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THE DYNAMICS AND NATURE OF THE
HIERARCHY OF VSG EXPRESSION
DURING *Trypanosoma brucei*
INFECTION

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Abstract

Trypanosoma brucei is a tsetse fly-transmitted kinetoplastid protozoan that parasitises a wide range of mammals in sub-Saharan Africa. The chronic infections typical of trypanosomes have a profile characterised by intermittent parasitaemic peaks, that become smaller and further apart. A key component that facilitates the survival of the parasite within the bloodstream of the host, and the generation of chronic infections, is antigenic variation. This process involves the inherent switching of the surface-expressed variant surface glycoprotein (VSG), and the new variable antigen types (VATs) in order to pre-empt the host immune response. There are approximately 1,000 genes in the *VSG* repertoire, and the observation that certain VATs appear at similar times within infections, has led to the conclusion that the expression of VSGs is semi-predictable and semi-ordered. This hierarchical system allows optimal use of the *VSG* repertoire, and is highly likely to be a significant factor in generating chronicity. It has been established that VSGs encoded by subtelomeric genes tend to appear early in infection, whilst those encoded by chromosomal internal genes tend to appear later.

The first aim of this thesis was to examine the timing of expression of a subset of VSGs within replicate infections in mice and cattle, and to link the timing of expression to the genetic locus of the silent, donor, *VSG* gene. The *VSG*s examined represented all *VSG* locus types, incorporating 1 bloodstream expression site (BES) gene (the transcription site for *VSG* genes in the bloodstream stage), 2 metacyclic expression site (MES) genes, 2 minichromosomal genes, and 2 chromosomal internal genes. The infections were initiated with pleomorphic trypanosomes that switch at a high rate, and are a close-to-field, 'wild type' strain. The infections in mice confirmed that there was a statistically significant difference in the timing of onset of VAT-specific immune responses across replicate batches of infections with two separate analyses; one by comparing the average time of onset of the immune response (General Linear Model [GLM]; $F_{6,106}=7.49$, $p < 0.0001$), and the second by ranking the onset of the immune response by sequence of appearance (GLM; $F_{6,112}=8.03$, $p < 0.0001$). The appearance of VSGs within a restricted period of time was confirmed by directly analysing the parasite population in cattle, using *VSG*-specific reverse transcriptase polymerase chain reaction (RT-PCR). These findings allow further dissection of the hierarchical expression of VSGs, and provide statistically significant confirmation of the existence of semi-ordered expression in high-switching pleomorphic trypanosomes for the first time.

A mathematical model was formulated (with the assistance of Dr. K. Lythgoe, University of Edinburgh) in order to simulate the dynamics of trypanosome infections. The model incorporated and built upon successful aspects of previous studies, and included measured biological parameters that are known to affect *in vivo* parasite kinetics. Manipulations were undertaken to investigate the effect, *in silico*, of differential *VSG* switching rates. The effect of varying the intrinsic rate of growth of the immune response was also analysed. The simulations indicated areas for potential further experimental studies. In particular, results suggested that the rate of growth of the immune response may be extremely important in shaping the profile, and duration, of an infection. The outcome of modelling whereby *VSG* switching was manipulated, suggested that there are distinct subsets of *VSGs*, which have differing probabilities in switching to each other. The significance of genomic position of the silent, donor, *VSG*, and also sequence homology between the donor *VSG* and the expressed *VSG* were investigated. The proportional influence of genomic locus and homology-driven switching was suggested to be the most important aspect to be elucidated, with respect to further resolving the hierarchical switching of *VSGs*.

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Author's Declaration

The mathematical model presented in Chapter 5 was formulated by Katrina Lythgoe, University of Edinburgh, in collaboration.

I declare that this thesis and the results presented in it are entirely my own work, except where otherwise stated.

