable test where all *T.b.rhodesiense* (isolated from humans) gave positive results and strains which had shown to be human non-infective, and presumed to be *T.b.brucei*, gave a negative BIIT result (Rickman & Robson, 1970). There were however, a few strains which gave ambiguous results in that the parasitaemia took much longer to develop and retests were negative. The tests were repeated a large number of times with the same results.

In vitro methods have been developed based on the BIIT, which consist of microscopically counting the percentage lysis caused after a certain time of incubation in human serum (Rifkin, 1978) or, as developed by Tomlinson *et.al.*

(1995), the use of a probe, acetoxymethyl (AM) ester of calcein, for measuring the viability of cells. AM-calcein is an uncharged, colourless and non-fluorescent ester which enters the trypanosomes and, in viable cells, is cleared by intracellular esterases resulting in the generation of the fluorescent free acid inside the cells. These *in vitro* methods allow comparison between stocks and/or clones but cannot determine whether a particular stock is capable of causing a parasitaemia in humans or other mammals. Brun *et.al.* (1981) described an *in vitro* culture system for bloodstream *T.brucei* in which *T.b.gambiense* and *T.b.rhodesiense* stocks could survive and grow in the presence of human serum while *T.b.brucei* was killed. This *in vitro* test consisted of a feeder layer of fibroblast-like cells from *Microtus montanus* embryos, culture medium and normal human serum (NHS). The cultures were maintained for 10 days after which HSS stocks were neutralised by NHS while HSR stocks showed continuous growth in the presence of human serum (Jenni & Brun, 1982). This method was however, not widely used since it has been proven difficult to reproduce in other laboratories.

It has been argued that it would be more appropriate to test resistance to human serum in metacyclic forms instead of in bloodstream forms since these are the stage which first encounter human serum when injected by a tsetse fly. Brun & Jenni (1987), carried out initial tests of metacyclic forms and the resulting bloodstream forms of representative stocks of *T.b.brucei, T.b.gambiense* and *T.b.rhodesiense* using the above method. It is open to question however, whether this test genuinely measures the HSS/HSR of metacyclics since within 1 day in culture, all of the metacyclic forms have transformed into bloodstream forms. However, it would be possible to analyse metacyclics by the BIIT but this has not been undertaken.

In conclusion, while there are reliable and reproducible methods for measuring the resistance or sensitivity of trypanosomes to lysis by human serum, it is unclear whether these fully reflect the ability of the trypanosomes to infect humans. A number of representative assays have been developed that vary in the degree of simplicity of screening but, given that human experiments cannot be undertaken, it is difficult to fully evaluate how well they reflect true human infectivity.

1.4.3 Intermediate resistance to human serum

A particular feature of HSS/R assays that has tended to be overlooked in recent years is that intermediate levels of resistance can be detected in some stocks or lines of trypanosomes. These intermediate levels of resistance have been attributed to the presence of unknown factors in the test animal, inaccuracy in the test method, artefacts or have simple been ignored (Targett & Wilson, 1973; Geigy *et.al.*, 1975; Mehlitz & Feddersen, 1987; Rifkin, 1984; Jennings & Urquhart, 1985). It is entirely possible however, that trypanosomes with intermediate levels of resistance represent a valid and specific phenotype. Rickman & Robson, (1980), found intermediate levels of resistance while developing the BIIT when two *T.brucei* stocks repeatedly showed infectivity for some, but not all, rats. Metacyclic forms can also exhibit intermediate resistance. When Brun & Jenni (1987), analysed stocks of *T.b.brucei*, *T.b.rhodesiense* and *T.b.gambiense* they found inconsistent results from resistance assays within a group of *T.b.rhodesiense* stocks. If these isolates were passaged in rodents the resistance disappeared completely whereas if they were cultivated in the presence of human serum they all became resistant.

A key feature in discussing findings of intermediate resistance is whether the stocks used for analysis are clones as uncloned stocks could be a mixture of distinct human serum sensitive and human serum resistant trypanosomes. An artificial model of such a mixed strain was tested by Hawking (1976) who mixed a very small number of cells from a highly resistant population with one million cells from a sensitive stock and could show that when no human serum was present infection was dependent on the size of the inocula but in the presence of human serum infection was instead dependent on the small number of resistant cells. Hawking explained his finding of sub-resistant strains as being due to the presence of a mixed population, and the level of resistance depended on the number of resistant individuals in it. All the trypanosomes used in the study were uncloned strains.

The existence of trypanosomes with intermediate levels of resistance may be explained by there being one or two genes responsible for resistance and the intermediate, or sub resistant, strain contains a mixture of cells without the resistance gene/genes but also a few with the resistance gene/genes (Hawking, 1976). Other possible explanations may involve gene amplification, the presence of several mechanisms for resistance or resistance being a particularly labile trait prone to mutations so that they are generated repeatedly from a sensitive stock. A spontaneous switching from sensitive to resistant would probably also cause strains to appear of an intermediate resistance but since converting a sensitive strain to a resistant strain or *vice versa,* seems to take at least 25-30 mouse passages (Rifkin *et.al.,* 1994; Ortiz *et.al.,* 1984; Rickman *et.al.,* 1982; Rickman & Kolala, 1980) a complete switching may appear unlikely. Alternatively, the whole population of cells may express an intermediate level of resistance between fully resistant and sensitive.

1.4.4 The trypanolytic effect of human serum on HSS trypanosomes

Trypanosomes which are sensitive to human serum will go through a series of morphological changes prior to lysis when incubated with human serum. The cytotoxic factors in the serum cause acute, irreversible damage to the plasma membrane and the cell will eventually die. The morphological changes are consistent with a colloid osmotic mechanism (Rifkin, 1984). First there is a lag phase when no visible damage can be seen on the trypanosomes. This lag phase usually lasts 20-40 minutes regardless of the serum concentration used. The morphological changes of cells incubated with NHS cannot be reversed even if they are exposed to serum for only 10 minutes, washed and placed in non-cytotoxic medium. After 2 hours these cells showed the same changes as cells which had been incubated with human serum for 2 hours (Rifkin, 1984). A decrease in protein synthesis starts after an approximately 30 minutes lag phase. After the lag phase the cells appear distorted and swollen with a prominent flagellum but they are still motile. Complete lysis occur within 2 hours when only a cell ghost with a prominent nucleus can be seen. When the ultrastructure of the cells was examined 20 minutes after the start of

incubation, a decrease in cytoplasmic staining with uranyl acetate and lead citrate was observed (Rifkin, 1984; Hawking *et al.* 1973). The cytoplasm becomes pale and vacuolated and finally appears to disintegrate. The structure of the mitochondrion, ribosomes and nucleus did not change however, and morphological changes in the cell membrane could only be observed late in the lytic process. Rifkin, (1984) demonstrated that lysis by human serum led to an osmotic imbalance and a rapid efflux of K^+. The K^+ efflux and the lysis could be prevented by the presence of low levels of sucrose or dextran during *in vitro* incubation and the cells remained of slender morphology with normal motility under these conditions. Rifkin gives two explanations for this apparent protection. Firstly, sucrose may be a competitive inhibitor of the trypanocidal factor, or, secondly, sucrose might simple block the cell swelling and lysis caused by osmotic imbalance without interfering with the trypanocidal factor at all (Rifkin, 1984).

1.4.5 Potential factors involved in human serum mediated lysis

HDL as a lytic factor (TLF1)

Rifkin (1978) was the first to isolate a potential candidate for the trypanocidal factor in human serum by showing that trypanolytic activity coincides with human high density lipoprotein (HDL). Human HDL was purified by density gradient centrifugation or size-exclusion chromatography and in both cases a fraction of each had the ability to kill trypanosomes. When the lytic ability of the fraction was compared with that of intact human serum it was discovered however, that the peak of trypanosome lytic activity overlapped, but did not actually coincide, with the eluted HDL. It was therefore suggested that the lytic factor is a minor part of the purified HDL or even a specific HDL serum subclass.

Human lipoproteins consist of neutral lipids with a shell of phospholipids and apolipoproteins. Their function is to transport insoluble cholesterol, cholesterol esters and triglycerides to peripheral tissues and to the liver. This will ensure adequate amounts of cholesterol for membrane assembly, steroid hormone synthesis and bile

acid production. There are four major classes of lipoproteins, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and chylomicrons and each is characterised by the addition of specific lipids and apolipoproteins (Illingworth, 1993).

Hajduk *et.al.* (1989) supported the idea that the lytic factor is a specific HDL serum subclass. Human serum was fractionated by gel filtration chromatography and the lytic factor was found only in a very minor subclass of HDL which is now named the trypanosome lytic factor 1 (TLF1). The majority of serum HDL was subsequently shown to be non-toxic to trypanosomes. TLF1 consists of very large HDL particles with an estimated size of 500 kDa (average size of human HDL is 250 kDa) and is unique in that it contains two apolipoprotein complexes, apoLI and apoLIII which are not found in any of the other four human lipoproteins mentioned. It does however contain apoAI and apoAII which can be found also in the non-lytic human HDL fraction and may indicate its close relationship with HDL. ApoAI, apoLIII and apoLI are, according to Tytler *et.al.* (1995), all needed for trypanosome lysis by reconstituted TLF1 particles but individually they cannot cause lysis. ApoLIII in particular appears to be essential for the formation of a cytotoxic particle and either apoAI or apoLI must also be present. ApoAII, on the other hand, is not required for lysis to take place. Human serum lipids were needed for a correct assembly of the TLF1 particle but may not be involved in lysis directly. A second set of data that support the idea that TLF1 is a trypanosome lytic factor is that antibodies produced against apoLI and apoLIII can inhibit lysis by purified TLF1 (Seed *et.al.* 1990). This is however not true for antibodies against apoAI and apoAII which have no effect on the lytic ability. This has been explained by competition for antibody binding between TLF1 and non-lytic HDL since the concentration of non-lytic HDL in serum is several hundred fold higher than the concentration of TLF1 (Seed & Sechelski, 1989).

Apolipoprotein as the sole toxic factor

In contrast to these data suggesting that several components of HDL are required for lysis, other studies have suggested that apoA-1 is the sole trypanosome lytic factor in

TLF1 (Owen *et.al.*, 1992; Gillet & Owen, 1992). Purified apoAI from human, baboon, sheep and cattle HDL was incubated with *T.b.brucei* and it was shown that human and baboon apoAI lysed trypanosomes both when it was free in solution and when associated with lipids (Gillet & Owen 1992). Sheep and cattle apoAI did not have any significant effect on *T.b.brucei*. Owen *et.al.* (1992) went on to test the trypanolytic effect of sera from transgenic mice expressing human apoAI levels similar to those found normal human sera. The authors report the paradox that sera from these mice had a raised ability to lyse trypanosomes *in vitro*, compared with control mice, but when trypanosomes were exposed to the same sera *in vivo*, the infected mice showed no resistance to the infection at all and developed patent parasitaemias at the same rate as normal mice. Furthermore, sera from patients with apoAI deficiency had no measurable activity against *T.b.brucei* (Owen *et.al.*, 1996). The view that human apoAI alone can cause lysis of *T.b.brucei* is not shared by a number of other investigators (Rifkin, 1991; Tomlinson, *et.al.* 1995). Rifkin (1991) tested extracted apoAI from human, rabbit and rat HDL on *T.b.brucei* but found no lytic activity either with purified free apoAI or with apoAI reconstituted with HDL lipids. Serum from transgenic mice expressing the human apoAI gene did not have any elevated trypanocidal effect in this study and the same is true for a study by Tomlinson *et.al.* (1995). None of the authors offer any explanation for these contradictory results and the only differences in the assays consists of the use of different strains of *T.b.brucei* and that Gillet & Owen (1992) used twofold higher concentrations of purified apolipoprotein which might increase its lytic ability.

Can serum from HDL deficient people cause lysis?

There are other data concerning the trypanolytic factors in human serum that have caused much controversy and one issue is whether serum from patients suffering from Tangier disease has the ability to lyse HSS trypanosomes. Tangier disease is a rare autosomal recessive disorder which causes a severe lack of HDL, not exceeding 5% of the normal concentration, and, if the trypanocidal factor is confined to the HDL fraction serum from these individuals, it would be predicted to lack the ability to lyse trypanosomes. Rifkin (1978) noted that serum from persons with diagnosed Tangier disease did indeed lack trypanocidal activity. But when a new fluorescence

based, viability assay was used to measure parasite death caused by serum from patients with Tangier disease (Tomlinson *et.al.*, 1995) the results did not support Rifkins (1978) finding. The percentage lysis of parasites was as high with serum from individuals with Tangiers disease as with normal human serum. These findings are in conflict with the idea that the lytic factor is always associated with HDL and has encourage the investigation into alternative lytic factors. Human HDL is itself a very heterogeneous particle and by examining various fractions of HDL, Lorenz *et.al.* (1994) found trypanolytic activity in several particles with different properties.

Another lytic factor? (TLF2)

Barth (1989) using a novel procedure, first isolated a cytotoxic particle from pooled normal human serum with a molecular weight >1000 kDa. The protein was composed of four polypeptides, none of which appear to be known apolipoproteins, according to size estimates, suggesting that the protein may not be part of HDL. This would support the idea that TLF1 is not the only fraction with trypanosome lytic activity. The isolated fraction had the same lytic effect on *T.b.brucei* as human serum used in a control assay. Owen *et.al.* (1996) identified the particle as the μ-heavy chain of IgM and two light chains of Ig but this work was not pursued further since Rifkin (1978) convincingly had shown that IgM is not important in trypanolysis. Hajduk *et.al.* (1994) were not convinced that the >1000 kDa protein is not a HDL particle and argued that the size of one of the proteins in the trypanocidal fraction was consistent with being apoAI because its initial size estimation had been wrong. The novel trypanolytic particle also loses its lytic activity when treated with anti-α-lipoprotein serum (Owen *et.al.*, 1996).

Other investigators have also found the presence of a trypanolytic >1000 kDa fraction distinct from HDL. Tomlinson *et.al.* (1995) size fractionated proteins from both NHS and Tangier patients serum and found two distinct peaks of lytic activity against *T.b.brucei*. One of the peaks correlated with the described >1000 kDa particle but contained no apoAI while the other peak resembled the HDL and very likely include the TLF1 fraction described by Hajduk *et.al.* (1989) and Tytler *et.al.*, (1995). The >1000 kDa fraction, which can be routinely separated by gel filtration and which

is thought not to be a lipoprotein has been named TLF2 by Raper *et.al.* (1996). Partially purified TLF2 binds specifically to *T.b.brucei* in a saturable manner (Tomlinson & Raper, 1996). This may suggest the presence of a specific TLF2 receptor. It must therefore be considered likely that human serum mediated lysis of *T.b.brucei* is not an exclusive property of human serum HDL, and that at least one other factor may be involved.

1.4.6 Possible mechanisms for the action of TLF

The search for the toxin within the TLF still continues. Smith *et.al.* (1995) identified the specific toxin as human haptoglobin related protein (*hpr*). Haptoglobin normally exists as an abundant serum protein which can bind haemoglobin after which it is removed from the blood by the liver. The *hpr* is similar to haptoglobin in that it can bind haemoglobin and it is also a component of the very high density subclass of HDL. When the NH_2-terminal sequences of apoLI and apoLIII were determined they were found to be identical to that of both haptoglobin and *hpr*. Further evidence for the importance of *hpr* in trypanosome lysis was that antibodies against human *hpr* could inhibit TLF1 mediated lysis (Smith *et.al.*, 1995). The proposed mechanism of lysis by *hpr* is as follows: the haptoglobin related protein in TLF1 binds haemoglobin and is endocytosed and incorporated into lysosomes. At this low pH, the *hpr*-haemoglobin complex has peroxidase activity which is damaging to trypanosomes as they lack catalase. The *hpr* cleaves peroxide present in the cell leading to the formation of hydroxyl radicals, disruption of lysosomal membranes, oxidative damage and ultimately cell death. Catalase was able to inhibit TLF1 mediated lysis thus supporting the model. Purified TLF1 shows a higher degree of lytic activity than the same amount of TLF1 diluted in human serum and it was further shown that the loss of inhibitor activity was associated with the loss of haptoglobin. Subsequently, haptoglobin was suggested as a natural inhibitor of TLF1 trypanocidal activity (Smith *et.al.*, 1995). Haptoglobin has on the other hand, been reported to have no effect on the lytic activity of TLF2 (Raper *et.al.*, 1996). An interesting observation is that many individuals living in areas of sleeping sickness

have genetically lower blood-haptoglobin levels than the average population (Pintera, 1971) but the trypanolytic effect of TLF1 may still be active. In addition, these people may have increased levels of *hpr* (S.Hajduk, personal communication, 1998).

Betchart *et.al.* (1989) were the first to suggest that trypanolysis involved binding of trypanolytic HDL to cell surface receptors followed by the uptake and transport of the particle to acidic intracellular compartments. Lorenz *et.al.* (1994) later provided evidence that acidic intracellular compartments are indeed involved in HDL-mediated trypanolysis. Weak bases that raise the intracellular pH and inhibit degradation of endocytosed macromolecules also inhibit trypanolysis. This is equally true for TLF1- and TLF2-mediated lysis which would imply that they both may need to enter an intracellular acidic vesicle to be cytotoxic. The same group also showed that *T.b.rhodesiense* can bind and accumulate the same amounts of radiolabelled HDL as the HSS *T.b.brucei* and therefore resistance can not be explained simply by a lack of ability to bind or internalise trypanolytic HDL particles (Lorenz *et.al.*, 1995). It may therefore be more likely that differences between HSS and HSR stocks are due to differences in intracellular processing of the endocytosed HDL-particle. This idea has been further developed by Hagar & Hajduk (1997) who demonstrated that both HSS and HSR *T.b.rhodesiense* were capable of receptor mediated endocytosis of macromolecules like TLF1 but HSR *T.b.rhodesiense* did not accumulate TLF at the same rate as HSS *T.b.rhodesiense*. HSR *T.b.rhodesiense* bound TLF in the flagella pocket but did not accumulate TLF intracellularly while HSS *T.b.brucei* both bound and accumulated TLF in endocytic vesicles thought to be lysosomes. Further support for this observation comes from the observation that TLF1 mediated lysis is temperature sensitive and is completely inhibited at $17^\circ C$ and below (Hajduk *et.al.*, 1989; Rifkin, 1978). Binding, but not endocytosis, does however occur, as cells incubated with TLF1 for a short time at $17^\circ C$ and then washed, will still lyse if the cells are moved to $37^\circ C$. Electron and fluorescence microscopy of trypanosomes incubated with gold-labelled TLF shows that TLF binds specifically in the flagella pocket and to the flagellum at $4^\circ C$, $17^\circ C$ and $37^\circ C$ but is only free or within large vesicles when the cells are at $37^\circ C$. These cytoplasmic vesicles disrupted before the onset of cell lysis (Hagar *et.al.*, 1994).

If a component of normal human serum is responsible for trypanosome lysis and is taken up by cells by a specific and saturable mechanism it would be predicted that the activity of this factor would decrease as parasite density increased. An early study by Rifkin (1983) could not confirm this prediction since, according to her data, binding appeared to be independent of trypanosome cell concentration. Several more recent studies do however, suggest that binding of TLF1 to trypanosomes is saturable and that TLF1 requires receptor mediated endocytosis (Hager *et.al.*, 1994; Lorenz *et.al.*, 1995; Ortiz & Seed, 1995). The study by Ortiz and Seed (1995) also implied that HSS clones removed more trypanolytic factors than HSR clones.

1.4.7 Individual differences in trypanolytic effect

It is interesting to note that sera from different individuals vary greatly in their level of lytic activity. It seems however as if it is only whole NHS from different individuals that differs in its ability to kill trypanosomes (Rifkin, 1978), while isolated TLF1 from different individuals had similar levels of killing activity (Hajduk *et.al.* 1989). Purified TLF1 normally has a more than 3-fold higher killing activity compared with the whole HDL fraction. When other fractions of HDL were mixed with TLF1 however, the killing activity reached the same levels as in human serum which would suggest that there is an antagonistic factor to TLF1 in human serum. It may be the presence of this inhibitory factor that accounts for individual variation in trypanosome killing activity. Smith & Hajduk (1995), conducted experiments indicating that haptoglobin is present in the inhibitory fraction of TLF1 in HDL and that haptoglobin on its own inhibits TLF1 in the same way as the whole lipoprotein-deficient fraction of human serum. It might be the ratio between haptoglobin and the haptoglobin-related protein in serum that determines an individuals capacity to kill trypanosomes. The only report arguing against this theory is that of Lorenz *et.al.* (1994) who found that the size and density of the trypanolytic factor differed considerable between individual serum donors. Individual variation in killing by TLF2 has not yet been investigated.

1.4.8 Trypanocidal factors in non-human mammals

When trying to identify the lytic factor in NHS it is potentially useful to make comparisons with trypanocidal factors found in animals which have co-evolved with African trypanosomes and which can tolerate infections with few or no signs of disease. Certain wild African animals can resist or control *T.brucei* parasitaemia partly due to the production of IgM, IgG1 and IgG2 directed against specific trypanosomal antigens (Rurangirwa, 1986). There may also be other innate, trypanocidal factors active against some strains which aid wild animals in tsetse infested areas in protection against trypanosomiasis (Mulla & Rickman, 1988).

Muranjan *et.al.* (1997) studied serum collected from cape buffalo which are known to be able to restrict infection to a low level parasitaemia by mechanisms not involving production of protective antibodies (Reduth *et.al.*, 1994). Cape buffalo serum has the capacity to kill *T.b.brucei*, *T.b.rhodesiense* and *T.b.gambiense in vitro*. A number of findings indicated that the trypanocidal factor was a protein with a molecular mass of 150 kDa and this has been identified as xanthine oxidase. Xanthine oxidase is an enzyme that converts purines such as hypoxanthine and xanthine to uric acid. Bloodstream trypanosomes rely on the catabolism of glucose to pyruvate for their energy supply. Hydrogen peroxide, which is generated during catabolism of oxypurine by the catabolic enzyme xanthine oxidase, directly inhibits trypanosome glycolysis. Resistance to trypanosome infection is therefore induced by the ability to accumulate large amounts of hydrogen peroxide in the serum which will lead to the lysis of trypanosomes.

The evidence that xanthine oxidase mediates lysis of trypanosomes was provided by immunodepletion experiments using an antibody to xanthine oxidase. The depleted sera had no lytic activity showing the importance of xanthine oxidase. The origin and secretory pathway of serum xanthine oxidase is not known but the enzyme is found in the cytosol in a variety of cells. Cow and mouse sera do not accumulate hydrogen

peroxide during catabolism of xanthine by xanthine oxidase and cows and mice are also susceptible to the trypanosome species mentioned above.

Few studies have been conducted on human serum killing of Trypanosoma species other than the *T.brucei* group. Verducci *et.al.* (1989) concluded that natural antibodies of the IgM class, not HDL, are responsible for lysis of *T.equiperdum*. Only human serum and not serum from laboratory animals, sheep, horses or cattle could agglutinate *T.equiperdum* and eventually caused lysis. This group were also unable to detect significant trypanocidal activity of purified human HDL either *in vitro* or *in vivo*.

1.4.9 The serum resistant associated gene

Since no biochemical differences could be found between resistant and sensitive strains of *T.b.rhodesiense* (Rifkin *et.al.*, 1994), attention has focused on finding genes responsible for changes in human serum sensitivity. Genes differentially expressed in the resistant and sensitive phenotypes from a common cloned line of *T.b.rhodesiense* (EATRO 3) were therefore studied by looking for differentially expressed mRNAs by subtractive hybridisation (De Greef *et.al.*, 1989). A transcript of 1.5 kb was found to be unique for all serum resistant sub-clones while it was absent in serum sensitive forms of the same stocks and VATs. This led the authors to suggest that a resistance-specific transcript was associated with serum resistance in *T.b.rhodesiense* and that switching from sensitive to resistant forms involved expression of a specific gene. Since expression of resistance or sensitivity could occur in subclones expressing the same VAT, switching between phenotypes was independent of VSG switching (De Greef *et.al.*, 1989). The resistance-specific cDNA (mRNA) was later sequenced and named the serum resistance-associated (SRA) gene (DeGreef & Hamers, 1994). The SRA gene encodes a protein similar in sequence to VSGs (De Greef *et.al.*, 1992) with the best fit in the carboxy-terminal part of the molecule. It was concluded that the resistant-specific mRNA codes for a protein with some homology with VSG (De Greef & Hamers, 1994) but with a smaller overall

size. The lack of an N-terminal leader sequence of the protein would, however, mean that it is not inserted into the membrane. Several related genes, or possible pseudogenes which are not actively transcribed, may also be present in the genome since Southern blot analysis showed a number of bands rather than the few predicted from the restriction map of the cloned gene. The SRA gene has also been identified in freshly collected *T.b.rhodesiense* isolates from infected patients with symptoms of East African trypanosomiasis, originating from various locations in Zambia (De Greef *et.al.*, 1992). *T.b.brucei* parasites, which are human serum sensitive, lacked the resistance specific transcript and so did *T.evansi* and *T.equiperdum*. Expression of the SRA gene could not be detected in the human serum resistant *T.b.gambiense* as might be predicted if a single mechanism is present in all stocks and subspecies (DeGreef *et.al.*, 1992). The resistance specific cDNA also failed to hybridise with RNA from other *T.b.rhodesiense* strains (ETat1.9 and ETat1.3) (Rifkin *et.al.*, 1994). Recent data does however, suggest that the SRA gene can confer serum resistance since transfection of SRA into the ribosomal locus of *T.b.brucei* resulted in resistance to human serum (Van Xong *et.al.*, 1998). The same authors also provided data suggesting that the SRA is an ESAG of the ETat1.10 expression site. The SRA gene appears to be developmentally regulated; procyclic forms do not express the SRA specific sequences despite being resistant to lysis by human serum.

1.5 GENETIC ORGANISATION AND GENETIC EXCHANGE

1.5.1 Ploidy and karyotype

Chromosomes in trypanosomes do not condense during cell division and therefore it has not been possible to make direct cytological observations of ploidy and karyotype. Instead, ploidy has been determined indirectly from electrophoretic patterns of isoenzymes, from Southern blotting of restriction digests with single copy gene probes and from the measurement of total DNA content relative to its kinetic complexity. Results from these studies in general conclude that the major part of the genome in bloodstream trypanosomes is diploid (Tait, 1980; Gibson *et.al.*, 1980;

Gibson *et.al.*, 1985; Borst *et.al.*, 1982). One important exception to this diploidy has been described for the VSG genes where only one allele per genome for each VSG gene is present and therefore they can be regarded as haploid, (Borst & Cross, 1982). An explanation for VSG genes being haploid might be their ability to change position within the genome together with the large number of different VSG genes present which makes them appear as different alleles (Tait & Turner, 1990). Mini and intermediate size chromosomes may also be exceptions to the general state of diploidy (Wells *et.al.*, 1987).

The ploidy of other life cycle stages has also been examined by comparison to the DNA contents within bloodstream form cells. Zampetti-Bosseler *et.al.* (1986) suggested that metacyclic stages are haploid after measuring the DNA content by microfluorometry and comparing it with other stages of the life cycle. This conclusion is probably wrong since Shapiro *et.al.* (1984), Kooy *et.al.* (1989) and Sternberg *et.al.* (1988) showed that bloodstream, metacyclics and procyclics all had identical DNA contents. Shapiro *et.al.* (1984) failed to find any haploid forms in bloodstream stumpy and slender forms or in metacyclics or procyclics when measuring DNA content by flow cytometry. Tait *et.al.* (1989) showed that the genetic consequences of haploidy, homozygosity of all heterozygous markers in metacyclic clones, were not observed. Therefore the metacyclics must be diploid and not a product of meiosis.

Trypanosome chromosomes can be visualised directly by pulsed-field gel electrophoresis (PFGE) and the chromosomes fall into three different classes which together form 80% of the genome with the reminder of the DNA in the kinetoplast. Mini-chromosomes are between 50 and 150 kb in size, intermediate chromosomes are between 200-1000 kb and large chromosomes are >1 Mb long. There are approximately 11 defined large chromosomes per genome and these have been shown to contain housekeeping genes and many VSG genes (Van der Ploeg *et.al.*, 1989; Melville *et.al.* 1998). The mini-chromosomes and intermediate-chromosomes vary in number between species and isolates but there are approximately 100 and 1-7 per genome respectively. The larger chromosomes are diploid but chromosomes

homologues show considerable variation in size. Such size variation can lead to non-homologous as well as homologous chromosomes being of the same size which can cause difficulty in interpreting PFGE results. The reason for this size difference is not known as yet but change in size is associated with meiosis (Tait *et.al.*, 1993) and, unlike many other pathogenic parasites, chromosome sizes remain constant at mitosis (Tait *et.al.*, 1996).

1.5.2 Genetic exchange in laboratory crosses

The first demonstration of genetic exchange between two different clones of trypanosomes in a laboratory cross was described by Jenni *et.al.* (1986). Mixed infections were produced in the laboratory by allowing tsetse flies to feed through a membrane on bloodstream trypanosomes from two different clones mixed in equal proportions. Flies producing metacyclic stages were subsequently fed on mice and the resulting bloodstream forms were passaged through another set of mice. Clones derived from this mixed infection, parental clones and clones derived from a separate cyclical transmission of each clone were all screened by isoenzyme electrophoresis for five enzymes for which the parental clones differed. It was found that the enzyme phenotypes of the single transmitted parental clones stayed the same after transmission through tsetse flies while some progeny of the mixed infection differed from either of the parental clones and were heterozygous at loci for which parental clones were homozygous and different. The genotype of parental and hybrid trypanosomes was also investigated by detection of RFLPs and with Southern hybridisation of DNA probes for VSG genes. It was observed that the parental clones were homozygous but different while the hybrid clones were heterozygous. The hybrid clones from this cross had identical phenotypes, genotypes and molecular karyotypes. These experiments clearly demonstrated that recombinant phenotypes and genotypes could be produced when two trypanosome clones were transmitted simultaneously through tsetse flies and thus that genetic exchange and hybrid formation had taken place.

Since this first cross, which provided evidence for the occurrence of genetic exchange in *T.brucei*, several subsequent crosses have been conducted in order to understand the mechanisms of genetic exchange (Sternberg *et.al.*, 1989; Turner *et.al.*, 1990; Gibson, 1989). The progeny from the crosses have been analysed with the two marker systems mentioned above, electrophoretic variants of specific isoenzymes and RFLPs of single and multicopy gene probes. Results of such experiments have shown that genetic exchange is a non-obligatory but frequent event. Approximately 40% of flies with mature infections produce hybrid trypanosomes but this figure can be highly variable. Results, in summary, show that the co-transmitted trypanosomes fall into one of three categories: one that is identical to one of the parent clones, one that has a novel combination of parental markers from both parents and one that is the result of self-fertilisation (Tait *et.al.*, 1996). The genotype of the progeny depends on whether the marker is homozygous or heterozygous in one or both parents. Hybrid formation was demonstrated by the inheritance of markers for which the parents are homozygous but different, by inheritance of markers for which parents are heterozygous or by inheritance of multiple markers for which at least one parent is heterozygous. Self fertilisation products were demonstrated when one class of progeny were similar to one of the parental clones except for being homozygous for one or more loci for which the parent is heterozygous. The finding that all possible combinations of alleles at several loci occurred is consistent with allelic segregation, independent assortment and recombination as would be predicted if the progeny were the offspring of a diploid Mendelian system (Sternberg *et.al.*, 1989; Gibson, 1989; Turner *et.al.*, 1990). The fact that some parental clones are cyclically transmitted without change would imply that mating is a non-obligatory event.

The nuclear DNA content of the first three hybrids was demonstrated to be greater than the nuclear DNA content of the parents (Paindavoine *et.al.*, 1986; Wells *et.al.*, 1987). The mean value of the DNA content was about 1.6 times the parental value. This could be interpreted in different ways, including the hybrids being tetraploid and loosing DNA during growth or hybrids being a result of the fusion of haploid gametes followed by amplification of part of the genome or, possibly, that the chromatin structure in the hybrids was different from that in the parents which would

result in the increased uptake of dye during cytofluorimetry. It could also be that these hybrids are aneuploid and have lost or gained one or more chromosomes (Tait & Turner, 1990). A review by Tait *et.al.* (1993) of the DNA content measured in 24 hybrid progeny by several investigators reveals that 21 of the hybrids have DNA contents equal to or between the parental values but the remaining three hybrids showed increased DNA levels. Hybrids with an increased DNA content have been found in other crosses as well (Gibson & Garside, 1991; Gibson *et.al.*, 1992). It is generally accepted that triploid trypanosome clones occur and such progeny are frequent in some crosses but not in others.

1.5.3 Chromosome and kinetoplast inheritance

The use of PFGE has led to the ability to separate and define pairs of homologous chromosomes and determine the inheritance of these chromosomes in progeny from genetic crosses. Wells *et.al.*, (1987) investigated the inheritance of mini-chromosomes in hybrid progeny and found that most, but not all, of these chromosomes were inherited from both parents which contributed to an increase in DNA content. It should however, be noted that the progeny class analysed were sub-tetraploid and thus may be atypical products of genetic exchange. Mini-chromosome inheritance in diploid F_1 progeny of a genetic cross have not been analysed.

Because of the small size of intermediate size chromosomes their pattern of inheritance would not be expected to have a major effect on DNA content. Inheritance of intermediate size chromosomes is difficult to analyse because they contain no housekeeping genes and no unique polymorphic markers which would enable detailed analysis. When analysing a hybrid clone by pulse field gel analysis, Sternberg *et.al.* (1988) showed that intermediate size chromosomes also segregate since the hybrid displayed a new combination of these chromosomes. The smaller size chromosomes do not segregate as diploid homologues and are perhaps best considered as aneuploid chromosomes which are inherited randomly. The larger

chromosomes are diploid and in general segregate in a Mendelian fashion in genetic crosses as discussed in detail in **section 1.5.2**.

Much less is known about the inheritance of kinetoplast DNA and maxi circles were first thought to be inherited uniparentally, with no apparent preference for either parent (Gibson, 1989; Gibson & Garside, 1991; Sternberg *et.al.*, 1989). Turner *et.al.* (1995) showed however, that maxicircles can be inherited biparentally as well. Minicircles are inherited from both of the parents. This was demonstrated when hybrid progeny were found to have minicircle networks which appeared to be a mix from the two parents (Gibson & Garside, 1990; Gibson *et.al.*, 1997). It has been suggested therefore that during the formation of hybrid progeny, the kinetoplasts from both parents fuse with subsequent stochastic segregation of both the maxi- and mini-circles.

1.5.4 Frequency and stage of mating

The lifecycle stage of *T.brucei* at which mating takes place has not yet been identified. It occurs between the time the fly ingests bloodstream cells and production of metacyclic forms in the fly's salivary glands. Procyclic, proventricular, epimastigote or metacyclic stages are all possible stages at which mating could take place. However, proventricular and metacyclic forms are thought not to divide (Vickerman, 1985) and it would be unusual for a non-mitotic cell to undergo meiosis. Schweizer & Jenni (1991) have published the only finding of a procyclic population with hybrid characteristics suggesting that hybridisation takes place in the posterior part of the fly midgut. Unfortunately, no clones from these hybrid procyclics could be derived and therefore no firm evidence for mating at this stage exists. In contrast, Gibson & Whittington (1993) conducted genetic crosses using parents into which selectable markers had been introduced and detected double resistant (i.e. hybrid progeny) cells amongst the metacyclic population but not amongst procyclics. These data infer that it is highly likely that genetic exchange occurs in the salivary glands rather than in the fly gut.

The frequency of mating in laboratory crosses has been analysed in two different ways; by looking at the proportion of metacyclic producing flies that produce hybrids and by looking at the proportion of clones at a single time point from a single fly that are hybrid. Sternberg *et.al.* (1989) used the first approach and determined the proportion of mixed infected flies that produced hybrid trypanosomes. Flies with early infections produced mainly parental stocks without mating but approximately 25 days after initial infection, recombinant progeny started to be transmitted. Hybrids, together with parental clones were produced and mating was shown occur at a high frequency. These observations were in agreement with an earlier study by Schweizer *et.al.* (1988) who used the second approach and investigated bloodstream form populations produced from a mix of two *T.brucei* clones after fly transmission. Of 23 tsetse flies examined, 9 trypanosome populations contained hybrid progeny which led to the conclusion that hybrid formation is a regular and frequent event.

Even though it was shown that mating occurs at a high frequency, Sternberg *et.al.* (1989) also demonstrated that it is not an obligatory event in the life-cycle of *T.brucei*. One or both of the parental stocks could pass through the fly without any genetic exchange taking place. The same observation was made by Gibson (1989) who used pulsed-field gel electrophoresis to study non-parental karyotypes from a cross between *T.b.rhodesiense* and *T.b.brucei* stocks. Again, the hybrid clones produced shared many characteristics with both parents and their genotypes confirmed that segregation and re-assortment of parental alleles had taken place.

To investigate the extent to which there might be genetic barriers to mating a study was conducted of progeny produced from mixed infections by crossing three stocks of *T.brucei*, (STIB 247L, STIB 386AA and TREU 927/4) in all possible pairwise combinations (Turner *et.al.*, 1990). The resulting analysis showed that F_1 hybrid progeny could be produced by all three combinations of stocks. The simplest explanations of these data are that mating is controlled by a mating type locus for which at least one stock is heterozygous or that there are no barriers to mating.

The above findings would predict that self fertilisation can occur as a result of genetic exchange and recombinant products of self fertilisation, "selfers", have now been identified (Tait *et.al.*, 1996; Gibson, 1997). Interestingly, self-fertilisation products have only been observed when cross-fertilisation also occurs, perhaps implying that the presence of two dissimilar stocks within a fly is required to trigger the mating process.

1.5.5 Genetic exchange in natural populations

The first indication that genetic exchange takes place between different trypanosome stocks came after a study of isoenzyme electrophoretic variation between stocks isolated from a single population (Tait, 1980). A high degree of polymorphism was found when 17 stocks of *T.b.brucei*, collected in Lugala, Uganda, were screened for electrophoretic variation in 19 enzymes. The observed genotype frequencies agreed with those predicted by the Hardy-Weinberg equilibrium and could not readily be explained by models based only on mutations thus providing strong evidence for trypanosomes being diploid, undergoing random mating and recombination between different loci occurring. However, while the presence of genetic exchange in laboratory populations of trypanosomes has been confirmed experimentally, the importance of genetic exchange in natural populations in the field is still uncertain. Knowing more about the nature and frequency of genetic exchange in natural populations of trypanosomes would aid the understanding of factors involved in inheritance and geographical distribution of medically important traits such as human infectivity and drug resistance (Tait, 1980; Tait, 1983; Gibson, 1980). Initial analyses of genetic exchange in the field yielded results which were consistent with a random mating pattern (Tait, 1980). Genotype frequencies from only 17 isolates were determined, and when Cibulskis (1988) subjected these data to more detailed statistical methods he found the sample size was too small to exclude the possibility of chance being the reason for the agreement with Hardy-Weinberg equilibrium. The number of expected recombinant genotypes was higher than the observed number and linkage between loci could not explain the absence of some

combinations. Tibayrenc & Ayala (1991) proposed that *T.brucei* has a clonal population structure and that this is well confirmed in most cases apart from some wild samples from East Africa. Several statistical methods were used to detect divergence from Hardy-Weinberg equilibrium frequencies and linkage disequilibrium with the null hypothesis being panmixia and random mating. It was found that there was an absence of recombinant genotypes with an over-representation of identical genotypes. Linkage disequilibrium was also found and these observations, when taken together, argue against a panmictic population structure where mating between individuals occurs at random. Instead, there is a non random association between alleles or genotypes at different loci. The study was however, mainly based on results from trypanosomes isolated from humans and cattle and the authors add that a higher genotype diversity was found in isolates from wild animals and tsetse flies and therefore recombination or clonal diversity may be higher in trypanosomes from these sources.

