Human infectivity trait in *Trypanosoma brucei*: stability, heritability and relationship to *sra* expression

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SUMMARY

Some *Trypanosoma brucei* lines infect humans whereas others do not because the parasites are lysed by human serum. We have developed a robust, quantitative *in vitro* assay based on differential uptake of fluorescent dyes by live and dead trypanosomes to quantify the extent and kinetics of killing by human serum. This method has been used to discriminate between three classes of human serum resistance; sensitive, resistant and intermediate. TREU 927/4, the parasite used for the *T. brucei* genome project, is intermediate. The phenotype is expressed in both bloodstream and metacyclic forms, is stably expressed during chronic infections and on cyclical transmission through tsetse flies. Trypanosomes of intermediate phenotype are distinguished from sensitive populations of cells by the slower rate of lysis and by the potential to become fully resistant to killing by human serum as a result of selection or long-term serial passaging in mice and to pass on full resistance phenotype to its progeny in a genetic cross. The *sra* gene has been shown previously to determine human serum resistance in *T. brucei* but screening for the presence and expression of this gene indicated that it is not responsible for the human serum resistance phenotype in the trypanosome lines that we have examined, indicating an alternative mechanism for HSR exists in these stocks. Examination of the inheritance of the phenotype in F1 hybrids for both bloodstream and metacyclic stages from two genetic crosses demonstrated that the phenotype is co-inherited in both life cycle stages in a manner consistent with being a Mendelian trait, determined by only one or a few genes.

Key words: *Trypanosoma brucei*, genome project, human infectivity, human serum resistance

INTRODUCTION

Trypanosoma brucei, has been divided into three sub-species, *T. b. brucei*, *T. b. rhodesiense and T. b. gambiense* based on a combination of geographical distribution, host specificity and the course of infection in humans (chronic or acute). *T. b. brucei* is solely infective to animals including domestic livestock and game, *T. b. rhodesiense* infects humans and animals in East and Southern Africa, while *T. b. gambiense* infects humans and domestic animals in West and Central Africa (Hoare 1972). An important aspect of research on this group of parasites has been to develop methods to distinguish these three morphologically identical sub-species so as to be able to identify the human infective sub-species in reservoir hosts and in the tsetse vector. Extensive analyses using a range of biochemical and molecular markers (Gibson 2002; Godfrey *et al* 1990; Matthieu-Daude *et al* 1995; Tait *et al* 1985) have largely been unable to identify consistent differences between the three sub-species with two exceptions; firstly, using both iso-enzyme and molecular markers, two distinct clades of *T. b. gambiense* could be defined (type 1 and 2) with type 2 being more closely related to West African *T. b. brucei* (Gibson 1986; Tait *et al* 1984). Second, in the Busoga focus in Uganda, it has been possible to define *T. b. rhodesiense* using multilocus genotypes as the sole criteria (Hide *et al* 1994; MacLeod *et al* 2000).

The early observation that non-human infective isolates (*T. b. brucei*) are lysed by human serum and human infective isolates (*T. b. gambiense* and *T. b. rhodesiense)* are not, led to the development of the blood incubation infectivity test (BIIT), which can identify human infective isolates based on their ability to resist the lytic effects of human serum (Rickman and Robson 1970a). These authors showed that, in addition to fully resistant and fully sensitive trypanosomes, a class of ‘subresistant’ parasite isolates could be defined (Rickman and Robson 1970b). However, given these authors were not using cloned lines of trypanosomes, it was unclear whether this phenotype was the result of mixtures of resistant and sensitive trypanosomes or a distinct phenotype.

The identification of the *sra* gene as exclusively expressed by human serum resistant (HSR) trypanosome lines and the demonstration that the gene can confer HSR when transfected into the bloodstream expression site (ES) of sensitive trypanosomes, has provided a further potentially valuable marker for the definition of human infective trypanosomes (Milner and Hajduk 1999; Xong *et al* 1998). The identification of *sra* as a determinant of resistance/sensitivity also solved the conundrum that human serum resistance is an unstable phenotype in at least some trypanosome stocks with different subclones being either sensitive or resistant. This variability of expression arises because the *sra* gene is expressed from an ES and thus expression of *sra* is linked to antigenic variation. There are multiple expression sites in the genome but the parasite uses only one ES at a time and switches use between ESs; which may or may not contain *sra* (Xong *et al* 1998).

A PCR based method for the detection of the *sra* gene has been developed and the presence of the gene has been shown to correlate strongly with the human serum resistant (HSR) phenotype in the Busoga focus, and so this method has been heralded as a means of defining human infective trypanosomes (Welburn *et al* 2001). However,

while it is clear that the *sra* gene is responsible for human infectivity in a large number of *T. b. rhodesiense* isolates (Gibson *et al* 2002; Welburn *et al* 2001), there are exceptions to this in trypanosome lines from East Africa (Rifkin *et al* 1994; Agbo *et al* 2003) and, critically, this gene is not present in West African *T. b. gambiense* isolates (De Greef and Hamers 1994; Radwanska *et al* 2002). Thus there must be alternative mechanisms determining human infectivity/human serum resistance other than those based on the *sra* gene.