Liam Morrison

Chapter 1

Introduction

1 Introduction

1.1 African trypanosomiasis

Trypanosomes are haemoflagellate protozoan parasites responsible for causing disease across wide areas of Africa, Asia and South America. Transmitted by biting vectors, the parasites infect many host species. In Africa, the implications are large with regards to human morbidity and mortality, and an even more serious situation exists among the domestic animal population. Large swathes of the sub-Saharan subcontinent have historically been rendered unsuitable for rearing of livestock, and periodic human epidemics have caused significant loss of life. In modern Africa, the human disease situation is worsening, with an estimated 60 million people living with the threat of the disease, and potentially between 300,000 and 500,000 people infected per annum. This susceptible population spans 37 sub-Saharan countries, 22 of which are among the least developed in the world (WHO).

The cost to the agricultural community is enormous, with 48 million cattle at risk, and an estimated potential cost of \$ 1–1.2 billion per year. 10 million km² of sub-Saharan Africa are inhabited by the tsetse fly (*Glossina* species) vector, and any significant farm animal industry is thus restricted in this vast area (Leak, 1999). 85% of the continent's poor live in rural areas, and 80% of the population as a whole rely upon agricultural output (FAO), so the impact of trypanosomiasis is far-reaching and profound.

Trypanosomiasis is one among a plethora of disease, and in particular parasite, threats to people and animals in sub-Saharan Africa. In these areas the threat to people and their livelihoods is significant, and hence increasing knowledge of the parasite, the disease, and the vectors is potentially an important step towards reducing the impact of these factors.

1.2 Life Cycle

African trypanosomes belong to the order Kinetoplastida, and group within the Salivaria (Hughes and Piontkivska, 2003). They are transmitted by an insect vector, and in the case of *T. brucei* the vector is the tsetse fly. Within both the human and tsetse host the parasite essentially has two types of form; the replicative that divides by mitosis, and the non-replicative, which are transmissive types pre-adapted for the next host or niche (see Figure 1.1, p.4, for a graphical summary of the life cycle).

When an infected tsetse fly feeds on a mammalian host, trypanosomes are deposited into the dermal connective tissue via the proboscis, from where they spread to the blood and lymph compartments. The first trypanosomes inoculated, the metacyclic form, serve to initiate the infection, and quickly give rise to the long slender bloodstream form. The long slender trypanosomes divide mitotically every 6 hours, and are entirely reliant on glucose present in the host blood, aerobic glycolysis metabolising the glucose to pyruvate in glycosomes, dedicated organelles (Opperdoes and Borst, 1977). Due to repression of mitochondrial function, oxidative phosphorylation does not occur. As the infection progresses, and the number of trypanosomes increases, an increasing proportion of the long slender trypanosomes differentiate to the non-replicating short stumpy form via intermediate forms. The trigger for this process is thought to be a density dependent mechanism (Vassella *et al.*, 1997). The short stumpy trypanosomes are pre-adapted to transmission to the fly.

Once ingested by the fly, which can be male or female, there is rapid differentiation in the tsetse posterior midgut to the mitotically dividing procyclic form, and metabolism switches from being carbohydrate-based to amino acid-based, with proline being the main substrate. The number of parasites in the midgut declines dramatically during the short stumpy to procyclic differentiation, in one study falling from 7.5×10^6 to 2×10^3 trypanosomes per midgut examined over the first three days post blood meal (Van Den Abbeele *et al.*, 1999). This number then rises over the next three days to a stable threshold of $2-5 \times 10^5$ procyclic parasites.

There is progression of the parasite population from the posterior to the anterior midgut as the procyclic population expands, and the parasites become elongated (Van Den Abbeele *et al.*, 1999), eventually becoming the long, mesocyclic form (Vickerman, 1985), which migrates forwards into the proventriculus (Vickerman, 1985; Van Den Abbeele *et al.*, 1999). Upon entering the proventriculus, there are further changes to longer post-mesocyclic cells. There is replication of DNA, and the post-mesocyclic cells make the journey to the foregut and proboscis. Based on staining with the DNA dye YOYO-1 iodide, the post-mesocyclic cells have been suggested to be tetraploid (Van Den Abbeele *et al.*, 1999), as measured by flow cytometry and fluorescence microscopy, but independent controls, *i.e.* cells of known DNA content, were lacking (the controls used were reference trypanosomes from tsetse dissections). Further differentiation to a long epimastigote cell then occurs, and it is thought this stage transfers to the salivary glands. This epimastigote divides asymmetrically to give 2 morphologically distinct daughter cells, one short and one long. The long daughter cell appears to play no further role, and dies. The short daughter