Cibulskis (1992) explained the apparent clonality by taking into account the prediction that certain strains may show host species preferences which would cause differential selection pressure for different strains and result in deviation from Hardy-Weinberg equilibrium when all stocks were pooled as a single population. This view was shared by Mihok *et.al.* (1990) who also described considerable diversity within an isolated focus and suggested this was caused by introduction of new zymodemes which stimulated genetic recombination. Cibulskis (1992) examined trypanosome samples collected over a 14 years period from man, cattle, game animals and tsetse flies in Lambwe Valley, Kenya, and found a significant difference in strain frequencies over even short geographical distances. The association between strains and the species of host mammal was also strong and this led the author to suggest that transmission of *T.brucei* was to some extent localised and that certain strains are adapted to different host species. The degree of genetic exchange does not seem to interfere with host association over this 14 years time scale which may be explained by selection pressure for particular transmission cycles. An important conclusion from this study was that whilst the data did not support panmictic random mating, neither could it be explained by a model of clonality.

Deviations from Hardy-Weinberg expectations, which would suggest some restrictions in mating, have been found in studies on other trypanosome populations (Hide *et.al.*, 1994, Stevens and Welburn, 1993). Hide *et.al.* (1994) found clear evidence for genetic exchange within the *T.b.brucei* population but much less in *T.b.rhodesiense* isolates, both collected in a confined location in south-east Uganda. Despite the occurrence of genetic exchange in both populations none of them fulfilled the criteria for random mating and certain stocks seem to occur more frequently than others. An epidemic population structure would however, involve random mating but with predominance of particular genotypes due to epidemic expansion. Stevens and Welburn (1993) examined isolates in this same area by isoenzyme analysis and also concluded that mating was not random. The hypothesis of all trypanosomes being clonal was tested using a collection of already published data which was subjected to analysis in order to detect association between genes at different loci (Maynard-Smith *et.al.*, 1993). This analyse could not fully support the clonality theory but instead suggested an "epidemic" population structure with frequent sex and recombination within the population as a whole but that this genetic exchange was masked by occasional epidemic clones arising. Stevens & Tibayrene (1995) studied *T.brucei* species isolated from the midguts of wild tsetse flies and found that mixed populations of trypanosomes are common. From an investigation of linkage disequilibrium within populations the authors concluded that the trypanosomes are not undergoing random mating and that epidemic clonality is not enough to explain the many genetic variants present in individual flies. The hypothesis by Cibulskis (1992) that certain *T.brucei* genotypes in certain transmission cycles are selected for may also explain why *T.brucei* in some circumstances may be regarded as clonal in the short term even though other population studies suggests that sex is occurring and leads to genetic exchange and diversity in the longer term.

1.5.6 Proposed mechanisms of genetic exchange

The cytological details of genetic exchange and the precise time and place of mating are still unclear but there appears to be an agreement that most data conform to a Mendelian model for the hybrid progeny being F_1 products in a diploid system. A rearrangement of chromosomes occurs with the segregation and independent assortment of alleles at different loci. It should be noted however that, from any single cross, the numbers of F_1 hybrid progeny analysed have been insufficient to determine statistically robust segregation ratios.

A classical Mendelian system, with meiosis producing haploid gametes which subsequently fuse, was suggested by Sternberg *et.al.*, (1988), Sternberg *et.al.*, (1989) and Turner *et.al.*, (1990). This would involve diploid parental stocks undergoing meiosis to produce haploid nuclei which fuse to produce diploid progeny. The data are also consistent with an alternative Mendelian model where fusion precedes meiosis. Either model would predict Mendelian inheritance of loci on the large chromosomes but would not explain inheritance patterns of mini-chromosomes or the elevated nuclear DNA content of some hybrids. The occurrence of triploidy may instead be explained by a failure in meiosis which leads to fusion of diploid and haploid nuclei, or three or more haploid nuclei may be present in one cell with subsequent fusion of multiple nuclei (Gibson, 1995).

A fusion model where parental stocks fuse to yield "sub-tetraploid" trypanosomes was proposed by Paindavoine *et.al.* (1986). Their suggestion came after a report on increased DNA contents in hybrid clones produced in the laboratory by Jenni *et.al.* (1986) and later analysed by Wells *et.al.*, (1987). The three hybrids analysed were found to have 50-60% higher DNA content than that of parental clones and DNA content was reported to be stable during serial passage through mice. The increase was accompanied by an almost doubling of minichromosomal DNA but this was not enough to explain the difference in total DNA content. Paindavoine *et.al.* (1986) then suggested that hybrids are subtetraploid and have resulted from fusion of diploid nuclei and they calculated the mean amount of hybrid DNA to be 175% of either

parent. The fusion was suggested to be followed by random loss of chromosome content in several stages to re-establish diploidy.

As larger number of hybrids from a variety of genetic crosses have been analysed over recent years, it has become clear that F_1 hybrid progeny are usually diploid and the "sub-tetraploid" model was based on an inadequate data set. It seems most likely that the process is essentially Mendelian with meiosis before or after fusion but to date there is no evidence to distinguish between these models.

1.6 AIMS OF STUDY

Some strains of *T.brucei* infect humans and cause African sleeping sickness whilst others are lysed by (a) lytic factor(s) in the human serum and can only survive in cattle or game animals. These strains are otherwise morphologically indistinguishable. Early studies have concluded that the lytic factor in human serum is separate from an immune response and does not involve the complement pathway. The underlying mechanisms for human serum sensitivity/resistance are not yet fully understood and more than one factor in human serum may be involved. The overall aim of this thesis was to investigate the inheritance and molecular basis for human serum resistance/sensitivity. This was made possible because of the availability of recombinant progeny from a cross between a human serum resistant and a human serum sensitive trypanosome strain. A molecular approach based on differential display RT-PCR was also used to complement the more biochemical approaches adopted by others. The objectives of this thesis was to:

- develop a reliable human serum sensitivity bloodstream form assay
- identify the number of loci and alleles determining sensitivity/resistance
- determine whether inheritance is dominant or recessive
- determine whether the gene putatively associated with human serum resistance (the SRA gene) is present and expressed in genetically competent stocks used as parents in inheritance studies.

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- identify candidate genes potentially involved in human serum sensitivity/resistance

A better understanding of these areas may lead to new ways of blocking human infectivity in trypanosomes causing human sleeping sickness.

Chapter 2

Materials and Methods

2.1 TRYPANOSOME STOCKS

Several different trypanosome stocks have been used in both the genetic and the molecular analysis undertaken in this study. Swiss Tropical Institute Basel (STIB) 247 was originally isolated from a hartebeest (*Alcelaphus buselaphus*) at Kongoni in the Serengeti Park in Tanzania during a sleeping sickness survey in November 1971 (Geigy & Kauffmann, 1973). At the time of the study, large numbers of animals in the park were investigated for the presence of trypanosomes and strains of *T.brucei* were isolated in rats before being preserved in liquid nitrogen. STIB 247 was also immediately tested with the BIIT and found to be lysed by human serum. STIB 386 was obtained from a human in Koudougou in the Ivory Coast in 1978 (Felgner *et.al.*, 1981). It was shown to be highly resistant to human serum and referred to as a type II *T.b.gambiense*. Trypanosomiasis Research Organisation (TREU) 927 comes from Kiboko in Kenya, where it was isolated from a tsetse fly in 1970 (Gobloed *et.al.*, 1973). All three stocks have been cloned and they are all pleomorphic and transmissible through tsetse flies. STIB 247 and STIB 386 are within 10 mouse passages of original isolate whereas TREU 927 is approximately 14 mouse passages from the original isolate. Virulence and parasitaemia characteristics are given in Turner *et.al.* (1995). The two subcloned lines, ETat1.2 and ETat1.10 are derived from East African Trypanosomiasis Organisation (EATRO) 3 which was originally isolated from a tsetse fly in Busoga district in Uganda, 1959.

The method for generating F_1 hybrid progeny from crosses has been described as have some of the F_1 progeny used in the current study (Sternberg *et.al.*, 1989; Turner *et.al.*, 1990). In addition, F_1 clones were obtained by optical cloning of bloodstream trypanosome stabilates. The stabilates had been made from trypanosome infected mouse blood where the infection had been initiated by the bite of a mixed infected tsetse fly. The optical cloning was carried out by Prof. Andy Tait. Recombinant

progeny from self-fertilisation events were also used in the study and the generation of these clones was described in Tait *et.al.* (1996).

2.2 TRYPANOSOME GROWTH AND ISOLATION

Bloodstream trypanosomes were grown in mice which had been infected with cryopreserved clones by intraperitoneal (i.p.) inoculation or by transferring infected blood from one mouse to another. Routinely, outbred adult female mice were used, either Hsd/Ola:MF1 or CFLP, both from Harlan Olac Ltd. Adult female Wistar rats (Harlan Olac Ltd) were used when larger numbers of trypanosomes were required and these were infected by i.p. inoculation of whole blood obtained from trypanosome-infected mice. The mice and rats were monitored by tail bleeding (Herbert & Lumsden, 1976) and trypanosomes harvested by cardiac puncture during the exponential phase growth, usually at a parasitaemia corresponding to approximately 1×10^8 trypanosomes/ml of blood. Bloodstream trypanosomes were then separated from blood cells by one of three methods; differential centrifugation (Ghiotto *et.al.* 1979), centrifugation on a Percoll density gradient (modified from Grab & Bwayo, 1982) or by ion exchange chromatography on a DEAE-cellulose column (DE-52, Whatman) by the method of Lanham & Godfrey (1970).

Purification by differential centrifugation

Purification of bloodstream trypanosomes from whole blood involved adding an equal volume of 1x Phosphate buffered saline (PBS) pH 7.4 and centrifugation at 200xg for 7 minutes at room temperature. The upper straw coloured layer, containing the trypanosomes, was then transferred to a new tube and the trypanosomes pelleted by centrifugation for 5 minutes at 2500xg at 4°C. After removal of the supernatant, the trypanosomes were resuspended in MEM + Earles salt w/o L-glutamine (Gibco-BRL) and the concentration measured using an improved Neubauer haemacytometer.

Isolation using DEAE-cellulose columns

Isolation of bloodstream trypanosomes by ion exchange chromatography on DEAE-cellulose columns was essentially carried out as previously described (Lanham & Godfrey, 1970). The method depends on differences in cell-surface charge so that the more negatively charged blood cells and platelets are absorbed onto the DEAE-cellulose while the less negatively charged trypanosomes are allowed to pass through. Blood from infected mice or rats was mixed with an equal volume 1x Phosphate buffered saline containing 1% glucose (PSG) pH 8.00 and centrifuged at 200xg for 7 minutes in order to remove most of the blood cells. The upper layer, containing the trypanosomes, was transferred into a new tube to be added to the column. DE-52 cellulose (Whatman) was stored in a suspension in PBS pH 8.0 and 1% azide and then washed with at least 5-6 volumes of 1xPSG pH 8.0 before use to remove the azide and to ensure a pH of 8.0. The PSG solution containing the trypanosomes was then gently added to the top of the column and the flow-through collected. Samples were placed on microscope slides and examined for trypanosomes at regular intervals. When all of the trypanosomes had been collected they were diluted in 1xPBS pH 7.4, centrifuged for 5 minutes at 1000g and then resuspended in 1xPBS pH 7.4 to return them to iso-osmotic neutral pH conditions and counted in an improved Neubauer haemocytometer.

Percoll purification of bloodstream trypanosomes

Percoll with a density of 1.130/ml (Pharmacia) was mixed with 2% (w/v) glucose and 8.55% (w/v) sucrose and the pH adjusted to 7.4 with solid HEPES. The percoll mixture was mixed with whole blood immediately after it was removed from the infected animal. 5 ml Percoll/sucrose/glucose and 2 ml PBS was used for every 1 ml of blood. The suspension was then centrifuged for 20 minutes at 30 000xg at 4°C and the trypanosomes collected from the upper phase. The cells were then transferred to a fresh tube and pelleted by centrifugation at 1000g for 5 minutes, resuspended and counted with an improved Neubauer haemocytometer.

Parasitaemia determination

Parasitaemias in mice and rats were monitored at least every other day by the rapid matching method of Herbert & Lumsden (1976).

Cryopreservation of bloodstream trypanosomes

Bloodstream trypanosomes were preserved as stabilates in plastic straws in liquid nitrogen. Infected mouse blood was obtained by cardiac puncture and immediately mixed with 7.5% DMSO in CBSS (Fairlamb *et.al.*, 1992) containing heparin at a concentration of 100U/ml. Using a Nalgene™ Cryo 1°C Freezing container the stabilates were allowed to freeze at 1°C per minute until they reached -70°C when they were transferred for storage in liquid nitrogen and assigned a unique identifier GUP number.

Optical cloning of bloodstream trypanosomes

Cloning of single trypanosomes was essentially carried out as described by McLintock *et.al.* (1990). A drop of infected blood was diluted in 100-200 µl guinea pig serum and, using a fine pin, a small drop of the suspension was added to wells of a humidified Terasaki tissue culture plate at 10°C. The wells where then examined for the presence of single trypanosomes using an inverted microscope. 10 µl of guinea pig serum was added to wells containing single trypanosomes and this was mixed with CBSS before being injected i.p. into a mouse. The mice where then monitored for at least 10 days for a parasitaemia and trypanosomes harvested by one of the methods described above.

2.3 *IN VITRO* HUMAN SERUM SENSITIVITY ASSAY

A range of conditions were examined in order to optimise the assay and details of these are given in **3.2.1**. In brief, a reliable assay which gave consistent results could not be developed even under optimal conditions. Following the investigation of the effect of the various factors involved, the most reliable assay was a lysis test monitored by microscopy. Bloodstream trypanosomes were purified from mouse or

rat blood by the methods described in **section 2.2** and the concentration estimated using a haemocytometer. The cells were then diluted in MEM + Earles salt culture medium (Gibco-BRL) and 25% (v/v) serum to a concentration of 1×10^7 cells/ml and incubated at 37°C for 4 hours. At the end of the incubation time at least 200 cells were counted and the percentage normal, 'tadpole' shaped and lysed cells recorded.

Serum

Serum for the development of the human serum sensitivity assays was obtained from five human volunteers. Whole blood was drawn by venous puncture and transferred from the syringe to a glass bottle where it was left to coagulate at room temperature for 2 hours. The clot was loosened from the glass surface and stored at 4 °C overnight. Serum could then be separated from remaining blood cells by centrifugation for 15 minutes at 3000g before being heat inactivated by incubation at 56°C for 30 minutes. The serum was then stored in single aliquots at -70°C until needed. For the standard *in vitro* HSS-assay only serum from one of the volunteers (Mike Turner), was used and this was always obtained after overnight fasting.

Blood from the volunteers was tested for the presence of circulating trypanosomal antigens by the card agglutination trypanosomiasis test (CATT) and all samples were found to be negative.

Guinea-pig serum (SeroTech) was used as a positive control serum for each assay performed.

2.4 *IN VIVO* HUMAN SERUM SENSITIVITY ASSAY

After the initial optimisation of the assay described in **section 3.2** it was essentially carried out in the following way: mice were infected with cryopreserved bloodstream trypanosomes of the stock or line to be tested and bled by cardiac puncture once the parasitaemia reached approximately 1×10^8 trypanosomes/ml and the clear majority of observed trypanosomes were slender forms. These were then purified from blood

cells by one of the methods described under 2.2, resuspended in MEM+Earles salt and counted using a haemocytometer. The cells were diluted with MEM+Earles salt and 25% serum to a final concentration of 1×10^7 cells/ml and incubated at 37°C for 4 hours. 100 cells were subsequently isolated by appropriate dilution in 1xPBS pH 7.4 and added to CBSS containing heparin at a concentration of 100U/ml before being injected i.p into mice. For standard *in vivo* human serum sensitivity assays, three outbred mice, Hsd/Ola:MF1 (Harlan Olac Ltd) or CFLP (Harlan Olac Ltd), were used for each assay, two for trypanosomes incubated with human serum and one for control cells incubated with guinea-pig serum. The mice were immunosuppressed using a dose of 150 mg/kg cyclophosphamide by i.p. injection 24 hours prior to trypanosome inoculation. The presence or absence of parasitaemia in the mice was then screened by tail bleeding at least every second day for a minimum of 14 days.

2.5 CONDUCTING TRYPANOSOME CROSSES

Crosses between cloned trypanosome populations, STIB 386 x STIB 247, STIB 386 x TREU 927 and STIB 247 x TREU 927, had all been produced prior to the study and products from crosses were available in the laboratory. The method for conducting the crosses has been published by Jenni *et.al.* (1986). Briefly, the two cloned trypanosome populations were fed as an equal mixture in whole infected blood to groups of tsetse flies. The flies were monitored for the presence of metacyclic stage trypanosomes by encouraging them to probe on warmed glass slides which then were examined under a phase contrast microscope. Infected flies were allowed to feed on mice and the resulting bloodstream trypanosomes were cloned and screened for the presence of non-parental phenotypes and genotypes. Alternatively, or in addition, the salivary glands of the fly were removed and metacyclic forms obtained, cloned and screened for their genotypes.

2.6 IDENTIFYING HYBRID PROGENY

Recombinant F1 trypanosome populations were identified using a range of polymorphic markers. The majority of the hybrids had been identified prior to this current study using electrophoretic variation detected by starch gel electrophoresis and/or isoelectric focusing and using probes detecting RFLPs (Jenni *et.al.*, 1986; Sternberg *et.al.*, 1988; Sternberg *et.al.*, 1989; Turner *et.al.*, 1990). A proportion of the hybrids were also identified after PCR amplification of the polymorphic minisatellite markers ms42 (Barrett *et.al.*, 1997), Tb292 and CRAM or the microsatellite marker JS-2 (Annette MacLeod, pers.comm) and were carried out by Annette MacLeod, University of Glasgow. Most of the previously identified hybrids were also rescreened with this new PCR-based method.

2.7 DNA ISOLATION

Total genomic DNA was prepared from purified bloodstream trypanosomes using a conventional method (Sambrook *et.al.*, 1989). Pelleted trypanosomes were resuspended in extraction buffer (50 mM Tris, 5 mM EDTA, 100 mM NaCl), 10% SDS and proteinase K and incubated at 37°C for 2 hours to lyse the cells. The organic solvents phenol and chloroform were then used to deproteinize the solution, first using phenol alone in a 1:1 v/v ratio and then a 1:1 mixture of phenol and chloroform, mixing and centrifuging at 10 000g between each step and collecting the aqueous supernatant. The DNA samples were finally concentrated by adding 1/10 volume 3M sodium acetate and 2.5 volumes 100% ethanol to the DNA solution, incubation at -70°C for a minimum of 30 minutes and then centrifugation to pellet the nucleic acid precipitate. The DNA was washed with 70% ethanol before being left to dry and resuspended in an appropriate volume of sterile ddH$_2$O or TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The concentration of the DNA was measured by ultraviolet absorbancy spectrophotometry (GeneQuant II, Pharmacia Biotech). Absorbance was measured at 260 nm at which wavelength an absorbance of 1.0 corresponds to 50µg of double stranded DNA per ml. The DNA was usually

digested with a restriction endonuclease before the concentration was measured to ensure that DNA was in solution.

Rapid DNA purification from whole blood

A rapid blood DNA miniprep method was used for DNA extraction when preparing control DNA from mouse and rat. This involved centrifuging the whole blood for 1 minute, discarding the supernatant, washing the cells with 1xSSC and then resuspending them in 1xSSC and 10% SDS. The protein was removed by shaking the suspension with phenol-chloroform in a 1:1 v/v mix. The mixture was centrifuged and the aqueous layer retained. 2.5 volumes of 100% ethanol was then added to precipitate DNA, the solution centrifuged to pellet the DNA which then was dissolved in ddH_2O. After another ethanol precipitation and wash, the pellet of DNA was resuspended in ddH_2O or TE buffer and kept at -20°C until needed.

2.8 RNA PREPARATION

Total RNA was isolated from bloodstream trypanosomes using the ready-to-use reagent TRIzol (Gibco-BRL) which is a monophasic solution of phenol and guanidine isothiocyanate. Basically, the method was carried out as recommended by the manufacturers. Bloodstream trypanosomes were isolated from infected mouse or rat blood on DEAE cellulose columns, with no visible contamination of blood cells or platelets, and pelleted by centrifugation. The pellet was then lysed in TRIzol reagent, approximately 1 ml reagent per $1x10^8$ cells, and incubated for 5 minutes at room temperature. 0.2 ml of chloroform per 1 ml of TRIzol was added followed by centrifugation at 10 000 g for 5 minutes to separate the solution into an aqueous phase and an organic phase. The RNA remains exclusively in the aqueous phase and was recovered by precipitation with isopropanol at room temperature followed by centrifugation at 10 000g for another 5 minutes. The RNA pellet was then washed with several volumes of 75% ethanol, air-dried briefly and dissolved in RNase free H_2O.

Preparation of RNase free equipment and RNase free H₂O

The introduction of RNases during RNA isolation and all work involving RNA was prevented in several ways. Sterile disposable plasticware was used whenever possible and all nondisposible glassware and plasticware were soaked in 0.5M NaOH for 10 minutes, rinsed in ddH$_2$O and autoclaved. RNase free pipette tips were prepared by filling tipboxes with H$_2$O, adding DEPC to 0.01% (v/v), leaving at 37°C overnight and then draining and autoclaving. RNase free H$_2$O was prepared by adding 2 drops of diethylpyrocarbonate (DEPC) (0.01% v/v) per litre and leaving the bottles at 37°C overnight before autoclaving. All solutions used for RNA work were either treated in this way or were prepared using DEPC-treated water. Electrophoresis tanks used for electrophoresis of RNA, were first cleaned with detergent, dried with ethanol and then filled with a solution of 3% H$_2$O$_2$. After 10 minutes at room temperature the electrophoresis tank was rinsed with water that had been treated with DEPC. Alternatively, plastic, stainless steel and glassware were treated with the ribonuclease decontaminant, RNase AWAY (Molecular Bio-Products) according to the manufacturers recommendations.

DNase treatment of total RNA

RNA isolated by TRIzol reagent is supposedly free of contaminating DNA, but it was sometimes necessary to treat the RNA samples with DNase to remove remaining DNA that was clearly present. This was particularly important prior to RT-PCR reactions and during the preparation of starting material for mRNA differential display RT-PCR. 1µg of RNA was mixed with 1µl 10xDNase I reaction buffer (500 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 1 mg/ml BSA, Gibco-BRL) and 1 µl DNase I (Amp grade, 1 U/µl) or 1 µl RQ1 RNase free DNase (1U/µl, Promega) and DEPC treated water to a final volume of 10µl. The tubes were then incubated for 15 minutes at room temperature before DNase was inactivated by the addition of 1 µl 25 mM EDTA solution and heat inactivated for 10 minutes at 65°C. Alternatively, the RNA was extracted with an equal volume of phenol:chloroform, followed by an equal volume of chloroform and then precipitated with ethanol to recover the RNA. Only phenol saturated in NET-buffer (100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) was used.

2.9 FIRST STRAND cDNA SYNTHESIS

Two different, but similar, protocols were used for the synthesis of first strand cDNA from total RNA. Initially, Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega) was used. Total RNA was stored in ethanol and 3M sodium acetate at -70°C and was pelleted by centrifugation before use. 5 μl of a glycogen solution (10 mg/ml) was added per tube of RNA solution as a carrier for RNA. 0.5 μg of oligo(dT)$_{15}$ primer (Promega) was added for each μg of RNA sample in a total volume of 15 μl DEPC-treated water. The solution was then heated to 70°C for 5 minutes to melt secondary structures within the template and immediately cooled on ice. To the solution was then added: 5 μl M-MLV RT 5x reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl$_2$, 50 mM DTT, Promega), 1.25μl each of dATP, dCTP, dGTP and dTTP, all at 10 mM, 25 units Recombinant RNasin®Ribonuclease Inhibitor (Promega), 200 units M-MLV RT and nuclease free water to a final volume of 25 μl. The reaction was finally incubated at 42°C for 60 minutes and either used immediately for second strand synthesis by RT-PCR, or stored for a limited time at -20°C.

An alternative method involved the use of components of the Gibco-BRL Superscript preamplification system for first strand cDNA synthesis. 1-5 μg total RNA was mixed with 0.5 μg oligo(dT)$_{15}$ primer (Promega) in a total volume of 12μl, incubated at 70°C for 10 minutes and chilled on ice. To this reaction 2 μl 10xPCR mix, 2 μl 25 mM MgCl$_2$, 1 μl 10 mM dNTP mix and 2 μl 0.1 M DTT were added and the reaction incubated at 42°C for 5 minutes before 200 units of Superscript II RT was added and incubation continued for a further 50 minutes. The reaction was terminated by heating at 70°C for 15 minutes and then chilled on ice. Finally 1 μl RNase H (2 units/μl) (Gibco-BRL) was added to each tube and incubated for 20 minutes at 37°C in order to digest the RNA template from the cDNA:RNA hybrid molecule before second strand synthesis. The cDNA was then either amplified directly in PCR reactions or stored at -20°C until needed.

2.10 GEL ELECTROPHORESIS

Agarose gels for electrophoresis of DNA or RNA were prepared as described in Sambrook *et.al.* (1989). Powdered agarose (Sigma, Promega or Gibco-BRL) was mixed with electrophoresis buffer (either 1xTAE or 0.5xTBE) and ethidium bromide to a final concentration of 0.5 μg/ml. An agarose concentration of 0.8-2.0 % (w/v) was used, the concentration depending on the size of DNA fragments to be separated. When appropriate, low-melting-temperature agarose (Agarose Prep from Pharmacia or NuSieve®GTG® agarose from Flowgen) was used instead of standard agarose. The slurry was then heated in a microwave oven until the agarose was completely dissolved after which the solution was cooled to 60°C and the warm agarose poured into a plastic electrophoresis tray and an appropriate sample well comb inserted. After the gel was completely set, the comb was removed and the gel mounted in an electrophoresis tank. The gel was covered with electrophoresis buffer with added ethidium bromide (0.5 μg/ml) and the DNA or RNA samples, mixed with a gel-loading buffer (0.25% w/v bromophenol blue, 30% glycerol in H_2O), loaded into the wells. The first well was usually loaded with a marker DNA of a known size, either a 1 kb DNA ladder with 13 bands (Advanced Biotechnologies) or a Lambda/Hind III molecular weight marker (Appligene). A voltage of 1-5V/cm was applied and the DNA was allowed to migrate towards the anode until the bromophenol blue had migrated the appropriate distance through the gel. The gels were then examined by ultraviolet light and photographed with a Polaroid camera or a Gel Imager.

2.11 RECOVERY OF DNA FROM AGAROSE GELS

Frequently there was a need to recover DNA from agarose gels and several different protocols were used for this purpose. The QIAEX II gel extraction kit (Qiagen) is based on dissolving agarose in the presence of a buffer and silica particles that will absorb the DNA. Non-nucleic acid impurities are then washed away and the DNA can be eluted from the silica particles with water. After elution the silica particles are

pelleted by centrifugation and the DNA recovered in the supernatant. The complete protocol is described in the QIAEX® II handbook. Another method used for recovery of DNA from agarose gels was the Wizard™PCR preps DNA purification system for rapid purification of DNA fragments (Promega). With this method, agarose containing the desired DNA fragment is melted in the presence of resin, to which it becomes bound, loaded onto a minicolumn and washed with isopropanol before the DNA is eluted from the column with water. The full protocol is described in Promega's Technical bulletin for the purification system. Fast recovery of DNA using spin columns was also used. GenElute™ Agarose spin columns (Supelco, through Sigma) were washed with TE buffer before the gel slice containing DNA was placed in the spin column and centrifuged for 10 minutes at 12 000 x g. The DNA was eluted in its original buffer and concentrated by ethanol precipitation in the presence of 3M sodium acetate before use. Another spin-column based method used was the QIAquick gel extraction kit (QIAGEN). Gel slices containing DNA were dissolved in the buffer provided, which generates the appropriate conditions for binding of DNA fragments, and loaded onto the silica membrane in the column. Impurities were washed away and the purified DNA could be eluted with elution buffer (10 mM Tris-Cl, pH 8.5). A complete protocol can be found in the QIAquick Spin Handbook (QIAGEN).

Genomic DNA was purified from possible contaminating enzymes, salts, proteins and other inhibiting factors using the Wizard™ DNA clean up system (Promega). The DNA sample was mixed and bound to a resin and then loaded onto a minicolumn and washed with isopropanol. The resin was dried and the DNA eluted with prewarmed water or TE buffer. For a complete protocol see Promega's Technical Bulletin for Wizard™ DNA clean up system.

2.12 RESTRICTION ENZYME DIGESTS

Restriction enzyme digests, with various restriction endonucleases, were performed prior to Southern blots, prior to estimating genomic DNA concentrations and in order

to cut out inserts from plasmid DNA. Restriction endonucleases were used together with their optimal enzyme assay temperature and buffers and details are given in **table 2.1**. A standard protocol was used in which 1 μg DNA was digested with 1μl enzyme in the presence of an appropriate restriction endonuclease buffer. The mixture was incubated, usually overnight, at a temperature optimal for the particular enzyme. Another 1 μl of enzyme was added the next morning and the digestion allowed to proceed for another 1-2 hours after which the products could be run on an agarose gel. Occasionally, the digest was carried out in a large volume in order to dilute possible contaminants and inhibitors and this was then followed by a phenol-chloroform extraction and ethanol precipitation. When high molecular weight DNA was digested, 10x restriction enzyme buffer was added to the DNA solution and left at 4°C, with gentle agitation from time to time, for several hours before the restriction endonuclease was added. The solution was stirred for several minutes before warming it to the optimal digestion temperature. The digestion was incubated for at least 2 hours before a second aliquot of enzyme was added and the solution stirred again and incubated for an additional 2 hours.

Restriction enzyme	Buffer	Optimal assay temp.
BamH I	50mM Tris-HCl, pH 8.0, 10mM MgCl$_2$, 100mM NaCl	37°C
Eco47 III	50mM Tris-HCl, pH 8.0, 10mM MgCl$_2$, 100mM NaCl	37°C
EcoR I	50mM Tris-HCl, pH 8.0, 10mM MgCl$_2$, 100mM NaCl	37°C
Hind III	50mM Tris-HCl, pH 8.0, 10mM MgCl$_2$, 50mM NaCl	37°C
Kpn I	20mM Tris-HCl, pH 7.4, 5mM MgCl$_2$, 50mM KCl	37°C
Pst I	50mM Tris-HCl, pH 8.0, 10mM MgCl$_2$, 50mM NaCl	37°C
Rsa I	50mM Tris-HCl, pH 8.0, 10mM MgCl$_2$	37°C
Sma I	20mM Tris-HCl, pH 7.4, 5mM MgCl$_2$, 50mM KCl	25°C
Taq I	50mM Tris-HCl, pH 8.0, 10mM MgCl$_2$, 50mM NaCl	65°C

Table 2.1. Restriction endonucleases, buffers components and optimal assay temperatures. The table lists restriction enzymes used in the study together with the recommended buffer components at 1x concentration. The optimal temperatures for assays with respective enzymes are also given.

2.13 SOUTHERN BLOTTING AND HYBRIDISATION

Genomic DNA was digested with restriction endonuclease(s) and separated by agarose gel electrophoresis in 1xTAE containing ethidium bromide to a final concentration of 0.2 μg/ml at a voltage of 4-6V/cm. The DNA in the agarose gel was fragmented in 250 mM HCl for 10 minutes at room temperature with gentle agitation, denatured in 1.5 M NaCl, 0.5 M NaOH for 30 minutes, washed in ddH$_2$O and neutralised in 1.5M NaCl, 0.5 M Tris-HCl, pH 7.5 for another 30 minutes. The gel was then transferred to a solid support capillary transfer system, using a piece of Whatman 3MM paper as the source of capillary action and overlaid with a nylon membrane, either Hybond™ -N , positively charged nylon membrane (Amersham, UK) or MSI nylon transfer membrane (Micron Separation Inc.). The membrane was overlaid with 3MM paper which had been wetted with 20xSSC and a stack of paper towels were placed on top followed by a glass plate and a 1 kg weight. 10x or 20xSSC (1xSSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) or SSPE (20x SSPE is 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7) was used as transfer buffer and the transfer completed in 12-16 hours. After blotting, the apparatus was dismantled, the positions of the gel slots marked on the filter and the filter washed in 2x or 6xSSC or SSPE. The blots were fixed either by UV-crosslinking, using the optimal setting of 1200 units in a UV-crosslinking oven, or by alkali fixation. The method for alkali fixation involves placing the membrane with the DNA side up on a pad of absorbent paper soaked in 0.4 M NaOH for 20 minutes. The membrane was then rinsed briefly in 6xSSC or SSPE. Filters that were not used immediately in hybridisation experiments were wrapped in SaranWrap and stored at 4°C until needed.

Hybridisation

Hybridisation to Southern blots was either carried out with radiolabelled probes or with probes labelled with fluorescein. For fluorescein labelled probes, the filter was prehybridised in a hybridisation buffer recommended by the Gene Images labelling

and detection system (Amersham; 5xSSC, 0.1% (w/v) SDS, 5% dextran sulphate and 20-fold dilution of a liquid blocking buffer supplied with the system). Prehybridisation was then carried out for at least 30 minutes at 60°C before the fluorescein labelled probe was added. The DNA probes were labelled with fluorescein-11-dUTP random primer labelling kit according to the manufacturers recommendations (Amersham). 50 ng of probe was routinely used which was estimated to give a final probe concentration of approximately 8 ng/μl. After addition of the probe, denatured at 100°C for 5 minutes, hybridisation was carried out overnight at 60°C and the filter washed at the same temperature, initially in 1xSSC, 0.1% SDS (w/v) for 15 minutes and then in 0.5xSSC, 0.1% SDS for another 15 minutes. Detection was achieved via an antibody conjugate labelled with alkaline phosphatase (Gene Images CDP-Star detection module, Amersham). After application of a detection reagent, the antibody catalyses a chemoilluminscent reaction with dioxetane substrate and the signal could then be captured on Medical X-ray film (Fuji).

For hybridisation of radiolabelled probes to DNA immobilised on nylon membranes, one of two alternative prehybridisation solutions were used; either Denhardts solution (6xSSC, 5xDenhardt's reagent, 0.5% SDS, 100μg/ml denatured, fragmented herring sperm DNA) or Church & Gilbert buffer (0.5 M Na_2HPO_4, pH 7.2, 1 mM EDTA, 7% SDS). The filter was prewetted in 6xSSC prior to hybridisation and prehybridised for at least 45 minutes at 60°C. The probes were labelled with α^{32}P-dCTP, 3000 Ci/mmole (NEN™ Life Science Products) either using the Prime-It™ II Random Primer Labelling kit (Stratagene) or the *redi*prime DNA labelling system (Amersham). For the Prime-It™ system, 25-50 ng of probe were labelled for each reaction following the instructions for the kit. The system relies on the ability of random hexanucleotides to anneal to several sites along the DNA template. The newly synthesised DNA was labelled by substituting a radiolabelled nucleotide for a non-radioactive one during the reaction with the use of the Klenow fragment of DNA polymerase I. In some experiments the probe was purified with NucTrap® Probe Purification Columns (Stratagene) using the Push Column Beta Shield Device (Stratagene) in order to remove unincorporated nucleotides from the probe. The *redi*prime DNA labelling system contains a buffered

solution of dATP, dGTP and dTTP, exonuclease free Klenow enzyme and random primers, all in a stabilised form in ready to use tubes. The working principle is the same as for Prime-It™ system but the amount of probe needed is less, usually 2-25 ng per reaction. The labelling was carried out as recommended in the Rediprime protocol. After labelling, the probe was denatured and added to the hybridisation buffer and filter and left to hybridise at 60°C overnight. The blots were washed, first in 5xSSC, 0.1% SDS for 15 minutes at 60°C and then in 2xSSC, 0.1% SDS for 15 minutes at 60°C. Additional washes at higher stringency were carried out as necessary. The filter was removed, wrapped in SaranWrap and exposed to X-ray film at -70°C for an appropriate length of time. Probes were prepared in various forms depending on requirements and were purified PCR products, PCR products in LMPA or whole midiprep or purified midiprep inserts from recombinant plasmids.

Removal of radiolabelled and fluorescein labelled probes from nylon membranes

Nylon membranes that had been kept wet at all stages during hybridisation, washing and exposure of X-ray film could be stripped, checked and reprobed. The filters were either immersed for at least 30 minutes in 0.4M NaOH at 42°C or added to a boiling solution of 0.1 or 0.5% SDS and allowed to cool to room temperature on a bench-top shaker. If the filters were still hot, the procedure was repeated.

2.14 NORTHERN BLOTTING AND HYBRIDISATION

Northern blotting was carried out essentially as described in Sambrook *et.al.* (1989). A 1.2% agarose gel was prepared by dissolving 1.2 g agarose in 10 ml 10xMOPS buffer (0.2 M MOPS, 80 mM sodium acetate, 10 mM EDTA) pH 8.0, 73 ml RNase free H_2O and 17 ml formaldehyde (38%). Total RNA was prepared and stored as described in section 2.8. Normally 5 μg RNA was resuspended in loading buffer (50% formamide (w/v), 6.7% formaldehyde (w/v) and 1xMOPS buffer in RNase free H_2O) and denatured at 70°C for 10 minutes. The samples were immediately chilled on ice and mixed with loading dye (40% (w/v) glycerol in water, 0.25% bromophenol blue) and loaded onto a prerun gel. An RNA marker, 0.28-6.58 kb, (Promega), was loaded in the first lane for size estimation of the RNA. A small

amount of ethidium bromide (1 μl of a 1 mg/ml solution) was usually added to the samples before electrophoresis to allow detection of undegraded RNA when viewed under ultraviolet illumination at the end of the run. 1xMOPS buffer was used as running buffer and the gel photographed with a ruler aligned with the marker to be able to calculate the size of the RNA species detected by hybridisation. The gel was washed in 10xSSC and then blotted directly onto a nylon filter using the same transfer technique, equipment and fixation protocols as for Southern blotting described in section **2.13**.

Slot blotting of RNA

To increase the amount of RNA/cm² of filter, a slot blotting technique was used when low-abundance mRNA was to be detected. The method for this was essentially as described in Sambrook *et.al.* (1989). A piece of nylon membrane, either Hybond™ -N+, positively charged nylon membrane (Amersham, UK) or MSI nylon transfer membrane (Micron Separation Inc.), was soaked in 20xSSC for at least 1 hour at room temperature. The slot manifold was cleaned with 0.1 M NaOH or with RNase AWAY (Molecular Bio-products) before use. Two sheets of Whatman 3MM paper, prewetted in 20xSSC, were placed on top of the vacuum unit and the nylon membrane on the bottom of the sample wells and the two parts clamped together. The slots were then washed twice with 10xSSC prior to adding the RNA samples. Samples of 5-10 μg RNA were prepared in a final solution of 50% formamide, 7% formaldehyde, 1xSSC and denatured at 68°C for 15 minutes before snap cooling on ice. The samples were mixed with 20xSSC and gently sucked through the filter, using a vacuum pump, which was then washed with 10xSSC. The filter was allowed to dry before the RNA was UV-crosslinked to the filter at 1200 units in a UV-crosslinking oven (Amersham).

Dot blotting RNA

Dot blotting was undertaken using the same nylon membranes as described above. 5-10 μg total RNA, resuspended in DEPC treated H_2O, was incubated at 65°C for 5 minutes in three volumes of a solution of 500 μl formamide, 162 μl formaldehyde (37%) and 100 μl 10x MOPS buffer. The samples were chilled on ice, 1 volume of

cold 20xSSC added and the RNA then spotted onto the filter in 2 μl aliquots, allowing it to dry between applications. The filter was dried and fixed as described above. All solutions used were rendered RNase free by DEPC treatment.

Hybridisation

Northern hybridisations were carried out in the same way as described for Southern blots in section **2.13**, except that a higher hybridisation temperature of 65°C was used in most experiments.