The question arises as to whether these other mechanisms are likely to determine a phenotype that is a stable and heritable trait. Given the experience with *sra* these issues should not be assumed; *sra* expression is unstable and although the gene is inherited, it's position in the genome is potentially variable by virtue of location in an ES. In this paper we report on the stability and heritability of the HSR phenotype in trypanosome stocks where it is not determined by *sra*.

MATERIALS AND METHODS

Parasite stocks and lines

Trypanosomes of five different genotypes were used in these studies as indicated in Table 1. Using the classical criteria of Hoare (1972), STIB 247 and TREU 927 were classified as *T. b. brucei,* ELIANE and STIB 386 are type 1 and type 2 *T. b. gambiense* respectively (Gibson, 1986), and the isogenic lines Etats 1.2 and 1.10 are *T. b. rhodesiense* clones derived from the stock EATRO 3 (Xong *et al*, 1998). STIB 247, STIB 386, TREU 927 and EATRO 3 were transmitted separately through tsetse flies and metacyclic forms of TREU 927 were cloned as previously described (Turner *et al* 1990). STIB 386 and TREU 927 were also rapidly syringe-passaged between mice for 49 passages before recloning to generate lines that were human serum sensitive (HSS) and HSR, 386S and GUTat 10.1, respectively (Turner *et al* 1991; van Deursen *et al* 2001). A sub-line of TREU 927 was selected for human serum resistance by two rounds of incubating cells in 25 % human serum for four hours and inoculation of mice with 3×10^7 cells – TREU 927R. We also used 74 F1 hybrid progeny from two genetic crosses, STIB 247 x TREU 927 and STIB 247 x STIB 386 described previously (Turner *et al* 1990; MacLeod *et al* manuscript in preparation). Six of the progeny were transmitted separately through tsetse flies to generate metacyclic forms.

In vitro h*uman serum resistance 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 (25^oC or 37^oC), serum concentration, trypanosome density, source of control serum, duration of incubation, parasite viability staining methodology (data not shown).

In our optimized assay, trypanosomes were isolated by differential centrifugation (Ghiotto *et al* 1979) rather than using anion exchange chromatography as the latter method always generated a 'background' of 5 – 20 % 'dead' cells. The cells were resuspended in a volume of 500 l of either 25% human or guinea pig serum (Serotec) in PBS at a concentration of $1x10^7$/ml and incubated at 37°C for 0, 2 and 4 hrs. The human serum was from a single individual after overnight fasting. The same donor was used for all assays. On completion of the incubation the suspension was

centrifuged at 900g for 5mins at 4°C, the pellet resuspended in 200 l of nucleic acid staining solution (1 in 500 dilution of Syto 10 and Dead Red dyes in PBS; Molecular Probes) and incubated in the dark at room temperature for 15mins followed by centrifugation at 900g for 5mins. The cell pellet was resuspended in 50 l of PBS followed by the addition of 400 l of 4% glutataldehyde and fixation for at least 15mins. At least 200 cells were scored as green or red using a fluorescence microscope and Zeiss filter set 9. These dyes stain the nuclei of viable cells green and those of dead cells red when they are viewed by fluorescence microscopy, thus allowing the number of viable and non viable cells to be measured. No double-labeled cells were detected and unlabeled cells comprised < 1% of the numbers counted in all assays.

In vivo *human serum resistance assay*

The assay we used was a minor variant of the Blood Incubation Infectivity Test (BIIT) (Rickman and Robson 1970a). Trypanosomes were separated from blood as above and diluted in MEM/Earles salts with 25% human serum from a single human donor to a final concentration of 1 x 10^7 cells ml^{-1}. After incubation for 2-4 hrs at 37°C, 100 cells, diluted in PBS, were inoculated intraperitoneally into each of two ICR mice, immunosuppressed by i.p. inoculation with 150 mgKg-1 cyclophosphamide 24 hrs previously. Mice were screened for patent parasitaemias 6 days a week for at least 2 weeks. A third mouse (positive control) in each experiment was inoculated with an equivalent number of parasites after incubation in 25 % guinea-pig serum under the same experimental conditions.

DNA Preparation and PCR

DNA was purified from bloodstream stage parasites using standard methods (Lanham and Godfrey 1970; Sambrook *et al* 1989). A PCR test of genomic DNA to verify the quality of the DNA stocks under investigation was carried out using primers TIM-C and TIM-D as described in MacLeod *et al* (1997). The PCR amplification of the *sra* gene was undertaken using the conditions and primers described in Radwanska *et al* (2002), Gibson *et al* (2002) and Welburn *et al* (2001). The products were separated on agarose gels and visualised under UV. The correct sized product for *sra* from STIB 247 was excised from the gel under UV illumination then loaded onto a Spin-X column (Costar) and centrifuged at 15,000 rpm for 5 min. One microlitre of a 1/20 dilution of the eluted DNA was amplified in a semi-nested reaction using primers SP-A, 5' ACAGCAACATCTCAGCGCTTTATGC and 538 (Welburn *et al* 2001). The PCR conditions were as follows: 95°C for 50 seconds, 58°C for 50 seconds and 64°C for 1 min for 30 cycles, using primer concentrations and the PCR buffer described elsewhere (MacLeod *et al* 1999). The products were cloned using the TA cloning kit (Invitrogen) and sequence determination carried out using the same primers and an ABI sequencer.