cell, however, attaches to epithelial cells in the salivary gland, where the trypanosomes divide rapidly (Van Den Abbeele *et al.*, 1999). There is then an intermediate premetacyclic stage (Vickerman, 1985), giving rise to the metacyclic trypomastigote, which is infective for the mammalian host. A proportion of this population of trypanosomes are then inoculated when the tsetse fly feeds. This salivary gland population remains as a transmission source within the salivary glands of the fly, initiating infections at each subsequent feed.

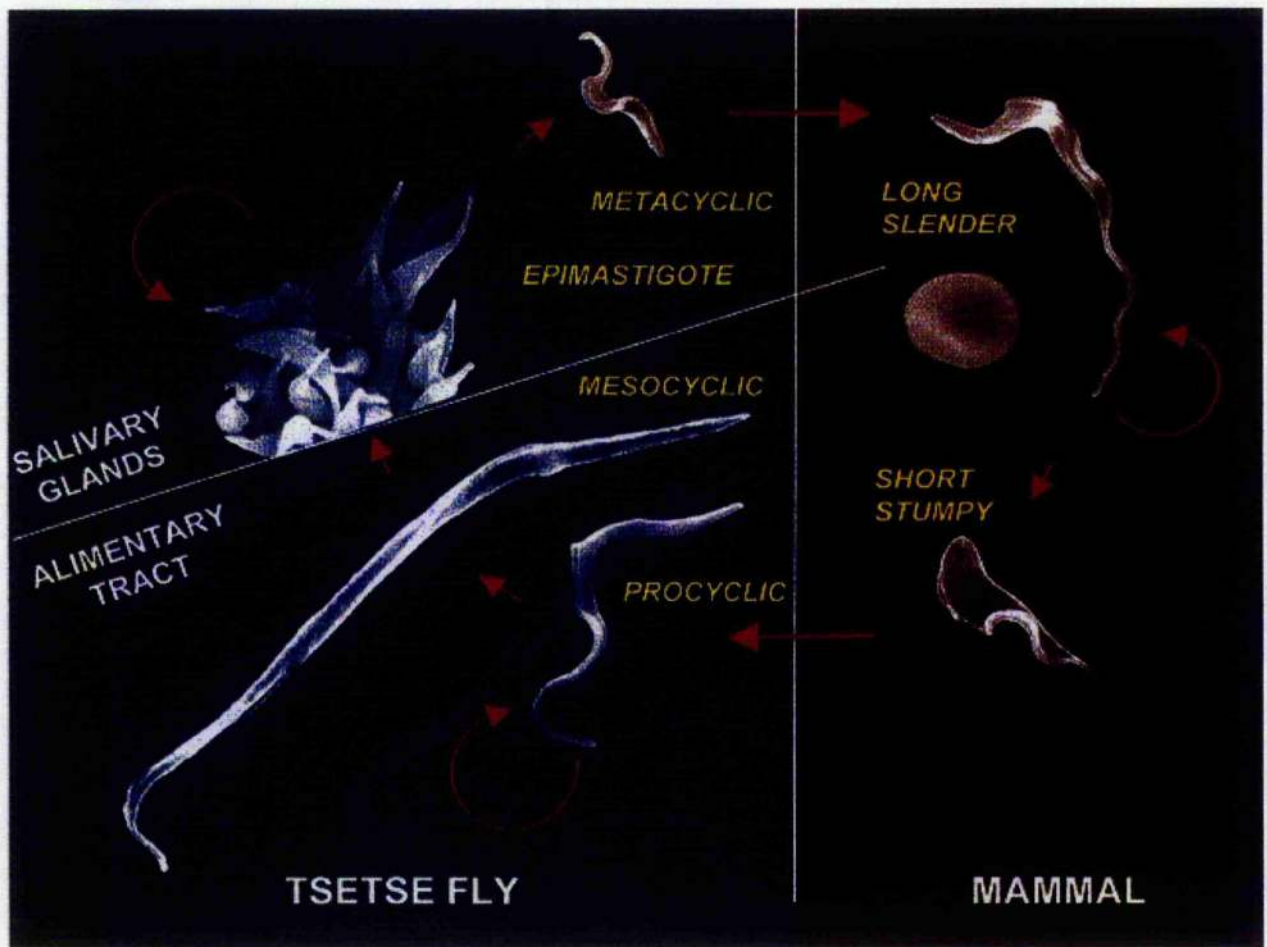


Figure 1.1 Life cycle of *Trypanosoma brucei*. From Barry and McCulloch, 2001. The long slender, procyclic and epimastigote cells are proliferative, while the short stumpy, mesocyclic and metacyclic stages are non-proliferative. Procyclic and mesocyclic trypanosomes occur in the tsetse midgut, whereas epimastigote and metacyclic cells reside in the salivary glands. Long slender and short stumpy trypanosomes are found in the mammalian bloodstream.

1.3 Trypanosome pleomorphism, monomorphism and differentiation

A large body of experimental data exists about *T. brucei*, encompassing many aspects of the parasite. The majority of molecular studies have been undertaken *in vitro*. The strains utilised have been passaged a significant number of times in laboratory rodents, and some lines have secondarily adapted to growing in culture medium. This serial passaging *in vivo*, and maintenance under *in vitro* conditions, has resulted in these trypanosomes losing the ability to be transmitted to the tsetse vector – they exist entirely as the replicating long slender bloodstream stage ('monomorphic' trypanosomes), and do not form the short stumpy fly transmissible stage (although with artificial manipulation differentiation to the procyclic form can be induced). The inability of the trypanosomes to establish chronic infections in laboratory animals results in