2.15 PCR AMPLIFICATION OF THE SRA GENE

The Polymerase Chain Reaction (PCR) was used to test for the presence or absence of the SRA gene in various stocks of trypanosomes. A range of synthetic oligonucleotide primers were designed which are all described in **section 5.2.1**. The optimal annealing temperature for each primer combination was determined by running reactions on a Robocycler PCR machine to cover the temperature range 50-70°C. For these control reactions ETat1.2 was used as DNA template before other stocks were included. Typically, 20 ng of template and 1μM of each of primer with Taq DNA polymerase, 5 units/μl (Advanced Biotechnologies) and 11.1x PCR buffer to give a final concentration in the reaction of 45 mM Tris Hcl, pH 8.8, 11 mM ammonium sulphate, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.4 μM EDTA, pH 8.0, and dATP, dCTP, dGTP and dTTP all at 1 mM, in a total volume of 10 μl. Optimal cycling conditions were usually found when performing the reactions at 29 cycles of 94°C for 50 seconds (denaturing), 55-65°C for 50 seconds (annealing) and 72°C for 2 minutes (extension). Nested PCR reactions were frequently needed in order to obtain strong signals. In these cases a final primer concentration of 0.2 μM was used instead of 10 μM and the number of cycles reduced to 24 during the first reaction. The second reaction was essentially as described above using a 1μl carry over as the DNA template. Occasionally, the reactions were carried out using the 10x reaction buffer IV with separate $MgCl_2$, supplied by Advanced Biotechnologies.

2.16 RT-PCR AMPLIFICATION OF THE SRA GENE

Reverse transcription PCR (RT-PCR) is a sensitive method for detection of mRNA sequences from cDNA and was used for identification of expression of the SRA gene. The cDNA was isolated from the various trypanosome stocks as described in section **2.9** and typically 1 µl of the reverse transcription reaction used for each RT-PCR reaction of 25 µl. The synthetic oligonucleotides that were used are all described in section **5.2.7** and the positions of the primers used for amplification within the SRA gene locus are shown in **figure 5.1**. The heat-treated reverse-transcriptase reaction product was usually used immediately for PCR amplification and only occasionally stored at -20°C before use. The number of PCR cycles required was 30 or less and the thermal cycle profile that worked well for amplification of the SRA gene in the control stock ETat1.2, was: denaturing for 1.5 minutes at 95°C, annealing primers for 1 minute at 55°C and extending the primers for 1 minute at 72°C. The same buffer was used for both reverse transcription and PCR; either Advanced Biotechnologies formula with separate $MgCl_2$ (buffer IV) or Promega 10x PCR buffer, depending on which Taq DNA polymerase was used. A control reaction with primers for the single copy triosephosphate isomerase (TIM) gene was always carried out simultaneously with each RT-PCR reaction. TIM is an enzyme found exclusively within the glycosome in *T.brucei* (Swinkels *et.al.*, 1986).

2.17 DNA SEQUENCING

The primary structure of PCR products and cloned cDNA from differential display RT-PCR were determined on an automated Applied Biosystems 373 DNA sequencer by using the Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems). Cycle sequencing reactions were prepared as recommended for the Dye Terminator Cycle Sequencing Ready Reaction Kit (The Perkin-Elmer Corporation) followed by ethanol precipitation. The automated sequencing was carried out by the Molecular Biology Support Unit, University of Glasgow.

2.18 ANALYSIS OF SEQUENCE DATA

Sequencing results were supplied in electronic form and usually downloaded directly into the Unix version of the Wisconsin GCG program. After editing of the sequence to remove vector sequence or removal of unsuccessful sequence stretches and the comparison of reverse and forward sequences, the sequence was submitted to a database search. FASTA and BLASTN were both used to perform nucleotide vs. nucleotide sequence comparisons and BLASTX was used to compare the translated product of an unknown sequence against translated nucleic acid and protein databases. Databases used include the GenBank (National Centre for Biotechnology Information, Bethesda, USA) the EMBL database (European Bioinformatics Institute, Cambridge, UK), the protein sequence databank SWISS-PROT (Centre Medical Universitaire, Geneva, Switzerland) and the African Trypanosome DNA database at the Parasite Genome Blast Server (http://www.ebi.ac.uk/parasites/parasite _blast_server.html). Other modifications and analyses were performed using the GCG program.

2.19 INDIRECT IMMUNOFLUORESCENCE

An indirect immunofluorescence antibody technique for bloodstream trypanosomes was used when determining the variable antigen type (VAT) of particular trypanosome lines. Thin blood smears were prepared with infected mouse blood displaying a parasitaemia above antilog 7.5 cells/ml. The smears were airdried and fixed in 70% ethanol for 1 hour at 4°C and could then be used either immediately or stored at -20°C in the presence of silica gel. After returning to room temperature the slides were rehydrated in a humid chamber for 10 minutes before an appropriately diluted first antibody was applied and again incubated in a humid chamber for 30 minutes at room temperature. All VAT-specific antibodies were provided by Mike Turner and usually needed to be diluted 1:100 or 1:50 in PBS before use. After

incubation the slides were tapped on the bench to remove most antibody solution and then washed twice in PBS/0.01% Tween for 5 minutes each. Excess PBS was removed by tapping the slides on the bench but care was taken to ensure the slide did not dry out. The appropriately diluted conjugated secondary antibody, anti-rat FITC (Sigma) or anti-rabbit FITC (SAPU), was then applied to the slides and incubated in a humid chamber for 15-30 minutes. When 10 minutes remained of the incubation time, 4,6-diamidino-2-phenylindole (DAPI) (0.01 mg/ml) was applied as a counterstain for DNA. The slides were washed briefly in PBS/0.01% Tween 20 before mounting with Citifluor antifadant (Citifluor Ltd) or in non-autofluorescent glycerol. The trypanosomes were then examined by immunofluorescence microscopy and the prevalence of the major VAT, in percentage, determined by counting 200 cells.

2.20 ANTIBODY MEDIATED COMPLEMENT LYSIS

An antibody mediated complement lysis assay for bloodstream trypanosomes was used when selecting for populations expressing particular VATs. To perform the assay, trypanosomes were separated from blood, resuspended in PSG, pH 8.05 at 4×10^6 cells/ml and incubated with VAT-specific antisera in double dilution's. The antisera, produced against a specific VAT, were diluted in guinea pig serum starting with 1/10 dilution and mixed with equal volume of trypanosome suspension in a disposable microtitre plate. The plate was then incubated at room temperature for between 30 minutes and 3 hours after which the surviving trypanosomes were cloned optically and injected i.p. into mice.

2.21 DIFFERENTIAL DISPLAY RT-PCR

Differential display RT-PCR was the major tool used to identify genes differentially expressed in a human serum resistant population compared with a human serum sensitive population. The method, invented by Liang and Pardee (1992), was carried

out using the RNAimage™ system produced by GenHunter. The method involves the reverse transcription of mRNAs with oligo-dT primers anchored to the beginning of the poly(A)tail and a PCR reaction in the presence of a second 10mer which is random in sequence. The PCR products were visualised on a denaturing polyacrylamide gel and any bands of interest, i.e. differentially displayed between the two populations, reamplified, ligated into a vector and cloned. The resulting cloned inserts were then screened by Northern blotting and analysed.

Preparation of starting material

The method of differential display depends on the isolation of intact RNA obtaining, free from chromosomal DNA contamination and the method used is described in section **2.8**. The selection of trypanosome lines of the same genotype but expressing differences in human serum resistance phenotype is described in **6.2.1**.

Reverse transcription of mRNA

One-base anchored oligo-dT primers were used for reverse transcription in order to avoid under-representation of certain mRNA populations. Therefore, three reverse transcription reactions were set up for each of the two RNA samples, each containing one of the different primers $H-T_{11}-G$, $H-T_{11}-A$ or $H-T_{11}-C$, RT-buffer and dNTPs. A complete protocol can be found in the RNAimage™ system information booklet. The reverse transcription reaction was carried out in a thermocycler at 65°C for 5 minutes, 37°C for 60 minutes, 75°C for 5 minutes with the addition of MMLV reverse transcriptase after 10 minutes at 37°C. The reactions were then either used immediately for PCR or stored at -20° for later use.

Polymerase chain reaction

24 different 5'-primers, which are short and arbitrary in sequence, were used in combination with the anchored primers to amplify a set of cDNA fragments from the 3' end of the mRNA. These random primers were all supplied with the RNAimage™ system and are 10 nucleotides long but sequence information is withheld. The PCR reaction was carried out in the presence of α-^{33}P dATP (2000 Ci/mmole) (DuPont-NewEngland Nuclear) so that the resulting PCR products could be visualised by

autoradiography. The PCR reactions were otherwise set up as recommended by GenHunter in the information booklet about RNAimage™. PCR reactions were carried out at 94°C for 30 seconds, 40°C for 2 minutes and 72°C for 30 seconds repeated for 40 cycles and followed by an extra extension time of 72°C for 5 minutes. After the reaction was complete, samples were usually used for the next step in the system but were occasionally stored at -20°C for later use.

Band separation

A 6% denaturing polyacrylamide gel, commonly used for DNA sequencing, was used for resolution of amplified cDNAs. The gel was prepared either by a standard polyacrylamide gel protocol (Sambrook *et.al.*, 1989) or the Sequagel™XR (National Diagnostics) was used and prepared according to the manufacturers instructions. After addition of 0.8 ml of 10% (w/v) ammonium persulfate for every 100 ml of gel casting solution, the solution was poured into the cleaned and siliconized gel casting cassette. The gel was polymerized for at least 2 hours or overnight. The cassette was then assembled into the electrophoresis apparatus and using 1xTBE as running buffer, the gel was prerun for 30 minutes and the wells carefully flushed before loading the samples. 3.5 µl of each sample was mixed with 2 µl loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.09% xylene cyanole FF, 0.09% bromophenol blue) and incubated at 80°C for 2 minutes before loading onto the gel. Each sample was prepared in duplicate and two samples from the human serum resistant trypanosome population and two samples from the human serum sensitive population, produced by the same primer combination, were loaded next to each other. Electrophoresis was then carried out at 60 W constant power until the xylene dye had reached the bottom of the gel. The gel apparatus was dissembled and the gel transferred to a piece of Whatmann 3MM paper. The gel was covered with plastic wrap and dried under vacuum on a gel dryer at 80°C for 2 hours and marked with fluorescence stickers (Stratagene) for later orientation and autoradiography.

Reamplification of cDNA probes of interest

Retrieval of differentially expressed bands from polyacrylamide gels was achieved by a boiling and ethanol precipitation method (Liang *et.al.*, 1993). After developing

the film, the autoradiogram was oriented with the gel and bands of interest marked with a needle at each of the four corners so that the same band could be cut out with a clean razor blade from the dried gel. The gel slice, along with the 3MM paper, was soaked in 100 µl ddH$_2$O for 10 minutes and boiled for 15 minutes. The supernatant was transferred to a new tube and 1/10 volume 3 M sodium acetate and 2.5 volumes 100% ethanol was added together with 5 µl glycogen(10 mg/ml). The DNA was precipitated at -70°C for at least 30 minutes followed by centrifugation to pellet the DNA; the pellet was washed once with ice-cold 85% ethanol. After dissolving the DNA in ddH$_2$O a portion of it was used for reamplification using the same primer set and PCR conditions, except with a higher dNTP concentration (20 µM instead of 4 µM) and no radioactive nucleotide added. A detailed protocol is included in the RNAimage™ booklet. The amplified PCR product was then run on a 1.5% agarose gel stained with ethidium bromide and in the few cases where no product could be visualised, another 40-cycle PCR amplification was carried out using a 1:100 dilution product as template. The reamplified cDNA was extracted from the agarose gel by one of the methods described in section **2.11** and the yield determined. In most cases the recovered DNA band was subsequently cloned, by the method in section **2.22**, before serving as a probe to confirm mRNA expression.

Confirmation of gene expression

The next crucial step was to confirm gene expression i.e. to identify false positive bands and remove them from the study. Initially, after comparing the sizes of the reamplified PCR product with the sizes of the original band on the DNA sequencing gel, the amplified cDNA was used as a probe in Northern blot experiments, see section **2.14**. Unfortunately, signals were rarely detected on the Northern blot, presumably because of low abundance of the mRNA, and all retrieved bands were therefore rescreened with Northern slot hybridisation for increased sensitivity, again as described in section **2.14**.

Sequencing analysis of the differentially expressed genes

After cloning of the differentially expressed cDNA fragments, they were sequenced by the automatic sequencing method described in section **2.17**. Cloning was

necessary because multiple DNA species were likely to have been present in the recovered DNA band and the differential display primers are too short to be used for direct sequencing. The sequence information was then used in searching for the identity of the differentially expressed genes in computer databases. In a few cases, a longer cDNA sequence was obtained by RT-PCR before meaningful sequence analysis could be carried out.

2.22 PRODUCTION OF RECOMBINANT DNA, TRANSFORMATION OF *E.COLI* AND CLONING

Ligation of PCR fragments into a vector was performed using the SureClone® Ligation kit from Pharmacia Biotech. The protocol uses a Klenow digestion, a column purification and a blunt-end ligation into the pUC 18 vector. The PCR product is prepared for ligation by the 3'-5' exonuclease activity of the Klenow fragment of DNA polymerase I to remove single base 3' overhangs on the fragments. Depending on the concentration of the gel purified PCR product and length of the fragment, approximately 300 ng was used in the blunt end kinase reaction in the presence of the Klenow fragment and polynucleotide kinase, as described in detail in the instruction booklet for the SureClone® Ligation kit. The reaction was allowed to proceed for 30 minutes at 37°C and the product purified by a phenol/chloroform extraction. The upper, aqueous layer was further purified on MicroSpin columns of Sephacryl® S-200 (Pharmacia Biotech) and a portion of the elute used to ligate into the vector. After calculating how much column purified sample was needed in the ligation reaction (in order to obtain the right ratio of ends) a ligation reaction with T4 DNA ligase, DTT solution and the dephoshorylated pUC 18 vector was set up as described in the instruction booklet for the SureClone® Ligation kit. The reaction was incubated at 16°C for 1.5-2 hours.

Transformation of competent E.coli *cells*

Transformation was performed either by heat-shocking JM 109 competent cells (Promega or Stratagene) which had been made competent by a modification of the

method of Hanahan (1983) or by electroporation of electrocompetent JM 109 cells (Stratagene) prepared in the laboratory. For heat-shock transformation of *E.coli*, frozen competent cells were thawed on ice and approximately 50 ng of ligated DNA added to 100 µl cells. 1 ng pUC 18 was also added to a separate portion of cells as a positive control for the competent cells. The tubes were left on ice for 10 minutes, heat-shocked at 42°C for 45 seconds and immediately placed on ice for 2 minutes. 900 µl cold LB or SOC medium was then added to each transformation and the suspension incubated for 60 minutes at 37°C on a shaker. Cells were then plated, undiluted or in a 1:10 dilution, onto agar plates containing ampicillin, 100 µg/ml, IPTG and X-gal. The pUC 18 vector expresses β-galactosidase and displays α-complementation in *E.coli* so that recombinants can be identified by screening with IPTG and X-gal which results in the colonies being blue or white; colonies that contain a non-disrupted β-galactosidase will appear pale blue while colonies that contain a plasmid will not be capable of α-complementation and therefore appear white. To each LB-agar plate 40 µl X-gal (20 mg/ml) and 4 µl IPTG (200 mg/ml) was added. The plates were only inoculated with the bacteria when all the fluid had disappeared and then incubated for 12-16 hours at 37°C. Bacteria for transformation by electroporation were grown overnight, diluted in LB and allowed to grow until the OD_{600} of the culture reached 0.6. The cells were then washed twice in cold sterile water, pelleted by centrifugation and resuspended in cold sterile water with 10% glycerol. Cells not used immediately were snap-frozen on dry ice and stored at -70°C until needed. Electrotransformation was performed using a GenePulser (Bio-Rad) in pre-chilled cuvettes using the suppliers conditions: 25 µF, 200 Ω and 2.5 kV. After transformation, 1 ml of SOC medium was added immediately and the solution incubated at 37°C for 1 hour to allow initiation of expression. Plating of the electrotransformed cells was as described above.

2.23 RECOMBINANT CLONE CHARACTERISATION

The presence and size of plasmid DNA inserts were determined directly by PCR before mini- or midipreparation of DNA and restriction analysis. Material from a

colony was transferred into PCR mix (14.9 μl H_2O, 2 μl 10x PCR mix, 1 μl 5 mM dNTPs, 1 μl each of primers M13 forward and M13 reverse (Cruachem Ltd), both at 10 pmole/μl and 0.1 μl *Taq* polymerase) with a toothpick. A stock of the colony was established by placing the remaining material on the toothpick directly into culture media or streaking out on a new plate. The insert was amplified by PCR; 94°C for 5 minutes followed by 25 cycles of 94°C, 1 minute, 55°C, 1 minute, 72°, 5 minutes and 72°C for 5 minutes. A portion of the amplified insert was analysed on an agarose gel stained with ethidium bromide including a control reaction of vector without insert for size comparison.

Mini and midipreps of plasmid DNA from each clone were prepared using QIAGENs plasmid purification system according to the protocol found in the QIAGEN plasmid handbook. The resulting DNA pellet was resuspended in TE buffer and the yield determined on a UV spectrophotometer followed by analysis on an agarose gel. A restriction enzyme digest was designed to cut out the insert from the vector. For pUC 18, double digestion with *EcoRI* and *BamHI* was used. After gel electrophoresis the insert could be isolated and purified and used as a probe in hybridisation experiments.

2.24 5'RACE

With the aim of obtaining full length cDNA, from the 3'end fragment isolated during differential display RT-PCR, one approach was to use rapid amplification of cDNA ends (RACE) anchored PCR with the aid of the 5'/3' RACE kit (Boehringer Mannheim). The method was first described by Frohmann *et.al.* (1988) and is based on the principle of first strand cDNA synthesis from total RNA using a nested series of gene-specific primers. The first strand cDNA was purified from unincorporated nucleotides and primers with the High Pure PCR Product Purification Kit (Boehringer Mannheim). Terminal transferase was then used to add a poly(A)-tail to the 3'end of the cDNA and this was followed by PCR amplification using a second gene-specific primer and an oligo dT-anchored primer. The PCR product obtained

was further amplified by a second, nested reaction with a third gene-specific primer and a pre-designed PCR anchor primer. In theory, this sequence of PCRs would result in a product corresponding to the full length gene sequence which can be sequenced and analysed. A detailed protocol for the method is published in the instruction book for the 5'/3' RACE kit.

Chapter 3

Development of an assay for human serum resistance/sensitivity

3.1 INTRODUCTION

A fundamental requirement for the study of serum resistance/sensitivity is that a reliable assay for experimentally measuring this trait is available. The test must rely on differential killing of resistant and sensitive *T.brucei* by the trypanolytic factors in human serum. This was originally carried out *in vivo* by the blood incubation infectivity test (BIIT), developed by Rickman & Robson (1970), and involved injecting trypanosome-infected rats with human serum to investigate if the human serum could clear the infection and, later, incubating purified trypanosomes with human blood and then injecting them into rodents. Since there were some concerns that the resistance of strains could change with variable antigen expression and with passage *in vivo* (Rickman, 1977; Van Meirvenne *et.al.*, 1976) *in vitro* tests were developed as described in **1.4.2**. The initial aim of this study was to establish a reliable *in vitro* bloodstream form human serum resistance assay to be used for all further analyses. Basically, this assay was to consist of incubating purified trypanosomes with human serum and then count the percentage of lysed cells compared with unaffected cells. A range of variables were examined and optimised but despite every effort to design a reliable and stable assay it was clear that cell viability could not be estimated from morphological appearance alone. A statistically significant difference in mean percentage lysis was observed between stocks that correlated with human serum sensitivity but the level of lysis varied too much between individual assays for it to be unambiguous. Instead, a more reliable *in vivo* human serum resistance assay, which involved incubating bloodstream trypanosomes with human serum, injecting them into mice and screening of the mice for the development of parasitaemia, had to be optimised and adopted.

3.2 RESULTS

3.2.1 Development of an *in vitro* HSS assay

In a series of preliminary experiments the following potential variables were investigated: serum from different individuals, the use of heat-inactivated/non heat-inactivated serum, incubation temperature, serum concentrations, trypanosome concentration, source of control serum, the morphology of the trypanosomes, incubation volume, scoring of live or fixed cells and the use of viability stains and dyes. When serum sensitive bloodstream trypanosomes were incubated with human serum, a whole range of deformed, swollen and 'tadpole' shaped cells started to appear after approximately 1 hour of incubation (depending on conditions used), for an example see **figure 3.8**. When measuring the percent lysis in the following experiments, these deformed cells are scored as lysed cells and only cells with an apparent normal morphology are scored as 'normal'.

Serum from different individuals

Serum from different individuals was tested for lytic activity since differences in activity between serum from different donors has previously been reported (Lorenz *et.al.*, 1994). Serum samples from five different volunteers were taken, three of them after overnight fasting. To evaluate the different sera, the time to maximum lysis and the percentage of trypanosomes lysed was determined. Two different stocks of trypanosomes were used for this purpose, STIB 247, which is regarded as human serum sensitive, and STIB 386, which has been shown to resist killing by human serum (Hide *et.al.*, 1994). Purified trypanosomes were incubated with the sera, non-heat inactivated, at a concentration of $1x10^7$ cells/ml in MEM+Earles salt and 25% serum at 37°C for various length of times. A graph of percent lysis with time is shown in **figure 3.1**. Individuals differed mainly in the time of onset of lysis but there was also a small difference in the percentage of lysed trypanosomes after 4 hours. The most significant difference in lytic effect was after 1.5 hours when serum from two of the donors failed to cause lysis greater than that of the control serum. The differences between donors was still significant after 3 hours but much less so

hours. There were larger differences between different sera for STIB 247 than for STIB 386. The assay was repeated three times for two of the sera and twice for the remaining three sera. Serum from the same individual was constant in its trypanolytic effect (data not shown) and during subsequent experiments (section 3.3 onwards) only serum from the same person was used.
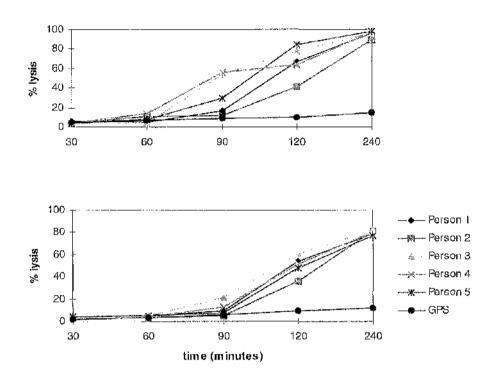
Figure 3.1. A comparison of the lytic effect of serum from five different individuals. Serum from five different individuals was tested both with STIB 247 bloodstream trypanosomes (upper graph) and with STIB 386 (lower graph). Purified trypanosomes were incubated with 25% non heat-inactivated serum at a concentration of 1×10^7 cells/ml at 37°C. The assays were performed twice for three of the sera and three times for two of the sera, the % lysis calculated and the mean values are shown. Guinea pig serum (GPS) was used as a negative control serum.

Heat-inactivated/non heat-inactivated serum

To remove complement components the serum was heat-inactivate prior to use. Serum was heated to 56°C for 30 minutes according to standard procedures. This process did not affect the trypanosome lytic properties of the sera because heat-inactivated sera was as active as unheated sera as shown in **figure 3.2.**

STIB 386   **TREU 927**

STIB 247
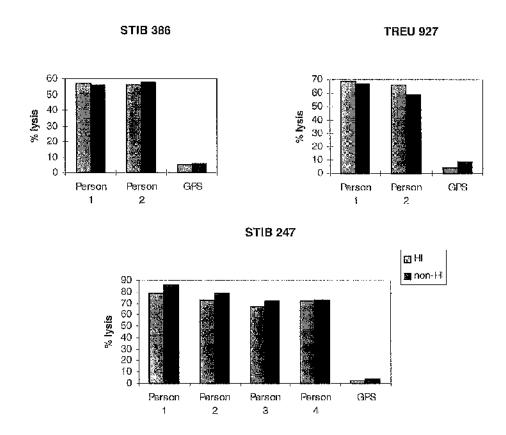
Figure 3.2. Comparisons of the lytic effect of heat-inactivated and non heat-inactivated human serum. Heat inactivated and non heat-inactivated sera were compared in an *in vitro* HSS-assay in order to detect possible differences in their lytic capacities. Comparisons were made using cells from the three trypanosome stocks STIB 247, STIB 386 and TREU 927 by incubating purified cells with 25% heat-inactivated or non heat-inactivated serum at a concentration of 1×10^7 cells/ml for 2.5 hours at 37°C. The assay was performed four times for STIB 247 and twice for STIB 386 and TREU 927 and the mean % lysis calculated and displayed above for serum collected from different individuals. The overall lysis for STIB 247 was 73% using heat inactivated serum and 77% lysis when incubated with non-heat inactivated serum.

Incubation temperature

Because of observations that TLF1 mediated lysis is temperature dependent and can be inhibited at 17°C (Hajduk *et.al.*, 1989; Rifkin, 1978) it was of interest to test lysis at different temperatures. The trypanocidal effect of heat-inactivated serum from the same individual was evaluated both at room-temperature (20°C) and at 37°C in the presence of 25% serum and with a cell concentration of 1×10^7 cells/ml. Only STIB 247 was used for this experiment which was repeated twice. In each case there was a clear, almost ten-fold, increase in lytic activity at 37°C compared with incubation at room-temperature, as shown in **figure 3.3**.
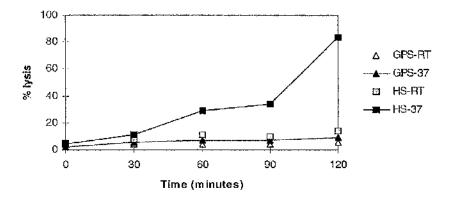
Figure 3.3. Comparison of trypanosome lysis at room temperature and at 37°C. STIB 247 cells were used for comparing the extent of lysis at two different incubation temperatures; room temperature (20°C) and 37°C. The *in vitro* HSS-assay involved incubating purified trypanosomes at a concentration of 1×10^7 cells/ml with 25% human serum (HS) for 2.5 hours at either room temperature (RT) or at 37°C. The assay was performed twice, the level of lysis determined and the mean values calculated and displayed in the above graph. Guinea pig serum (GPS) was used as negative control serum.

Serum concentrations

The percent lysis obtained in an assay may depend on the concentration of serum present. Heat-inactivated serum was used to examine the percentage lysis for serum concentrations ranging from 10% to 50% (v/v). There was an increased percentage lysis with increased serum concentration between 10% and 30% but a limited increase in percentage lysis above 30% (**figure 3.4**). With 20% serum there was a sufficient and reliable difference between lysis by human serum and lysis by the control serum. The differences between the two negative control sera are discussed below.
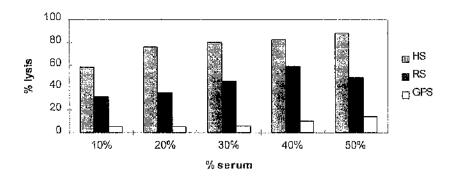
Figure 3.4. Different serum concentrations and their effect on percentage trypanosome lysis. Serum concentrations between 10% and 50% were used in an *in vitro* HSS assay and the % lysis calculated for each serum concentration. STIB 247 trypanosomes were purified and incubated with 25% serum at a concentration of 1×10^7 cells/ml for 2.5 hours after which the % lysis was calculated. HS; human serum, RS; rabbit serum; GPS; guinea pig serum, the latter two were used as negative control sera.

Trypanosome concentration

Viability of bloodstream trypanosomes in *in vitro* cultures is affected negatively if they reach concentrations that are too high. To investigate the effect of different trypanosome concentrations in the HSS assay, trypanosome concentrations were varied between 1×10^6 cells/ml and 5×10^7 cells/ml using 25% heat-inactivated serum in the assay. As shown in **figure 3.5** there was a general trend apparent with all three stocks towards a direct correlation between parasite density and percentage of parasites lysed by human serum. This correlation was also observed for trypanosomes incubated with controls using either guinea-pig or rabbit serum, indicating that MEM+Earles salt alone could not support the survival of the cells at higher cell concentrations, resulting in non-specific lysis. At a concentration of 1×10^7 cells/ml there was a significant difference in percentage lysis between cells incubated in human serum compared with cells incubated with the guinea pig control serum. For STIB 247 the average percentage lysis always exceeded 70% while lysis in the guinea pig control serum never reached more than 8%. For STIB 386 the trend towards higher lysis with higher cell concentrations was less clear than for STIB 247 and trypanosomes were generally more resistant to killing by both human serum and

control sera at higher cell concentrations. Similar results were observed for TREU 927.

STIB 247

STIB 386   **TREU 927**
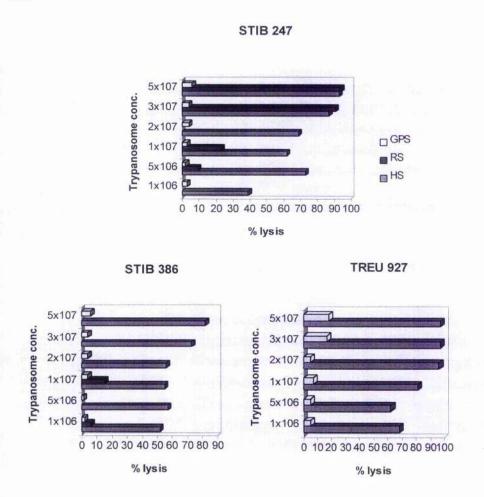
Figure 3.5. Comparisons of percentage lysis at different trypanosome concentrations. Six different trypanosome concentrations ($1x10^6$, $5x10^6$, $1x10^7$, $2x10^7$, $3x10^7$ and $5x10^7$ cells/ml) were used in an *in vitro* HSS assay in order to identify differences in the resulting percentage lysis. Purified trypanosomes from the three stocks STIB 247, STIB 386 and TREU 927 were used in individual assays using guinea pig serum (GPS) and/or rabbit serum (RS) as control serum by incubating the cells, in the various final concentrations, with 25% human serum (HS) for 2.5 hours at 37°C.

Control serum

Serum from rodents and rabbits have been reported to lack the trypanocidal factor (Hajduk *et.al.*, 1989) and should therefore serve as a good negative control serum when testing for the trypanosome killing effect of human serum. Initially, rabbit serum was chosen as the control serum for all the *in vitro* human serum sensitivity assays since it was available in the laboratory. Unfortunately, this rabbit serum had the ability to cause an unacceptable high level of background lysis, at a trypanosome

concentration of 1×10^7 and a serum concentration of 20-25%, rabbit serum often caused more than 20% lysis, as shown in **figures 3.4, 3.5** and **3.6**. It was therefore desirable to identify an alternative serum with a lower non-specific effect upon the trypanosomes. The lytic effect of both fresh and heat-inactivated guinea-pig serum was thus compared with lysis by rabbit serum and the results in **figure 3.6** show that guinea-pig serum caused less lysis than rabbit serum. The percent lysis caused by guinea-pig serum was usually under 5% and never reached more than 10%.
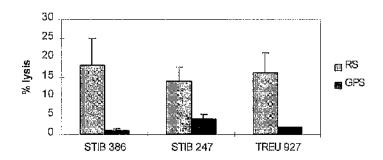
Figure 3.6. A comparison of guinea-pig serum and rabbit serum as potential negative control sera. Both guinea pig (GPS) and rabbit sera (RS) were tested and their lytic abilities assessed in an *in vitro* HSS assay. Trypanosomes from STIB 386, STIB 247 and TREU 927 were purified and incubated with 25% serum for 2.5 hours at a concentration of 1×10^7 cells/ml at 37°C. n= 3, $\overline{x}$ + 1SD;

Morphology of bloodstream trypanosomes

As has been noted by others (Rickman & Robson, 1972; Rifkin, 1978) there is a great variability in susceptibility to lysis depending on the parasitaemia of the donor animal. During a rising parasitaemia the majority of trypanosomes are slender form trypomastigotes and these are more sensitive to lysis than the stumpy bloodstream forms seen during lag phase parasitaemia and subsequent peaks of parasitaemia as shown in **table 3.1**. Stumpy forms are relative resistant to lysis and to minimise variation due to a mixture of morphological types all subsequent experiments were carried out with bloodstream trypanosomes during log phase parasitaemia when slender forms predominate.

	% lysis	
	STIB 386	**STIB 247**
Predominantly slender forms (>85%)	52	86
Predominantly stumpy forms (>85%)	27	54

Table 3.1. A comparison of the percentage lysis caused by human serum of slender and stumpy form trypomastigotes. The susceptibility of two morphologically different stages of bloodstream trypanosomes to human serum was tested by comparing the % lysis for the two forms in an *in vitro* HSS-assay. Slender forms were harvested during log phase growth while stumpy form were purified after the initial peak of parasitaemia. The proportion of slender and stumpy form trypanosomes in each population were determined according to the criteria of Hoare (1970) from Giemsa stained blood smears. The cells were then incubated with 25% human serum for 2.5 hours at 37°C after which the percentage lysis was determined.

Incubation volume

The total incubation volume is another factor which might influence the percentage lysis and incubation volumes from 250 µl to 2 ml were compared for variability in lysis. Using a standard assay with 1×10^7 cells/ml, 25% serum and a 2 hours incubation time at 37°C in eppendorf tubes, only a very minor difference could be observed, implicating a slight trend towards higher percentage lysis under smaller volumes (**figure 3.7**). Even though the difference is not statistically significant in these experiments, it was considered best to perform subsequent assays in a standard volume of 0.5 ml.
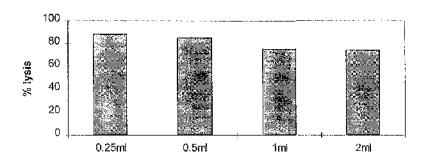
Figure 3.7. A comparison of percentage lysis in different incubation volumes. STIB 247 cells were used to investigate if the total incubation volume during an *in vitro* HSS-assay had an effect on the % lysis. Cell were purified and incubated in eppendorf tubes at a concentration of 1×10^7 cells/ml for 2.5 hours in 25% human serum at 37°C. The difference between the various incubation volumes ranged from 74% lysis in 2 ml to 88% lysis in 0.25 ml.

Counting live cells/fixed cells

A potentially important variable is the method by which the lysed cells were counted. Using unfixed cells, inaccuracies could arise firstly as a result of the time taken to count the cells leading to the time of incubation being variable and secondly, because live cells are much more readily observed than those undergoing lysis. The latter could lead to underestimates of the percentage lysis. These potential problems could be overcome by fixing the cells at specific time points during the incubation. A preliminary experiment was therefore conducted to compare the results of counts of fixed and unfixed cells. STIB 247 and STIB 386 trypanosomes, at a concentration of 1×10^7 cells/ml, were incubated with 25% serum at 37°C for 1 hour, 2 hours, 3 hours and 4 hours after which one sample was fixed in an equal volume of 1% formaldehyde in PBS and a second sample left unfixed. The percent lysis of the samples were then calculated after differential counting of the trypanosomes using phase-contrast microscopy. No differences between fixed and unfixed samples could be noticed once the incubation time was longer than 2 hours (**table 3.2**). It is possible that the reason for this is that the majority of cells are lysed within the first 2 hours and therefore the exact time point for counting is only of importance during the first 2 hours because after that time the majority of lysis has already taken place.

Stock	Treatment	Serum source	Time (min)			
			60	120	180	240
386	Fixed	HS	12	27	65	78
"	"	GPS	nd	6	13	14
"	Unfixed	HS	4	22	68	72
927	Fixed	HS	8	38	53	52
"	"	GPS	10	10	17	12
"	Unfixed	HS	17	40	55	55

Table 3.2. A comparison of percentage lysis observed when counting unfixed or fixed trypanosomes. Bloodstream trypanosomes from the two stocks STIB 386 and TREU 927 were tested for their resistance/sensitivity to human serum in a standard *in vitro* HSS-assay and the percentage lysis assessed either by counting the cells directly under a phase-contrast microscopy or after they had been fixed in 1% formaldehyde. The assay was repeated twice and the mean % lysis for cells incubated in human serum (HS) and cells incubated in control guinea pig serum (GPS) are shown for four different incubation times. nd, not determined.

3.2.2 Viability stains and dyes for an *in vitro* HSS assay

While there was no difficulty in distinguishing between lysed and normal, healthy trypanosomes, there was a whole range of slightly deformed, swollen and "tadpole" shaped cells which started to appear after 1 hours incubation of human serum sensitive cells in human serum. The extent to which these cells of abnormal morphology are viable or non-viable is unclear and cannot be readily determined either by infectivity tests or by phase-contrast microscopy. Four different staining methods were tested as markers for parasite viability: rhodamine 123, acridine orange, bis-benzimide and MitoTracker Green FM (Molecular Probes).

Rhodamine 123

Rhodamine 123 is a fluorescent, cell-permeant relatively non-toxic stain that is readily sequestered by active mitochondria without morphological distortion. Trypanosomes were stained with rhodamine 123 by diluting a 10 mM rhodamine:DMSO stock solution in MEM to a final concentration of 100 μg/ml and mixing with the trypanosome suspension at 1/10 of the total volume. Both STIB 386 and STIB 247 were used but in different experiments. The solution was incubated for 10-30 minutes at 24-37°C and the cells then centrifuged and resuspended in MEM. This method was modified after Ronot *et.al.* (1986). Observation by fluorescence microscopy with appropriate filters (Zeiss filter-set 09 which gives maximum emission at green wavelengths) showed the mitochondria of trypanosomes stained with rhodamine 123 as bright yellow-green. Dead cells did not stain at all while "tadpole" shaped cells all stained with bright green mitochondria. The staining was however not completely discriminatory and there were always some motile cells that did not become stained and often dead (i.e. immotile) cells had a faint green stain which was difficult to distinguish from "tadpole" shaped cells.

Acridine Orange

Acridine orange accumulates in acid structures e.g. DNA, RNA and lysosomes and is well retained in the mitochondrion. The mitochondrial uptake of acridine orange is not dependent on membrane potential and all live cells will fluorescence. High purity

acridine orange was diluted to a stock concentration of 0.01% (w/v) in sterile saline and 1 µl of this stock mixed with 100 µl of a trypanosome suspension. Both STIB 386 and STIB 247 were used in separate experiments. The cells were then immediately viewed by fluorescence microscopy using Zeiss filter-set 09 which gives maximum emission at green wavelengths. Acridine orange appeared slightly toxic to bloodstream trypanosomes at this concentration and made them very sensitive to UV-light so that they died if viewed for too long. Live cells fluorescenced with a bright green mitochondrion and nucleus while dead cells had a yellow mitochondria and nucleus. Tadpole shaped trypanosomes all had yellow mitochondria. The green and yellow colours were however, rather hard to distinguish which made differential counting time-consuming and unreliable.

MitoTracker Green FM

MitoTracker Green FM is a commercial mitochondria-stain, produced by Molecular Probes, which was developed to be retained in cells after cell fixation and permeabilisation. When the MitoTracker probe enters an actively respiring cell it is oxidised and sequestered in the mitochondria where it reacts with accessible thiol groups on peptides and proteins to form an aldehyde-fixable conjugate which is fluorescent. The MitoTracker was prepared as recommended by the manufacturer; one ampoule, 50 µg, was dissolved in 75 µl pure anhydrous DMSO to give a 1 mM stock solution, diluted to a working concentration of 200 nM, mixed with pelleted cells, which had been incubated with human serum, and incubated for 15-45 minutes at 37°C. The cells were then washed in HBSS, fixed in 1% formaldehyde for 15 minutes at 37°C and finally washed twice in PBS before being viewed by fluorescence microscopy using Zeiss filter-set 09. Live cells gave a bright green staining of the kinetoplast but the whole cell surface became stained to some degree. The staining appeared to be the same after fixation with either formaldehyde or paraformaldehyde or no fixation. Procyclic trypanosomes gave the same fluorescence pattern and intensity as bloodstream trypanosomes. Dead cells did not stain at all and very sluggish, "tadpole" shaped cells stained as live cells. The percentage live cells identified when stained with MitoTracker Green was slightly higher than when counted without staining; on average 59% instead of 51% for STIB 386 and 32%

instead of 24% for STIB 247, n = 2. It was very convenient to be able to work with fixed cells and smears stained with MitoTracker Green were useful for counting live cells but a counterstain for dead cells was also required because in fixed preparations it was extremely difficult to detect dead and lysed cells.