Northern analysis

Total RNA was prepared using the Trizol TM reagent (Gibco-BRL) according to the manufacturer's instructions. The isolated RNA was treated with amplification grade DNAse 1 to remove contaminating DNA. For northern blots, 4 g total RNA was denatured in formaldehyde and separated by agarose gel electrophoresis (Sambrook *et al* 1989). After separation, the RNA was transferred to Hybond nylon membrane using standard protocols, prehybridized in 7% SDS, 0.5M sodium phosphate pH 7.2, 1mM EDTA, at 65°C for 2hrs followed by overnight hybridization with a random

primed probe (Sambrook *et al* 1989). The probe for the *sra* gene was prepared by PCR amplification of genomic DNA from ETat1.10 using the primers and conditions described by Welburn *et al* (2001). In order to generate a tubulin probe, a 630bp fragment was amplified from DNA using the primers TUB 3' GGGAATTCTTTCGCATCGAACATCTGCTGC and TUB 5' GGGAATTCCCCCGACAACTTCATCTTTGGA, under the following conditions: 95°C for 50 seconds, 55°C for 50 seconds and 64°C for 1 minutes for 30 cycles.

RESULTS

HSR phenotypes using in vivo *assays of bloodstream forms*

An *in vivo* human serum resistance assay was used to determine the serum resistance profile of three stocks used as parents in genetic crosses. Using an inoculum of 100 parasites per mouse, STIB 247, previously reported as human serum sensitive (Brun and Jenni 1987) failed to infect any of 10 mice and STIB 386 infected all 10 mice as expected. TREU 927, however, infected only 2 out of 8 mice, an outcome previously described for other field stocks and termed 'subresistant' (Hawking 1973; Rickman and Robson 1970b). At a higher inoculum (1000 cells), STIB 247 still failed to infect any of 8 mice whereas TREU 927 now infected all 8 mice. Equal numbers of trypanosomes were incubated with guinea pig serum prior to inoculation in TO mice, as controls, which resulted in all mice becoming infected. The results are summarized in Table 1.

HSR phenotypes using in vitro assays

We first analysed the serum resistance profile of the stocks STIB 386, TREU 927 and STIB 247 in this assay to compare with the *in vivo* data. The fluorescent staining of these stocks is shown in Figure 1, and the percentage death depicted graphically in Figure 2A. To verify this new assay we also tested a number of reference lines. The HSR stock, ELIANEand three isogenic lines of *T.b. rhodesiense*, EATRO 3, Etat 1.2 and Etat 1.10, the latter two having previously been described as being HSS and HSR, respectively (De Greef and Hamers 1994; Rifkin *et al* 1994; Xong *et al* 1998). These results are summarized in Table 1. The results for these lines are also shown in Figure 2A. Lines Etat 1.2 and STIB 247 were examples of the HSS phenotype class, with a substantial loss of viability (>90%), while ELIANE and Etat 1.10 were resistant to human serum with only ~18% loss of viability over a four hour incubation period. However, TREU 927 and EATRO 3 both showed intermediate kinetics of lysis over the 4hr time course. These data are consistent with those obtained with the *in vivo* assay and confirm that intermediate resistance is a definable phenotype, distinct from either resistance or sensitivity. To address the formal possibility that the intermediate phenotype could be due to a mixture of cells either expressing or not expressing human serum resistance, a 1:1 mixture of HSR (STIB 386) and HSS (STIB 247) trypanosomes was assayed as before and resulted in ~50% lysis and different lysis kinetics compared with those obtained for the intermediate phenotype (figure 2A). Based on these results, we conclude that in addition to the phenotypes of human serum sensitivity and resistance, a third phenotype of intermediate resistance can be defined by both *in vivo* and *in vitro* assays.

To determine if the human serum resistance phenotypes are stable through the course of chronic infections, we initiated infections of STIB 247, STIB 386 and TREU 927 in groups of mice and at weekly intervals assayed populations of trypanosomes. Over

a five week period for STIB 247 and a three week period for each of the other two stocks, the HSR phenotypes were unchanged for each stock (data not shown).

Human serum resistance in metacyclic trypanosomes

To determine whether the human serum resistance phenotype was the same in metacyclic forms as in bloodstream forms, we transmitted EATRO 3, STIB 247, STIB 386 and TREU 927 through tsetse flies, isolated metacyclic forms directly from the salivary glands and determined their human serum resistance profiles using the *in vitro* assay. The results, presented in Figure 2B and summarized in Table 1, are directly comparable to those in Figure 2A and show that the metacyclic forms of each stock had the same resistance phenotype as the bloodstream stages. EATRO 3 and TREU 927 were intermediate, STIB 386 was resistant and STIB 247 was sensitive to lysis by human serum. In all cases, the percentage lysis was slightly higher for metacyclic than for bloodstream forms. This can be attributed to the method of isolating metacyclic forms as they will be contaminated with pre-metacyclic trypanosomes which would be lysed by the alternative pathway of complement.

To further investigate the intermediate phenotype of stock TREU 927, single metacyclic forms of the parasites were optically isolated from the salivary glands of tsetse flies and amplified in mice. The resultant sub-cloned bloodstream populations were analyzed for human serum resistance using the *in vitro* assay and all had an intermediate phenotype equivalent to that observed for TREU 927 before fly transmission (data not shown). These results show that the intermediate phenotype is a genuine third phenotypic state which is stable after fly transmission and subsequent expansion in mice.