exponential growth of the parasites until death of the host, occurring in mice in a matter of days. The loss of ability to form the short stumpy stage is indubitably an important factor – this differentiation process being suggested as a mechanism of self-limitation of growth of the trypanosome population (Vassella *et al.*, 1997).

The morphology of the short stumpy cells is distinct in several ways, as the flagellum is shorter and the kinetoplast is in a more posterior position, and the mitochondrion is more cristate – this being a pre-adaptation for the tsetse host, where the mitochondrial respiratory chain is necessary for survival (van Weelden *et al.*, 2003). The stumpy form is cell-cycle arrested at G₀ or G₁ (Vassella *et al.*, 1997), and has a finite lifespan unless taken up by the tsetse vector; the *in vivo* half life in mice has been calculated as 24-36 hours (Black *et al.*, 1982), in a separate study as 48-72 hours (Turner *et al.*, 1995), and *in vitro* as 48 hours (Reuner *et al.*, 1997). The cell-cycle arrest occurs at a threshold level, and is independent of the host immune response as it demonstrably occurs *in vitro* (Reuner *et al.*, 1997) and in immunosuppressed animals (Black *et al.*, 1985). There has been speculation of the existence of a 'stumpy induction factor' (SIF), which induces the cell cycle arrest and differentiation of cells (Vassella *et al.*, 1997). The precise ingredient(s) involved have not been identified, although in one study it was determined that the factor must be less than 500 Da and is stable in *in vitro* conditions, and was speculated to be either a trypanosome-derived pheromone or catabolite (Vassella *et al.*, 1997). It has been elucidated that it is via cAMP signalling that cell cycle arrest occurs. The addition of a cell-permeable cAMP analogue induced cell cycle arrest *in vitro* – the exit from the cell cycle was a necessary precursor to the other morphological and metabolic events