Bis-benzimide

Bis-benzimide has been used for staining the nucleus of non-viable *Cryptosporidium* (Brown *et.al.*, 1996) and this dye turned out to work equally well for staining the nucleus of dead trypanosomes. 0.01 mg bis-benzimide was mixed with 100 µl trypanosomes suspension in MEM immediately before viewing the cells by fluorescence microscopy using filter set 00 which gives optimal emission of blue light. All immotile and lysed trypanosomes stained with a bright blue nucleus whereas none of the normal, live cells or the tadpole shaped cells stained at all.

3.2.3 Lysis under optimal *in vitro* HSS-assay conditions

Taking the results from each of these preliminary experiments and using the optimal conditions for each variable, a set of standard incubation conditions was decided upon. The following standard incubation conditions were subsequently used. Trypanosomes were harvested during log-phase parasitaemia and purified by differential centrifugation or by DEAE-cellulose purification. The cells were then incubated in 25% heat-inactivated serum (v/v) from a single donor, using guinea-pig serum as control serum, at 37°C for 2.5 hours. Cell concentration was kept at 1×10^7 cells/ml in a total reaction volume of 0.5 ml in sealed 1.0 ml eppendorf and the cells were placed on ice immediately after incubation. The percentage lysed cells was then counted using phase-contrast microscopy without prior fixation, counting 200 cells at 400x magnification. Human serum sensitive trypanosomes started to become affected after 1 hours incubation, after 2 hours the majority of cells had become swollen, 'tadpole' shaped or lysed and within 3 hours all of the human serum sensitive cells had lysed (**figure 3.8**).
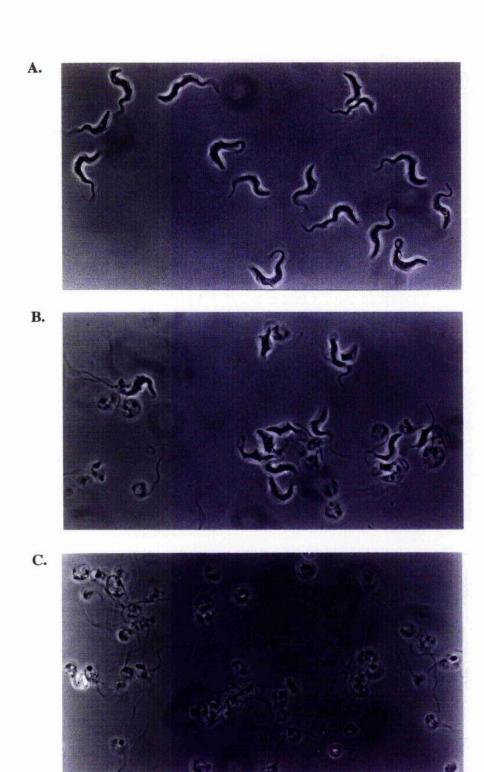
Figure 3.8. Phase-contrast photomicrographs of *T.brucei* (ETat1.2S) incubated at 37°C in 25% human serum for (**A**) 0 min, (**B**) 60 min and (**C**) 120 min.

The standard assay was repeated 18 times for STIB 386, 16 times for STIB 247 and 8 times for TREU 927 and the combined results are shown in **figure 3.9**.
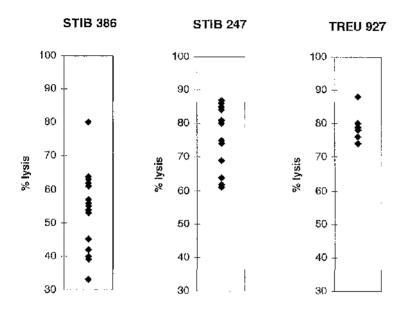
Figure 3.9. Percentage lysis after standard *in vitro* HSS-assay for each of the trypanosome stocks STIB 386, STIB 247 and TREU 927. Purified cells were incubated with 25% serum for 2.5 hours at 37°C at a final concentration of 1×10^7 cells/ml. For STIB 386 $\bar{x}\pm$ SD = 52.2±11.4 (n=18), for STIB 247 $\bar{x}\pm$SD = 76.4±9.5 (n=16) and for TREU 927 $\bar{x}\pm$SD = 79.2±3.8 (n=8). A comparison between STIB 386 and STIB 247 using Mann-Witney U-statistics indicated a significant difference (U=16.5, p<0.05) but the considerable variation between individual assays made it too unreliable for further use.

The mean percentage lysis for STIB 386 was 52% but varied between 33% and 80% for different assays, equivalent to a SD of 11.44, despite identical conditions. The variation in percent lysis was also observed for the other two strains with STIB 247 showing a mean lysis of 76% with individual assays varying between 61% and 87% (SD 9.50) and TREU 927 varying between 74% and 88% with a mean lysis of 79% (SD 3.83). In addition, two *T.b.gambiense* stocks were tested as biological controls since *T.b.gambiense* is known to be highly resistant to human serum. Both these *T.b.gambiense* isolates, ELIANE and BIM, showed only 5% lysis after 2 hours incubation in human serum, 11% after 4 hours incubation and after 19 hours incubation 68% of the cells had a normal morhpology as observed by phase-contrast microscopy. The *T.b.gambiense* isolates shows a much higher degree of serum resistance than the other three trypanosome stocks. Two conclusions can be drawn

from the *in vitro* HSS-assay data. Firstly, that STIB 386 was more resistant to killing with human serum than the other two stocks and secondly that there was considerably variability between individual assays such that, as a potential screening assay for F_1 progeny of genetic crosses, it was too unreliable for the purposes of this analysis.

3.2.4 Development of an *in vivo* HSS-assay

The variation between replicates of *in vitro* assays of the same trypanosome strain under identical and optimal conditions, as defined in **3.2.3** was too large and investigations into developing an *in vivo* human serum sensitivity assay were therefore initiated with the prediction that this should produce more reliable results. The results from the *in vitro* assays were used to help develop the *in vivo* assay. Starting conditions for the *in vivo* assay were: the use of purified bloodstream trypanosomes, harvested during log-phase parasitaemia, incubation at a final concentration of 1×10^7 cells/ml in 25% serum from a single donor and incubation at 37°C. Other parameters had to be reinvestigated since they were not applicable to an *in vivo* assay.

3.2.5 Comparisons of potential variables for an *in vivo* HSS assay

The *in vivo* human serum sensitivity assay was planned to involve incubating purified bloodstream trypanosomes with human serum for a fixed length of time and injection of the trypanosomes i.p. into mice which were then monitored for the development of parasitaemias. A set of variable parameters were investigated for the development of the assay: the number of trypanosomes to be injected, the duration of incubation with human serum, the number of days needed for monitoring the mice and the choice of control serum.

Number of trypanosomes for injection

It is clear that some cloned lines of *T.brucei* are stable and sensitive to lysis by human serum, such as STIB 247, some are stable and resistant whilst others can switch phenotype (see **section 1.4.1**). The stability of phenotype had not been determined for STIB 386 or TREU 927. There is the potential, therefore, for the numbers of trypanosomes in an inoculum to influence the assay results and so this variable was investigated. The apparently human serum sensitive strain TREU 927 might, for example, include a small proportion of human serum resistant cells which could give rise to a parasitaemia in mice if a large inocula was used.

Results of preliminary experiments (**table 3.3**) indicated that when a total of either 100 or 1000 cells were used for inoculation into mice, with an incubation time of 2.5 hours, patent parasitaemias for STIB 386 but not for STIB 247 were obtained. The situation with the (presumably) human serum sensitive TREU 927 was ambiguous in that inoculations of 100 trypanosomes in some cases was enough to cause a parasitaemia whereas 1000 trypanosomes in all cases gave rise to an infection in the mice.

Inocula size/mouse	Trypanosome line	No. of mice developing patent infection /no. inoculated	
		HS	GPS
1000	STIB 386	8 / 8	4 / 4
"	STIB 247	0 / 8	2 / 2
"	TREU 927	8 / 8	2 / 2
100	STIB 386	8 / 8	4 / 4
"	STIB 247	0 / 8	2 / 2
"	TREU 927	3 / 8	2 / 2

Table 3.3. The number of mice that developed a patent parasitaemia when injected i.p. with either 100 or 1000 trypanosomes which had been incubated with human serum. Mice were injected i.p. with either 100 or 1000 trypanosomes which had been incubated with 25% human serum or with control guinea pig serum for 2.5 hours at 37°C and monitored for parasitaemia for 20 days. These are the combined data of 2-4 replicate experiments.

Incubation time

The length of the time trypanosomes are incubated with human serum before being injected into mice will affect the degree of damaged caused to them and their ability

to initiate an infection. To investigate this effect, purified bloodstream trypanosomes were incubated with 25% human serum for various lengths of time at 37°C. 100 cells from each sample were then injected i.p. into mice and the mice monitored for the development of a parasitaemia for at least 20 days. The human serum resistant line, STIB 386, was capable of infecting mice in all cases after it had been incubated in human serum for 2, 3 or 4 hours but cells incubated for 6 or 8 hours could no longer cause an infection (**table 3.4**). A F_1 hybrid from STIB 247 x TREU 927 gave rise to a parasitaemia in 2 of 8 mice after 2 hours incubation in human serum but 0 of 6 mice after 4 hours incubation. TREU 927 infected 2 of 8 mice after either 2 or 4 hours incubation. The guinea pig serum negative controls all gave rise to parasitaemias even after 8 hours incubation.

Stock	Serum source	No. of mice developing patent parasitaemia / no. inoculated at different incubation times (hours)				
		2	**3**	**4**	**6**	**8**
STIB 386	HS	12 / 12	3 / 3	12 / 12	0 / 6	0 / 6
"	GPS	4 / 4	1 / 1	4 / 4	2 / 2	2 / 2
F_1 247x927	HS	2 / 8	-	0 / 6	-	-
"	GPS	2 / 2	-	2 / 2	-	-
TREU 927	HS	2 / 8	-	2 / 8	-	-
"	GPS	2 / 2	-	2 / 2	-	-

Table 3.4. The proportion of mice which developed parasitaemias after injection with trypanosomes which had been incubated with human serum. Mice were inoculated with 100 trypanosomes which had been incubated with 25% human serum or guinea pig serum for 2 to 8 hours. The *in vivo* HSS-assays were repeated 2 to 4 times in 2 to 4 mice for each assay and one control mouse, injected with cells incubated in guinea pig serum instead of human serum, was included in each assay.

Immunosuppression of mice

One concern was that small numbers of viable trypanosomes inoculated into immunocompetent mice might not always generate a detectable parasitaemia if the immune response cleared infections before they become patent. This possibility would apply in particular to STIB 247 which grows more slowly than the other stocks (Turner *et.al.*, 1995). Evidence suggesting this possibility is that incubation of STIB 247 in the control guinea pig serum on several occasions failed to produce a parasitaemia (data not shown). To avoid any potential interference of the mouse immune system with the assay it was therefor considered more reliable to use only

immunosuppressed mice for the standard *in vivo* HSS-assay. The mice were immunosuppressed with Cyclophosphamide (0.2 mg/g) given 24 hours prior to infection.

3.2.6 The reliability of an *in vivo* HSS assay under optimal conditions

The optimal assay conditions were determined as follows: purified trypanosomes were incubated at a concentration of $1x10^7$ cells/ ml with 25% human serum from a single donor for 4 hours and 100 trypanosomes subsequently inoculated by i.p. injection into two immunosuppressed mice. For each assay one control mouse was also used which was inoculated with 100 cells, treated exactly the same way as described above, apart from being incubated with guinea pig serum instead of human serum. The mice were than monitored for the presence of a parasitaemia at least every other day for 14 days (Turner *et.al.*, 1986; Scott, *et.al.*, 1996). To test the reliability of the assay it was carried out repeatedly in the three parental stocks STIB 247, STIB 386 and TREU 927. During six separate repeats of the assay using STIB 386, all mice (12) developed a parasitaemia within 6 days of infection. For STIB 247, the assay was repeated five times but none of the mice (10) become infected even though all of the control mice developed a parasitaemia within 7 days of infection. TREU 927 reacted as a semiresistant strain where in three of six assays one of two mice became infected while in the other three assays none of the mice become infected.

3.2.7 The effect of human serum compared with purified TLF

To further assess the reliability of the *in vivo* HSS assay a comparison of the lytic effects of human serum and purified TLF was performed. The purified TLF was kindly provided by Dr. Jayne Raper at NYU Medical Centre, USA, and was prepared as described in Raper *et.al.* (1996). At the time of purifying and aliquoting the TLF, the activity was measured as 1 unit per μl where 1 unit is the amount required to kill

50% of human serum sensitive trypanosomes (ETat1.9) in 2 hours when incubated at a concentration of 1×10^6 cells/ml in 200 µl. To conduct this comparison purified trypanosomes at 1×10^6 cells/ml were incubated with amounts of TLF between 0.25 µl and 8 µl diluted in 1xTBS in a total volume of 200µl for 2 hours at 37°C and 100 cells then injected into mice. Also, the percent lysis was determined by phase-contrast microscopy and the data are presented in **table 3.5**.

TLF (µl)	% lysis				
	STIB 386	**STIB 247**	**TREU 927**	**ETat1.2 (S)**	**ETat1.10 (R)**
0.25	35	64	82	26	nd
0.5	nd	nd	79	48	1
1	70	60	75	43	3
2	68	77	100	44	5
4	67	100	95	60	12
8	nd	nd	90	55	10
HS	52	79	76	63	7
GPS	2	1	5	4	2

TLF (µl)	No. of mice developing patent parasitaemia / no. inoculated mice				
	STIB 386	**STIB 247**	**TREU 927**	**ETat1.2 (S)**	**ETat1.10 (R)**
1	2 / 2	0 / 2	0 / 2	2 / 2	1 / 2
4	2 / 2	0 / 2	0 / 2	1 / 2	1 / 2
HS	2 / 2	0 / 2	nd	nd	nd
GPS	1 / 1	1 / 1	1 / 1	1 / 1	1 / 1

Table 3.5. The lytic effect of purified TLF *in vitro* and *in vivo*. The lytic effect of purified TLF was compared with the lytic effect of human serum (HS) by conducting HSS-assays both *in vitro* (upper table) and *in vivo* (lower table). Different amounts of TLF was used and guinea pig serum used as negative control. nd, not determined.

ETat1.2 and ETat1.10 were used as biological controls, the former is human serum sensitive and the latter is human serum resistant. ETat1.2 were able to infect a proportion of the mice after incubation with TLF and the same was true for ETat1.10. Incubation of STIB 386 with 4 µl TLF, which is four times the amount of TLF which is expected to kill 50% of the trypanosomes within 2 hours, was still able to infect mice. For STIB 247 none of the mice became infected after inoculation with cells incubated with 1µl TLF, which was the lowest concentration of TLF used. TREU 927 was also TLF sensitive as determined by the *in vivo* HSS-assay as non of the

mice developed a parasitaemia. In summary, TLF *in vivo* defines STIB 386 as resistant and STIB 247 and TREU 927 as sensitive but TLF does not discriminate ETat1.2 from ETat1.10. Apart from the results with TREU 927 and ETat the results are the same as obtained with human serum.

For TLF *in vitro* STIB 386, STIB 247 and TREU 927 essentially show no difference when incubated with more than 1 μl TLF although TREU 927 and STIB 247 are marginally more sensitive. There is a very good discrimination between the resistant ETat1.10 and the sensitive ETat1.2. at all concentrations. When incubated with 0.25 μl TLF, all five clones give similar results to the experiment *in vivo*.

3.3 DISCUSSION

From the experiments described in this chapter, it was concluded that an *in vitro* HSS-assay could not be used for reliable measurement of the resistance or sensitivity to human serum of various stocks or lines of trypanosomes and that only an *in vivo* assay could provide clear results. Previous *in vitro* tests used by other groups were either performed on metacyclic trypanosomes, using a culture technique described by Brun & Jenni (1987), or on bloodstream cells either using a fluorescence-based assay originally described by Tomlinson *et.al.* (1995) (see section **1.4.2**) or directly by phase contrast microscopy by counting the proportion of lysed blood stream trypanosomes (De Greef *et.al.*, 1989; Hajduk *et.al.*, 1989; Rifkin, 1978). Since we were interested in human serum resistance/sensitivity in bloodstream trypanosomes, which possibly may be different from resistance/sensitivity in metacyclics, the culture technique assay was not an option. Also, the assay needed to be applicable to a substantial number of F_1 recombinant progeny of genetic crosses and individual transmission of each of these through tsetse flies would be labour intensive. The fluorescence-based assay, on the other hand, is virtually identical to counting live and lysed trypanosomes, only less laborious. One of the reasons why the research groups using this method have not encountered problems may be that they are working on 'pure' human serum sensitive stocks, which have been in the laboratory for more

than 30 years and only rarely switch between sensitive and resistant phenotypes which may not be the case with recently fly-transmitted stocks. This assay is not used for testing unknown stocks for their sensitivity to human serum but as an aid when studying the lytic component/s in human serum and it is the percentage of lysed cells that is of primary interest. For those purposes, the *in vitro* HSS-assay may give sufficient information. The main problem with the *in vitro* assay is the difficulty in knowing which cells are viable and infective and which cells have lost their infectivity. There were no morphological criteria which were able to distinguish infective cells from non-infective cells and which could be identified by phase contrast microscopy.

It was interesting to note that serum from different individuals differed in its lytic activity and that the main difference was the time for the onset of lysis. Differences between individuals have been observed previously (Rifkin, 1978; Lorenz *et.al.*, 1994). After 1.5 hours incubation time, lysis could vary between 12% and 56% for serum from two individuals while the final amount of lysis only varied between 89% and 97% after 4 hours. Since the lytic factor in human serum has not been clearly identified yet, it makes it difficult to speculate about the reasons for this difference. It has been suggested that haptoglobin-related protein is the toxin in the trypanocidal factor (Smith *et.al.*, 1995) and that its activity can be inhibited by haptoglobin (Smith & Hajduk, 1995). Differences in the onset of lysis may therefore depend on differences in the level of haptoglobin in the serum. A recent paper by Muranjan *et.al.* (1998) however, rejects the idea of the importance of haptoglobin and argues that the addition of haptoglobin to TLF1 does not affect the trypanolytic activity. All of the blood samples for serum preparation was unfortunately not drawn from fasting individuals and another explanation for the variation in lytic effect may be dietary variation resulting in variations in the overall concentration of HDL in the serum and therefore in the lytic activity. It was clear that this lytic activity was much enhanced at 37°C compared with room temperature, possible because the trypanocidal factor needs to be internalised into the trypanosomes by an active process. This process also appears to be inhibited if the cells are placed on ice or at 4°C where they can remain intact in the presence of human serum for at least 24 hours (own observations).

The trypanosome concentration in reaction tubes also affected the degree of lysis. It is however, reasonable to expect more lysis with higher trypanosome concentrations probably because of limited nutrient availability. In the absence of human serum there was more lysis when cells were kept at high concentrations in MEM+Earles salt. During these experiments, with various trypanosomes concentrations, it became evident that rabbit serum was a less than ideal negative control serum. The lysis occurring during incubation with rabbit serum could reach the same levels as lysis caused by human serum especially as trypanosome concentrations increased. A comparison with guinea pig serum clearly showed that rabbit serum caused an almost tenfold increasion of cell death when STIB 386 cells were used. It is not clear why the rabbit serum exhibited such a trypanocidal effect, especially since other investigators have found that it caused very little lysis (Rifkin, 1978; Rifkin, 1984). The rabbit serum I used all came from the same batch and had been prepared in the laboratory and no other sources were tried so it is not possible to determine whether this was a general feature or specific for this particular batch of serum.

The use of dyes to assess cell viability offered no apparent improvement over phase-contrast microscopy and could not distinguish between cells capable of infecting mice and non-viable trypanosomes. There was no consistent pattern between different dyes. For a viability stain to be useful both lysed and live cells have to be stained so that the percentage can be counted without having to change the field. Bis-benzimide stained dead cells very clearly but was not a good counterstain because it obviously can not be used on fixed cells which would have been needed if MitoTracker was going to be useful. Rhodamine 123 stained tadpole shaped cells as live cells while acridine orange stained them as dead. When these tadpole shaped cells were injected into mice they were in some cases capable of causing an infection which would implicate that some of them should be regarded as live cells while others are dead. The only advantage with fixing the cells before counting percentage lysis was the convenience of being able to count them at a later stage but in practice it made no difference to the accuracy of the test.

By counting live and lysed cells, and regarding any change in morphology as non-viable, it was not possible to clearly distinguish a human serum sensitive population from a serum resistant population. There was an overlap in the range of percentage lysis between the clearly resistant STIB 386 and the clearly sensitive STIB 247. It is possible that if time was less limiting a way of solving this problem would have been found but operationally the *in vivo* assay proved to be more reliable. Most of the conditions found as optimal for an *in vitro* assay were also used in the *in vivo* HSS-assay, only the number of trypanosomes needed for inoculation into mice and the length of the incubation time had to be optimised. The number of cells for inoculation into mice was initially thought to be of importance because TREU 927, originates from a location in Kenya with no history of sleeping sickness and would thus generally be regarded as a human serum sensitive *T.b.brucei* but this stock behaved differently in the assay from the serum sensitive STIB 247. If, however, we accept the concept that TREU 927 is of intermediate phenotype, as has been previously reported for some field isolates (Rickman & Robson, 1980; Brun & Jenni, 1987), the inoculum dose is less important. Even inoculation of 1×10^6 cells of STIB 247 incubated with human serum for 2 hours did not result in a parasitaemia whereas a low inocula of STIB 386 always gave rise to parasitaemias. These data suggest that the *in vivo* assay is reliable, at least for STIB 247 and STIB 386. The incubation time is probably not a critical variable in that STIB 247 and STIB 386 gave reliable results irrespective of duration of incubation in the range of 2-4 hours.

To compare the lytic effect of whole serum upon trypanosomes with the lytic effect of a defined and purified component of the trypanolytic factor both *in vitro* and *in vivo* assays were carried out in the presence of TLF2 instead of human serum. The activity of TLF2 was measured as a volume required to kill 50% of human serum sensitive stocks after 2 hours at 37°C. No assumptions were made about how much activity was needed to kill 100% of cells and it was therefore necessary to assay using a dose titration of TLF2. There was a reasonable agreement between the results of the *in vivo* test with human serum and TLF2 again supporting the reliability of the *in vivo* assay. The only in-consistency was with the ETat1.10 trypanosome line which, in *in vitro* assays was clearly resistant and yet failed to infect half of the mice

after incubation with TLF2 in the *in vivo* test and so could not be distinguished from the sensitive ETat1.2.

Chapter 4

Genetic inheritance of human serum sensitivity/resistance

4.1 INTRODUCTION

The three cloned trypanosome stocks STIB 247, STIB 386 and TREU 927 have previously been crossed in all pairwise combinations (Turner *et.al.*, 1990) and a number of hybrid progeny identified and cloned. Progeny from these crosses were originally identified by screening for isoenzyme and RFLP markers for which the parents were different and/or showed allelic segregation. The majority of the hybrids used in this project have also been re-screened by PCR analysis of the polymorphic minisatellite markers Cram, ms-42 and Tb-292 to further confirm that they are recombinants. The F_1 progeny show allelic segregation at loci heterozygous in one of the parents and recombination between alleles at different loci which is consistent with the occurrence of meiosis and a Mendelian segregation of alleles. Even though mating is not obligatory (Sternberg *et.al.*, 1989), it is clear that it occurs and therefore genetic exchange may influence the development of new virulent trypanosome strains with different host specificities. The availability of characterised hybrids provides the possibility of studying the genetic basis for human serum resistance/sensitivity. Recombinants can also arise by self-fertilisation (Tait *et.al.*, 1996) and, by marker and karyotype analysis, four clones from STIB 247 and one clone from TREU 927 have been identified as the products of self fertilisation. This chapter describes the genetic analysis and the inheritance of human serum resistance/sensitivity and explores the genetic basis of this trait. The main aim was to reveal important information about the number of loci and alleles involved and to determine the dominance relationships between segregating alleles. The F_1 progeny of crosses between STIB 247 (sensitive), STIB 386 (resistant) and TREU 927 (intermediate) were analysed for their resistance to human serum. From the pattern of inheritance it has been possible to suggest a model for the inheritance of human serum resistance and, more importantly, refute a number of models for inheritance which are incompatible with the results. The parental stock TREU 927 and some of the F_1

progeny exhibit an intermediate resistance phenotype which is defined in the assay as a proportion of trypanosomes surviving lysis by human serum. As a result of detecting F_1 progeny clones of intermediate resistance, the simplest possible model is based on 3 alleles, a codominant sensitive and resistant allele and a recessive sensitive wildtype allele, all at a single locus.

4.2 RESULTS

4.2.1 Human serum resistance in the F_1 progeny from crosses

Recombinant progeny from the three crosses STIB 247 x STIB 386, STIB 247 x TREU 927 and STIB 386 x TREU 927 were all analysed for their resistance or sensitivity to human serum by the *in vivo* HSS-assay (Chapter 3). The initial aim was to analyse all the available progeny and this was to a large extent achieved. Since TREU 927 turned out to be of intermediate resistance and difficult to score, most effort was concentrated on the products of the STIB 247 x STIB 386 cross. All assays involving STIB 247 were conducted in immunosuppressed mice to exclude interference of the mouse immune system with the growth of the trypanosome population.

Recombinants from a cross between STIB 247 and STIB 386

A total of 28 hybrids from a cross between the resistant STIB 386 and the sensitive STIB 247 were analysed for resistance or sensitivity to human serum. Nine of these hybrids were the first bloodstream population of metacyclic clones from the tsetse fly while the remaining 19 were obtained by cloning trypanosomes from the bloodstream infections initiated by tsetse flies containing mating products. These were all diploid recombinants of distinct genotypes identified by marker analysis (Annette MacLeod, pers.comm). The results of the *in vivo* HSS-assay showed that thirteen of the hybrids were of a sensitive phenotype, eight of them of a resistant phenotype and seven of an intermediate, semi-resistant phenotype, as shown in **table 4.1**.

Trypanosome cross STIB 386 x STIB 247

Clone	No. of mice developing parasitaemia / no of mice inoculated		Total no. of assays	HSR/HSS
	HS	GPS		
Parental STIB 386	12/12	6/6	6	Resistant
Parental STIB 247	0/10	5/5	5	Sensitive
F9/45 mcl 2	4/4	2/2	2	Resistant
F9/45 mcl 7	0/2	1/1	1	Sensitive
F9/45 mcl 9	4/4	2/2	2	Resistant
F9/45 mcl 10	4/8	4/4	4	Intermediate
F9/45 mcl 11	2/2	1/1	1	Resistant
F9/45 mcl 12	1/2	1/1	1	Intermediate
F492/50 mcl 12	2/2	1/1	1	Resistant
F492/50 mcl 13	0/2	1/1	1	Sensitive
F492/50 mcl 24	2/2	1/1	1	Resistant
F492/50 bcl 1	0/2	1/1	1	Sensitive
F492/50 bcl 2	0/2	1/1	1	Sensitive
F492/50 bcl 3	0/2	1/1	1	Sensitive
F492/50 bcl 4	0/2	1/1	1	Sensitive
F492/50 bcl 5	0/2	1/1	1	Sensitive
F492/50 bcl 6	1/2	1/1	1	Intermediate
F492/50 bcl 7	0/2	1/1	1	Sensitive
F492/50 bcl 8	0/2	1/1	1	Sensitive
F492/50 bcl 13	0/4	2/2	2	Sensitive
F492/50 bcl 14	2/2	1/1	1	Resistant
F492/50 bcl 15	0/2	1/1	1	Sensitive
F492/50 bcl 16	0/4	2/2	2	Sensitive
F492/50 bcl 17	1/2	1/1	1	Intermediate
F492/50 bcl 18	2/2	1/1	1	Resistant
F492/50 bcl 19	1/2	1/1	1	Intermediate
F492/50 bcl 20	0/2	1/1	1	Intermediate
F492/50 bcl 21	1/2	1/1	1	Intermediate
F492/50 bcl 23	2/2	1/1	1	Resistant
F492/50 bcl 25	0/2	1/1	1	Sensitive

Table 4.1. The results of *in vivo* HSS-assays of hybrid progeny from a cross between the human serum resistant STIB 386 and the human serum sensitive STIB 247. The clone identification number is based on fly number, number of days post-infection for dissection and whether it is a metacyclic clone (mcl) or a bloodstream clone (bcl). From a total of twenty-eight hybrid progeny, thirteen were sensitive to human serum, eight were resistant and seven were semi-resistant.

Intermediate resistance is defined by the ability of a trypanosome stock, after incubation with human serum, to cause an infection in only a proportion of the mice inoculated. Since the intermediate phenotype was a rather unexpected finding, most clones identified as possessing this phenotype were re-assayed several times to verify the reproducibility of the initial result (**tables 4.1** and **4.2**). The results were reproducible leading to the conclusion that that was a genuine, definable phenotype.

F₁ progeny from a cross between STIB 247 and TREU 927

Ten of the hybrid progeny from a cross between the human serum sensitive STIB 247 and the intermediate resistant TREU 927 were screened for their resistance or sensitivity to human serum by the *in vivo* HSS-assay. Six of the hybrids were scored as human serum sensitive, two as human serum resistant and two as intermediate resistant as shown in **table 4.2**.

Trypanosome cross STIB 247 x TREU 927

Clone	No. of mice developing parasitaemia / no of mice inoculated		Total no. of assays	HSR/HSS
	HS	GPS		
Parental STIB 247	0/10	5/5	5	Sensitive
Parental TREU 927	3/12	6/6	6	Intermediate
F974/70 mcl 4	0/2	1/1	1	Sensitive
F532/72 mcl 1	2/2	1/1	1	Resistant
F532/72 mcl 2	0/2	1/1	1	Sensitive
F532/72 mcl 3	0/2	1/1	1	Sensitive
F532/72 mcl 4	2/2	1/1	1	Resistant
F532/72 mcl 5	2/6	3/3	3	Intermediate
F532/72 mcl 6	0/2	1/1	1	Sensitive
F532/72 mcl 7	2/4	2/2	2	Intermediate
F532/72 mcl 8	0/2	1/1	1	Sensitive
F532/72 mcl 9	0/2	1/1	1	Sensitive

Table 4.2. The results of *in vivo* HSS-assays of hybrid progeny from a cross between STIB 247 and TREU 927. Ten of the hybrid progeny from a genetic cross between the human serum sensitive STIB 247 and the semi-resistant TREU 927 were analysed for their resistance or sensitivity to human serum. Six of them were sensitive to human serum, two resistant to human serum and two of an intermediate resistance. All the hybrids tested were from metacyclic clones and none of them prepared from bloodstream clones. The results from both of the intermediate human serum resistant clones were repeated and confirmed.

F₁ progeny from a cross between STIB 386 and TREU 927

STIB 386 has been shown to be resistant to human serum while TREU 927 has, in this project, proved to be of an intermediate human serum resistance. Hybrid progeny from a cross between the two stocks were analysed for their sensitivity or resistance to human serum and the results are shown in **table 4.3**. Of the seven hybrids tested, four were sensitive to human serum and three were resistant to human serum. There were no clones with an intermediate resistance among the tested hybrids.

Trypanosome cross STIB 386 x TREU 927

Clone	No. of mice developing parasitaemia / no of mice inoculated		Total no. of assays	HSR/HSS
	HS	GPS		
Parental STIB 386	12/12	6/6	6	Resistant
Parental TREU 927	3/12	6/6	6	Intermediate
F246/56 mcl 6	0/2	1/1	1	Sensitive
F296/44 bcl 1	2/2	1/1	1	Resistant
F296/44 bcl 2	4/4	2/2	2	Resistant
F296/44 bcl 3	0/4	2/2	2	Sensitive
F296/44 bcl 9	2/2	1/1	1	Resistant
F296/44 bcl 12	0/2	1/1	1	Sensitive
F296/44 bcl 13	0/2	1/1	1	Sensitive

Table 4.3. The results from *in vivo* HSS-assays of 7 hybrids from a genetic cross between the human serum resistant STIB 386 and the intermediate human serum resistant TREU 927. Four of the hybrids proved to be sensitive to human serum while the other three were resistant to human serum.

Recombinants from self-fertilisation events

A further five clones which previously had been characterised as selfing products (Tait *et.al.*, 1996) were also tested for their sensitivity to human serum. These clones were identified as the products of self-fertilisation based on marker analysis. They are identical to one of the parental stocks used in the crosses except that they are homozygous for a proportion of the loci which were heterozygous in the original parental stock. Self-fertilisation products can be very informative when determining parental genotypes as they can be used to determine if an individual is heterozygous or homozygous for the allele in question. Four of the selfing products were from STIB 247 (Tait *et.al.*, 1996) while one of them was identified as a TREU 927 selfer (MacLeod, unpublished). All four selfing products from STIB 247 were sensitive to human serum while the TREU 927 selfing product was of an intermediate resistance (**table 4.4**). Thus, no segregation of the sensitive phenotype of STIB 247 was observed nor of the intermediate resistance of TREU 927.

Self-fertilisation products from STIB 247 and TREU 927

Clone	No. of mice developing parasitaemia / no of mice inoculated		Total no. of assays	HSR/HSS
	HS	GPS		
Parental STIB 247	0/10	5/5	5	Sensitive
F492/50 mcl 10	0/4	2/2	2	Sensitive
F492/50 mcl 11	0/4	2/2	2	Sensitive
F492/50 mcl 15	0/4	2/2	2	Sensitive
F492/50 mcl 16	0/4	2/2	2	Sensitive
Parental TREU 927	3/12	6/6	6	Intermediate
F296/46 mcl 1	1/4	2/2	2	Intermediate

Table 4.4. Results of *in vivo* HSS-assays of recombinants produced after self-fertilisation of STIB 247 and TREU 927. All of the selfing products from STIB 247 turned out to be sensitive as the parent and the only selfer from TREU 927 was also of the intermediate phenotype as its parental source.

4.2.2 Suggested models for inheritance of human serum resistance

The F_1 progeny from all three crosses demonstrated segregation of the human serum resistance phenotype but the limited number of selfing products screened showed no such segregation. When analysing the inheritance pattern for the human serum resistance trait only the results from the genetic cross between STIB 386 and STIB 247 were initially considered. This was mainly because a cross between a pure resistant and a pure sensitive clone could be more informative than a cross involving an intermediate resistant stock and also because a large number of hybrids had been cloned and were available for analysis while only a small number were available from the other two crosses. The results of the analysis of the STIB 247 x STIB 386 cross show that segregation of the resistance phenotype occurs in the F_1 progeny providing evidence that the HSR trait is genetically determined. There are a number of classical inheritance models which can be refuted on the basis of the three segregating phenotypes found (human serum sensitive, human serum resistant and intermediate, semi-resistant, phenotype) and the numbers in each class. The ratio of the numbers of progeny in each phenotypic class can be compared to theoretical ratios based on different models for the parental genotypes. Assumptions can be made about the number of alleles involved, the number of loci and the dominance

good fit with the observed ratios of phenotypes of F_1 progeny but the refuted models can be rejected with confidence. Only the simplest models that fit the data are considered in this section. Such models can subsequently be tested against the data to determine whether they fit and then tested by the analysis of offspring from a backcross or from a F_2 generation.

In order to explain the existence of an intermediate resistant phenotype there has to be either a specific allele coding for intermediate resistance or a codominance relation between a resistant and sensitive allele which would result in an intermediate phenotype. The simplest model that fits the data involves a codominant S^S and S^R allele and a recessive sensitive wildtype allele, s^+. For the human serum sensitive STIB 247 to express a sensitive phenotype under these circumstances it would have to be $S^S s^+$ and the resistant STIB 386 would have to be $S^R s^+$. The potential resulting hybrid progeny from this cross are shown in a Punnett square in **figure 4.1**.

Parents:		STIB 247 sensitive $S^S s^+$	STIB 386 resistant $S^R s^+$	
Gametes:		S^S and s^+	S^R and s^+	

$S^S s^+ \times S^R s^+$

	S^R	s^+
S^S	$S^S S^R$	$S^S s^+$
s^+	$s^+ S^R$	$s^+ s^+$

F_1:	Phenotypes	Genotypes	Genotypic ratio	Phenotypic ratio
	Sensitive	$S^S s^+$ $s^+ s^+$	1	2
	Resistant	$S^R s^+$	1	1
	Intermediate	$S^S S^R$	1	

Figure 4.1. The simplest possible model for inheritance of the human serum resistance trait. The model involves three alleles at a single locus, the S^S and S^R alleles being codominant while the third allele is a recessive wildtype sensitive allele, s^+. 1/2 of the hybrid offspring will theoretically become human serum sensitive, 1/4 will become human serum resistant and 1/4 will be of an intermediate resistant phenotype. The model has a good fit with the observed phenotypic ratio as proven by a χ^2 test which is shown in **figure 4.2**.

It can be seen that four different genotypes can arise, two of them expressing the same phenotype, human serum sensitivity, one being serum resistant and one having a potentially intermediate resistant phenotype. The observed and expected numbers of progeny are compared with a χ^2-test (see calculations in **figure 4.2**). The calculated χ^2 is 0.214 and the critical value from a theoretical distribution at 5% with d.f.=2 is 5.991. This means that there is no significant deviation and therefore a good fit with the model. A schematic picture of the proposed inheritance model is illustrated in **figure 4.3**.

Chi-square test

Phenotype	Observed number	Expected number
Sensitive	13	$(1/2) \times 28 = 14$
Resistant	8	$(1/4) \times 28 = 7$
Intermediate	7	$(1/4) \times 28 = 7$
Total	*28*	*28*

$$\chi^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

$$= \frac{(13-14)^2}{14} + \frac{(8-7)^2}{7} + \frac{(7-7)^2}{7} = 0.214$$

d.f. = 3-1 = 2
Table of Chi-square 5% critical value at 2 d.f. = 5.991

Figure 4.2. Chi square test for the proposed inheritance model. Comparison of the observed and expected results from the cross between STIB 247 and STIB 386 when assuming an inheritance model consisting of a codominant sensitive and resistant allele and a recessive sensitive allele. The model has a good fit with expected results since the calculated χ^2 is significantly less than χ^2 from table at 5% critical value and 2 degrees of freedom.
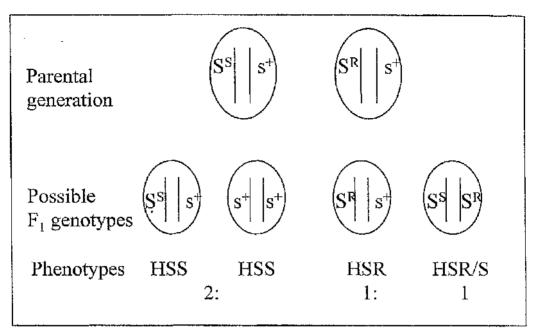
Figure 4.3. A schematic view of the proposed genetic model for the inheritance of the human serum resistance trait. This is the simplest possible model which has a good fit with the observed distribution of phenotypes after a cross between a human serum sensitive stock and a human serum resistant stock and it can be confirmed by a χ^2-test.