Stability of human serum resistance

The instability of the human serum resistance phenotype on passaging in mice has been previously described for some T. *b. rhodesiense* stocks, including EATRO 3 (De Greef and Hamers 1984; Hajduk *et al* 1989; Rifkin *et al* 1994; Xong *et al* 1998). The resistance phenotypes of STIB 247, TREU 927 and STIB 386 are stable through the natural life cycle of the trypanosomes, i.e. in chronic infections and on fly transmission, but it is possible that their phenotype could change on serial bloodstream syringe passage. To investigate human serum resistance stability in these stocks we examined their ability to firstly, change phenotype on long term passaging, followed by sub-cloning and second, under selection for resistance. The line GUTat 10.1, which is being used for the *T. brucei* genome sequencing project, changed HSR phenotype from intermediate to resistant (Table 1). Also, just two rounds of selection of TREU 927 for 4 hours in human serum caused an equivalent change in phenotype, to generate the line TREU 927R. We attempted to select HSR STIB 247 lines in the same manner, but without success. Long term passaging of STIB 386 and sub-cloning led to identification of a sub-clone that was sensitive to lysis by human serum (STIB 386S, Table 1). These results indicate that the HSR phenotype is a variable trait in the intermediate and resistant stocks, but is invariant in the sensitive stock, STIB 247. To investigate this last result further, 11 different lines which were sensitive to human serum, as determined by the *in vitro* assay, were selected in human serum in an attempt to generate resistant lines. No resistant trypanosomes were isolated (data not shown).

Presence/absence of the sra gene

In order to test if the *sra* gene is present in the stocks under investigation in this study, we attempted to amplify this gene by PCR, using the PCR test described in Welburn *et al* (2001). A sequence homologous to the *sra* gene, which has been identified in TREU 927 on chromosome II and shown to be a VSG pseudogene (Campillo and Carrington 2003), was amplified from all stocks using this test. Since this generates a larger sized product than the *sra* gene it acts as an internal control for PCR amplification (figure 3A). The 743bp product derived from the *sra* gene was amplified from Etat 1.10 and Etat 1.2 as expected. The PCR assay failed to amplify *sra* from TREU 927 and STIB 386, although a series of products was amplified from STIB 247. To identify if any of these products was from the *sra* gene, semi-nested PCR was performed on gel-extracted DNA using primers SP-A and 538. A single product of the expected size (576bp) was obtained. To establish the relationship between the amplified products and the *sra* gene sequence, this amplified fragment was cloned and sequenced. The fragment obtained from STIB 247 showed 99.5% identity to the published sequence (data not shown). This level of homology suggests that the PCR product from STIB 247 is the *sra* gene. Further attempts to amplify the *sra* gene from TREU 927 and STIB 386 using alternative primer combinations (Gibson *et al* 2002; Radwanska *et al* 2002) failed to produce a product, although amplification of the expected product in the positive controls was observed (Table 1). As a positive control to demonstrate that the DNA samples contained amplifyable DNA, the *tim* gene was amplified by PCR from each stock under the conditions described previously (MacLeod *et al* 1997). The expected sized product was obtained for all samples (data not shown). These data suggest that TREU 927/GUTat 10.1 and STIB 386 do not contain the *sra* gene despite being HSR.

Expression of the sra gene in bloodstream forms

In order to test whether the *sra* gene in STIB 247 was expressed, northern blot analysis was undertaken. Etat 1.10 and Etat 1.2 were used as positive and negative controls (Xong *et al* 1998). TREU 927,lineTREU 927R, STIB 386 and STIB 386S were also included in this analysis. The probe was a 575bp fragment of the *sra* gene amplified from Etat 1.10 genomic DNA, which was sequenced to confirm it was part of the *sra* gene (data not shown). The results, shown in figure 3B(i), revealed a strong hybridization signal for Etat 1.10 but no signal for ETAT 1.2 or from STIB 247. Similarly, no signal was observed with either STIB 386 (either resistant or sensitive) or TREU 927 (either resistant or intermediate), suggesting that the human serum resistance expressed by these stocks is not determined by the *sra* gene. A tubulin probe was used as a control for loading (figure 3B(ii)). The results are summarized in Table 1.

Inheritance of HSR

An investigation into the inheritance of the phenotype was undertaken to determine if the ability to resist the lytic effects of human serum is a trait that is inherited in a Mendelian fashion. The HSR phenotypes of a series of F1 hybrids (bloodstream forms) from two genetic crosses (previously described in Tait *et al* (2002) were analyzed using the *in vitro* assay. Some of the results are presented in figure 4A and indicate that all the progeny from the STIB 247 x STIB 386 cross and the STIB 247 x TREU 927 cross fall into one of three discrete categories either sensitive, resistant or intermediate, and not a range of different levels of resistance; thus the phenotype segregates in the cross. For the STIB 247 (sensitive) x STIB 386 (resistant) cross, 8

were HSR, 14 were HSS and 12 were intermediate and for the STIB 247 (sensitive) x TREU 927 (intermediate) cross, 10 progeny were HSR, 11 were HSS and 17 were intermediate in phenotype. It is clear, therefore, that the phenotype is inherited in a manner consistent with being a Mendelian trait, and that only one or a few genes determine the phenotype. As STIB 247 is invariably sensitive to human serum, the resistant hybrids presumably inherited the ability to resist human serum from the other parent, either TREU 927 or STIB 386. Therefore, TREU 927 can confer full resistance as well as intermediate resistance to its progeny and STIB 386 can pass on intermediate resistance as well as full resistance to its progeny.
In order to determine if the same phenotype is expressed in metacyclic form hybrids from the STIB 247 x TREU 927 cross were transmitted through tsetse flies and metacyclics tested. The human serum resistance profiles were the same for metacyclic forms as for bloodstream forms, indicating that the inherited phenotypes are stable on fly transmission and that the same gene(s) are likely to be responsible for both bloodstream and metacyclic HSR (figure 4B).