associated with differentiation to the short stumpy form (Vassella *et al.*, 1997). It has been suggested that the inability of monomorphic strains to differentiate to the short stumpy stage is due to an undefined defect in this signalling pathway. This hypothesis has been supported by a study showing that this differentiation can occur *in vitro* with monomorphic trypanosomes by incubating them with the same cAMP analogues, although the concentrations of analogue required were four times as high, and the time taken for differentiation was three times as long (Breidbach *et al.*, 2002). These data suggest that the signalling pathway is intact in monomorphic trypanosomes, but the sensitivity to the putative SIF is very much reduced to a level that is incompatible with the ability to self-regulate growth.

1.4 Antigenic variation

African trypanosomes exist extracellularly in the mammalian host, in blood vessels and tissue spaces. This poses particular problems for a parasite; although the environment is very rich in readily available metabolite (glucose in the case of the trypanosome), the parasite is constantly exposed to all facets of the host immune response. African trypanosomes have circumvented this obstacle by developing a system of antigenic variation. The key to antigenic variation in trypanosomes is the variant surface glycoprotein (VSG). The VSG forms a coat that cloaks underlying invariant antigens, and in addition interferes with non-specific immune mechanisms (Overath *et al.*, 1994). However the VSG is in turn the primary target of the specific immune response, and antigenic variation is the method by which new antigenically distinct variants of the protein are introduced.

Antigenic variation as a distinct process has been identified in several classes of organism – bacteria (*Borrelia burgdorferi*-(Barbour, 2003)) and more relevantly parasites, *Plasmodium* (Kyes *et al.*, 2001), *Anaplasma* (Brayton *et al.*, 2002) and *Giardia* (Nash, 2003) being well studied examples. Genetic drift of antigens occurs widely; that is, gradual evolution of antigens through accumulation of point mutations, meiotic crossovers or other background cellular events and processes (notably HIV and influenza, see (Craig and Scherf, 2003)). Antigenic variation is distinct from this in that it is a system that has evolved specifically to evade the immune response. In order to accomplish this, the rate of antigen replacement has to be equal to or greater than that of the immune response rate of antigen recognition and removal. This high rate, and in addition specifically directed cellular resources and machinery, distinguishes antigenic variation from antigenic drift.

This concept of a subset of genes with higher mutation rates is similar to the idea of 'contingency genes' in bacteria (Moxon *et al.*, 1994; Deitsch *et al.*, 1997), whereby increased variability at particular loci is an evolutionary adaptation to changes in environmental stress.

Obviously antigens that undergo antigenic variation are those that are exposed to the immune system, and are therefore surface expressed, for example *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1), encoded by *var* genes in *P. falciparum* (Kyes *et al.*, 2001), Major Surface Protein 2 (MSP-2) in *Anaplasma marginale* (Meeus *et al.*, 2003), and the VSG in trypanosomes. Trypanosomes are unique however in unicellular parasites, in that they spend the all of their time extracellularly within the mammalian host bloodstream. Parasites such as *Anaplasma* and *Plasmodium* spend a considerable portion of their life cycle intracellularly. Trypanosomes have a concomitantly increased necessity for a highly efficient system.

The trypanosome invests a large amount of its genome to this process, there being potentially 1,000 *VSG* genes and pseudogenes. Indeed 101 distinct variants were isolated in a single study (Capbern *et al.*, 1977). This capacity for variation is undoubtedly one of the keys to the chronic course of infection characteristic of the disease, both extending the lifetime of the parasite population within individual hosts, and enhancing the probability of transmission to the tsetse vector.