Another model which has a good fit with the observed ratio of the phenotypes involves an allelic series with three alleles in the dominance hierarchy $S^S > s^R > s^I$, where S^S is a completely dominant allele coding for serum sensitivity and s^R is a partially dominant allele for serum resistance and s^i is a completely recessive allele for intermediate resistance. This model has as good fit as the previously suggested model, as defined by χ^2 values and a model involving a specific allele for intermediate resistance might be helpful when considering the actual function of the gene. The model is regarded as another possibility since the precise function of gene/genes conferring human serum resistance/sensitivity is still unknown as discussed in section 4.3. The parental genotypes in this model would be $S^S s^I$ for STIB 247 and $s^R s^I$ for STIB 386 and the resulting hybrid progeny would have four different genotypes, two of which would express a human serum sensitive phenotype ($S^S s^R$ and $S^S s^I$), one that would express human serum resistance ($s^R s^I$) and one that would express the intermediate human serum resistance phenotype ($s^I s^I$) (**figure 4.4**).

Parents:		STIB 247	STIB 386	
		sensitive $S^S s^I$	resistant $s^R s^I$	
Gametes:		S^S and s^I	s^R and s^I	

$S^S s^I \times s^R s^I$

		s^R	s^I
S^S		$S^S s^R$	$S^S s^I$
s^I		$s^I s^R$	$s^I s^I$

F$_1$:	Phenotypes	Genotypes	Genotypic ratio	Phenotypic ratio
	Sensitive	$S^S s^R$	1	2
		$S^S s^I$		
	Resistant	$s^R s^I$	1	1
	Intermediate	$s^I s^I$	1	1

Figure 4.4. A second possible model for the inheritance of human serum resistance trait. The allele combination shown here is as equally valid as the first model. It involves a single allele coding for an intermediate resistant phenotype and it has just as good fit as the first suggested codominance model, confirmed by a χ^2 test.

4.2.3 Refuted inheritance models

The two models, described above, are the simplest possible scenarios that have a good fit with the numbers found in each phenotypic class. As already mentioned, there are many more classical models which do not fit the data and which can be refuted on the basis of the three phenotypes identified and the segregation ratios. The refuted models are important because it is difficult, without backcrosses or F$_2$ data, to prove the proposed models that fit the data, however, it is much more straightforward to prove that other models cannot fit the data. The refuted inheritance models will be dealt with one at a time including discussion of the reason for them being unable to explain the observed inheritance pattern.

Two allele models at one locus

All two allele models can be refuted because none of the models can ever lead to three classes of phenotype. This general point can be illustrated by an example where one of the phenotypic traits, resistance or sensitivity to human serum, is considered

as being determined by a dominant allele. If sensitivity to human serum is a dominant trait then the sensitive STIB 247 could be either heterozygous ($S^{S}s^{R}$) or homozygous ($S^{S}S^{S}$) for this trait while the resistant STIB 386 would be homozygous recessive ($s^{R}s^{R}$). A cross between the two would result in either all sensitive hybrid offspring or resistant and sensitive offspring in a 1:1 ratio as illustrated in **figure 4.5**.

Parents:		STIB 247	STIB 386		
		sensitive $S^{S}S^{S}$ or $S^{S}s^{R}$	resistant $s^{R}s^{R}$		
Gametes:		S^{S} or S^{S} and s^{R}	s^{R}		

A. $S^{S}S^{S} \times s^{R}s^{R}$

F₁:	Phenotypes	Genotypes	Genotypic ratio	Phenotypic ratio
	Sensitive	$S^{S}s^{R}$	1	1

B. $S^{S}s^{R} \times s^{R}s^{R}$

F₁:	Phenotypes	Genotypes	Genotypic ratio	Phenotypic ratio
	Sensitive	$S^{S}s^{R}$	1	1
	Resistant	$s^{R}s^{R}$	1	1

Figure 4.5. A genetic model for the cross between STIB 247 and STIB 386 assuming that sensitivity to human serum is a dominant trait and that two alleles are involved at a single locus. There are two different possibilities since the sensitive STIB 247 can have either the gametes SS or the gametes Ss. In the first instance, part **A**, all the progeny would be of a sensitive phenotype while in the second assumption, part **B**, half of the F₁ would be sensitive and the other half resistant.

No two allele model could explain the results obtained since only two phenotypes would be possible and these phenotypes would be in a 1:1 ratio or all progeny would be identical. It would not make any difference if resistance was determined by a dominant allele instead of sensitivity. It would still only give rise to two different phenotypes in a 1:1 ratio or all the progeny would be resistant instead of sensitive.

The predicted outcome of the crosses would not be any different if the recessive allele was a null allele rather than a functional recessive allele, as considered in the example above. Depending on which is the dominant allele one of the phenotypes,

resistance or sensitivity to human serum, would have to be regarded as being determined by a functional allele so that a lack of its expression would result in the other phenotype. It is therefore clear that inheritance of the human serum resistance trait can not be explained by simple dominant and recessive alleles but must involve alleles with different dominance relationships.

Another refuted two allele inheritance model involves codominance where alleles can express their products independently in heterozygotes so that a heterozygote will express an intermediate phenotype. If this was the case, a cross between a resistant and sensitive phenotype could give rise to intermediate, semi-resistant progeny but all of the progeny would be intermediate and no pure resistant or sensitive hybrids would be obtained. This is because the sensitive or resistant phenotype of the parental stock would have to be homozygous for the resistant and sensitive allele respectively. This model can therefore be refuted on the basis of predicting only one phenotypic class for the offspring.

Instead of the alleles being codominant, they could be partially dominant so that the F_1 progeny would express both alleles. If this was the case, and there were only two alleles at a single locus, then the human serum sensitive STIB 247 could be either homozygous for an incompletely dominant allele conferring sensitivity ($S^S S^S$) or homozygous for a recessive sensitive allele ($s^S s^S$). The human serum resistant STIB 386 would have to be either homozygous for an incompletely dominant resistant allele ($S^R S^R$) or homozygous for a recessive resistant allele ($s^R s^R$). In both models all of the hybrid offspring would be semi-resistant, as in the case of codominance.

Even though there is no distinct dividing line between the outcome of codominance and incomplete dominance and there is no easy way to define which is involved, it can be concluded that none of these models would be able to explain the observed phenotypes since the F_1 generation would consist of only one phenotype and not the three phenotypes observed.

Three allele models at a single locus

In the next set of models three different alleles with two levels of dominance are postulated, all defining the trypanosomes reaction to human serum. Some of these models can be refuted because they only generate two classes of progeny and others because they give segregation ratios which do not agree with those observed. One of the models that can be postulated is the model described in **figure 4.1**. The possible number of F_1 genotypes with a multiple allele model is defined by the formula $n(n+1)/2$ where n is the number of alleles involved. In a three allele model this will mean a possible 6 different genotypes while the number and ratios of phenotypes will vary depending on the dominance relationships between the different alleles.

As a different example, there can be two codominant alleles, conferring resistance and sensitivity, and a recessive wild type resistant (r^+) allele (**figure 4.6**).

Parental genotypes						
STIB 247	STIB 386	F_1	Phenotypes	Genotypes	Genotypic ratio	Phenotypic ratio
$R^S R^S$ x $R^R R^R$			Intermediate	$R^S R^R$	1	1
$R^S R^S$ x $R^R r^+$			Intermediate	$R^S R^R$	1	1
			Sensitive	$R^S r^+$	1	1
$R^S R^S$ x $r^+ r^+$			Sensitive	$R^S r^+$	1	1
$R^S r^+$ x $R^R R^R$			Intermediate	$R^S R^R$	1	1
			Resistant	$R^R r^+$	1	1
$R^S r^+$	$R^R r^+$		Resistant	$R^R r^+$	1	2
				$r^+ r^+$	1	
			Sensitive	$R^S r^+$	1	1
			Intermediate	$R^S R^R$	1	1
$R^S r^+$	$r^+ r^+$		Resistant	$r^+ r^+$	1	1
			Sensitive	$R^S r^+$	1	1

Figure 4.6. The predicted genotypic and phenotypic outcome for a cross between STIB 247 and STIB 386 assuming codominance and a recessive resistant wild type allele. In this model there are three alleles, a recessive wild type r^+ allele and two codominant R^S and R^R. alleles which would result in an intermediate resistant phenotype. It is only the fifth allele combination that possible could give rise to hybrid offspring producing all three of the observed phenotypes. The ratio of these phenotypes can however, be used to reject the model when tested in a χ^2-test.

There are two possible genotypes for the human serum sensitive STIB 247 and three different genotypes for the resistant STIB 386. Only one of the combinations could result in the three observed phenotypes in a 2:1:1 ratio, with the resistant phenotype being the most frequent but this model does not have a good fit with the observed ratio ($\chi^2 = 7.714$, d.f = 2, p < 0.05)

In the alternative model, the wild type allele (s^+) allele is recessive to the S^S allele and there is a codominance relationship between S^S and S^R. Again, there are six different possible allele combinations for a cross between STIB 247 and STIB 386. Five of them can be refuted on the basis of the number of phenotypic classes found (**figure 4.7**) but the sixth possible combination is the model shown to have a good fit with the observed data (section 4.2.2 and **figure 4.1**).

Parental genotypes						
STIB 247	STIB 386	F_1	Phenotypes	Genotypes	Genotypic ratio	Phenotypic ratio
$S^S S^S$ x	$S^R S^R$		Intermediate	$S^S S^R$	1	1
$S^S S^S$ x	$S^R s^+$		Intermediate	$S^S S^R$	1	1
			Sensitive	$S^S s^+$	1	1
$S^S s^+$ x	$S^R S^R$		Intermediate	$S^S S^R$	1	1
			Resistant	$S^R s^+$	1	1
$s^+ s^+$ x	$S^R S^R$		Resistant	$S^R s^+$	1	1
$s^+ s^+$ x	$S^R s^+$		Resistant	$S^R s^+$	1	1
			Sensitive	$s^+ s^+$	1	1

Figure 4.7. The possible genotypic and phenotypic outcome of a cross between STIB 247 and STIB 386 when assuming a recessive wild type (s^+) allele that is recessive in relation to the two codominant S^S and S^R alleles. This assumption can lead to six different crossing combinations with regard to the parental phenotypes. The five combinations shown here will only result in one or two phenotypes in the offspring and can therefore be rejected as a model which could fit the observed results. There is a sixth possible parental genotype combination but since this has a good agreement with the results from the observed data it was discussed in **section 4.2.2.**

When considering three allele models at a single locus and with two levels of dominance another possibility is that the third allele is an allele for intermediate human serum resistance (s^I). Codominance between two of the three alleles is postulated but the outcome of codominance including an allele for intermediate human serum resistance cannot be predicted since the phenotype that would arise from such a cross is not known. The putative hybrid offspring might display various degrees of sensitivity to human serum but the *in vivo* HSS-assay is probably not sensitive enough to be able to detect this. The only codominance situations discussed here will, therefore, involve a codominant S^S and S^R allele and a recessive s^I allele; on this basis STIB 247 would have to be either $S^S S^S$ or $S^S s^I$ and STIB 386 $S^R S^R$ or $S^R s^I$. It has to be assumed that hybrid offspring from such a cross, which are expressing the intermediate resistant phenotype, could arise from two different allele combinations so that both the two codominant alleles S^S and S^R together and $s^I s^I$ would produce intermediate resistant trypanosomes. Even under this assumption there is only one combination that can produce all the three phenotypes that were observed but the ratio of the phenotypes is not consistent with those obtained since the intermediate resistant phenotype is the most common in a 2:1:1 ratio ($\chi^2 = 8.786$, d.f = 2, p<0.05).

Complex models

There are other more complex models that can also either explain the observed data or can be refuted as models for the inheritance data. These may involve two or more loci, more than three alleles or have several levels of dominance. These are all much more difficult to test and only the general points will be discussed.

If it is assumed that there are three alleles: a human serum sensitive allele (s^s), human serum resistant allele (s^r) and an intermediate resistant allele (s^i) there can also be three levels of resistance. These three alleles can relate to each other in nine different ways: s^s can be completely dominant over both the other alleles while the s^r allele is dominant over s^i. Alternatively, the dominance relationships could be any of the following: $s^s > s^i > s^r$ or $s^r > s^s > s^i$ or $s^r > s^i > s^s$ or $s^i > s^s > s^r$ or $s^i > s^r > s^s$. In addition, there can be a situation of codominance, with s^s and s^r being two

codominant alleles but the other two possibilities, where s^s and s^i are codominant or s^r and s^i are codominant will not be discussed since in neither case can the phenotype of the possible genotypes be predicted. The possible genotypes of the parental trypanosomes and their F_1 offspring are described in **table 4.5** but their corresponding phenotypes will depend on the relationships between the alleles and so cannot be defined.

Possible gametes:	s^s, s^r and s^i		
Possible genotypes:	$s^s s^s$	$s^s s^i$	$s^r s^i$
	$s^s s^r$	$s^r s^r$	$s^i s^i$

Possible phenotypes: HS sensitive: HS resistant: HS intermediate resistant

Possible genotypes of parental stocks with different dominance relations between alleles:

	HSS STIB 247	HSR STIB 386
Allele series $s^s > s^r > s^i$	$S^s S^s$ or $S^s s^r$ or $S^s s^i$	$s^r s^r$ or $s^r s^i$
Allele series $s^s > s^i > s^r$	$S^s S^s$ or $S^s s^r$ or $S^s s^i$	$s^r s^r$
Allele series $s^r > s^s > s^i$	$s^s s^i$ or $s^s s^i$	$S^r S^r$ or $S^r s^i$ or $S^r s^s$
Allele series $s^r > s^i > s^s$	$s^s s^s$	$S^r S^r$ or $S^r s^i$ or $S^r s^s$
Allele series $s^i > s^s > s^r$	$s^s s^s$ or $s^s s^r$	$s^r s^r$
Allele series $s^i > s^r > s^s$	$s^s s^s$	$s^r s^r$ or $s^r s^s$
Codominance s^s and s^r	$S^s S^s$ or $S^s s^i$	$S^r S^r$ or $S^r s^i$

Table 4.5. Possible gametes, genotypes and phenotypes of parents and F_1 progeny assuming three alleles with different dominance relationships between alleles. Six possible genotypes can result from the three alleles as indicated in the top part of the table. A cross between a sensitive and a resistant stock determined by these alleles could ultimately give rise to the three phenotypes; sensitive, resistant and intermediate resistant hybrids. The bottom part of the figure shows the possible genotypes of the parental strains when the three alleles show the dominance hierarchy indicated to the left.

If the parental stocks have three alleles, 9 possible allele combinations can occur, three of which are homozygous and six of which are heterozygous. There are however only 6 different unique combinations since each of the heterozygotes appears twice. The next assumption is that S^s is the dominant allele and that s^i is recessive to both the s^s and s^r allele. STIB 247, which is human serum sensitive, would then have to be either $S^s S^s$ or $S^s s^r$ or $S^s s^i$ while the resistant STIB 386 have to be either $s^r s^r$ or $s^r s^i$. Only one of the six combinations of the assumed parental genotypes could possibly give rise to an intermediate phenotype. The intermediate phenotype could be formed if STIB 247 is assumed to be $S^s s^i$ and STIB 386 $s^r s^i$ and

as this genetic model could potentially explain the observed results it was described and discussed in section 4.2.2. None of the other five possible models could explain the observed phenotypes in the F_1 generation.

For the second allelic series, $S^S > s^I > s^R$, the possible combinations would produce three possible F_1 phenotypes: hybrids of only the sensitive phenotype, sensitive and resistant phenotypes in a 1:1 ratio or sensitive and intermediate resistant hybrids in a 1:1 ratio. The third allelic series where S^R is the completely dominant allele, s^S the partially dominant allele and s^I the completely recessive allele, one of the possible parental combinations could give rise to all three observed phenotypes. The three classes would appear in a 2:1:1 ratio with the most frequent class being the human serum resistant phenotype but when comparing the numbers with the observed data there was a significant difference ($\chi^2 = 7.714$, d.f. $= 2$, $p < 0.05$). The other combinations would produce only resistant offspring or resistant and sensitive hybrids in a 1:1 ratio. The remaining three possible allelic series cannot lead to the production of the three phenotypic classes observed and therefore do not fit with the observed results described in section **4.2.1**. It is possible that human serum resistance is determined by alleles at more than one locus so that resistance and sensitivity are inherited independently. The different loci could then either all be coding for sensitivity or all for resistance and an intermediate phenotype could arise when more than one of the alleles was inherited and expressed. Several of the possible combinations involving two alleles at two loci could produce the three phenotypic classes human serum sensitive, human serum resistant and intermediate resistant phenotypes but none of them in the 2:1:1 ratio that was observed. Models involving more than two loci are more difficult to test and with multiple alleles at each locus, the potential number of F_1 phenotypes would be large.

4.2.4 Application of the proposed inheritance model

The suggested model for inheritance of human serum resistance (described in **figure 4.1**), which involves three alleles at a single locus; codominant human serum

sensitive and human serum resistant alleles and one recessive sensitive wild type allele, can be used to predict the outcome of the other crosses. Hybrid offspring from STIB 247 x TREU 927 and STIB 386 x TREU 927 were analysed for their sensitivity or resistance to human serum and the results were given in section 4.2.1. Only a small number of F_1 from these crosses were actually analysed but if the model is applied it can test whether it is consistent with the available data. From the model, the serum resistant STIB 386 is predicted to have the genotype $S^R s^+$ and STIB 247 is predicted to be $S^S s^+$. An intermediate resistant trypanosome stock, like TREU 927, would in this model have the allele combination $S^S S^R$. A cross between STIB 247 and TREU 927 would subsequently result in a F_1 generation with human serum sensitive, human serum resistant and intermediate resistant phenotypes in a 2:1:1 ratio (**figure 4.8**).

Predicted model:

Parents:

		STIB 247 sensitive $S^S s^+$	TREU 927 intermediate $S^S S^R$	
F_1:	Phenotypes	Genotypes	Genotypic ratio	Phenotypic ratio
	Sensitive	$S^S S^S$	1	2
		$S^S s^+$	1	
	Resistant	$S^R s^+$	1	1
	Intermediate	$S^S S^R$	1	1

Preliminary results from the STIB 247 X TREU 927 cross:

	Phenotypes	Observed number	Expected number	Proportion Obs.	Exp.
F_1:	Sensitive	5	4.5	0.556	0.500
	Resistant	2	2.25	0.222	0.250
	Intermediate	2	2.25	0.222	0.250

Figure 4.8. Predicted model for the inheritance of human serum resistance applied to a STIB 247 x TREU 927 cross. The model suggested in figure 4.1 is here tested on a cross between STIB 247 and TREU 927 assuming the genotypes predicted by the model. There is a good fit between the suggested model and the results obtained from a rather limited number of hybrid progeny tested for their human serum resistance/sensitivity.

The observed ratio is in fact very close to the predicted ratio of phenotypes from this cross (**table 4.2**) which is 5 sensitive, 2 resistant and 2 intermediate resistant. A chi-square test can confirm that there is no significant difference between the observed data and those expected ($\chi^2 = 0.112$, d.f. = 2, p < 0.05). There are however, problems with using chi-square tests with such small numbers as other ratios could probably still suggest agreement.

The model was also applied to the cross STIB 386 x TREU 927 where seven F_1 progeny had been tested for their reaction to human serum. The comparison of the predicted numbers with those observed was less similar; the observed results were 3 resistant hybrids and 4 sensitive hybrids while the predicted result is human serum resistant, human serum sensitive and intermediate human serum resistant offspring in a 2:1:1 ratio (**figure 4.9**).

Predicted model:

Parents:		STIB 386 resistant $S^R s^+$	TREU 927 intermediate $S^S S^R$	
F_1:	Phenotypes	Genotypes	Genotypic ratio	Phenotypic ratio
	Resistant	$S^R S^R$	1	2
		$S^R s^+$	1	
	Sensitive	$S^S s^+$	1	1
	Intermediate	$S^S S^R$	1	1

Preliminary results from the
STIB 386 X TREU 927 cross:

F_1:	Phenotypes	Observed number	Expected number	Proportion	
				Obs.	Exp.
	Sensitive	4	1.75	0.571	0.250
	Resistant	3	3.5	0.429	0.500
	Intermediate	0	1.75	0	0.250

Figure 4.9. Application of a proposed model for inheritance of human serum resistance with progeny from STIB 386 x TREU 927. The inheritance of human serum resistance/sensitivity trait in a cross between STIB 386 and TREU 927 is tested by comparing the predicted results from the proposed model (**figure 4.1**) with observed results. There is not a good fit with the model but reservations must be made for the small number of progeny tested for this cross.

The main point is however, not how good the fit to the model is for these crosses, since the number is too small for such conclusions, but that the model for this trait can be used to predict the outcome of a cross which can then be tested experimentally. If further progeny were generated and scored for their HSS phenotype, the predicted segregation could be tested.

4.3 DISCUSSION

The results in this chapter have been presented without considering the actual function of the gene or genes that determine human serum resistance. The aim was to approach the subject from a genetic perspective and determine the inheritance of this trait. It is clear from the inheritance data in this chapter that resistance to human serum is inherited as a stable trait and that genes determining human infectivity are transmitted to approximately 25% of the offspring from the STIB 247 x STIB 386 cross. This can have serious implications in the field and even though there is some debate about the frequency of sexual reproduction in natural populations (Tait, 1980; Cibulski *et.al.*, 1988; Tibayrenc & Ayala, 1991; Hide *et.al.*, 1994), a rare event can still be important and result in the generation of new human infective strains in an area.

Most research on the reaction of *T.brucei* with human serum has focused specifically on the resistance trait. Here a mechanism is proposed involving not only resistance to human serum but also sensitivity to human serum as a functional process. It is not only a *lack* of resistance that makes a trypanosome strain sensitive to human serum but there is a specific gene product that confers sensitivity. In the proposed model for inheritance of resistance/sensitivity to human serum there are three different alleles at a single locus, two of these potentially translate into polypeptides which confer sensitivity while the third allele codes for resistance. One of the sensitivity alleles, s^+, is recessive to the other two alleles, S^R and S^S, which are codominant when expressed together. This is a simple model to explain the occurrence of the intermediate resistant phenotype since codominance implies an

independence of allele function so that a hybrid product would express a phenotype which is a mixture of the two alleles, in this case intermediate in their resistance. The only proposed mechanism of resistance of *T.b.rhodesiense* to human serum was described by Hager & Hajduk (1997) and involves the presence of cell-surface receptors which bind and internalise TLF, transport it to lysosomes and lead to their disruption resulting in complete cell lysis. Both the human serum resistant and human serum sensitive trypanosomes were shown to bind TLF in the flagellar pocket but the resistant trypanosomes failed to endocytose the TLF and therefore could avoid lysis. Assuming that this model is correct and that a specific receptor is responsible for the endocytosis that leads to resistance and sensitivity to human serum, a functional explanation of the inheritance model can be suggested. If the receptor is present and fully functional it would have the capacity to internalise a toxic factor leading to cell lysis i.e. the trypanosome would be human serum sensitive. The receptor would, in this case, be the result of one of the genotypes $S^S s^+$ or $s^+ s^+$. A non-functional receptor would, on the other hand, be the gene product of allele combinations $S^R S^R$ or $S^R s^-$ and the combination $S^S S^R$ might result in an unstable gene product and a receptor which is not fully functional or a lower affinity receptor which leads to lower levels of lysis The second possible inheritance model, outlined in **figure 4.4**, could be explained in very similar terms so that S^S is coding for the presence of a fully functional receptor, s^R for a non-functional receptor and s^I for a receptor which is present but has partially lost its function. An alternative model with identical functional consequences is that the gene product of the locus is a molecule required for internalisation of the TLF complex with the receptor present on both resistant and sensitive cells. The described SRA-gene (DeGreef & Hamers, 1994) however, cannot be a receptor as it does not have the appropriate sequence for membrane insertion. A critical question that arises from this model is how to unravel the true nature of the intermediate resistant phenotype. An intermediate phenotype was defined as a trypanosome isolate that after incubation with human serum was able to infect a proportion of mice in a group. TREU 927 was shown to express the intermediate resistant phenotype and it is worth considering a few other observations about this strain. There was a difference in the outcome of the *in vivo* HSS-assay when 50, 100, 1000 or 1×10^6 cells were injected into mice after exposure to human

serum in that when a higher number of cells were injected more of the mice developed a parasitaemia. The length of the incubation in human serum before inoculation did not affect the outcome of the *in vivo* assay ; the results were the same after 15 minutes, 30 minutes, 1 hour, 2 hours or 4 hours incubation. Conceivably, there could be two mechanisms involved in determining intermediate resistance; either there are differences in expression of resistance within the population so that individual cells are either resistant or sensitive, and thus some aliquots contain only sensitive trypanosomes while others contain one or more resistant trypanosomes. However, as it is not known how sensitive the *in vivo* HSS assay is and whether it would be able to detect only one remaining trypanosome among a number of lysed cells. Alternatively, each individual trypanosome has the ability to be either resistant or sensitive. To my knowledge, there have been no experiments that have addressed this question and it would indeed be hard to image how this could be done since not even single cell experiments would be able to tell the two events apart. The data presented in this chapter, together with the above observations are, however, compatible with the second explanation i.e. each cell may be either resistant or sensitive to human serum. If this is considered in light of the proposed receptor mediated uptake model by Hagar & Hajduk (1997), intermediate resistant cells may have a partially dysfunctional receptor for the toxic serum factor and the source of the toxin may be of importance. It has been suggested that the toxin in normal human serum is human haptoglobin-related protein which has been found both in TLF1 and TLF2 (Tomlinson & Raper, 1998), so that a TLF receptor would have to have a low affinity in order to bind both of these molecules. An intermediate resistant phenotype might have receptors with even lower affinity due to being a variant or it might be unstable so that it sometimes manages to bind and internalise the toxic factor but other times fail to complete this process.

When discussing a three allele system with two codominant alleles there were two parental genotypes which were deliberately not included because their existence cannot be tested for in the assay used. These genotypes both involved an intermediate resistant allele, S^I, which was codominant with either a sensitive allele (S^S) or a resistant allele (S^R). Only speculation about the resulting phenotypes from such a

cross can be made since the *in vivo* HSS assay would not be sensitive enough to separate these phenotypes. Likely phenotypes would include a grading of sensitivity to human serum so that some progeny are more resistant than other resistant stocks and some sensitive isolates more sensitive than other sensitive stocks. The idea does not fit with the proposed model since in order for the S^I allele to be expressed at least one of the parental strains must have this allele but both STIB 247 and STIB 386 are very clearly sensitive and resistant respectively. It is however, interesting to note that among the hybrid offspring there were clones that most certainly were more resistant than other resistant clones from the same cross in that they established a patent parasitaemia after 2-3 days while most resistant strains were not detected until after 5-6 days, implying that differences in numbers of infective organisms amongst the hybrids of resistant phenotype. This was not confined to single experiments but could be repeated and the trypanosomes still showed the same growth pattern. Unfortunately, the *in vivo* HSS-assay is not sensitive enough to be able to pick up minor differences in resistance but it would not be surprising if there was some grading of sensitivity. The allele combination $S^R S^R$ might be more resistant than $S^R s^+$ and $S^S S^S$ more sensitive than $S^S s^+$ in the model described in **figure 4.1**. Even when a completely sensitive strain is incubated with human serum there are individual cells that seem morphologically intact and may differ in their response to the toxic factor in human serum and, similarly, among the resistant STIB 386, 10-20 % of the cells were usually lysed in contact with human serum. What is important in this discussion is that there are not only strains which are clearly sensitive or resistant to human serum but there are also strains which exhibited at least one level of intermediate resistance to human serum.

The refuted models are in a way more important than the actual suggested model in that they can be rejected with a great deal of certainty while the suggested model, as stressed earlier, should be seen only as the simplest possible explanation of observed results. There are more complex models in which more than one locus is involved and where more than three alleles are involved. Models can be proposed with 2 alleles at two different loci, which can be postulated to control two different receptors, both necessary to confer sensitivity or where one locus codes for resistance

and the other for sensitivity and there are combinations which can give rise to all three observed phenotypes. None of these combinations would however give a 2:1:1 ratio with human serum sensitive as the most abundant segregant but the intermediate phenotype, in all cases, would be the most frequently observed.

The most direct way to test the proposed model of inheritance, would be to attempt a series of backcrosses. A cross between an F_1 hybrid and one of the parental types could be used to determine whether the parent is homozygous or heterozygous for a certain allele and the predicted results compared with the observed results. Backcrosses in *T.brucei* have been achieved (Gibson *et.al.*, 1995), which implies that hybrid genotypes are sexually competent and that backcrosses can be obtained. In a planned backcross experiment, which unfortunately was delayed because of problems with the tsetse fly colony and lack of time, one of the human serum resistant F_1 from the STIB 247 x STIB 386 cross would be crossed with the parental STIB 247. If the model is correct then the resistant F_1 progeny would be of the genotype $S^R s^+$ and the backcross progeny would segregate as sensitive, resistant and intermediate resistant in a 2:1:1 ratio (**figure 4.10**).

Predicted results from a backcross between the parental STIB 247 and a HSR F_1 progeny

Parents:		STIB 247 sensitive $S^S s^+$	F_1 progeny resistant $S^R s^+$	
F_1:	Phenotypes	Genotypes	Genotypic ratio	Phenotypic ratio
	Sensitive	$S^S s^+$ $s^+ s^+$	1 1	2
	Resistant	$S^R s^+$	1	1
	Intermediate	$S^R s^+$	1	1

Figure 4.10. Suggested approach for confirming the proposed genetic model by conducting a backcross between one of the parents and a hybrid offspring. The outcome of such a testcross can be predicted and subsequently compared with observed results to show the probability of the model. A proposed backcross to confirm or reject the proposed inheritance model in **figure 4.1** is outlined.

There are five human serum resistant F_1 offspring which could be used for a backcross. The locus identified by the microsatellite marker JS-2 could be used to detect tsetse flies with mixed infections and half of the progeny clones would be genotypically distinct from both STIB 247 and the F_1 progeny clones used in such a cross. Backcrosses would also be extremely informative when investigating TREU 927's intermediate phenotype in that its proposed genotype can be tested i.e. a cross between TREU 927 and one of the intermediate resistant hybrid progeny would result in only intermediate resistant recombinants. It was hoped that the self-fertilisation test of STIB 247 would be useful in confirming its genotype. A heterozygous genotype would have segregated into three genotypes in which 50% of the progeny would be homozygous for one allele or the other. But as the proposed genotype of STIB 247 is *ss*, all segregants would be sensitive and so non informative. The four products of self-fertilisation from STIB 247 were sensitive which is consistent with the proposed genotype. TREU 927 selfers however, would be informative as there would be a segregation into intermediate, sensitive and resistant phenotypes. Unfortunately, only one TREU 927 selfer was available so no conclusion could be made.

As STIB 386 does not express the SRA gene (Chapter 5), the locus identified by the genetic analysis is unlikely to be the SRA gene. This leads to the conclusion that there are several different genes that can determine human serum resistance. The genetic approach illustrated here, can be very useful in identifying loci as it makes no assumptions about the mechanism of human serum resistance. Genetic mapping of the human serum resistance trait could be undertaken to determine the chromosomal region(s) that differ between human serum sensitive and resistant trypanosome lines. Work is under way to identify a complete set of universal chromosomal markers for the *T.brucei* genome (Melville *et.al.*, 1998; Dan Masiga, pers.comm., 1998) and when a genetic linkage map has been fully established, the inheritance pattern of specific markers could be compared with observed inheritance pattern of the human serum resistance trait. This could subsequently lead to the actual localisation of the human serum resistance locus, if the map position is determined and a single gene determines human serum resistance. Once a specific region on the genetic map had

been identified, closely linked physical markers could be used to clone the region identified.

Chapter 5

SRA gene expression and its lack of correlation with serum resistance in *T.brucei*

5.1 INTRODUCTION

In a number of stocks the human serum resistance trait in *T.b.rhodesiense* is unstable. Resistant populations can be transformed to become sensitive and resistance can be selected in sensitive populations by exposing them to human serum in small doses (Rifkin *et.al.*, 1994; Rickman & Kolala, 1980). Several attempts have been made to explain the resistance trait and since a change in serum sensitivity was often associated with a change in the expressed VSG, it was thought that the VSG was involved in mediating resistance. Rifkin *et.al.* (1994) compared human serum resistant and human serum sensitive *T.b.rhodesiense* clones expressing the same VSG but could find no differences in structure or biochemical properties of the VSG in the two clones. Similarly, De Greef *et.al.* (1989) studied differences in cDNA sequences of VSG genes from sensitive and resistant forms and the relative abundance of VSG molecules but could not correlate resistance to any difference in VSG expression.

One approach used to investigate the molecular basis for human serum resistance was by subtractive hybridisation to identify RNAs differentially expressed between serum sensitive and serum resistant isogenic lines expressing the same VSG. This method was employed by De Greef *et.al.* (1989) who found a 1.5 kb transcript which was present only in the resistant forms. This resistance-specific transcript was found in four *T.b.rhodesiense* clones which all were derived from the ETat repertoire and in one uncloned stock from Tanzania. It was, however, not expressed in one *T.b.gambiense* isolate or in one *T.b.brucei* isolate tested. Southern blot analysis indicated that the Serum Resistant Associated (SRA) gene was present in the sensitive *T.b.rhodesiense* line but not expressed. In a subsequent paper, four uncloned *T.b.rhodesiense* isolates from Zambia, two *T.b.brucei* isolates, two

T.b.gambiense isolates, one *T.evansi* and one *T.equiperdum* isolate were analysed and all of the Zambian samples shown to express the SRA gene while sequences related to the SRA-transcript were present, but not expressed, in the other members of the *Trypanozoon* subgenus (De Greef *et.al.*, 1992). The SRA-transcript was later shown to code for a protein that has homology with VSG. The best homology was found in the carboxy-terminal part of the molecule which is the domain with the highest degree of sequence conservation between VSG genes. The N-terminal leader sequence, forming the elongated structure of VSG genes, was however, missing in the SRA gene. The genomic clone, isolated by probing genomic libraries with the SRA c-DNA clone, was shown to contain a pseudogene since no open reading frame could be found but belonged to the same gene family (De Greef & Hamers, 1994).

This chapter describes the investigation of the presence and expression of the SRA gene in seven different *T.brucei* clones including the three genetically competent stocks used in the inheritance studies (Chapter 4). The objectives of this study were to, firstly, investigate whether the SRA gene was present in STIB 247, STIB 386 and TREU 927 and, if so, whether it was expressed. Secondly, to determine whether expression of SRA was associated with human serum resistance in these stocks and thirdly, to potentially follow the inheritance of SRA presence/expression into the F_1 to investigate whether it co-segregated with the serum resistance phenotype. The presence of the SRA gene was investigated both by PCR and by Southern blotting. Sequences related to the SRA gene were detected in all clones tested by Southern analysis. However, sequencing of the PCR product suggested a degree of sequence variation at the site of one primer so that in one stock no PCR product could be obtained at all and in another case only the 3'end of the fragment could be amplified with primers designed for the SRA gene sequence. The expression of the SRA gene was studied by Northern hybridisation and by RT-PCR and it was established that its expression could not be correlated with human serum resistance in the clones studied here. From those data, it can be concluded that resistance to human serum is determined by more than one mechanism which may differ between isolates from different areas and that the inheritance of the serum resistance/sensitivity phenotype described in Chapter 4 does not involve differences in SRA expression.

5.2 RESULTS

5.2.1 Analysis of the SRA gene by PCR

Total genomic DNA was isolated from bloodstream forms of STIB 247, STIB 386, TREU 927 and ETat1.2 and used as DNA templates for PCR analysis of the SRA gene as described in **2.15**. The ETat1.2 trypanosome line was derived from the stabilate bank in Glasgow. It was used as a positive control because SRA was originally identified from ETat1.2 from the stabilate bank in Antwerp. Typically 20 ng DNA was used in each 10 μl reaction. The published SRA gene sequenced was accessed in the GenBank™Data Bank, accession number Z37159, and four sets of oligonucleotide primers were designed from this sequence. The sequences of the eight primers are shown in **table 5.1** and the positions of the sequences in the published sequence are shown in **figure 5.1**.

Oligo name	Oligo sequence	No. of bases
MHT	5' > CAA AAG CAG CAA CCA TAT TCA G < 3'	22
MHS	5' > TAG CAA GGC GAA CCG AAA G < 3'	19
2SRA1	5' > CGA ACG ACA GTT AAC GTA ACA < 3'	21
2SRA2	5' > AGC CAA TTT CAT ATC CAC AAG < 3'	21
SRA5	5' > CGC TTT GAC GAA GAG CCC GTC AAG < 3'	24
SRA6	5' > CCT TCC CAT TCG TAT TCT TGT G < 3'	22
SRA7	5' > ATT CTG CAG CAG GCG TCA C < 3'	19
SRA8	5' > TTC CGC ATT ATA TGT GCA C < 3'	19
TIM A	5' > GCG TAG TGG CCT CCA CCT TTG TTC < 3'	24
TIM B	5' > AAC ACC CCC TAT TGT TTC CTC TCC < 3'	24
TIM E	5' > TGC CGT TGA GTG GGT GAA GAT AGC < 3'	24

Table 5.1. Oligonucleotides used as primers for SRA gene analysis. Eight different primers were designed for PCR analysis of the SRA gene and used in various combinations. Their names and sequences are outlined above. Primers for the TIM gene were used as positive controls for the PCR reactions.

```
Z37159  Length: 1395

    1   CGCTATTATT AGAACAGTTT CTGTACTATA TTGACACACC TCTAAGAATC

   51   ACAATAGCAA GGCGAACCGA AAGCOAACGA CAGTTAACGT AACAGCA*ATG

  101   CCCCGAAATT CGGGCCGGAC AACAAGTACC TTGGCGCTCC CTGGCCCTAA

  151   ACCTGCTGGC AGTGCCTGTA TCGCCCAGTG GCAC CGCTTT GACGAAGAGC

  201   CCGTCAAGAA GGTTTGCAAA GTAGAAAAAA ACTTAGCCAGA CGTCGCAGGA

  251   ATCCCTTTGG CCAAAATAAA CAACCTGATA AAACAAGTAT CGGCAGCAAC

  301   CGAAGCGCAA GCAAGAATGA CCTTGGCCGC CGCAAGCACA GACCACAGCA

  351   ACATCTCAGC GCTTTATGCC GCGGCCGTCAA ACATAGTGAC AAGATGCGTA

  401   CTCAACGCAG TCCACCCTCT TACAAGTCTT GCGCCAATAG CCTTAACTGC

  451   AGCGTCCAAC GGAGCCAAAA CCAGTGGGCA CATCTCAGAA GTAATCGACA

  501   TTCTGCAGTA GGCGTCAC AA GGTAACACAG AAGGAAAGTG CATACTGAAA

  551   AGCGGCGGCG CTACAACAAC AGTAGCAATA AGGCAACTTT ACAACAAAAT

  601   AGGGGACCTA CAAAAACAAA CGACCAACAA CTCCGCCACC ACCGTCACCG

  651   AAGTACTCGA ACACATTCTA AAACAAGAAG CGCTCAAGGA AGCGCTACTT

  701   TCAATCGTGA AAAAACCAAA AGGGGCGCCA GACAAAACAG CAGCAGATGA

  751   ATTGCTCACC GCCCTAATCA ACGGCGTGGT GCCAAACAGC ACAGCACAGA

  801   CCCAAAAATT AAAGGAGAAA ATTCTAAACA CCTTGGTCCC CAAGCTTGTG

  851   GAAGGCTCAA AAAGCCAAGT AAAACTAAGG ATTCTGAACT ACCCGGCAAA

  901   AATACACAAA ACCAAACTCC TATCAATCCA AGAGTTAAAA ACCCGAGTGG

  951   AGCCTCAATC TACCACTGAA AGCTCCAACC ACCAGGTGCC CACCAAGCAG

 1001   GCACAGGAGG CATTTTGTAA CGCAATTGCC GACGACAAAG ACAAGTGTAA

 1051   CAATGAGACA CGATGCAGTT ACGATGACAG CAAAGGCTCA GACAAAAA

 1101              AAA GCAGAAGCAA ATTCCCGCACC TGCAACCCAA    Primer MHS
                                                            Primer 2SRA1
 1151   CCTCAAGGGG GAGTGAACGA AGCAACAACA GGAAATTGTA AAGGGAAACT   Primer SRA5
 1201   CGAACCCGGA TGCACCAAGG CACAAGAATA CGAATGGGAA GGAAAAGAAT   Primer SRA7
 1251   CCAAAGATTC AAGTTTTCTT GTGGATATGA AATTCGCTCT CAATATGGTT   Primer     (complementary)
                                                            Primer SRA6 (complementary)
 1301   GCTGCTTTTG TGGCCTTTCT GTTT*TAATTT TCACCTCTTT TGAAAGAACT   Primer 2SRA2 (complementary)
 1351   TTCCTCTTTC ATATACTTTA ACACATTTTC ATGAATTTGT CAAAA          Primer MHT (complementary)
```

Figure 5.1. The published SRA sequence for ETat1.2 and primers designed for PCR. The SRA sequence of ETat1.2 (GenBank accession number Z37159) indicating the positions of sequences used to design primers for PCR analysis of the presence of the SRA gene in other stocks. A total of eight different primers were designed for the published SRA gene and used both for PCR analysis and for sequencing reactions. The stop- and start-codon are marked with *.