DISCUSSION

The BIIT assay (Rickman and Robson 1970a) was the first technique which could differentiate *T. b. brucei* from *T. b. rhodesiense*, by assaying the ability of trypanosomes to survive in human serum and subsequently infect mice. A number of *in vitro* assays have been developed since then that discriminate trypanosome lines as HSR or HSS (c.f. Brun and Jenni 1987; Hajduk *et al* 1989; Tomlinson *et al* 1995). In this paper, we describe a simple, robust quantitative method which directly measures the survival of trypanosomes in human serum and that discriminates the response to human serum into three phenotype classes – sensitive, resistant and intermediate. The intermediate phenotype is defined by a slower rate of parasite killing than that observed with the sensitive phenotype. The comparison of our *in vitro* and *in vivo* data suggests that the intermediate phenotype may correspond to the ‘subresistant’ class previously described using the BIIT (Rickman and Robson 1970a; Hawking 1977)

What is the biological significance of the intermediate phenotype? At first glance, the similarity between the intermediate and HSS phenotypes is such that the intermediate phenotype could be considered a slower form of HSS. However, three lines of evidence demonstrate that the distinction between sensitive and intermediate is biologically important. Trypanosomes with intermediate sensitivity were capable of becoming fully resistant by (1) rapid selection, (2) by long term passaging and recloning and (3) can pass on a resistant phenotype to progeny in a genetic cross between parents of intermediate and sensitive phenotypes.

Our discrimination of three phenotype classes and the demonstration in this paper and by others (De Greef and Hamers 1994; Hajduk *et al* 1989) that lines of trypanosomes can be developed that differ in phenotype from that of the stocks from which they originated calls into question the biological validity of the subspecies definitions used for *T. brucei*. This is because the classical criterion to discriminate *T. b. brucei* from *T. b. rhodesiense* and *T. b. gambiense* is the ability to infect humans (Hoare 1972). In light of this ambiguity we denote some of the subspecies definitions in Table 1 with question marks. How the subspecies definition issue might be resolved is important but falls outwith the scope of this current paper.

We have shown that the serum resistance phenotype is stable through the course of chronic infections, after cyclical transmission through tsetse flies and is the same in both bloodstream and metacyclic forms. The only occasions in which we have found the trait to be variable is under selection pressure and in the artificial laboratory situation of long-term serial passaging between mice (for intermediate and resistant, but not sensitive, lines). This variability has been noted before (De Greef and Hamers 1994; Hajduk *et al* 1989) and indeed has been exploited extremely successfully to create isogenic pairs of sensitive and resistant lines, comparison of which has led to the identification of a gene, *sra*, that can determine resistance/sensitivity (De Greef and Hamers; 1994, Xong *et al* 1998; Milner and Hajduk 1999). It is possible that the mechanism of variability is common to many stocks irrespective of whether a *sra* or non-*sra* mode of action is employed to determine human serum resistance.

The identification of *sra* as a determinant of resistance in *T. b. rhodesiense* has resulted in the use of a simple PCR assay to distinguish between human serum sensitive and resistant trypanosomes (Gibson *et al* 2002; Radwanska *et al* 2002; Welburn *et al* 2001), as opposed to the lengthy and costly BIIT (Rickman and Robson 1970a). This PCR test is based on the assumption that human serum resistant trypanosomes have the *sra* gene and non-human infective trypanosomes do not which appears to hold true for the majority of isolates tested from east and southern Africa (Gibson *et al* 2002; Welburn *et al* 2001; Agbo *et al* 2003), but not for west and central Africa (Berberof *et al* 2001; Radwanska *et al* 2002).

In order to determine the relevance of the *sra* gene to the human serum resistance phenotypes described here, the PCR tests of Gibson *et al* (2002) and Welburn *et al* (2001) were used to determine if the *sra* gene is present in the stocks which were identified as HSR, HSS and intermediate. The HSS stock, STIB 247, tested positive for the *sra* gene (confirmed by sequence analysis) whereas the HSR and intermediate stocks, STIB 386 and TREU 927 were both negative. The sequence homologous to the *sra* gene, which has been identified in TREU 927 and is present in all isolates tested here, is a pseudogene it cannot be responsible for the HSR phenotype (Campillo and Carrington 2003). This clearly indicates that a test for the presence/absence of the *sra* gene, while being a good approximation, is not an absolute test to predict the phenotype of HSR and that alternative mechanisms for HSR exist not only in West African stocks (Berberof *et al* 2001; Radwanska *et al* 2002) but also in the East African TREU 927 isolate. The lack of correlation between human serum resistance and expression of the *sra* gene was further confirmed by northern analysis, which demonstrated that neither STIB 386 nor TREU 927 lines expressed the *sra* gene or a related *sra* homologue. The HSR phenotype of these stocks is also expressed in the metacyclic stage which suggests that the gene determining resistance is unlikely to be expressed from a bloodstream expression site, as is the case for the *sra* gene, as bloodstream expression sites are not thought to be transcribed at the metacyclic stage (Graham *et al* 1990). It is formally possible that low level transcription of the *sra* gene could occur to the extent that it confers HSR but is not detectable by northern analysis. However, the fact that no *sra* gene was detected by three independent PCR tests would suggest that the gene is not present in these stocks and so cannot be expressed or is so divergent that it cannot be amplified by a PCR test or detected by hybridisation. It is possible that both the *sra* mechanism and the alternative mechanism of HSR are present and functioning in the same