1.5 The VSG

The key to the antigenic variation system is the VSG, one species of which enshrouds the parasite at any one time, forming a dense 12–15 nm thick coat. The VSG is a dimer, and there are approximately 10 million copies per cell (Carrington *et al.*, 1991). These tightly packed proteins prevent access to invariant proteins by the host's specific immune response. Indeed, in a recent study it has been established that single domain antibodies derived from camels, which are much smaller than antibodies produced in other mammalian hosts due to the lack of light chains, do gain access to the underlying invariant molecules (Stijlemans *et al.*, 2003), unlike the dimer (IgG) and pentamer (IgM) immunoglobulins in other mammals. Low molecular weight compounds are able to pass between the VSG molecules, but the great majority of endo- and exocytosis occurs in a specialised invagination of the cell membrane around the base of the flagellum, the flagellar pocket. This is the only area of the surface membrane without a complex mesh of pellicular microtubules, which preclude endocytosis (Webster and Russell, 1993). The

flagellar pocket is not accessible by the cellular arm of the host's immune response. It is exposed to immunoglobulins, but results of recent work undertaken *in vitro* suggest that any response raised against the invariant proteins could possibly be ineffective, as anti-VSG antibodies are cleared by endocytosis (Pal *et al.*, 2003), which has been defined further as specifically via clathrin-mediated endocytosis (Grunfelder *et al.*, 2003). However, whether this would be the case *in vivo* is yet to be demonstrated.

VSGs are composed of 400-500 amino acids, and consist of two domains, the N-terminal domain (350-400 amino acid [aa] residues) and the C-terminal domain (50-100 aa residues), separated by a 'hinge region' (Johnson and Cross, 1979; Carrington *et al.*, 1991). So far, there are 3 types (A-C) of N-terminal domain and 4 (1-4) of C-terminus, as revealed by alignment of the amino acid sequences of 19 VSGs (Carrington *et al.*, 1991). These types are determined by the pattern of arrangement of conserved cysteine residues (Cross, 1984; Carrington *et al.*, 1991), which contribute significantly to tertiary structure by the formation of disulphide bridges. The N-terminal domain is the portion that contains the coding region for the epitopes exposed to the immune system and is therefore much more variable in sequence, whereas the sequence identity is much higher between the C-terminal domains. On average, overall sequence identity between VSGs is 20% (Carrington and Boothroyd, 1996). VSG 'families' have been identified where identity can be as high as 70% (Field and Boothroyd, 1996), and even in these cases antibodies against the variants are not cross-reactive. Despite often significant differences in sequence, VSG molecules fold into a similar three-dimensional structure, as determined by X-ray crystallography (Blum *et al.*, 1993). Although this has been examined for only 2 VSGs, the primary sequence identity was 20%, and less than this when aligned on the basis of tertiary structure.

The VSG is secured to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor, which is added to the protein as a post-translational modification. During this process a conserved carboxy-terminal hydrophobic tail extension (Boothroyd *et al.*, 1980) is removed from the precursor peptide, and the GPI is covalently linked. This membrane form (mf) VSG is then transferred to the cell surface by exocytosis in the flagellar pocket, and the protein diffuses across the membrane (Overath *et al.*, 1994). The VSG GPI anchors in trypanosomes have extensive carbohydrate side chains, and there is a suggestion that this forms a protective glycocalyx that contributes to the barrier nature of the VSG coat (Ferguson, 1992). Other invariant proteins are also GPI anchored to the trypanosome surface, in particular invariant surface glycoproteins (ISGs) 65 and 75, the transferrin receptor, and the protein encoded by the serum resistance associated (SRA) gene. These

have also been suggested to occur as dimers (Carrington and Boothroyd, 1996), and indeed transferrin is encoded by *ESAG6* and *ESAG7*, and expressed as a heterodimer (Steeverding *et al.*, 1994). These surface proteins have been proposed to have a similar tertiary structure to that of the VSG (Carrington and Boothroyd, 1996).