Initially, oligonucleotides MHS (5' TAG CAA GGC GAA CCG AAA G 3') and MHT (5' CAA AAG CAG CAA CCA TAT TCA G 3'),which flank the coding region, were tested on the four clones but annealing temperatures between 55°C and 70C° typically produced several DNA fragments of sizes ranging between 0.5 kb and 1.2 kb when separated on 1% agarose gel, stained with ethidium bromide and visualised by ultra violet transillumination (**figure 5.2**). For ETat1.2 a single specific

band of the predicted size was produced at annealing temperatures reaching 70°C but for STIB 386 and STIB 247 there were several bands apparent even at higher annealing temperatures. TREU 927 produced a single band at higher temperatures but of a smaller than expected size (0.9 kb).
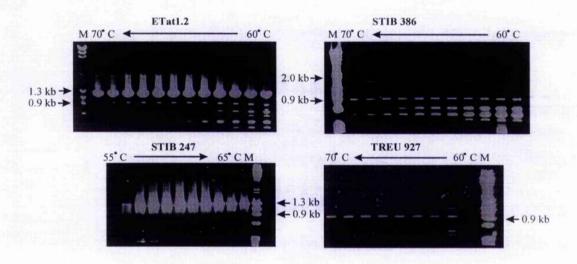
Figure 5.2. PCR amplification using a range of annealing temperatures with primers MHS and MHT designed from sequences flanking the SRA gene. PCR products were separated on a 1% agarose gel and visualised by UV transilluminator. PCR amplification with SRA primers for ETat1.2 DNA produced a major band 1.2 kb in size but there were also several smaller fragments being amplified even at 65°C annealing temperature. The same primers, when used with STIB 247, amplified a DNA fragment of approximately 1.2 kb in size and smaller fragments were only produced at annealing temperatures lower than 55°C. For TREU 927 and STIB 386 there was a range of, predominantly smaller, fragments being amplified with this primer combination even at higher annealing temperatures.

PCR amplification of the SRA locus was subsequently carried out using nested primers. Typically, reactions were first carried out with primers MHS and MHT under the following cycling conditions: 96°C for 50 s, 55°C for 50 s and 70°C for 2 min for a total of 24 cycles. A 1 µl sample of the product was transferred to a fresh tube and subjected to a second PCR amplification using primers 2SRA1 (5' CGA ACG ACA GTT AAC GTA ACA 3') and 2SRA2 (5' AGC CAA TTT CAT ATC CAC AAG 3') (see **figure 5.1**) under the same conditions, or with an annealing temperature at 52°C, for a further 30 cycles. Nested PCR amplification with the described primers resulted in a strong specific band of 1.2 kb in size from ETat1.2 and STIB 247 as shown in **figure 5.3**. The size of the resulting PCR product from

TREU 927 was slightly larger, approximately 1.5 kb in size and no product could be obtained from STIB 386. Control PCR was carried out using primers flanking the TIM locus to confirm that no PCR inhibitors were present in DNA isolated from STIB 386 and this gave rise to a fragment of expected size (**figure 5.3b**). Primers 2SRA1 and 2SRA2 could alone give rise to PCR products from all DNA samples mentioned apart from STIB 386 (data not shown).
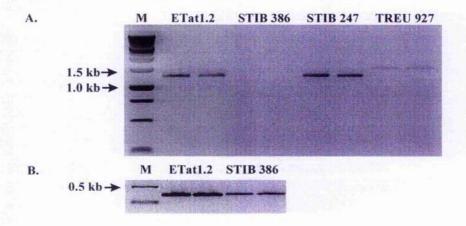
Figure 5.3. PCR amplification of the SRA gene locus using nested primers. (A) DNA from ETat1.2, STIB 247, STIB 386 and TREU 927 was isolated and used for PCR amplification with nested primers, MHS and MHT followed by 2SRA1 and 2SRA2. The products were separated on a 1% agarose gel, stained with ethidium bromide and visualised by ultraviolet illumination. M, 1 kb DNA marker. (B) TIM primers E and B were used in control PCR to verify the quality of DNA isolated from STIB 386 since PCR amplification with primers designed for the SRA gene failed.

To investigate further the presence of a sequence similar to the SRA gene in STIB 386 other primer combinations were used. Primers SRA5 (5' CGC TTT GAC GAA GAG CCC GTC AAG 3') and SRA6 (5' CCT TCC CAT TCG TAT TCT TGT G 3') produced two separate bands of 1.2 kb and 1.4 kb respectively in ETat1.2 but there was a complete absence of any product from STIB 386 (**figure 5.4**). A possible reason for the second band is the presence of a second sequence homologous to the primers used.
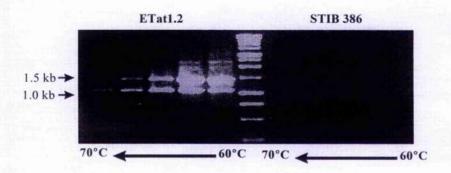
Figure 5.4. PCR amplification of the SRA gene sequence using primers SRA5 and SRA6. Primer combination SRA5 and SRA6 was used to amplify SRA related sequences in ETat1.2 and STIB 386 in order to investigate the presence of a SRA gene in STIB 386. Fragments were amplified from ETat1.2 gDNA but not from STIB 386 gDNA. The DNA preparation was the same as that used for the TIM control reaction in **figure 5.3**

Primer combination SRA7 (5' ATT CTG CAG CAG GCG TCA C 3') and SRA8 (5' TTC CGC ATT ATA TGT GCA C 3') were also tested in order to find sequence homology with the published SRA gene. Annealing temperatures between 55°C and 68°C were tested and this produced one or several segments in ETat1.2 at all annealing temperatures tested but no product could be detected from STIB 386 (data not shown).

PCR analysis of the SRA locus in STIB 247, STIB 386, TREU 927 and ETat1.2 suggested that a sequence related to the published SRA gene was present in ETat1.2, STIB 247 and TREU 927 but that the sequence was either absent from STIB 386 or sufficiently divergent that the primers designed from the ETat1.2 sequence were unable to prime. To investigate which explanation was more likely, Southern blots were performed with DNA from the cloned stocks used for PCR analysis.

5.2.2 Analysis of the SRA gene by Southern hybridisation

The probe used for Southern analysis was the purified product from the nested PCR reaction of ETat1.2 SRA (section **5.2.1**) labelled with fluorescein. Southern hybridisation was conducted as described in section **2.13** using digested genomic

DNA derived from ETat1.2, STIB 386, STIB 247 and TREU 927 and hybridising sequences were detected in all four cloned stocks (**figure 5.5**).
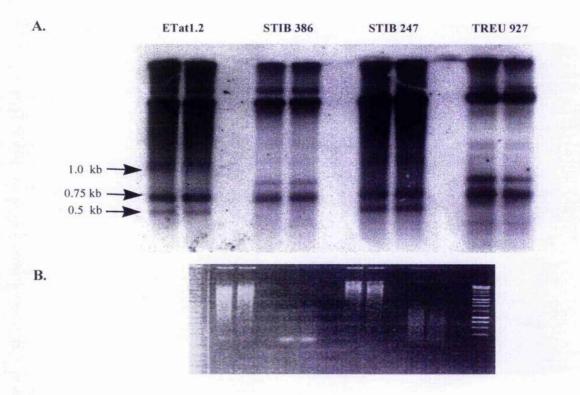
Figure 5.5. Southern hybridisation of restriction digested genomic DNA from four trypanosome stocks with the SRA gene probe. (A) Southern blot analysis of 1μg of genomic DNA digested with *TaqI* from ETat1.2, STIB 386, STIB 247 and TREU 927 with the fluorescein labelled PCR product purified from ETat1.2 after a nested amplification using primers MHS and MHT followed by 2SRA1 and 2SRA2. (B) Ethidium bromide stained gel prior to Southern blotting which reveals some DNA to have been only partly digested, particularly in ETat1.2 and STIB 247.

The pattern of hybridising fragments was however, not identical for the four cloned stocks when probed with the SRA gene probe. Apart from hybridisation with DNA left in the wells and with undigested DNA at around 20 kb, strong hybridisation can be seen with two DNA fragments. For ETat1.2 and STIB 247 the size of these fragments are 0.75 kb and 0.5 kb respectively. For STIB 386 and TREU 927 the size of hybridising bands are 0.95 kb and 0.8 kb. The restriction enzyme *TaqI* is expected to cut twice within the published SRA gene resulting in three fragments of the lengths 495 bp, 61 bp and 824 bp. The 61 bp fragment is probably too small to result in strong binding with the probe although a very faint band of a small size is just

visible in the lanes of TREU 927 and STIB 386 DNA. The hybridisation pattern is thus as expected for ETat1.2 and STIB 247 while for STIB 386 and TREU 927 one of the fragments is considerable larger than expected. The simplest explanation for this is that a modification has occurred at the 5'end of the gene since the fragment size at the 3'end is unchanged compared with the control ETat1.2. For the published SRA gene, more bands were detected after Southern blot analysis than expected on the basis of a restriction map (De Greef & Hamers, 1994). The authors explained this with the existence of several related sequences. In summary, it appears that the described SRA gene is present in ETat1.2 and in STIB 247 and that a sequence related to the SRA gene can be found in STIB 386 and TREU 927.

5.2.3 Sequence analysis of the PCR products from the SRA locus

In order to verify the identity of PCR products obtained in ETat1.2, STIB 247 and TREU 927, when using primers designed for the published SRA gene, the amplification products were cut from the agarose gel, purified and sequenced. Only single pass sequencing was conducted for most of the PCR fragment so some level of variation from the published SRA gene sequence was expected. From the band recovered from ETat1.2, 1050 bp were successfully sequenced and this showed an overall 98% identity with the published SRA gene which has a total length of 1230 bp within the open reading frame (**figure 5.6**).

```
        sra     CCCTATTATT ACAACAGTTT CTGTACTATA TTGACACACC TCTAAGAATC ACAATAGCAA GGCGAACCGA AAGCGAACGA
     Etat1.2    .......... .......... .......... .......... .... ..... .......... .......... ...GTACAA
     247sra     .......... .......... .......... .......... .......... .......... .......... ..........
     927sra     .......... .......... .......... .......... .......... .......... .......... ..........

                                        *
        sra     CAGTTAACGT AACAGCAATG CCCCGAAATT CGGGCCGGAC AACAAGCACC TTGGCGCTCC C..TGGCCCT AAAGCTGCTG
     Etat1.2    ..GTTAACGT AACAGCAATG CCC.GTAATT CGGGCCGGTC AACAAGTACC TTGGCGCTCG CGCTGGCCCT AAAGCTGCTG
     247sra     .......... .......TNTG CCC.GAAATG CCGGCNGGAC AACAAGTAC. CTGGCGCTCG CGCTGGCCCT AAAGCTGCTG
     927sra     .......... .......... .......... .......... AACAAGTAC. TTGGCGCTCG CGCTGGCCCT AAAGCTGCTG

        sra     GCAGTGCCTG TATCGCCCAG TGGCACCGC. TTTGACGAAG AGCCCGTCAA GAAGGTTTGC AAAGTAGAAA AAAACTTAGC
     Etat1.2    GCAGTGCCTG TATCGCCCAG TGGCACCGCC TTTGACGAAG TGCCCGTCAA GAAGGTTTGC AAAGTAGAAA AAAACTTAGC
     247sra     GCAGTGCCTG TATCGCCCAG TGGCCCCGCC TTTGACGAAG AGCCCGTCAA GAAGGTTTGC AAAGTAGAAA AAAACTTAGC
     927sra     GCAGTGCCTG TATCGCCCAG TGGCACCGCC TTTGACGAAG AGCCCGTCAA GAAAGTTTGC AAAGTATAAA AAAACTTATC

        sra     AGACGTCGCA GGAATCGCTT TGGCCAAAAT AAACAACCTG ATAAAACAAG TATCGGCAGC CAAGCAAGAA
     Etat1.2    AGTCGTCGCA GGAATCGCTT TGGCCAAAAT AAACAACCTG ATAAAACAAG TATCGGCAGC AACCGAAGCG GAAGCAAGAA
     247sra     AGACGTCGCA GGAATCGCTT TGGCCAAAAT AAACAACCTG ATAAAACAAG TATCGGCAGC AACCGAAGCG GAAGCAAGAC
     927sra     AGACGTCGCA GGAATCGCTT TGGCCAAAAT AAACAACCTG ATAAAACAAG TATCGGCAGC AACCGACGCG GAAGCAAGAA

        sra     TCACCTTGGC CGCCGCAAGC ACAGACCACA GCAACATCTC AGCGCTTTAT GCCGCGGCGT CAAACATAGT GACAAGATGC
     Etat1.2    TGACCTTGGC CGCCGCAAGC ACAGACCACA GCAACATCTC AGCGCTTTAT GCCGCGGCGT CAAACATAGT GACAAGATGC
     247sra     TGACCTTGGC CGCCGCAAGC ACAGACCACA GCAACATCTC AGCGCTTTAT GCCGCGGCGT CAAACATAGT GACAAGATGC
     927sra     TGACCTT... .......... .C AC...... .......... .......... .......... .......... ..........

        sra     GTACTCAACG CAGTCCACGC TCTTACAAGT CTTGCGCCAA TAGCGTTAAC TGCAGCGTCC AACGGAGCCA AAACCAGTGG
     Etat1.2    GTACTCAACG CAGTCCACGC TCTTACAAGT CTTGCGCCAA TAGCGTTAAC TGCAGCGACC AACGGAGCCA AAACCAGTGG
     247sra     GTACTCAACG CAGTCCACGC TCTTACAAGT CTTGCGCCAA TAGCGTTAAC TGCAGCGACC AACGGACCCA AAACCAGTGG
     927sra     .......... .......... CAATT TTAGCGACAG CAGGNGAAAC AGCGATC AA ACCCAAG
```

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       sra   CGGCGGTACA ACAACAGTAG CAATAAGG.C AACTTTACAA CAAAATAGGG G.ACCTAGAA AAA.CAAACG ACCAACAACT
   Etat1.2   ......TACA ACAACACTAC CAATAAGG.C AACTTTACAA CAAAATAGGG G.ACCTAGAA AAA.CAAACG ACCAACAACT
   247sra    ......GTACA ACAACAGTAG CAATAAGGGC AACTTTACAA CAAAATAGGG GGACCTAGAA AAAACAAACG ACCAACAACT
   927sra    .......... ..........                   AAATGGATGG CCGAATTGGA GGACCTAGCA GAAACAATGG A  AACAAAA

       sra   GCGGCACCAG CGTGACCGAA GTACTCGAAC ACATTCTAAA ACAACAACGG CTCAACGAAG CGCTACTTTC AATCGTGAAA
   Etat1.2   GCGGCACCAG CGTGACCGAA GTACTCGAAC ACATTCTAAA ACAACAAGCG CTCAAGGAAG CGCTACTTTC AATCGTCAAA
   247sra    GCGGCACCAG CGTGACCGAA GTACTCGAAC ACATTCTAAA ACAACAAGCG CTCAAGGAAG CGCTACTTTC AATCGTGAAA
   927sra    GCCGCGGCGG C.TG.CCGUA GTAGCC                                 CG C          CTAAACTTAC AAT    TA

       sra   AAACCAAAAG GGGCGCCAGA CAAAACAGCA GCAGATGAAT TGGTCACCGC GCTAAT.CAA CGGCGTGGTG CCAAACAGCA
   Etat1.2   AAACCAAAAG GGGCGCCAGA CAAAACAGCA GCAGATGAAT TGGTCACCGC GCTAAT.CTA CGGCGTGGTG CCTCATAGC.
   247sra    AAACCAAAAG GGGCGCCAGA CAAAACAGCA GCAGATGATT TGGTCACCGC GCTAATXCTA CGGCGAGGAG ..........
   927sra    AAAACAAAAG            AAACAGCC GCGCA         GGTCGCCA                      TT AAGAAAGA

       sra   CAGCACAGAC CCAAAAATTA AACCACAAAA TTCTAAACAC CTTCGT       CCCCAAGCTT G  TGGAAGG CTC  AAAAA
   Etat1.2   .......... ......TTG TTGGAGACA. TTTTAAACAC CTTCGT        CCCCAAGCTT G  TGCAAGC CTC  AAAAA
   247sra    .......... ..........    AAATTT TTTNAAACAC CTTCGTTTCC CCCAAAGCTT TGTTGGAAGC TTCNAAAAAA
   927sra                                        AAAAAC ATTTG           CAGCT

       sra   GCCAA GTAA AACTAAGGA   TTCT GAAG TACCCGGG A AAAATACA G AAAAGCAAAC TCG  TATCA AT CCAAGAG
   Etat1.2   GCCAA GTAA AACTAAGGA   TTCT GAAG TACCCGGG T AAAATACA G AAAAGCAAAC TTG  TATCA AT CCAAGAG
   247sra    GCCAAAGTAA AATTAAAGGA TTTCTNGGAG TACCCGGGGA AAAATACAAG AAAAGCAAAC TNTGTTATCA ATTCCAAGAG
   927sra    .......... ..........  .......ACA  TTCCC                        AGC                CCCAGC

       sra   TTAAAAACCC GAGTGGAGCC TGAATCTAGC ACTGAAACCT GCAAGCAGCA GGTCGCCACC AACCAGGCAC AGGAGGCATT
   Etat1.2   TTAAAAACCC GAGTGGAGCC TGAATTTAGC ATTGAAAGTT GCAAGCAGCA GGTCGCCACC AACCAGGCAC AGGAGGCATT
   247sra    TTAAAAACCC GAGTGGAGCC TGAATCTAGC ACTGAAAGCT GCAAGCAGCA GGTCGCCACC AACCAGGCAC AGGAGGCATT
   927sra         ACCC GT     AGTC                  CA GCCAACACCA A    ACC AA  GGAAAA AGGAGGCATT

       sra   TTGTAACGCA ATTGCCGAC  GACAAAGACA AGTGTAACAA TGAGACACGA TGCAGTTACG ATGACAGCAA AGGCTCAGAC
   Etat1.2   TTGTAACGCA ATTGGAGAC  GACAAAGACA AGTGTAACAA TCAGACACGA TGCAGTTACG ATGACAGCAA AGGTTCAGAC
   247sra    TTGTAACGCA APTGGCGAC  GACAAAGACA AGTGTAACAA TGAGACACGA TGCAGTTACG ATGACAGCAA AGGCTCAGAC
   927sra    TTGTAACGCA ATTGGCGACC GACAAAGACA AGA.TAACAA TGAGACNCGA TGCAGTTAGG ATGACACCAA NGGTTCAGAC

       sra   AAAAAGTGCA CATATAATGC GGAAAAAGCA GAAGCAAATG GGGCACCTGC AACGCAACCT CAAGGGGGAG TGAACGAAGC
   Etat1.2   AAAAAGLGCA CATATAATGC GGAAAAAGCA GAAGCAAATG GGGCACATGC AACGCAACCT CAAGGGGGAG TGAACGAAGC
   247sra    AAAAAGCGCA CATATAATGC GGAAAAAGCA GAAGCAAATG GGGCACCTGC AACGCAACCT CAAGGGGGAG TGAACGAAGC
   927sra    GAAAAGCGCA CATATAATGC GGAAAAAGCA GAAGCAAATG GGCCACATAC AACGCAACTT GAAGGGGAAG TGAACGAAKC

       sra   AACAACA.GG AAATTGTAAA GGGAAACTGG AACCCGGATG CACCAAGGCA CAA.GAATAC GAATGGGAAG GAAAAGAATC
   Etat1.2   AACCACA.GG AAATTGTA.. .......... .......... .......... .......... .......... ..........
   247sra    AACAACA.GG AAATTGTAAA GGGAAACTGG AACCCGGATG CACCAAGGCA CAA.GAATAC GGATGGGAAG GG........
   927sra    ANCAACANGG AATTTGTAAA GGGAAACCGG AACCCGAATG CACCAAGGCT CCCTGAATGC AAATTGGAAA GTGAGCAATG

       sra   CAAAGATTCA AGTTTTCTTG TGGATATGAA ATTGGCTCTG AATATGGTTG CTGCTTTTAT GGCCTTTCTG TTTTAA TTTT
   Etat1.2   .......... .......... .......... .......... .......... .......... .......... ..........
   247sra    .......... .......... .......... .......... .......... .......... .......... ..........
   927sra    CGGCAGCTTA AATTTNCAA. CGCCN..... .......... .......... .......... .......... ..........