organism. Indeed, the clone EATRO 3 has an intermediate phenotype and so, presumably, is expressing resistance from the alternative mechanism, (as expression of the *sra* gene confers full resistance), while its derivative, Etat 1.10 is fully resistant by expressing *sra*.

These results presented here clearly demonstrate that the human serum resistance phenotype of the stocks under study is not due to the expression of the *sra* gene but due to an alternative mechanism. To determine the genetic basis of this mechanism, the inheritance pattern of resistance was investigated by the analysis of a series of F1 hybrids from two genetic crosses, STIB 247 x TREU 927 and STIB 247 x STIB 386. It would have been interesting to investigate inheritance in the cross TREU 927 x STIB 386 but unfortunately there are too few F1 hybrids available to be informative (Turner *et al* 1990). For both crosses, the F1 hybrids fell into one of three phenotypes, fully resistant, fully sensitive or intermediate, demonstrating that this phenotype segregates in the hybrids and is potentially amenable to linkage analysis. A genetic map of TREU 927 is currently being generated with the first two chromosomes, I and II, having been completed (El Sayed *et al* 2003; Hall *et al* 2003). Thus it should be possible to identify markers in the genetic map which cosegregate with the phenotype, and so the region of the genome harbouring the gene which determines human serum resistance can be located. A similar analysis of a cross STIB 386 x STIB 247 should allow us to map and identify the gene(s) responsible for the HSR phenotype in West African human infective trypanosomes.

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FIGURE LEGENDS

Figure 1. Three stocks of *T. brucei* parasites incubated for four hours in human serum and stained using Syto 10/Dead Red. **A**, STIB 247 trypanosomes were killed by human serum and stained red; **B**, STIB 386 cells were largely resistant to human serum and stained green and **C**, TREU 927 cells gave an intermediate result.

Figure 2. A quantitative analysis of human serum resistance/sensitivity in lines of *T. brucei*. **A**, the kinetics of killing of bloodstream TREU 927 (▲) is intermediate between that of STIB 247 (□, *T.b. brucei*) and STIB 386 (▼, *T.b. gambiense* type 2) and different from the kinetics of killing of a 1:1 mixture of resistant and sensitive bloodstream trypanosomes (●). **B**, the kinetics of killing of metacyclic forms of STIB 247 (□), STIB 386 (▼), TREU 927 (▲) and EATRO 3(●). Average values are shown, n = 3, 2SE < 10% in all cases. Lysis in parallel incubations in Guinea-pig serum as negative controls <10% in all cases.

Figure 3. A, PCR amplification of the *sra* gene using primers and conditions described in Welburn *et al* (2001). PCR products from the lines STIB 247, Etat 1.10, Etat 1.2, TREU 927, TREU 927R, STIB 386R and STIB 386S were separated on a 1% Seakem agarose gel and visualized by eithidium bromide staining. The 1085bp product is the related VSG pseudogene and the 743bp band is the *sra* gene (Campillo and Carrington 2003). **B** (i), The *sra* gene probe was hybridized with a northern blot of total RNA (4 g per track). Exposure time was 4 hours. **(**ii), Ethidium stained gel of the RNA prior to northern blotting. (iii) The northern blot was reprobed with a tubulin probe as a positive control.

Figure 4. **A**, The kinetics of killing of bloodstream form F1 hybrids from a STIB 247 x TREU 927 cross. **B**, The kinetics of killing of metacyclic forms of the same hybrids. F532/63bcl3(◆) and 5(□), F532/72mcl1(■), 4(▲), 5(▼) and 9(○). Average values are shown, n = 3, 2SE < 10% in all cases. Lysis in parallel incubations in Guinea-pig serum as negative controls <10% in all cases.

Table 1 Human serum resistance phenotypes and *sra* expression and genotypes of trypanosome lines. STIB 386S is a subclone of STIB 386, GUTat 10.1 a subclone of TREU 927 and Etats 1.2 and 1.10 are subclones of EATRO 3. Human serum resistant clones are defined here as *T. b. rhodesiense* and human serum sensitive clones as *T. b. brucei*. A = wild animal, H = human, T = tsetse, S = sensitive, I = intermediate, R = resistant. '?' indicates equivocal subspecies definition as discussed in the text.