       sra   CACCTCTTTT GAAAGAACTT TGCTGTTTTGA TATACTTTAA CACATTTTCA TGAATTGGTG AAAA
   Etat1.2   .......... .......... .......... .......... .......... .......... ....
   247sra    .......... .......... .......... .......... .......... .......... ....
   927sra    .......... .......... .......... .......... .......... .......... ....
```

Figure 5.6. Comparison of the published SRA gene sequence and the sequence of PCR derived SRA fragments from ETat1.2, STIB 247 and TREU 927. A direct comparison with the DNA fragment produced by nested amplification of SRA related sequence from ETat1.2 and the published SRA gene sequence within the open reading frame showed 98% identity with the published gene. A line up with the PCR amplified product from STIB 247 and the published SRA gene show a 98% identity between the sequences across the 1080 bp sequenced. Nested PCR was also used to produce a fragment related to the SRA gene in TREU 927 and alignment with the published SRA gene sequence revealed a good homology at the 3'end of the sequence, close to 95%, but a comparison between the 5'ends of the sequences show much more divergence. Dots indicate missing sequence while gaps indicate gaps in sequence alignment. Start and stop codons are marked with *.

Similarly, the PCR product recovered from STIB 247 showed 98% identity with the published SRA gene sequence when 1076 bp of the PCR product were sequenced (**figure 5.6**). This is convincing evidence that the fragments obtained from PCR amplification using primers for the SRA gene in Etat1.2 and STIB 247 are true homologues of the SRA gene. The sequence obtained from TREU 927 showed major differences at the 5'end compared with the published SRA sequence while a stretch starting from the 3'end and ending at bp 992 show 95% identity with the SRA gene (**figure 5.6**). As discussed earlier, the 3'end of the SRA sequence has high homology

with the carboxy terminal part of sequenced VSG genes (De Greef and Hamers, 1994) and it is also at this end of the gene that TREU 927 show the best fit with the SRA gene.

5.2.4 Analysis of the expression of SRA by Northern hybridisation

Having established the presence of a SRA gene in STIB 247 and ETat1.2, identified a closely related sequence in TREU 927 and a homologue (by Southern hybridisation) in STIB 386, it was important to test for expression of the gene in these stocks to investigate the possibility of a correlation between SRA expression and resistance to lysis. RNA was isolated from the human serum sensitive line of ETat1.2, from ETat1.10 which is a human serum resistant line, and from STIB 386 (human serum resistant), STIB 247 (human serum sensitive) and TREU 927 (intermediate resistant). RNA was then separated by gel electrophoresis on agarose gels before blotting. The ETat1.10 RNA preparation was a kind gift from Etienne Pays and used as a control since this was the trypanosome line from which the SRA gene had originally been described. Initially, a PCR product comprised of the major part of the SRA open reading frame from ETat1.2 (primers MHS and MHT followed by 2SRA1 and 2SRA2) was used to probe the Northern filter. This resulted in strong hybridisation with an approximately 1.4 kb RNA fragment in the lanes of the human serum resistant ETat1.10 in addition, with a slightly larger fragment in a clone of STIB 247 which had been passaged in mice for approximately 80 times (STIB 247x80) but not in ETat1.2(S), STIB 386, STIB 247 or TREU 927 (**figure 5.7**).

STIB 247x80 was tested for human serum sensitivity by the *in vivo* HSS-assay used in this study and found to be serum sensitive. A SRA homologue was not present in any of the other stocks tested.

A.   **ETat1.10R**   **ETat1.2S**   **STIB 386**   **STIB 247**   **STIB 247**   **TREU 927**
passaged

B.
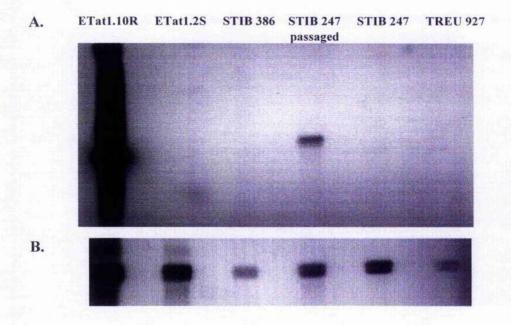
Figure 5.7. Northern hybridisation of mRNAs with the SRA gene probe. (A) 5µg of total RNA was separated by agarose gel electrophoresis in the presence of formaldehyde/formamide, blotted onto Hybond N⁺ membrane and hybridised to SRA PCR product derived from ETat1.2. (B) Tubulin was used as control probe to verify the presence and quality of RNA in each lane.

It was suspected that the hybridising fragment in the passaged line of STIB 247 could be a VSG gene on the basis that the SRA gene has some homology with the VSG gene family and the band is the approximately correct size for a VSG gene transcript. This possibility was tested by indirect immunofluoresence using VAT-specific antisera. It was confirmed that STIB 247x80 was expressing the GUTat 8.1 VAT which was not expressed in any of the other lines but labelled 99% of the trypanosome population in STIB 247x80 (data not shown). PCR with a miniexon primer and a specific VSG primer designed to base-pair with a conserved region in the 3' untranslated region of VSG mRNAs (Carrington *et.al.*, 1991) further verified the expression of a single gene of the size expected for a VSG gene (**figure 5.8**).
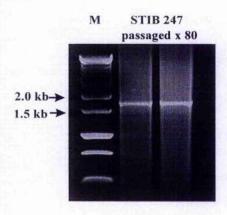
Figure 5.8. RT-PCR amplified VSG gene expressed in a passaged line of STIB 247. The fragment hybridising with STIB 247x80 RNA was suspected to be a VSG gene and the resulting RT-PCR product using miniexon primer (5' AAC GCT ATT ATT AGA ACA GTT TCT G 3') and VSG specific primer (5' GTG TTA AAA TAT ATC A 3') is shown above. The following conditions were used: 96°C for 5 min followed by 30 cycles of 96°C for 1 min, 42°C for 1 min and 70°C for 2 min and an additional extension time of 5 min at 70°C.

A comparison of the sequences close to the C-terminal ends of SRA and VSG genes show particular high homology in the last 200 bp of the translated region and to test if this region alone was responsible for hybridisation PCR products, which lacked this region, were generated from ETat1.2 to be used as a probe in Northern blots. The prediction was that this PCR product could generate specificity of hybridisation to the SRA gene and less cross reactivity to the putative GUTat8.1 VSG gene. Primers SRA5 and SRA8 were combined in reactions together with purified SRA from ETat1.2 as DNA template. This was predicted to generate a 900 bp product which would be 200 bp shorter at the 3'end compared with the previous probe. The amplified fragment was subsequently run on an agarose gel, purified and used in Northern hybridisation experiments. The SRA gene probe lacking the 3'end no longer bound to RNA derived from the passaged line of STIB 247 but hybridisation still occurred with a 1.4 kb size fragment in the human serum resistant line ETat1.10. Again, no hybridisation could be observed in any of the other trypanosomes clones used (**figure 5.9**). Hybridisation of this probe was also observed to RNA of a human serum resistant ETat1.2 clone (kindly provided by Etienne Pays) (data not shown). Northern blots and hybridisations, both with the whole SRA gene and with the SRA gene minus its C-terminal end, were repeated several times, each showing identical results. It can be concluded that, of the trypanosome stocks tested, only the human

serum resistant ETat1.10 and ETat1.2 expressed the SRA gene and not the human serum resistant STIB 386, the human serum sensitive STIB 247, the passaged line of STIB 247 or the intermediate resistant TREU 927.

ETat1.10R	ETat1.2S	STIB 386	STIB 247 passaged	STIB 247	TREU 927

A.

B.
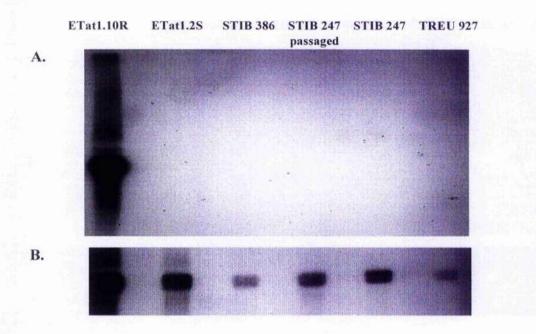
Figure 5.9. Northern blot analysis of mRNAs probed with the SRA specific PCR probe lacking its C-terminal end. (A) Total RNA derived from both human serum resistant and human serum sensitive trypanosome stocks were probed with PCR amplified SRA gene after approximately 200 bp had been removed from its 3'end. (B) β-tubulin from *T.brucei* was again used as positive control probe.

5.2.5 Analysis of the SRA gene by RT-PCR

RT-PCR was used as an additional tool for evaluating the expression of the SRA gene in human serum resistant and human serum sensitive cloned stocks of trypanosomes. The same set of primers were used as for the other PCR analyses described in section 5.2.1. To ensure the quality and integrity of RNA and synthesised cDNA, a control reaction with primers for the TIM locus was always performed for each batch of cDNA prior to any experiment. Primers TIM A and TIM B were routinely used for the control to produce a 1.1 kb PCR product. Control reactions with primer combination TIM E and TIM B, resulting in a 0.4 kb fragment,

were initially used but it was observed that the cDNA was sufficiently intact to produce the smaller product but failed to provide an adequate template for the 1.1 kb amplification product in two of five cDNA preparations (**figure 5.10**). Using primers for the larger fragment therefore provided a more rigorous control of cDNA quality.
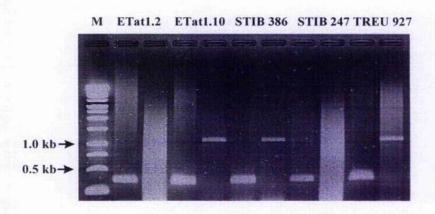
Figure 5.10. Example of positive control of the quality of cDNA prior to use in RT-PCR. cDNA synthesised from RNA derived from cloned trypanosome stocks was tested with TIM primers prior to use in RT-PCR experiments. Two sets of primers were used, TIM E and TIM B which was expected to produce a 0.4 kb band and TIM A and TIM B expected to produce a 1.1 kb band. In each pair of tracks the first shows the result using primers E and B and the second, the results using primers A and B. M, 1 kb DNA ladder (13 bands). PCR conditions were as follows: 96°C for 50 s, 64°C for 50s and 72°C for 2 min for a total of 30 cycles.

RT-PCR was performed on cDNA derived from the human serum resistant ETat1.2 and ETat1.10, from human serum sensitive ETat1.2, STIB 386, STIB 247 and TREU 927 and the results obtained are shown in **figure 5.11**. Only the human serum resistant ETat1.10 and ETat1.2 gave an ethidium bromide stained fragment of 1.2 kb but none of the other clones tested gave rise to an amplified product even though the same batch of cDNA could produce the 1.1 kb band using TIM A and TIM B primers. Primer combinations MHS/MHT and 2SRA1/2SRA2 were also tested in a nested amplification and both sets of primers produced DNA fragments for the human serum resistant ETat1.2 and ETat1.10 only (data not shown).

Figure 5.11. RT-PCR amplification of the SRA locus from RNA isolated from cloned stocks of trypanosomes. Ethidium bromide stained agarose gel separation of the RT-PCR amplified products obtained from cDNA using the primers 2SRA1 and 2SRA2. RT-PCR was performed under the following cycling conditions: 96°C for 50 s, 50°C for 50 s and 72°C for 2 min for a total of 30 cycles.

5.3 DISCUSSION

Genomic analysis of the SRA gene has shown that it is present in STIB 247, ETat1.2, ETat1.10 and that a similar sequence can be found in TREU 927 and in STIB 386. The expression of the SRA gene has previously been confirmed in the ETat repertoire of *T.b.rhodesiense* which all are descendants of a strain isolated from *G.pallidipes* in Uganda in 1959.

SRA gene expression was clearly not associated with human serum resistance in the genetically competent stocks investigated in this study. An SRA homologue was identified in all three stocks but it was expressed in none of them, including the serum resistant stock STIB 386. STIB 386 was isolated from a man in Ivory Coast and initially characterised by Mehlitz *et.al.* (1982) as a group 2 *T.b.gambiense* because it was observed to be more virulent in mice than other *T.b.gambiense* isolates but it was also shown to be highly resistant to human serum whereas other group 2 *T.b.gambiense* isolates are usually subresistant or even sensitive to human serum (Mehlitz *et.al.*, 1982). Richner *et.al.* (1989) did however, find variable resistance in human serum resistance tests on STIB 386 metacyclic forms. Further evidence for STIB 386 being a group 2 *T.b.gambiense* comes from its ability to be

passaged through tsetse flies in the laboratory (Jenni *et.al.*, 1986) since this has not been achieved with group 1 *T.b.gambiense* isolates. Data from Hide *et.al.* (1990, 1991, 1994, 1997) suggests that groups of *T.b.rhodesiense* isolates from different parts of Africa are very distinct and that they may have originated independently. This observation provides credibility to the suggestion that more than one mechanism conferring human serum resistance in *T.brucei* may exist. Human serum resistance could be determined by the expression of SRA in stocks from East Africa but not those from West Africa and so might not be responsible for human serum resistance in either group 1 or group 2 *T.b.gambiense*. Another mechanism may therefore determine resistance in STIB 386 and it is possible that multiple mechanisms for human serum resistance has evolved in different areas of Africa. The lack of SRA gene expression in STIB 247 and TREU 927 is what would be expected given that these stocks are sensitive to human serum.

It is clear from the sequencing data that the SRA locus identified in ETat1.2 (Glasgow line) and STIB 247 are both homologous to the published SRA gene sequence. It is however, less clear what the implications of the variation in sequence found in TREU 927 would have on the resulting gene product. The related gene in TREU 927 is larger than the SRA gene and the highest degree of similarity is found at the 3'end which is the part of the SRA gene that show strong homology with published VSG gene sequences. It is a possibility that the PCR product obtained from TREU 927 is a VSG gene. Findings that would support this suggestion are the size of the fragment, which is in the same size range as described for VSG genes, the Southern blots that show hybridisation of the SRA gene with DNA from TREU 927 only when the 3'end is present (data not shown) and the fact that sequence analysis showed some divergence from the SRA gene sequence. The related SRA sequence in STIB 386 was not analysed because of time constraints. Since no PCR product could be obtained from this stock, other methods would have to have been adopted to clone SRA gene homologues. De Greef & Hamers (1994) proposed the presence of SRA pseudogenes which are no longer transcribed and which show differences in sequence from the actively transcribed gene. The same group also detected more bands in Southern blot analysis than expected on the basis of the restriction map of

the cDNA clone and suggested the presence of several related sequences. The Southern blots performed on DNA from ETat1.2, STIB 386, STIB 247 and TREU 927 did not however, reveal the existence of any bands in addition of those expected from the restriction map of SRA. The extra bands identified by De Greef & Hamers (1994) could also be alternative functional forms of a gene family. Further analysis of the SRA homologues in the stocks studied here would have included sequencing of the entire open reading frame (in both directions) and analysis to determine gene copy number but in view of the lack of association of SRA gene expression with human serum resistance in these stocks it was decided not to give priority to this area of the project.

The importance of the SRA gene in conferring human serum resistance in the ETat repertoire and some other uncloned stocks of *T.b.rhodesiense* from East Africa has now been confirmed by transfection of SRA from ETat1.10(R) into a serum sensitive stock, Antat1.8 (Van Xong *et.al.*, 1998). The SRA gene was inserted into the ribosomal locus of the *T.b.brucei* clone and this transformed the population from human serum sensitive to human serum resistant without changing VSG expression. These authors have also presented evidence that the SRA gene is an ESAG in the ETat1.10 expression site and that this expression site is only active in human serum resistant clones. This could explain the earlier observations of a potential correlation between the change in expression of human serum resistance/sensitivity and VSG switching (Van Meirvenne *et.al.*, 1976). If the change in VSG expression is by gene conversion i.e. there is no change in expression site, then the SRA will not change and so the resistance/sensitivity will remain the same. But if there is a switch to another expression site, lacking an SRA-ESAG, the switch in VSG will be associated with a change from resistance to sensitivity. The localisation of the SRA gene to an expression site could have implications for the epidemiology of trypanosomes capable of causing an infection in humans. The trypanosomes would be limited to expression sites containing SRA genes as any trypanosomes (in humans) expressing non SRA-containing expression sites would be lysed by serum. Antigenic variation in trypanosomes is usually brought about by gene conversion of the VSG gene in an active expression site or switching between expression site. If trypanosome infections

in humans would be limited to a subset of expression sites containing SRA, and lacking ESAGs 1-5, this may have quantitative consequences for the number of VSGs the trypanosomes can use. However, the suggestion that SRA genes are only expressed in VSG expression sites has yet to be formally proven.

If expression of the SRA genes turns out universally to be expressed in *T.b.rhodesiense* but not in *T.b.gambiense* and *T.b.brucei* it could potentially be used as the first definitive marker to distinguish between *T.b.gambiense* and *T.b.rhodesiense*.

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Chapter 6

Genes isolated by differential display RT-PCR as potentially important in human serum resistance

6.1 INTRODUCTION

Human serum resistance in *T.brucei* is believed to be mediated by the SRA gene described by De Greef and Hamers (1994) which encodes a VSG like protein. But while this may be the case with some *T.b.rhodesiense* isolates it is not necessarily a universal means of conferring human serum resistance in *T.brucei* as discussed in Chapter 5 in this thesis. Other mechanisms, which also result in trypanosomes expressing resistance to human serum, may have evolved separately in various parts of Africa. In principal it could be postulated that cells expressing resistance express a specific gene sequence that confers resistance on cells that would otherwise be sensitive or alternatively, one could postulate the converse i.e. that cells are resistant but only rendered sensitive by the specific expression of a gene determining sensitivity. The ability to obtain sensitive parasite lines by rodent passage of some resistant stocks suggests that the expression of any gene determining sensitivity or resistance can also vary in its expression during the course of vegetative growth in a non-immune host.

One approach available to try and identify differentially expressed genes is RNA display which can be used to identify differences at the level of messenger RNA. RNA display is a technique in which random small fragments of expressed sequences from two different populations are compared in order to identify sequences expressed in only one of the populations (Liang & Pardee, 1992). A schematic representation of differential display is illustrated in **figure 6.1**. The success of the method is dependent on the expressed RNA being exactly the same in the two populations apart from the trait being studied.

Total RNA extraction of selected HSS and HSR trypanosome lines expressing the same VSG

Reverse transcription with A-, C- or G-anchored oligo dT primers

ACUGGGUAGCCAAAAA
GTTTTT

First strand cDNA

ACUGGGUAGCCAAAAA
CCCATCGGTTTTT

PCR with anchored primer and an arbitrary primer, incorporating ^{33}P

Double stranded cDNA fragment

ACTGGGTAGCCAAAAA
TGACCCATCGGTTTTT

Gel electrophoresis on 6% denaturing polyacrylamide gel followed by autoradiography

Differentially expressed bands displayed

Cut out differentially expressed bands
Extract DNA and reamplify

Clone

Confirm differential expression by Northern blotting

(RT-PCR or probe library to obtain complete cDNA)

Sequence, screen databases to identify genes

Figure 6.1. Schematic representation of differential display RT-PCR used for identifying possible HSR/HSS genes. Total RNA from two different populations of trypanosomes (A and B) are reverse transcribed using an anchored oligo-dT primer to produce single stranded DNA. A second primer, random in sequence, is then used in combination with the anchored primer to amplify a range of mRNAs from their 3'end. These cDNA fragments are displayed on a DNA sequencing gel. A DNA band of interest can be cut from the gel, amplified by PCR, cloned and sequenced. Finally, its sequence can be compared with sequences in data banks directly or used as a starting point for RT-PCR to obtain a longer cDNA fragment before DNA sequence analysis.

A complication when using bloodstream form *T.brucei* is that the trypanosomes used for analysis need to be expressing the same VSG gene (as well as being isogenic) otherwise the majority of differentially expressed transcripts would be the result of differences in VSG gene expression. The aim of this study was to identify a cDNA fragment that is expressed in only the serum sensitive or serum resistant trypanosomes lines. This was based on the assumption that human serum resistant trypanosomes express a transcript that human serum sensitive trypanosomes do not express or vice versa. To achieve this, a trypanosome line that was capable of expressing differences in human serum resistance phenotype was chosen and clones expressing the same VAT were selected. Total RNA was isolated from the expanded populations of these clones and analysed with the RNAimage™system (GenHunter). A total of nine differentially expressed fragments were identified from the approximately 10 000 expressed fragments analysed. These fragments were isolated, cloned and rescreened on Northern blots. These cloned inserts which were confirmed as being differentially expressed were sequenced and analysed for potential sequence homology with previously identified DNA sequences. Cloning of the nine bands resulted in sixteen different cloned fragments of which four showed expression patterns associated with human serum resistance or sensitivity. Three of these were specifically expressed in human serum sensitive trypanosomes and one was specifically expressed in human serum resistant trypanosomes. Two of the sensitive specific clones were related to EST sequences from a cDNA library of a cloned *T.b.brucei* bloodstream population. These sequences have not yet been fully characterised and time did not permit further investigation into the structure and function of these genes/gene fragments. The third sensitive specific clone was a fragment of a gene for ribosomal RNA. The resistance specific clone was unrelated to any other sequences in the database and attempts to obtain a longer sequence, which would aid identification, have not been successful.

6.2 RESULTS

6.2.1 Selection of a trypanosome line expressing differences in human serum resistance phenotype whilst expressing the same VAT

A crucial requirement for differential display RT-PCR to be successful was to produce trypanosome lines which differed in their expression of human serum resistance but were identical in all other respects. The human serum resistant stock STIB 386 was examined because it had been found, while screening clones for their resistance/sensitivity to human serum, that some of the clones derived from an extensively mouse-passaged line of STIB 386 (GUP 2944) no longer expressed the resistant phenotype and had become sensitive to human serum. One of these human serum sensitive subclones (GUP 3179) had been cloned at the second peak of parasitaemia and characterised as expressing the GUTat 9.2 VAT while another subclone from GUP 2944, which had been cloned at the first peak of parasitaemia and previously shown to express the GUTat 9.1 VAT, retained complete resistance to human serum (**figure 6.2**). This series of clones and subclones had been made prior to my arrival in the laboratory (Turner *et.al.*, 1991).

STIB 386
Human serum resistant

STIB 386 passaged x 49
human serum resistant/sensitive

Subclones expressing different VATs:

GUTat 9.1	HSR
GUTat 9.2	HSS
GUTat 9.3	HSR
GUTat 9.4	HSS/R
GUTat 9.5	HSS

Sub-sub clones:

GUTat 9.1	HSS
others	HSS
others	HSR

Figure 6.2. Selection of trypanosome stocks for differential display RT-PCR. A passaged line of STIB 386 was chosen as the starting material for differential display RT-PCR and clones from this screened for their human serum resistance/sensitivity until a stable resistant and a stable sensitive clone was found. These were selected to express the same VAT, GUTat 9.1. HSR; human serum resistant, HSS; human serum sensitive, HSS/R; human serum sensitive/resistant.

The two clones were thus ideal candidates for differential display RT-PCR if they could be selected to express the same VSG. Antibody mediated complement lysis assays and IFATs using rabbit anti-9.1 antiserum confirmed that 95 % of the cells of GUP 2979 expressed GUTat 9.1. The serum sensitive GUP 3179 was however, shown by both IFAT and antibody mediated complement lysis to consist of a mixture of trypanosomes expressing different VATs of which approximately 40% were expressing the GUTat 9.2 variant and, encouragingly, 10% still expressing GUTat 9.1. To produce a serum sensitive population expressing the GUTat 9.1 VSG, GUP 3179 was incubated with rat anti-9.2 antiserum so that trypanosomes expressing this VSG were lysed and the remaining cells used for cloning. Trypanosomes derived from the clones were again screened for their VAT expression by IFAT and their sensitivity/resistance to human serum until a clone positive for GUTat 9.1 but still sensitive to human serum was identified. *In vivo* HSS-assays and IFAT were repeated three times with identical results using a clone negative for both GUTat 9.1 and GUTat 9.2 as a negative control. To prepare RNA from each of the HSR and HSS subclones of GUTat 9.1 trypanosomes populations were expanded in mice and rats, checking by IFAT after each step and by HSS-assays *in vivo* before purifying the cells on DE-52 cellulose columns and isolating RNA as described in section **2.8**.

6.2.2 Preparation of starting material

The integrity of isolated RNA was checked by running a sample on an agarose gel before use which also permitted detection of any contaminating chromosomal DNA. The isolation of intact RNA that is free of DNA is crucial for the success of the differential display methodology as the subsequent randomly primed PCR will amplify DNA. All samples were treated with DNase I to ensure that the mRNA preparations were free of DNA since DNA contamination of RNA preparations isolated using the Trizol method was frequently observed. The DNase treated RNA preparations were reverse transcribed using three different one-base anchored oligo-dT primers and the resulting cDNA checked in a PCR reaction using primers for the

TIM gene as positive control for the quality of the cDNA. Only samples that gave rise to amplification products longer than 500 bp were used in the RT-PCR that followed. By using different sets of primers it has been calculated that most of the genes expressed in a cell may be systematically detected (Liang et.al., 1994). 24 different arbitrary primers were used in conjunction with the three different oligo-dT primers resulting in 72 RT-PCR reactions for each sensitive and resistant population. The total number of PCR amplified fragments for each of the sensitive and resistant populations was estimated to be approximately 10 000. If the size of the diploid nuclear genome in *T.brucei* is estimated to be 54 Mb (Melville et.al., 1998) and it is assumed that 10-15 % of the genome is expressed at a given time, this represents a total of 5.4 kb of mRNA. Since the size of the amplified fragments varies between 50 and 350 bp the sum of the length of fragments amplified by each primer-combination is around 10 kb which would theoretically mean that the majority of mRNAs in the genome should be represented even if some redundancy occurs. The coverage of the majority of mRNAs can also be argued on the basis of the size of the fragments amplified. If we assume an average size of 3 kb per mRNA and know that RT-PCR only amplifies 2-10% of the total length of any mRNA; an amplification of 10 kb of sequence would represent at least 100 kb of mRNA. Similarly, if one assumes that each cell expresses 5-10 000 different mRNAs, the sampling of 10 000 fragments is a reasonable sample size. The major potential problem with these arguments is the different levels of abundance of different mRNAs leading to more abundant mRNAs being better represented than low abundant mRNAs.

The arbitrary primers are only 10 nucleotides in length and so require a low annealing temperature (40°C) for priming. This low temperature may create some spurious products that are not reproducible and could lead to false positives. To avoid this, all RT-PCR reactions were undertaken in duplicate using two different RNA preparations run side by side on the sequencing gel. Only bands which were differentially expressed in both pairs of lanes were considered as potential differential display products. Since the use of different thermostable DNA polymerases under the same PCR conditions have been shown to amplify different cDNA populations (Haag & Raman, 1994), the same Taq DNA polymerase was used

throughout the experiments. The amplified subset of mRNA 3' termini from the cDNA preparations were then displayed on 6% denaturing polyacrylamide gels similar to those used for DNA sequencing.

6.2.3 Band separation and retrieval

DNA sequencing gels were used in order to obtain high resolution of a large number of amplified cDNAs and routinely 100 - 120 bands were separated in each lane on the gel. Because ^{33}P-dATP had been incorporated into the PCR reaction, the bands could be visualised after drying the gel and autoradiography. The autoradiographs were carefully studied and a total number of nine differentially expressed band were identified as shown in **figure 6.3**. Only those differentially amplified products that appeared in both lanes of the two separately isolated RNA samples were considered for further analysis. It was interesting to note that differentially expressed bands were found originating both from the human serum resistant and the human serum sensitive population. Bands 001, 002, 003, 004 and 008 were all produced from the serum sensitive population while 005, 006, 007 and 009 came from trypanosomes expressing the human serum resistant phenotype (**table 6.1**).

Band	Oligo-dT anchored primer	Arbitrary primer	Size of fragment (bp)	Origin of the band
001	H-T$_{11}$-C	H-AP-26	300	sensitive
002	H-T$_{11}$-C	H-AP-26	app. 200	sensitive
003	H-T$_{11}$-G	H-AP-25	app. 200	sensitive
004	H-T$_{11}$-G	H-AP-25	413	sensitive
005	H-T$_{11}$-G	H-AP-8	app. 100	resistant
006	H-T$_{11}$-G	H-AP-9	app. 300	resistant
007	H-T$_{11}$-G	H-AP-9	160	resistant
008	H-T$_{11}$-G	H-AP-15	224	sensitive
009	H-T$_{11}$-G	H-AP-13	188	sensitive

Table 6.1. The generation of nine differentially displayed cDNA fragments in a comparison of human serum sensitive and resistant trypanosome lines. The cDNA fragments were found to be differentially displayed on polyacrylamide DNA sequencing gels. The original primer combinations used for generating these bands, their approximate size and their origin from human serum resistant or human serum sensitive trypanosome RNA are shown.
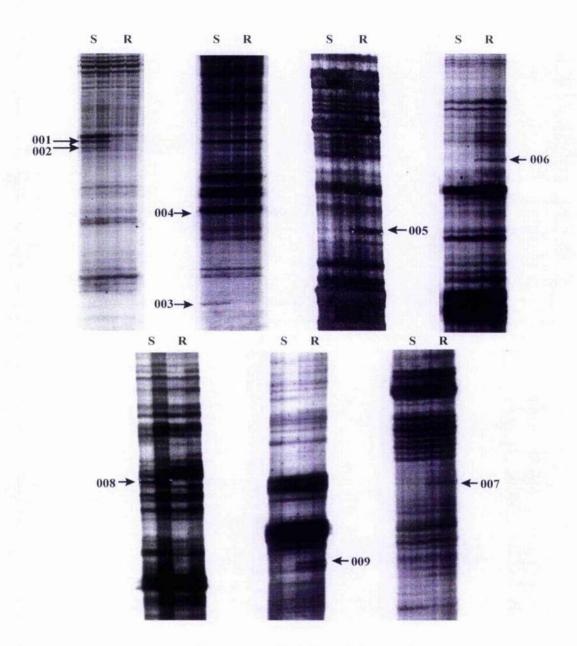
Figure 6.3. Examples of differential display RT-PCR products including the nine differentially displayed bands. Two independent RNA preparations were made from a human serum sensitive (S) and two from a human serum resistant trypanosome population (R) which were isogenic and expressing GUTat 9.1 before being subjected to RT-PCR with 24 different arbitrary primers coupled with three different one-base anchored oligo-dT primers. α-^{33}P dATP was incorporated into the PCR reaction so that the products could be visualised on a denaturing gel after autoradiography. All nine differentially displayed bands (001-009) that were found during the experiment are shown in the examples above.

Each of the nine bands was cut from the gel, reamplified by PCR using the original primer combination and the products separated on agarose gels. This allowed the sizes of the fragments to be more accurately measured (**figure 6.4** and **table 6.1**)
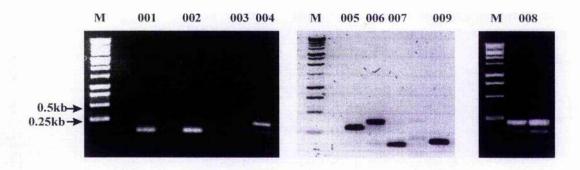
Figure 6.4. Recovered and reamplified differentially displayed DNA fragments. The nine differentially displayed bands were cut from the sequencing gel, reamplified by PCR and are shown after separation on a 1% agarose gel stained with ethidium bromide. M, 1 kb DNA ladder (13 bands).

The nine bands were each recovered from the agarose gel and cloned into a pUC18 vector. Some of the original fragments gave rise to cloned products with more than one insert size. Even though great care was taken only to cut out the specific differentially displayed band from the original sequencing gels, without touching the edges of the next, it is possible that DNA from adjacent bands may have contaminated the reactions. The bands may also overlap each other on the gel and it is therefore impossible to avoid contamination with DNA from other fragments. It was therefore necessary to screen several clones with different sized inserts for each differential display fragment to determine which one corresponded to the differentially expressed sequence. Midiprep samples of the cloned bands were either digested with suitable restriction enzymes to cut out the insert, or direct PCR amplification of plasmid inserts was performed to identify clones with inserts (**figure 6.5**).
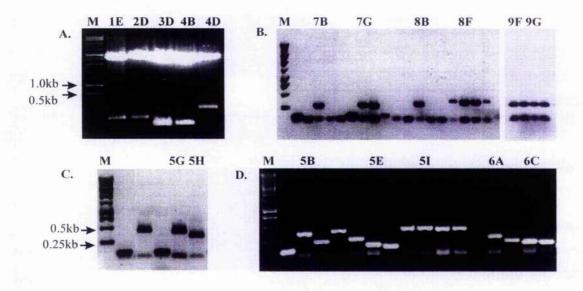
Figure 6.5. Different sized inserts identified in cloned fragments isolated from differential display. The presence of multiple DNA fragments present in some excised bands gave rise to plasmid inserts of different sizes as shown in the pictures above. Panel (A) is the result of midiprep digest where the top bands correspond to the plasmid and the lower bands are the plasmid inserts. Panels (B), (C) and (D) are the results of direct PCR screening of colonies and the bottom bands here are primer dimers. The number and letter refer to clone names for the colonies that where picked. Unlabelled tracks are either the products from colonies lacking an insert or contain inserts of identical size to colonies that have already been picked. Band 004 produced clones of two different sizes, band 005 three different sizes and band 006 two different sizes. The other recovered fragments, 001, 002, 003, 007, 008 and 009, resulted in inserts of a single size. M, 1 kb DNA ladder (13 bands).

A single size insert was found in clones from bands 001, 002 and 003 (1E, 2D and 3D, **figure 6.5a**) but two insert sizes were obtained from fragment 004 (4B and 4D, **figure 6.5a**). Three different sizes were found from fragment 005 (5B, 5E and 5I, **figure 6.5d**). Clone 5G and 5I were later shown to originate from the same fragment and clone 5H and clone 5B also had a common origin. Two different sizes were found from 006 (6A and 6C) but a successful recovery was only achieved with clone 6C. From the recovered and reamplified fragments 007, 008 and 009 only one size of insert was found.

6.2.4 Screening differentially displayed bands

cDNA fragments which are apparently differentially expressed on a differential display gel require checking to distinguish differential display products from PCR

artefacts. This is usually carried out by Northern blotting before cloning to establish whether the cDNA represents a true difference in expression between samples tested. Since the number of bands to be examined was rather low however, and in order to have a larger quantity of DNA for use as probes, the cDNAs of interest were cloned before screening. The screening was conducted using two approaches. Firstly, amplified inserts from each cloned fragment were used as probes onto Northern blots prepared from total RNA isolated from the human serum resistant and the human serum sensitive trypanosomes populations. Unfortunately, this approach proved unreliable because of limits in the sensitivity of detection of signals, presumably because of low abundance of the mRNAs. Secondly, bands were screened by Northern slot blots which allowed larger amounts of RNA to be concentrated in smaller areas and thus increase the chance for detecting signals. The integrity and level of RNA on slotblots and Northern blots was controlled by hybridising the filters with a trypanosome β-tubulin gene probe as a positive control. A summary of the results of screening each clone is provided in **table 6.2**. The clones originating from band 004 (004B),003 (003D) and 009 (009G) hybridised strongly with RNA from both the human serum resistant and the human serum sensitive trypanosomes using both approaches and these clones were therefore excluded from further analysis. Similarly, clone 002D, clone 005I (same size as 005G), clone 005B (same size as 005H) and clone 006C all hybridised with the two different RNA preparations when examined by slot blot hybridisation even though no signal could be detected after standard Northern blotting. Two clones, of the same size, obtained from band 007 (007B and 007G) both failed to give rise to any signal at all by either Northern or slot blot analysis. The expressed fragment from this band could have been potentially interesting to follow up, but further analysis was difficult because of time constraints and the most obvious explanation is that they result from a PCR artefact and are therefore of no interest.

cDNA clone	origin	Northern blot		Slot blot	
		HSR	HSS	HSR	HSS
001E	HSS	-	--	-	+
002D	HSS	-	-	+	I
003D	HSS	+	+	+	+
004B	HSS	+	+	+	+
004D	HSS	-	+	-	+
005G	HSR	-	-	+	+
005H	HSR	nd	nd	I	+
005B	HSR	nd	nd	+	I
005I	HSR	nd	nd	+	+
005E	HSR	-	-	+	-
006C	HSR	nd	nd	+	+
007B	HSR	-	-	-	-
007G	HSR	-	-	-	--
008B	HSS	-	--	nd	nd
008F	HSS	-	-	-	+
009G	HSR	+	+	+	+

Table 6.2. Summary of screening of clones originating from differentially displayed fragments by Northern and Northern slot blot analysis. Sixteen clones were produced from the nine differentially displayed bands excised from the DDRT-PCR gel and screened for their expression in human serum sensitive (HSS) and human serum resistant (HSR) trypanosome populations. (+) signal present, (-), no signal could be detected. Sequencing of the clones later showed that clone 005G and clone 005I were from the same fragments and so were clone 005H and 005B, 007B and 007G and 008B and 008F. nd, not determined.

The clones 001E, 004D, 005E and 008F (same size as 008B) all showed clear differences in their hybridisation pattern between the RNA derived from human serum resistant and human serum sensitive trypanosome populations as shown in the representative slot blot data in **figure 6.6**. Clones 001E, 004D and 008F show high expression in HSS trypanosomes but little or no expression in HSR trypanosomes while clone 005E shows increased expression in the HSR trypanosomes. Northern slot blotting was repeated for all four clones with similar results. Clone 004D and 001E occasionally produced a faint signal in the slot containing human serum resistant trypanosome RNA but the signal was always stronger with the slot containing RNA from the serum sensitive trypanosome population. The signal produced in slots probed with clone 008F were consistently rather faint but no signal was ever detected with RNA from serum resistant trypanosomes. Hybridisation with clone 005E always produced a strong signal with the human serum resistant RNA and no signal could be seen in the RNA from serum sensitive trypanosomes. Further

analysis of these four clones was subsequently undertaken with the aim of establishing their relationship to human serum resistance/sensitivity.
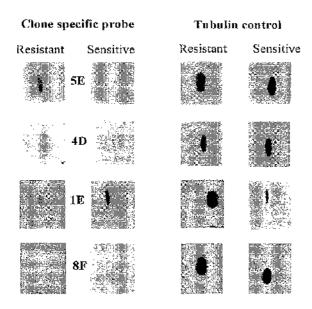
Figure 6.6. Screening of differentially displayed fragments by Northern slot hybridisation. Sixteen clones were screened for hybridisation with Northern slot blots with total RNA from serum sensitive and serum resistant trypanosome populations. The four blots shown above produced a significant difference in intensity of signal for one of the populations whereas the remaining clones either produced a signal for both of the blots or failed to produce a signal altogether. Trypanosome β-tubulin was used as a positive control.

6.2.5 Characterisation of differentially displayed clones

The four clones, each derived from a differentially amplified product, were tested to determine whether they were transcripts of *T.brucei* DNA on Southern blots, sequenced and analysed for database similarities.

Southern blot confirmation

Genomic DNA isolated from ETat1.2, STIB 386, STIB 247, TREU 927 together with DNA from the human serum resistant and human serum sensitive derivatives of STIB 386 and mouse was digested with *TaqI*, run out on an agarose gel, transferred to a nylon filter and hybridised with the purified insert of clone 004D. This resulted in strong hybridisation with trypanosome DNA in all lanes whereas no signal could be detected in the lanes of mouse DNA (**figure 6.7a**). There are two common bands of approximately 1.2 kb and 0.8 kb in size in all stocks apart from the lanes

containing ETat1.2 DNA where only the 0.8 kb band is present. It is possible that the hybridising sequence in ETat1.2 is slightly different and no *TaqI* site is present in the genomic region covered by the probe or there could be two genes with a high degree of sequence homology present of which only one copy is present.

Clone 001E was used to probe filters with genomic DNA digests with *EcoRI* and *TaqI* from two different trypanosome stocks, TREU 927 and ILtat1.2 (data not shown) and with DNA from STIB 386 digested with *PstI*, *BamHI* and *TaqI* which all resulted in specific hybridisation with two DNA fragments (5.0 kb and 4.5 kb for *PstI*, 6.0 kb and 4.0 kb for *BamHI* and 0.75 kb and 0.2 kb for *TaqI*, **figure 6.7b**). The trypanosome origin of the clone 008F was also confirmed since it clearly hybridised with genomic DNA isolated from STIB 386 cut with *PstI* and *BamHI* (**figure 6.7c**). Hybridisation of clone 005E onto filters containing DNA from TREU 927 and ILtat1.2 digested with *TaqI* and *EcoRI* was however, only just detectable (data not shown) probably because this insert was only 56 bp in size. The data suggests that the three clones 001E, 004D and 008F represent different genes and also confirms that they are of trypanosome origin.
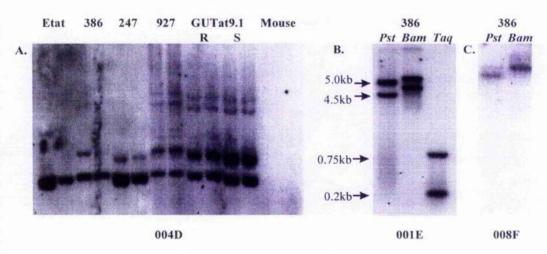
Figure 6.7. Southern blot analysis of DNA from trypanosome isolates probed with three differentially displayed fragments. Three different Southern blots were prepared with 1μg DNA isolated from various trypanosome clones and probed with three of the cloned differentially displayed fragments. (**A**) genomic DNA from ETat1.2, STIB 386, STIB 247, TREU 927 and the GUTat 9.1 human serum sensitive (S) and human serum resistant (R), used for RT-PCR differential display, were digested with *Taq I* for Southern blot analysis and probed with the fluoroscein labelled 004D clone. (**B**) genomic DNA from STIB 386 was digested with three different restriction enzymes, *PstI*, *BamHI* and *TaqI* and analysed by Southern blotting probed with the [32]P-labelled 001E clone. (**C**) genomic DNA prepared from STIB 386 was digested with *PstI* and *BamHI*, blotted and probed with the [32]P-labelled 008F clone.

Sequence analysis of the differentially expressed fragments

Fragments amplified with random primers and detected on differential display gels are usually rather short and the four products that were sequenced here ranged from 50 bp to 413 bp in length. Each of the four clones was sequenced in both directions and sequencing quality checked by comparing both strands using GCG BESTFIT. Each pair of sequences showed >99% identity. Each of the four sequences was then used for FASTA and BLAST searches but because of the short length of clone 005E (50 bp, **figure 6.8a**) the results of these searches were not meaningful for this clone.

005E Length: 50

5'   1 AAGCTTTTAC CGCGCCGTTA TATCTTGTTC TTACQAAAAA AAAAAAGCTT 3'

001E Length: 300

5'   1 AATTCGAGCT CGGTCACCAA GCTTGCCATG GCAGGNAAGG AATAGCTGGG

    51 GCTGCTGAGG TACGTCGGCG TGTGGCAGCA CAGAGGTCGT TTGTGCGGGA

   101 TGTGCAGTTA CAATGGGGAG CAAAACTCGA TTGACAGTCA TGGAGGTGGA

   151 CATTACATGT GTTACTGGTG TGGCTCTGTT TTACGTGATG CCCTGCACCC

   201 TTTTCATTGC ACAGTGTGCA AAAGAGTGAT ATTTTTCAGG TACTGCTGTT

   251 GTTATTGTTG TTCCGCTATG GATTGAGTGC TTGTGAAAAA AAAAAAGCTT 3'

C.   004D Length: 413

5'   1 AAGCTTTCCT GGAGCGGGGATTGATTTCGT GCCAACGGCA CTCGTACACG

    51 AGTTGTTCGG TCACTGAGCA CAACGTTACA CCGTTTTGTT AGGAAAGTGA

   101 AGGTGGTCGG CGCACTTCTC CCTTTTTTGG GGTTTGTGTG TTTTACCGGC

   151 TTATCTGAAA AGGGGCAACA GAgAACCTGG GATGATATTC CAAAAAAAAA

   201 AAGCTTAAGC TTTCCTGGAG CGGGGAAGGA TTTCgTGCCA ACGGCACTCG

   251 TACACGAGTT GTTCGGTCACTGAGCACAAC GTTACACCGT TTTGTTAGGA

   301 AAGTGAAGGT GGTCGGCGCA CTTCTCCCTT TTTTGGGGTT TGTGTGTTTT

   351 ACCGGCTTAT CTTGAAAAGG GGCAACAGAg AACCTGGGAT GATATTCCCA

   401 AAAAAAAAAA CTT 3'

163

D.   008F Length: 224

5' 1 AGAGCTTACG AAACGCAACG ATaTNCGCAA GCGGCACCCT TCGGCTTCAT

   51 CCTGTGTACG AAGCATCAAA CAACAAATAT TACGTGA GTC CTGTGGTGCA

  101 TGTGTCAATG CCCACAGGGA ACTCTACCTGTGA TCACATAA TTTTTNCAT

  151 NAGGGGAAAA TACNGTTTCC CTtGGCGTgc CTTCACTCAT GTaTATCTAT

  201 CCGCTCCCCA AAAAAAAAAA CCTT 3'

Figure 6.8. Sequences of clone 005E, clone 001E, clone 004D and clone 008F.(A) Both strands of clone 005E was sequenced using M-13 forward and reverse primers for the pUC18 vector and the sequence of the entire vector insert obtained. There are no putative stop codons within the short sequence. **(B)** Sequence of clone 001E after automatic sequencing in both directions. **(C)** Sequence of clone 004D. An open reading frame can be found between the start codon and stopcodons at bp 263, 305 and 365, double underlined. The stop codon at bp 298 is in another reading frame. **(D)** Sequence of clone 008F insert including three possible stop codons.
The region complementary to the sequence of the anchored oligo-dT primer, possible stopcodons and start codons are underlined.

The insert in clone 001E was 300 bp in length **(figure 6.8b)**. As expected, the sequence complementary to the oligo-dT anchored primer is present at the 3'end and several possible stop codons in all three reading frames can be identified. FASTA searches in the GenEMBL database did not reveal any similarity to other sequences in the database with the best score being Human DNA sequence from PAC 79C4 but with only 63% identity over a 105 bp region. BLASTN and BLASTX searches in the African Trypanosome DNA database found at the Parasite Genome Blast Server (http://www.ebi.ac.uk/parasites/parasite_blast_server.html) showed that the expressed sequence fragment from clone 001E was 99% identical to *T.brucei* cDNA from bloodstream forms of ILTat1.1 over a 250 bp region **(figure 6.9)**. This transcript has unfortunately not been further characterised but is a result of randomly sequenced ESTs from *T.brucei*. A map of the sequence reveals two *Taq1* restriction sites, one at position 4 and another at position 127. This is consistent with the genomic Southern blot using clone 001E **(figure 6.7b)** which shows hybridisation with two different fragments, approximately 1.1 kb and 0.4 kb in size for STIB 386 digested with *TaqI*, demonstrating two *TaqI* restriction sites. The third fragment is too small to result in detectable hybridisation.

```
EMBL:TBA16319 AA016319 T3554 Bloodstream form of serodeme ILTat1.1 Trypanosoma
brucei brucei cDNA 5'.
Length = 471
Minus Strand HSPs:
Score = 1244 (343.7 bits), Expect = 2.0e-100, P = 2.0e-100
Identities = 250/252 (99%), Positives = 250/252 (99%), Strand = Minus / Plus

Query:   286 TCACAAGCACTCAATCCATAGCGGAACAACAATAACAACAGCAGTACCTGAAAAATATCA 227
             |||||| ||||||| ||||||||||·||||||||||||||:||||||||||||||||| |||
Sbjct:    20 TCACAAGCACTCAACCCATAGCGGAACAACAATAACAACAGCAGTACCTGAAAAATATCA 79

Query:   226 CTCTTTTGCACACTGTGCAATGAAAAGGGTGCAGGGCATCACCTAAAACAGAGCCACACC 167
             |||||||||:||| ||||||||||||·||||:|||||||||||||:|||||||||| |||||:|||
Sbjct:    80 CTCTTTTGCACACTGTGCAATGAAAAGGGTGCAGGGCATCACCTAAAACAGAGCCACACC 139

Query:   166 ACTAACACATGTAATGTCCACCTCCATGACTGTCAATCGAGTTTTGCTCCCCATTGTAAC 107
             |||||||| |||||·|||||||||||||| |||||||||| |||||||||||||||| ||
Sbjct:   140 ACTAACACATGTAATGTCCACCTCCATGACTGTCAATCGAGTTTTGCTCCCCATTGTAAC 199

Query:   106 TGCACATCCCGCACAAACGACCTCTGTGCTGCCACACGCCGACGTACCTCAGCAGCCCCA 47
             |||||||| |||||!||||||||||||||||||| ||||||||| |||||||||!||||||| ||
Sbjct:   200 TGCACATCCCGCACAAACGACCTCTGTGCTGCCACACGCCGACGTACCTCAGCAGCCCCA 259

Query:    46 GCTATTCCTTNC 35
             ||||||||||:| |
Sbjct:   260 GCTATTCCTTCC 271
```

Figure 6.9. Alignment of the insert of clone 001E and a randomly sequenced EST from bloodstream *T.brucei* ILTat1.1. The insert of clone 001E (query) showed 99% identity with *T.brucei* cDNA from bloodstream forms of ILTat1.1 (subject) over a 250 bp region, starting at nt 35 and ending at nt 286 as defined in **figure 6.8b**.

The insert of clone 004D was 413 bp in length (**figure 6.8c**). The best scores in GCGs FASTA search in the GenEMBL database was *T.brucei* small ribosomal RNA with 96.5 % identity over a 200 bp region. The same region is also 96% identical to another 200 bp stretch of *T.brucei* large ribosomal subunit (**figure 6.10**).

```
EMBL:TBRRNA X14553 Trypanosoma brucei gene for ribosomal RNA large subunit
Length = 4511
Plus Strand HSPs:
Score = 937 (258.9 bits), Expect = 1.9e-74, Sum P(2) = 1.9e-74
Identities = 193/200 (96%), Positives = 193/200 (96%), Strand = Plus / Plus

Query:     7 TCCTGGAGCGGCGAATGATTTCGTGCCAACGGCACTCGTACACGAGTTGTTCGGTCACTG 66
             | ||| ||||||:||||| ||||||| |||,||||||||||||'||||||||||||||||' ||||
Sbjct:  1658 TACTGGAGCGGGGAAGGATTTCGTGCCAACGGCACTCGTACACGAGTTGTTCGGTCACTG 1717

Query:    67 AGCACAACGTTACACCGTTTTGTTAGGAAAGTGAAGGTGGTCGGCGCACTTCTCCCTTTT 126
             ||||||||||||:|||||||||||||| ||||||||:|||||||||||||||||| |||||||||
Sbjct:  1718 AGCACAACGTTACACCGTTTTGTTAGGAAAGTGAAGGTGGTCGGCGCACATCTCCCTTTT 1777

Query:   127 TTGGGGTTTGTGTGTTTTACCGGCTTATCTGAAAAGGGGCAACAGAGAACCTGGGATGAT 186
             ||||||||||||| ||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct:  1778 TTGGGGTTTGTGTGTTTTACCGGCTTATCTGAAAAGGGGCAACAGAGAACCTGGGATGAT 1837

Query:   187 ATTCCAAAAAAAAAAAGCTT 206
             ||||||||||| ||| |' |
Sbjct:  1838 ATTCCAAAAAGAAATTGCAT 1857
```

Figure 6.10. Sequence comparison of the insert of clone 004D and the *T.brucei* gene for large subunit ribosomal RNA. The cloned insert of 004D (query) showed 96% identity with a *T.brucei* gene for ribosomal RNA (accession number X14553) (subject) over 200 bp.

There is a similar percent identity with other *T.brucei* ribosomal RNA sequences over the same region and a high degree of sequence homology with ribosomal RNA from other organisms (other *Trypanosoma* species, Leishmania species and *Crithidia fasciculata* among others). BLASTX searches in the SWISSPROT database showed a tentative sequence match with a putative galactose-1-phosphate uridylyltransferase in the translated 5' region of the fragment but it is most likely that the insert in clone 004D is the product of contaminating ribosomal RNA. The start codon has not been identified with complete confidence and it is possible that the open reading frame identified within the sequence is not the correct one as even the smallest ribosomal RNA gene is at least 700 bp long compared with the 413 bp of clone 004D.

The amplified cDNA product 008F was again relatively short (224 bp) (**figure 6.8d**). Using the African trypanosome DNA database this clone was found to be 93% similar to a fragment randomly sequenced from a sheared genomic library from *T.b.rhodesiense* (expressing M-VAT 4) over 110 bp at the 3'end (**figure 6.11**).

```
EMBL:TBB352 B07352 G432T3 MVAT4 sheared genomic library Trypanosoma brucei
rhodesiense genomic clone G432.
Length = 336
Plus Strand HSPs:
Score = 544 (150.3 bits), Expect = 3.1e-71, Sum P(2) = 3.1e-71
Identities = 110/112 (98%), Positives = 110/112 (98%), Strand = Plus / Plus

Query:      9 CGAAACGCAACGATATTCGCAAGCGGCACCCTTCGGCTTCATCCTGTGTACGAAGCATCA 68
               || ||||||||||||| |||:|||| |||||||||||:||| ||||||||||:||||||||
Sbjct:    102 CGCAACGCAACGATATTCGCAAGCGGCACCCTTCGGCTTCATCCTGTCTACGAAGCATCA 161

Query:     69 AACAACAAATATTACGTGAGTCCTGTGGTGCATGTGTCAATGCCCACAGGGA 120
               ||||||||||||||||||||||:||| |||||:||||||||||:|||||||||
Sbjct:    162 AACAACAAATATTACGTGAGTCCTGTGGTGCATGTGTCAATGCCCACAGGGA 213
```

Figure 6.11. Sequence alignment of the insert of clone 008F and an EST identified in *T.b.rhodesiense* **expressing M-VAT4.** Clone 008F showed 98% identity with a randomly sequenced fragment in *T.b.rhodesiense* (accession number B07352) over a 112 bp region.

This was also confirmed for the translated sequence resulting from a BLASTX search again in the African trypanosome DNA database. **Table 6.3** summarises the information about the four differentially expressed clones identified in this study,

with information regarding the size of the fragments, the database matches and expression.

Clone	Expression	Size (bp)	Identification or homology	Acc. no. of homology	Length-% identity
001E	HSS	300	EST from *T.brucei* ILTat 1.1 bloodstream forms	T3554	250-99
001RT	HSS	506	EST from *T.brucei* ILTat 1.1 bloodstream forms	T3554	220 - 98
005E	HSR	50	Unknown		
005RT	HSR	476	Unknown repeat sequence		
004D	HSS	413	*T.brucei* gene for ribosomal RNA large subunit	X14553	193 - 96
008F	HSS	224	EST from *T.b.rhodesiense* M-VAT4	B07352	110 - 98
008RT	HSS	364	EST from *T.brucei* ILTat 1.1 bloodstream forms, cDNA 5'	T3551	74 - 98

Table 6.3. Summary of data on clones derived from differential display RT-PCR products and from putative full length open reading frames. Clones with the putative ORFs are denoted RT and length-% identity is the percent identity over the length of region (bp). The expression column indicate whether the clone is specifically expressed in the serum resistant (HSR) or the serum sensitive (HSS) trypanosomes population.

Obtaining full length cDNA sequences

The cDNA sequences resulting from differential display RT-PCR were in general rather short and, since they were all produced using one-base anchored oligo-dT primers, only the 3' end of each transcript was amplified. Even though the untranslated region at the 3'end in *T.brucei* is relatively short and there is a lack of introns in the *T.brucei* genome, the cloned cDNA fragments were clearly not long enough to generate sequence data that could be fully informative in terms of identifying open reading frames or homologues in the database. Consequently, there were clear potential advantages to be gained from obtaining longer sequences of coding regions. One approach available to obtain longer fragments is by RT-PCR using the presence of a conserved spliced leader sequence at the 5'end of every trypanosome mRNA. This 39 nt spliced leader sequence, or mini-exon sequence, is common in all mature trypanosome mRNA molecules but was first identified when studying VSG genes (Borst, 1986) and has since been used in conjunction with

primers for a conserved region at the 3'end to amplify VSG genes by PCR (Carrington *et.al.*, 1991). A primer for the spliced leader sequence together with specific primers designed from each clone sequence of the 3'end of each cDNA fragment was used to amplify the full length cDNA. The primers used for RT-PCR of differentially sequences of clones 001E, 008F and 005E are listed in **table 6.4.**

Primer	Length	Sequence (5' - 3')
Miniexon	25	AACGCTATTATTAGAACAGTTTCTG
Miniexon-2	21	TACTATATTGGTATGAGAAGC
1E-1	21	TCACTCTTTTGCACACTGTGC
1E-2	23	GCAATGAAAGGGTGCAGGGGATC
1E-3	20	CACCACTAACACATGTTATG
8F-1	21	GGCATTGACACATGCACCACA
8F-2	21	GGATGAAGCCGAAGGGTGCCG
5E-1	21	GAACAAGATATAACGGCGCGG
5E-2	19	AACGGCGCGGTAAAAGCTT

Table 6.4. List of primers used for RT-PCR amplification of differentially expressed cDNA fragments.

Clone 004D was not pursued any further because it appeared to encode ribosomal RNA. All newly synthesised cDNA was quality controlled before use by RT-PCR amplification with primers for the TIM locus and only cDNA where the 1.0 kb fragments could be amplified were used in further RT-PCR experiments.

Nested RT-PCR reactions using the miniexon primer and a clone 001E specific primer 1E-1 followed by the primers miniexon-2 and 1E-3, consistently amplified a 500 bp fragment from the cDNA obtained from the human serum sensitive STIB 386 whereas this fragment was not amplified from serum resistant trypanosomes (**figure 6.12a**).
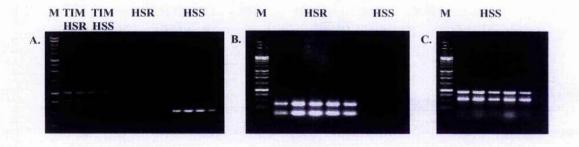
Figure 6.12. RT-PCR products amplified from RNA isolated from human serum sensitive or resistant trypanosomes. Primers were designed for three of the differentially displayed fragments, 005E, 001E and 008F, and used together with the miniexon sequence to amplify the expressed sequence from the cDNA. The resulting RT-PCR products were run on a 1% agarose gel and stained with ethidium bromide. Primers for the TIM gene were used as positive control reactions for RNA isolated from the human serum resistant (HSR) and human serum sensitive populations (HSS) of GUTat 9.1. (**A**) 001E. (**B**) 005E. (**C**) 008F.

RT-PCR using a range of annealing temperatures with the miniexon primer and the 5E-1 primer (specific for clone 005E) repeatedly resulted in several amplification products from both a human serum resistant cDNA template as well as from a serum sensitive cDNA template (results not shown). This problem was overcome by a further amplification step using nested primers, miniexon-2 and 5E-2, which resulted in a 0.9 kb product and a 0.6 kb product exclusively in lanes from human serum resistant cDNA (**figure 6.12b**).

Two fragments were amplified from cDNA derived from human serum sensitive trypanosomes using primer combination miniexon and 8F-1 followed by miniexon-2 and 8F-2. The primer combination only produced a product from cDNA isolated from the human serum sensitive trypanosomes population (**figure 6.12c**). It is possible that amplification of two different size products, instead of the expected single product from 005E and 008F, reflects the diploid state of *T.brucei* so that the two products represent transcripts of different lengths from two alleles of the same gene. STIB 386 would then be interpreted as being homozygous for the sequence in clone 001E and heterozygous for the sequences in clone 005E and clone 008F. There is also the possibility that there are two copies of the gene or multiple transcript of a single gene. The overall impression of the size of the products that were amplified by

RT-PCR, is that they are all rather short compared with the average size of mRNAs which is around 3 kb.

Apart from RT-PCR, another approach used in an attempt to obtaining full gene sequences from the short cDNA fragments was the use of 5'RACE. With this method cDNA synthesis is carried out starting from a gene specific primer at the 3'end and after capping the transcripts with dATP at the 5'end, PCR with a second gene specific primer and an oligo dT-anchored primer would ultimately result in a full cDNA sequence. The 5'RACE method was tested with primers specific for the three sequences identified in clones 001E, 005E and 008F but despite several trouble shooting attempts the PCR reactions never produced anything other than long smears of DNA products.

The PCR products differentially amplified from cDNA using primers specific for 001E, 008F and 005E were produced in larger amounts, purified and prepared for cloning. Unfortunately, cloning of the entire 0.9 kb amplification product from clone 005E was unsuccessful despite several attempts whereas cloning of the approximately 0.6 kb fragment resulted in a 476 nt long plasmid insert. The sequence of this insert (005RT) revealed that the cloned RT-PCR product consisted of a series of repeats of the primer sequence coupled to the 5' miniexon sequence. Sequencing from both directions showed a 98% similarity between the strands and the 5'end of the original clone 005E sequence can be found at the 3'end of the 005RT transcript (**figure 6.13**).

005RT Length: 476

5'   1 G AGAAGCTTT TACCGCGCCG TT ACAAGCTT TTACCGCGCC GTT ACAATCT

      51 TTTACCGCGC CGCT ACTATA TTGCTATGAG AAGC TTTTAC CGCGCCGTT A

     101 CAAGCTTTTA CCGCGCCGCT ACAAGCTTTT ACCGCGCCGT T ACAAGCTTT

     151 TACCGCGCCG TT ACAAGCTT TTACCGCGCC GTT ACAAGCT TTTACCGCGC

     201 CGTT ACAAGC TTTTACCGCG CCGTT ACAAG CTTTTACCGC GCCGTT ACAA

     251 GCTTTTACAG CGCCGTT ACG CTTTTACCGC GCCGTT ACGC TTTTACCGCG

     301 CCGTT ACGCT TTTACCGCGC CGTT ACGCTT TTACCGCGCC GTT ACGCTTT

     351 TACCGCGCCG TT ACGCTTTT ACCGCGCCGT T ACGCTTTTA CCGCGCCGTT

     401 ACGCTTTTAC CGCGCCGTTA CGCTTTTACC GCGCCGTTAC GCTTTTACCG

     451 CGCCGTT ACG CTTTTACAGC GCCGTG 3'

Figure 6.13. Sequence of the RT-PCR product of clone 005RT. The RT-PCR product amplified with a specific primer for the differential display fragment clone 005E and a miniexon sequence was subjected to automatic sequencing. The miniexon primer and the 005E clone sequence found within the insert are both underlined in red and the repeat units are underlined in black.

None of the restriction enzymes *PstI*, *BamHI* or *TaqI*, cuts within the sequence but all three digests nevertheless hybridised with the 005RT clone as two different bands (6.0 kb and 3.5 kb for *PstI*, 7.0 kb and 5.0 kb for *BamHI* and 2.0 kb and 0.9 kb for *TaqI*) (**figure 6.14b**). It is possible that 005RT is a PCR artefact resulting from dimerisation of one primer and the miniexon but it is also possible that it is a genuine sequence of a minisatellite with the repeat being within a gene or in the 3' UTR. The observation of two product of different size in the RT-PCR (**figure 6.12b**) and two bands in the Southern blot (**figure 6.14b**) could then be explained by two alleles with different numbers of repeats. To be able to tell if this is a polymorphism in a minisatellite repeat a genomic clone would need to be isolated and sequenced and the 0.9 kb band would also need to be cloned and sequenced.
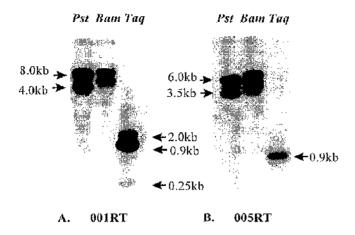
Figure 6.14. Southern blot analysis of two extended differential display products. DNA was prepared from STIB 386 and digested with the three enzymes *PstI*, *BamHI* and *TaqI* before Southern blot analysis. (A) shows the filter hybridised with ^{32}P labelled clone 005RT while (B) depicts the Southern blot filter hybridised with clone 001RT.

The insert derived from the cloned RT-PCR product of clone 001E (001RT) was 506 nt long and both the miniexon primer and the specific primer sequence could be identified within the sequence. The 5'end of the original cDNA fragment 001E can also be found within clone 001RT at its 3'end **(figure 6.15)**. When the 001RT clone was used to probe a Southern blot filter of *PstI*, *BamHI* and *TaqI* digested genomic DNA from STIB 386, two restriction fragments were detected in each of the digests **(figure 6.14a)**. Only the restriction enzyme *PstI* cuts within the sequence and it is possible that the larger fragment in the *PstI* digest consists of two bands. The hybridisation pattern is the same as the one observed after probing an identical Southern blot with the smaller fragment 001E **(figure 6.7b)** apart from an extra band for *TaqI* which could be explained by the probe hybridising with a larger fragment of the gene. The Southern blots strongly suggests that 001E and 001RT are part of the same gene.

001RT Length: 506

5'   1 AACGCTATTA TTAGAACAGT TTCTG TACTA TATTGTGATA CCTAACTCCT

    51 CTCGGTACCA GAGGCCGCAC TCACGTCTGC ACACGGGTTG TGGTGCAATG

   101 GTGAACCTGC ATG AGCTGGG AGGGAAGCAA GGCTGCGGGG GACCTCAATT

   151 TACTCCTGTG GGAGTGTCGT TGACACCGCT GTTGCAGTAT TTCTCGCTGC

   201 TCGTTCATGT AGTTGGAAAG CTACCCGGAT TTGCTGCCGG GTTTGTGACC

   251 GTGTGGCTAG TTTACATTAT GTTTATAGGG CGTGATGTGC TTTTGAACAC

   301 GTGCGTTCCT GTTGCTGGGT TCCACATTAC TGCACTGTTA CTGCTGCGTG

   351 AACTATTGAC ACCATGGCAc GAAGGAATAN CTGGGGCTGC TGCAGGTACG

   401 TCCGCGTGTG GCAGCACAGA AGTCGTTTGT GCGGGATGTG CAGTTCAATG

   451 GGGGAACAAA ACTCCAT TGA CAGTCATGGA AGTGGACA TAA CATGTGTTA

   501 CTGGTG   3'

Figure 6.15. Sequence of the RT-PCR product of clone 001RT. An RT-PCR product was obtained by combining a specific primer for the differentially displayed fragment clone 001E and a miniexon primer. The amplified product was cloned and both strands sequenced. The original fragments 001E (underlined, bold), the miniexon primer, a start codon (bp 111-113) and two possible stop codons (bp 467-470 and 487-490) are underlined.

A FASTA search in the GenBank database with 001RT sequence only results in one hit, a *D.melanogaster* fsh membrane protein, with 62% identity in a 155 bp overlap. A BLASTN search in the African trypanosome DNA database at the Parasite Genome Blast server site, does result in a nearly perfect sequence match (98% over 220 bp) with the 5' end of an anonymous *T.brucei* cDNA from the bloodstream stage of serodeme ILTat1.1 (accession number T3554). This transcript is the result of random sequencing of ESTs and has not been further characterised. The cDNA from the database does not include the miniexon sequence which would have started 153 bp upstream from the end of the sequenced fragment when compared with 001RT (**figure 6.16**). Amino acid alignment in all 6 open reading frames similarly identified the serodeme ILTat1.1 5' end cDNA as the best match. There are two possible open reading frames within the sequence, both with the same start codon but with two different stop codons resulting in a 127 aa or 120 aa translated sequence respectively.

```
EMBL:TBA16319 AA016319 T3554 Bloodstream form of serodeme ILTat1.1 Trypanosoma
brucei brucei cDNA 5'.
Length = 471
Minus Strand HSPs:
Score = 1075 (297.0 bits), Expect = 6.8e-124, Sum P(2) = 6.8e-124
Identities = 217/220 (98%), Positives = 217/220 (98%), Strand = Minus / Plus

Query:   372  CAGCAGCCCCAGNTATTCCTTCGTGCCATGGTGTCAATAGTTCACGCAGCAGTAACAGTG  313
              : |||||||||| ||||| ||| |||||||||||||||| ||||||||||||||:||||:
Sbjct:   249  CAGCAGCCCCAGCTATTCCTTCCTGCCATGGTGTCAATAGTTCACGCAGCAGTAACAGTG  308

Query:   312  CAGTAATGTGGAACCCAGCAACAGGAACGCACGTGTTCAAAAGCACATCACGCCCTATAA  253
              ||:|||||||||||||| |||!||||||||||||||||| |||||||||||||||:|||||
Sbjct:   309  CAGTAATGTGGAACCCAGCAACAGGAACGCACGTGTTCAAAAGCACATCACGCCCTATAA  368

Query:   252  ACATAATGTAAACTAGCCACACGGTCACAAACCCGGCAGCAAATCCGGGTAGCTTTCCAA  193
              |||||||||||||||||||| |||||||||||||||||||||||||||||||||||:||||
Sbjct:   369  ACATAATGTAAACTAGCCACACGGTCACAAACCCGGCAGCAAATCCGGGTAGCTTTCCGA  428

Query:   192  CTACATGAACGAGCAGCGAGAAATACTGCAACAGCGGTGT  153
              || |||||||||||||||||||||||| ||||||||||||||
Sbjct:   429  CTACATGAACGAGCAGCGAGAAATACTGCAACAGCGGTGT  468
```

**Figure 6.16. Sequence comparison of the cloned insert 001RT and a cDNA from *T.brucei*
ILTat1.1 (T3554).** The cloned RT-PCR product 001RT showed a 98% sequence identity over 220 bp
with a randomly sequenced cDNA fragment from *T.brucei* expressing ILTat1.1.

Sequencing of the RT-PCR product obtained from human serum sensitive
trypanosome cDNA using miniexon primer and specific primers for the 008F
transcript resulted in a 364 nt long sequence (008RT). Sequencing of both strands
confirmed the quality of the sequence with 99% identity over the entire insert
between the complementary strands. The miniexon primer and the primer for the
008F fragment cannot be identified with certainty within the sequence (**figure 6.17**).

```
008RT Length: 364

    1 ACCTATCTCA GCGATCTGTC TATTTCGTTC ATCCATAGTT GCCTGACTCC

   51 CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC TGGCCCCAGT

  101 GCTGCAATGA TACCGGCGAGA CCCACGCTCA CCCGGCTCCAG ATTTATCAGC

  151 AATAAACCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT CCTGCAACTT

  201 TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT

  251 AGTTCGCCAG TTAATAGTTT GCGCAACGTT GTTGCCATTG CTACAGGCAT

  301 CGTGGTGTCA CGCTCGTCGT TTGGTATGGC TTCATTCAGC TCCGGTTCCC

  351 AACGATCAAG GCGA
```

Figure 6.17. Sequence of the RT-PCR product 008RT. An RT-PCR product was obtained from the
HSS population of trypanosomes by using a specific primer for the differential display fragment 008F
and a miniexon primer. The resulting clone 008RT was sequenced in both directions.

It is a concern that the full sequence of the 008F clone cannot be found within the 008RT sequence. But whereas clone 008F shows similarity at its 3'end with a sequence identified in a sheared genomic library from *T.b.rhodesiense* expressing metacyclic VAT-4, clone 008RT is basically identical to a *T.brucei* cDNA 5'end from bloodstream form of serodeme ILTat1.1 **(figure 6.18)**.

A.

```
EMBL:TBB352 B07352 G432T3 MVAT4 sheared genomic library Trypanosoma brucei
rhodesiense genomic clone G432.
Length = 336
Plus Strand HSPs:
Score = 544 (150.3 bits), Expect = 3.1e-71, Sum P(2) = 3.1e-71
Identities = 110/112 (98%), Positives = 110/112 (98%), Strand = Plus / Plus

Query:     9 CGAAACGCAACGATATNCGCAAGCGGCACCCTTCGGCTTCATCCTGTGTACGAAGCATCA 68
             !! ||||||||||| ||  |||||||||||:|||| |||:|||||||||||||||||||:||
Sbjct:   102 CGCAACGCAACGATATTCGCAAGCGGCACCCTTCGGCTTCATCCTGTGTACGAAGCATCA 161

Query:    69 AACAACAAATATTACGTGAGTCCTGTGGTGCATGTGTCAATGCCCACAGGGA 120
             | ||||||||||| |||||:||||||||||||:||| |||||:|||||||||||
Sbjct:   162 AACAACAAATATTACGTGAGTCCTGTCGTGCATGTGTCAATGCCCACAGGGA 213
```

B.

```
EMBL:TBA52885 AA052885 T3551 Bloodstream form of serodeme ILTat1.1 Trypanosoma
brucei brucei cDNA 5'.
Length = 275
Plus Strand HSPs:
Score = 361 (99.8 bits), Expect = 9.8e-28, Sum P(2) = 9.8e-28
Identities = 73/74 (98%), Positives = 73/74 (98%), Strand = Plus / Plus

Query:   428 CGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTC 487
             ||!||||| ||||||||||||:|||:|||||||||||:||||||||| ||||||||||||
Sbjct:   178 CGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTC 237

Query:   488 GCCAGCTGGCGTTA 501
             ||||||||| ||| |
Sbjct:   238 GCCAGCTGGCGTAA 251
```

Figure 6.18. Sequence alignment of the cDNA produced from differential display fragment 008. (A) The cloned insert 008F (query) showed 98% identity with a cDNA fragment sequenced from *T.b.rhodesiense* (accession number B07352) (subject) over a 112 bp region **(B)** the RT-PCR product which stemmed from the same fragment (query) showed 98% similarity over 74 bp with an EST sequenced from *T.brucei* expressing ILTat1.1 (accession number T3551) (subject).

This sequence is not the same as that showing homology with clone 001RT and therefore represents a distinct gene. A BLASTX search in the African trypanosomes DNA database also matches the 008RT translated sequence with the 5' cDNA sequence identified in *T.brucei* ILTat1.1. **Table 6.3** lists the clones analysed with

information regarding the origin, the size of inserts obtained and any database matches.

6.2.6 Characterisation of genes potentially important in human serum resistance

From the original nine differentially displayed bands that were identified and the 13 different cloned inserts derived from them, four proved to be of interest regarding human serum resistant/sensitive trypanosome lines. Two of the resulting clones from these cDNA fragments, 001RT and 008RT, showed a high degree of identity with ESTs which had been generated from clones randomly selected from a cDNA library of *T.brucei* ILTat1.1. Unfortunately, both of the sequences generating high scores with the differentially expressed clones had not been characterised and had not been shown to be related to any other sequences in the database. From the high homology found with the sequences in the database, both when comparing either nucleotide or translated sequences, it can be concluded that each pair of sequences are identical. Clone 001RT and clone 001E, from which 001RT is derived, are both clearly the same as T3554. The other clone, 008RT, which is the RT-PCR product derived from clone 008F, is identical to the 5' region of T3551 whereas the 3' end is very similar to a genomic sequence in B07352. The sequence of clone 004D matches the sequence of various trypanosome ribosomal genes, especially *T.brucei* ribosomal DNA for the small ribosomal sub-unit. It is difficult to envisage how ribosomal gene products might determine resistance or sensitivity to human serum. Clone 005E itself was too short to give any indication concerning the likely homologues and a longer sequence of clone 005E was not obtained since the cloned RT-PCR product for this fragment most likely was the result of mispriming during PCR although the possibility that this represents a real transcript cannot be totally excluded

6.3 DISCUSSION

The decision to use RT-PCR differential display to investigate differences in expressed sequences was taken since, at the time, it seemed to be the method that would yield the most interesting results in a limited time and had the potential to identify sequences unique to the serum resistant and/or serum sensitive trypanosome population by covering the majority of expressed genes as well as being sensitive enough to pick up rare mRNAs. Other methods such as subtractive hybridisation, differential screening, representational difference analysis and RADES-PCR have been used successfully by other groups in other contexts (De Greef *et.al.*, 1989; Zhang *et.al.*, 1996; Lisitsyn, 1995; Murphy & Pelle, 1994; Stevens & Tibayrenc, 1994) but these methods all have limitations and drawbacks not found in DDRT-PCR. This is not to say that DDRT-PCR has weaknesses as well as strengths. The principal drawback is that it makes an assumption that the phenotype is determined by differences at the transcriptional level. If the human serum resistance trait was determined at, for example, a post-translational level, then DDRT-PCR would not be able to detect it. Other drawbacks are the large number of primer combinations required in order to cover a wide representation of mRNAs, the high numbers of false positives identified and the short size of the differential display fragments. The high number of false positives is often caused by the presence of more than one product in a particular excised band but there may also be other reasons such as mispriming and competition for the PCR primers. It is recommended that screening of the excised and re-amplified fragment is carried out directly by Northern blotting (Liang & Pardee, 1995) but if the excised fragments contain other overlapping bands of non differentially expressed sequences this could result in an ambiguous result with Northern blotting. On this basis it was decided to clone the fragments before screening. In hindsight, it would have been better to have screened the fragments with a reverse Northern blotting procedure instead of screening the fragments one by one. If the cDNA fragments from differential display had been dot blotted onto duplicate filters and probed with labelled cDNA made from RNA samples from the resistant and sensitive trypanosomes it would have saved both a lot of time and much less RNA would have been needed. It is often claimed that DDRT-PCR is a very

sensitive method of detecting low abundance mRNAs (Liang *et.al.*, 1995; Bauer *et.al.*, 1993) but these transcripts are the most difficult to screen for. Northern blots containing 5 μg total RNA per track were in some cases insufficiently sensitive to produce a signal when hybridised with the differentially displayed fragment and even slot blots with the same or higher amounts of RNA produced only faint signals and in one case failed to produce a signal altogether. It is likely that these clones correspond to rarer mRNAs. If time had permitted a quantitative reverse transcriptase-PCR, could have been used to verify if the clones are expressed at lower levels. The number of false positives encountered was however, not a major problem in these experiments when compared with other published results where sometimes very large number of clones have to be screened to separate out the false positives (Wang & Feuerstein, 1997; Callard *et.al.*, 1994; Utans *et.al.*, 1994). In this study, the major drawback was the short size of the differentially displayed fragments and, as the reverse PCR primer is anchored next to the poly(A) tail, most of the differentially displayed products probably correspond to 3' untranslated regions. This is less useful than coding sequence for comparison against EST or gene sequence data since these usually do not contain the untranslated 3' sequence. Besides, even homologous genes often differ to a large extent in their untranslated region compared with their coding region so that sequence comparisons may be potentially misleading until longer, preferably full length sequences have been obtained.

The use of the miniexon primer in order to obtain full-length cDNA fragments was chosen mainly because no full-length cDNA libraries were available and time was too short to produce a new library. The RT-PCR products that resulted from these amplifications were also comparatively short when compared with the average mRNA size in *T. brucei*. This could imply either that the miniexon was mispriming to another sequence or that the gene products determining resistance/sensitivity are small in size. Another issue that has been problematic is the reproducibility of the differential display autoradiographs. While it was rather straight forward to identify bands that were reproducible in repeat reactions in lanes next to each other, it turned out to be more difficult to reproduce the same banding pattern when the same primer combination was used again and run on a separate gel. When comparing

autoradiographs from two such repeat experiments it was easy to identify the major bands while a lot of the weaker bands could not be found on the repeat gel indicating that bands corresponding to rare mRNAs may be less reproducible. This would then lead to lack of detection of some less abundant transcripts which may well be as important as genes with a high level of expression.

Of the four cDNA fragments that passed the screening steps three were specific for the serum sensitive population and one was specific for the resistant population. But without more information about the four clones, it is difficult to speculate about the function of the genes/gene fragments. Clone 004D, which has a 200 nucleotide long stretch in common with a *T.brucei* ribosomal gene, seems an unlikely candidate to be determining human serum sensitivity even though it seems to be differentially expressed in the two populations. Clone 008RT and clone 001RT are both identical to ESTs randomly sequenced from *T.b.brucei* and the possibility cannot be excluded that they are part of the same gene since it is likely that the RT-PCR product 008RT contains more 5'end sequence than 001RT. Homologue of 008 is also to a *T.b.rhodesiense* EST but to my knowledge the trypanosome clone used for making the cDNA library has not been tested for its resistance or sensitivity to human serum so it is possible that this cloned stock of *T.b.rhodesiense* also is HSS. It is unfortunate that these ESTs have not been further characterised and that a cDNA library was not available. It is however, encouraging that these two clones were found to be specific for the serum sensitive trypanosomes since *T.b.brucei* by definition is a human serum sensitive sub species. The resistant specific clone 005E was initially very interesting since it hybridised specifically with RNA from the serum resistant population and not at all with RNA from the sensitive population. Attempts to produce a longer sequence were first thought to be successful but subsequently it was shown that the amplified sequence only contained primer repeats most likely caused by the primer priming itself! It is therefore still uncertain what role the 005E fragment may have in human serum resistance. However, further investigation of this transcript is worth following given the Southern blot data which argue against the RT-PCR product being an artefact.

The finding of differentially displayed cDNA fragments from both human serum sensitive and human serum resistant origin is in agreement with our genetic interpretation of the inheritance data (Chapter 4). A preliminary interpretation of this is that both sensitive and resistant phenotypes can result from the presence of a transcript and not only the lack of a transcript. However, it would require further analysis to examine whether the phenotype of the F_1 progeny clones correlated with the expression of the RNA display fragments.

Further work with the three clones 001RT, 005E and 008RT would have involved continued attempts to obtain the 5'end of the genes especially since the RT-PCR products amplified here were suspiciously short. Ideally, the cloned cDNA probe would be used to screen a cDNA library made from human serum resistant and sensitive STIB 386 mRNA. The 5'RACE method should also be suitable for recovering the 5' upstream sequence of the gene fragments if more effort was put into trouble shooting the procedure. If full gene sequences could be obtained it would simplify Northern blot experiments since probes would be longer. RNA from several different human serum resistant and serum sensitive isolates could be hybridised with the gene to determine its significance in human serum resistance. A genetic test would also be useful to further investigate if the three clones 008RT, 005RT and 001RT are responsible for conferring resistance or sensitivity to human serum. Parental trypanosomes and F_1 progeny could be screened either by RT-PCR or by Northern hybridisation to look for co-segregation with phenotype. Initial experiments by Northern hybridisation were undertaken but the results were ambiguous and difficult to interpret. The intensity of the bands on the resulting autoradiographs seemed to suggest that clone 001RT is expressed at higher levels in the serum sensitive clones than in the serum resistant clones and also that 008RT is overexpressed in the serum sensitive trypanosome clones. The experiments were carried out with the parental STIB 386, STIB 247, TREU 927 and with five hybrid trypanosome clones, two of which were human serum sensitive in an *in vivo* HSS-assay and three which were scored as serum resistant. However, if the autoradiographs were exposed for a long time (>5 days) a signal could be detected in all of the lanes regardless of whether the RNA was derived from a serum sensitive or

a serum resistant clone. It would be interesting to carry out quantitative RNA analysis for the expression of these clones in order to establish if there are real differences in RNA expression between the serum resistant and the serum sensitive trypanosome populations. In addition, a genomic library from STIB 386 could have been made and used to screen with the differential display fragments in order to obtain full length genomic sequences.

Identification of the biological functions for any differentially expressed genes would be the most interesting next step. This could be based on two approaches. Firstly, by expressing the encoded protein and analysing the gene product in resistant and sensitive cells and secondly, by using transfection to demonstrate whether the resistant or sensitive phenotype was determined by the identical gene. A third approach could be by identifying homologues with genes of known function in the database but as human infectivity is likely to be specific to trypanosomes rather than a common feature of many organisms, this is perhaps the approach least likely to be successful.

Chapter 7

General Discussion

Human African trypanosomiasis was almost extinct and under control by the 1960's but today there is a serious resurgence of the disease in sub-Saharan Africa. Large areas of central Africa, the southern Sudan and Uganda, Congo-Zaire and Angola, are suffering from resurgence and epidemics of trypanosomiasis (Smith *et.al.*, 1998). The reasons for the re-emergence include widespread civil unrest and wars, poor health financing, lack of control programmes and insufficient advances made in diagnosis, treatment and vector control.

A basic requirement for trypanosomes to be able to infect humans is that they can survive and replicate in the hostile environment of human blood and withstand lysis by a trypanosome toxic factor found in human serum. The mechanisms involved in determining whether a trypanosome strain is human serum resistant or sensitive are not fully understood but more knowledge in this area could aid in finding new ways of blocking infection by human infective trypanosomes. The emphasis of this study was to determine if, and how, human serum resistance is inherited and to initiate studies on identifying the gene/s involved in determining the resistance/sensitivity trait.

The trypanolytic factor for serum sensitive trypanosomes is contained within the HDL fraction of human serum (Rifkin, 1978; Hajduk *et.al.*, 1989; Tomlinson *et.al.*, 1995; Gillet *et.al.*, 1991; Seed & Sechelski, 1989) but their is still some controversy about the precise nature of the lytic factor. Hajduk *et.al.* purified TLF1 from a minor subclass of HDL, which are large, high density particles with a molecular mass of 500 kDa and later defined the lytic component within TLF1 as haptoglobin-related protein (*hpr*). Raper *et.al* (1996) opposed this suggestion since their experiments showed that the lytic activity of TLF1 was inhibited by haptoglobin present in the NHS and concluded that another lytic factor, TLF2, was the main trypanolytic factor. TLF2 has however, recently been shown to also contain *hpr* (Tomlinson & Raper,

1998) and it now seems likely that *hpr* is the major trypanolytic factor and whether it is part of TLF1 or TLF2 becomes less important. The experiments showing that *hpr* in TLF1 was inhibited by haptoglobin were undertaken *in vitro* but experiments *in vivo*, involving injecting trypanosomes together with haptoglobin into mice, show a much less dramatic effect (S. Hajduk, pers.comm., 1998). Haptoglobin levels may however, be important for differences in trypanolytic activity seen between sera from different individuals so that the greater the ratio of *hpr* to haptoglobin, the greater the resistance against trypanosome infection. In addition, African Americans have, when compared to Caucasians, lower levels of haptoglobin and higher levels of *hpr* (Smith *et.al.*, 1995) which would offer better protection against trypanosomiasis. This research area requires further investigation to firmly establish whether haptoglobin has an antagonistic effect on trypanosomes by *hpr*.

The difference between the subspecies *T.b.brucei* and *T.b.rhodesiense* is subtle since they are separated solely on their susceptibility to human serum and the assumption that trypanosomes can be neatly classified as either resistant or sensitive to human serum should be abandoned. The development of a reliable assay for measuring sensitivity and resistance to human serum highlighted this issue since it was not possible to distinguish human infective and human non-infective trypanosomes clones only by their morphological appearance when exposed to human serum. It was difficult to determine which cells were viable and infective and which ones had lost their infectivity using phase contrast microscopy observations alone. Not all cells appeared to respond to the lytic factor in human serum at the same time and there were always some cells that appeared more resistant than others. It is possible that there is a grading of susceptibility to TLF within *T.brucei* and this would explain why there was a significant spread in the percentage lysis between individual assays. Another possibility is that the phenotype of the trypanosomes is unstable so that the resistance, or sensitive phenotype is rapidly being switched on and off. This would be a reasonable explanation if the resistance/sensitivity trait was in a VSG expression site, as suggested for the SRA gene (Van Xong, 1998) since VSG switching also occurs at a high rate. The reason for the successful application of *in vitro* assays by other groups is probably because they are working on trypanosome

strains that have been adapted to a laboratory environment and have become inadvertently selected for more homogenous expression of the HSS phenotype whereas STIB 386, STIB 247, TREU 927 and the hybrid progeny produced from them, have all recently been transmitted through tsetse flies.

The *in vivo* HSS assay proved to be a more reliable test for distinguishing between serum sensitive and serum resistant clones since there could be no ambiguity to whether the trypanosomes were able to cause a parasitaemia or not and the results for the parental clones gave identical results when repeated a large number of times. For TREU 927 a proportion of mice became infected during each assay and this, together with the inheritance data, led to the conclusion that intermediate resistance to human serum is a specific phenotype. Further evidence for this was the observation that if the number of cells injected into mice was increased an infection was established in all mice. This result implies that a proportion of the trypanosomes are viable. Intermediate resistance was a finding which has been mentioned, but not explained, in several reports (Geigy *et.al.*, 1975; Rifkin, 1984; Gibson & Mizen, 1997). The sensitivity of the *in vivo* HSS assay has not been measured but it may reasonably be assumed that if there was only one intact trypanosome remaining, it could still give rise to a patent parasitaemia. A single trypanosome is capable of producing an infection as demonstrated when conducting optical cloning of trypanosome stocks where only one trypanosome is injected into mice although cloning efficiency is usually around 50-60%. Based on the *in vivo* HSS-assay, this would imply that 1/100 trypanosomes resist lysis in TREU 927 while 0/100 resist lysis in STIB 247 and > 1/100 resist lysis in STIB 386. This could further be tested by adding various ratios of STIB 386 cells to STIB 247 cells during an *in vivo* HSS-assay and investigate whether this would result in resistance.

In conclusion, there is still much work that needs to be done before we have a full understanding as to why some trypanosomes resist lysis by human serum while others do not. The basic requirement to be able to test trypanosomes for their sensitivity and resistance was carried by the *in vivo* assay described in Chapter 3. It would however had been an advantage if the *in vitro* test could have been enhanced

and made more sensitive and reliable. It is possible the MitoTracker dye in combination with counter staining with DAPI could have been optimised but since it is likely that human serum resistance exhibits a continuum of relative susceptibility, a more sensitive assay would have been required. In a broad sense, *in vitro* tests will measure the behaviour of the vast majority of cells in a strain while the *in vivo* infectivity assays will also measure the presence and behaviour of a small minority of resistant cells. At present, an *in vivo* assay is the best way to define the line between resistance and sensitivity in recently fly-transmitted trypanosomes. In terms of the mechanism of intermediate resistance, one could consider two testable models. First that TREU 927 binds the lytic factor less effectively than STIB 247 or second, that internalisation of the postulated TLF receptor complex occurs more slowly and leading to less lysis in a given time period.

A possible problem that can arise with the reliability of the *in vivo* HSS assay is that when scoring the F_1 progeny there is a risk of over-scoring the number of sensitive progeny. Progeny that can cause a parasitaemia are clearly resistant but there is a possibility that clones which fail to infect in one test could have resulted in infection if a larger number of mice had been used. For some of the intermediate resistant F_1's, the assay were repeated with identical results but a few intermediate resistant clones were sensitive in a second assay. Thus as intermediate resistance sometimes could be scored as sensitive this would lead to an over estimate of the number of sensitives. Since this is likely to affect only a few of the progeny scored, it would not alter the ratio significantly. However, given that the ratio of sensitive to intermediate resistant progeny is critical, all F_1 progeny clones should be tested using a larger number of mice.

The SRA gene expression, which was demonstrated by DeGreef & Hamers (1994) to coincide with human serum resistance in the ETat repertoire, was not expressed in the serum resistant line, STIB 386. Milner *et.al.* (1997) have also identified a resistant specific transcript which has 92% homology with the SRA gene. This transcript was isolated from a different *T.b.rhodesiense* strain isolated in Tanzania whereas the ETat strains originate from Uganda. Thus, both of these come from areas

in East Africa which are not far apart while the STIB 386 was isolated from a man in the Ivory Coast and has been characterised as a group 2 *T.b.gambiense* (Mehlitz *et.al.*, 1982) even though Hide *et.al.* (1990) found it to be indistinguishable from East African *T.b.rhodesiense* or *T.b.brucei* stocks. The results suggest that the mechanism of human serum resistance found in STIB 386 does not involve the SRA gene and therefore that resistance can be conferred by more than one mechanism. It is possible that gene/s involved in human serum resistance have evolved independently at different locations in Africa and that several different mechanisms are present. The strain responsible for sleeping sickness in the Busoga area in Uganda was shown by Hide *et.al.* (1994, 1996) to have been the same from at least the 1960s until today so that this human infective strain has arisen only once and is very stable in the Busoga focus. Human infective strains in the Zambian focus are very similar to each other but markedly different from those in Busoga suggesting that human infectivity has arisen more than once and that the mechanisms conferring resistance are likely to differ.

Since the SRA gene is not responsible for resistance in the clones used in this study it would be of much interest to find the gene/s which are involved in conferring resistance and sensitivity in these stocks. Initial studies to identify the genes were undertaken by DDRT-PCR and at least two fragments were identified as specific for the human serum sensitive population. The next step would have been to obtain the full length gene sequence from which these fragments were derived, either by continued efforts to make that 5'RACE method work, by PCR walking, by designing new primers to optimise the RT-PCR approach or, more directly, by screening a genomic library with the cloned RT-PCR products obtained. A full gene sequence could verify that the fragments really are differentially expressed since probing Northern blots with a full gene sequence would result in stronger, more defined hybridisation. In addition, a database search with the full gene sequence would possibly identify homology with genes from other organisms which might give clues about the function of the gene. At a later stage, the encoded protein could be expressed and the gene product analysed in sensitive and resistant trypanosome

populations or the gene used to transfect resistant cells and screen for changes in sensitivity/resistance expression.

It was shown in Chapter 4 that human serum sensitivity was inherited in a 2:1:1 ratio of sensitive: resistant: intermediate resistant in the F_1 progeny when a resistant and a sensitive strain were crossed. A model for inheritance was proposed which involves three alleles at a single locus; a codominant sensitive and resistant allele and a recessive sensitive allele. This model was suggested because it is the simplest model that fits the inheritance data and it can also account for the finding of intermediate resistance. There are several ways to further test the proposed inheritance model and the first would have involved conducting a series of backcrosses as discussed in **section 4.3**. Another way would have been to cross F_1 progeny of different resistance phenotype to produce a F_2 generation. The phenotypes of such a cross could then be compared with the expected ratio of phenotypes. Crossing hybrid progeny with a sensitive phenotype would be less informative since the model suggests two different genotypes for this phenotype. The identification of a larger number of TREU 927 selfer products could also aid in testing the model. TREU 927 selfers would be informative because they are predicted to segregate into intermediate, sensitive and resistant phenotypes in a ratio which would be predicted by the proposed genotypic model.

The suggested inheritance model is also consistent with a functional model. Hager & Hajduk (1998) demonstrated that TLF resistant trypanosomes can bind TLF in their flagellar pocket but that they fail to internalise it whereas sensitive trypanosomes can both internalise and deliver TLF the lysosome where it acts to destroy phagolysosome integrity. Conflicting data, however, suggests that resistance to TLF is the result of retention of TLF in a prelysosomal compartment and that internalisation of TLF is the same in resistant and sensitive trypanosomes (Coppi & Raper, 1997). Which of these conclusions is correct is not of major importance for the model presented here since a receptor that binds TLF or a molecule that aids in delivering the TLF to the lysosome can be considered as equivalent in terms of providing a model. Taking into account the suggested codominance and that STIB

386 would have to have a sensitive recessive allele, functional models in which the product of the locus is a single polypeptide or where there is an absence of this single polypeptide and which results in internalisation/no internalisation or alternatively, delivery to lysosomes/no delivery to lysosomes cannot explain the proposed genotypes and phenotypes of the inheritance model. Instead, a model based on different receptor affinities might explain the genetic results. A single polypeptide could have two binding sites with different affinities for internalisation or binding of TLF. If both of the sites are bound by TLF with high affinity lysis would occur (STIB 247) but if only the low affinity binding site was present (as for STIB 386) the result would be only partial lysis. If instead the high affinity binding site was present this would again result in partial lysis but this would be more than for STIB 386, i.e. as for the intermediate resistant phenotypes. Another model suggests a dimeric receptor or delivery molecule where the resistant receptor has a very low affinity. Again, the presence of one of each or two of the same of the higher affinity receptors would result in lysis whereas the presence of one resistance and one low affinity receptor would result in resistance (STIB 386) and an intermediate resistant phenotype would arise when an allele for the high affinity receptor was present together with the very low affinity receptor. These models could be tested by examining the affinity of the parental and F_1 progeny clones for human serum and the rate of internalisation of the receptor complex. The identification of the ligand/s and receptor/s for TLF will aid our understanding of the mechanisms of trypanosome resistance to human serum.

It is clear that human serum resistance can be inherited into previously human serum sensitive trypanosome lines, as shown in Chapter 4, and this raises the question of how important this is in the epidemiology of human trypanosomiasis as the human infective genotype may spread to non human-infective trypanosomes by genetic exchange. As discussed in Chapter 1, it has been shown that genetic exchange does have a role in the generation of diversity of trypanosome populations even though both clonality and genetic exchange can be detected in different populations (Hide *et.al.*, 1997; Stevens & Tibayrenc, 1996; Tibayrenc, 1997). A study by Hide *et.al.* (1994) suggested that the human infective population and the

non human-infective population had different levels of genetic exchange so that the non human-infective population was more clonal than the human infective group which showed evidence of frequent genetic exchange. This may however, be explained by the theory proposed by Maynard Smith *et.al.* (1993) which involves an epidemic population structure in which there is frequent recombination within all members of the population but with occasional epidemics when a highly successful individual arises and reproduces rapidly to produce an epidemic clone. A complex genetic system may however, exist since both triploidy and self fertilisation events have been described in *T.brucei* (Gibson, 1989; Tait *et.al.*, 1996) and genetic exchange between human and non human trypanosomes in the field has not been directly observed. There might be some natural reasons for why sex in trypanosomes has not been observed more often. Genetic exchange is thought to take place in the tsetse fly and for this to occur the fly needs to be infected with at least two trypanosomes strains. Since tsetse flies mainly become infected during their first blood meal (Maudlin & Welburn, 1988) the fly would have to feed on a host with a mixed infection. This might not be as unlikely as has been thought (Gibson, 1995) as a recent study in the Ivory Coast revealed that approximately 40% of all trypanosome infections were mixed and that there was a significantly higher prevalence of mature *T.brucei* infections than had previously been reported (Masiga *et.al.*, 1996).

Further investigation into the inheritance of human serum resistance and the genes involved, as well as enhancing our understanding of *T.brucei* genetics, should help to clarify the mechanism of human serum resistance and may provide insight into new areas of preventing human infection.

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