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Table 1

Stock	Host	Subspecies	*HSR assay*			*SRA assay*	
			Bloodstream forms		*Metacyclic forms*	*Presence of SRA gene by PCR*	*Expression of SRA by northern analysis*
			In vivo	In vitro	In vitro		
STIB 247	A	*T.b. brucei*	S	S	S	+	-
STIB 386	H	*T.b.gambiense*, type 2	R	R	R	-	-
STIB 386S	H	T.b.gambiense, *type 2?*	S	S		-	-
TREU 927	T	T.b. brucei*?*	I	I	I	-	-
TREU 927R	T	T.b. rhodesiense*?*	R	R		-	-
GUTat10.1	T	T.b. rhodesiense*?*		R			
ELIANE	H	*T.b.gambiense*, type 1		R		-	
EATRO 3	T	*T.b. rhodesiense*?		I	I	+	-
Etat 1.2	T	T.b. brucei*?*		S		+	-
Etat 1.10	T	*T.b. rhodesiense*?		R		+	+

Figure 1

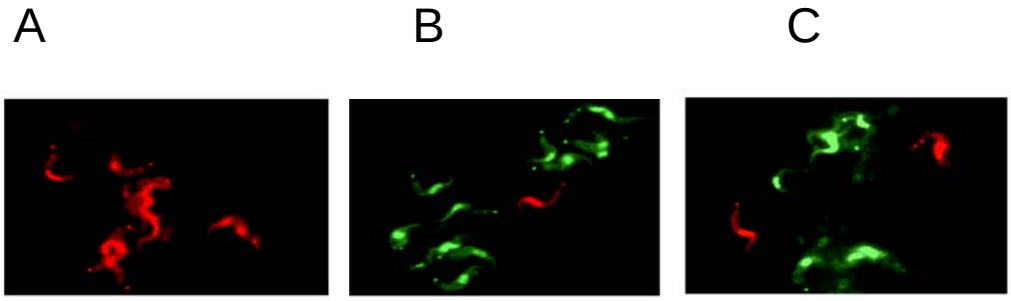
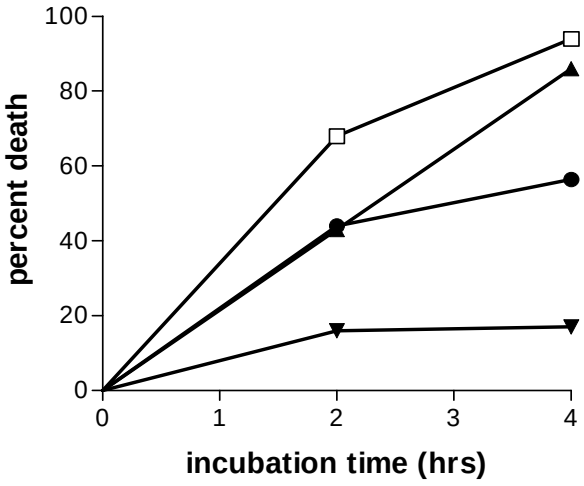


Figure2

A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60
40
20
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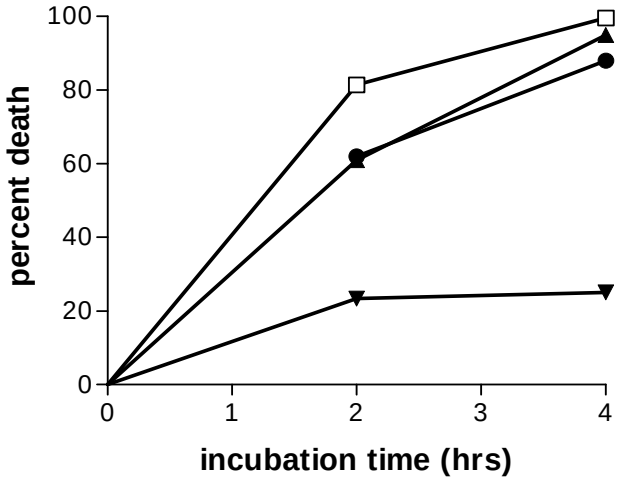
percent death
0
1
2
3
4
incubation time (hrs)

B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percent death
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1
2
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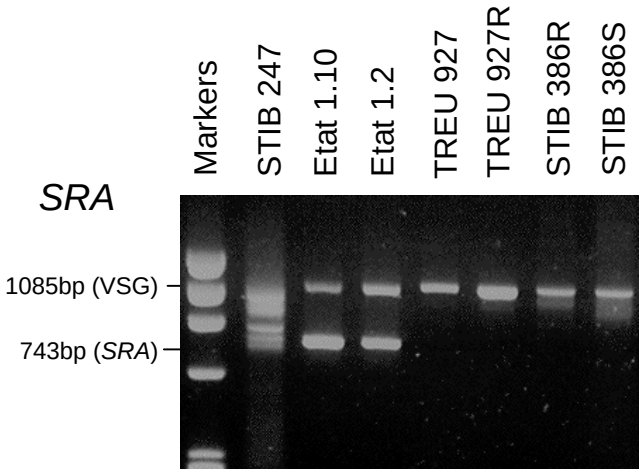
incubation time (hrs)

A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STIB 386S
SRA
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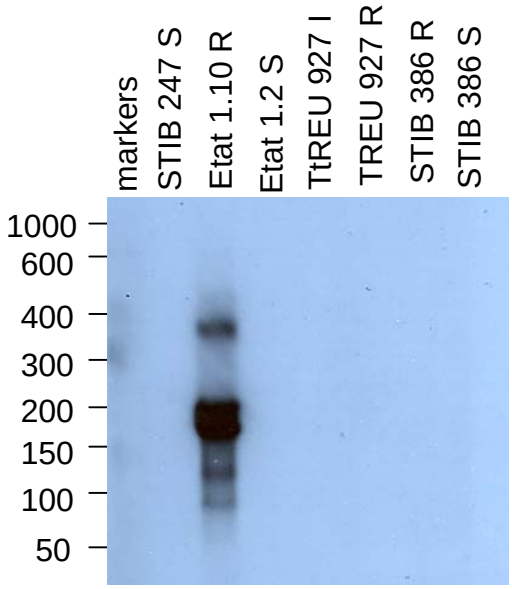
743bp (SRA)

B i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STIB 386 S
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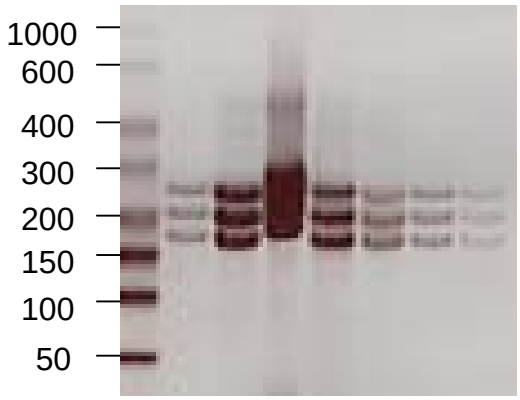
300
200
150
100
50

ii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iii

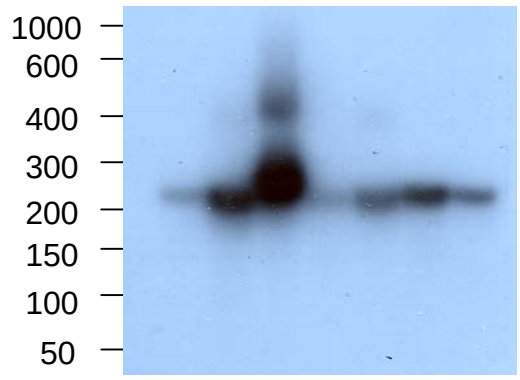
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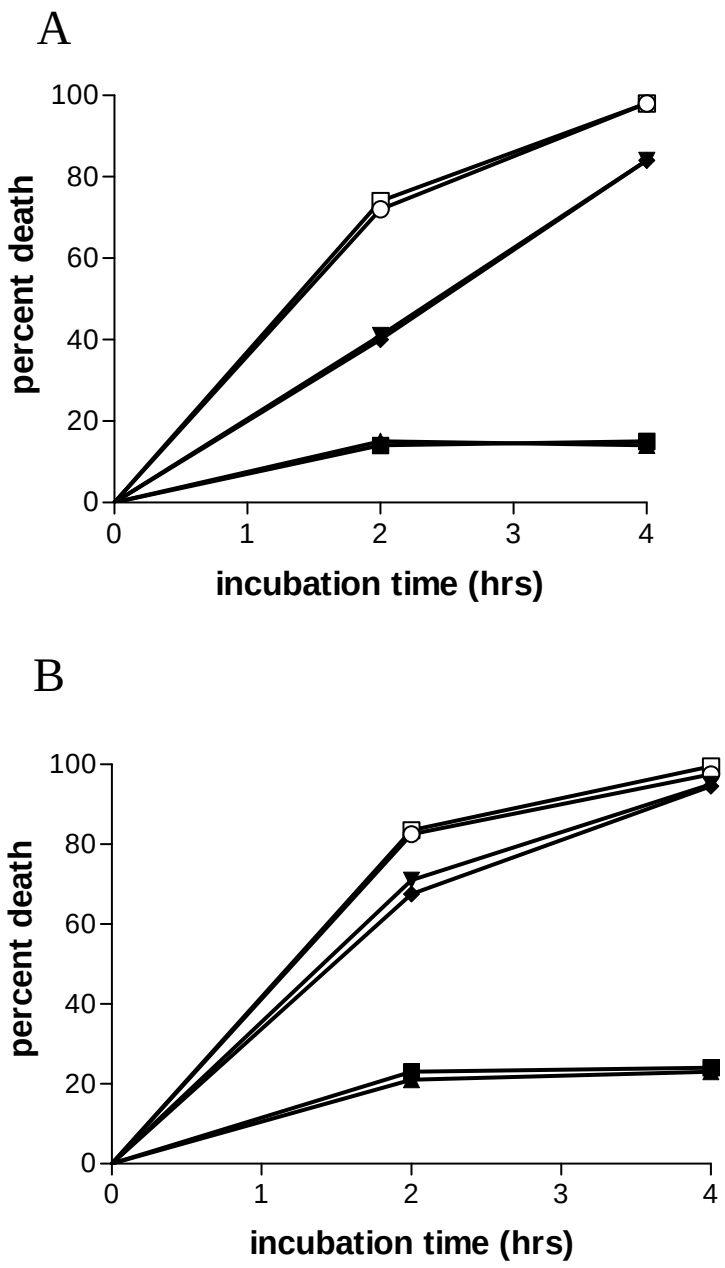
Figure 4


A
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60
40
20
0
percent death
0
1
2
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4
incubation time (hrs)
B
100
80
60
40
20
0
percent death
0
1
2
3
4
incubation time (hrs)