Upon uptake by the tsetse fly and arrival in the tsetse midgut, expression switches from the VSG to procyclin (Roditi *et al.*, 1989). The VSG is slowly shed, and there is a period of transition during which there is a mixed coat, leading to a gradual changeover, resulting in the trypanosome membrane never being exposed (Ziegelbauer and Overath, 1990). The procyclic molecules are not as densely packed as the VSG, as they are narrow, extended molecules (Treumann *et al.*, 1997) less broad than the GPI anchor carbohydrate structure (Ferguson *et al.*, 1993). This glycosylation is also suggested to exist as a dense protective glycocalyx for the procyclic insect stage (Ferguson, 1994). Procyclin has several putative functions, possibly protecting against digestion in the insect gut or playing a tropism role, in determining the location for growth and differentiation (Roditi *et al.*, 1998). There are 4 different types of procyclin, varying in the number of internal repeats; there being 3 types with differing numbers of dipeptide EP repeats (EP 1-3) and one with pentapeptide GPEET repeats. The relative proportion of these isotypes changes as the trypanosomes progress through the cycle in the tsetse, perhaps suggesting different functional roles (Vassella *et al.*, 2001)

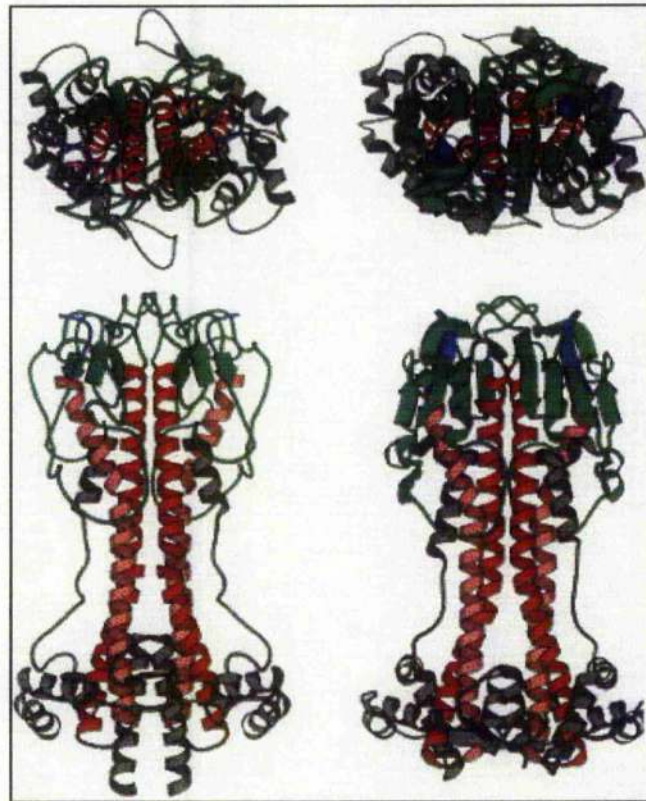


Figure 1.2 Illustration of VSG homodimer structure, as determined by X-ray crystallography (from Blum *et al.*, 1993). On the left is the MITat 1.2 VSG, and on the right the ILTat 1.24 VSG. Red or pink = α helices, green = β sheets. The top views, which are head-on, illustrate the portion of the VSGs exposed to the host's immune response. The sequence identity between the two genes was 20%, demonstrating the structural conservation despite sequence divergence. (adapted from <http://www.bio.cam.ac.uk/~mc115/page12.htm>; M. Carrington)

1.6 Pleomorphism, monomorphism and relative antigenic switching rates

A significant feature of monomorphic trypanosomes is that they have a relatively low switching rate of the VSG coat. They switch at a frequency of $1 \times 10^{-6} - 1 \times 10^{-7}$ switches/cell/generation (Lamont *et al.*, 1986). Compared with more 'wild-type' strains, which can have switching rates as high as 1×10^{-2} switches/cell/generation (Turner and Barry, 1989), this is a significantly decreased rate, and indeed is comparable to background mutation rates and mitotic recombination events. Whether this difference in switching rate is due to the reduced necessity for a high switching system, deriving from absence of

selection pressure during long term *in vitro* maintenance, and repeated short-term *in vivo* passages, is unclear. However, it is possible that the reduction in switching rate is linked to the adaptation in the first instance, and the trypanosomes that have been heavily selected for by the passaging process inherently switch at a lower rate. It has been suggested that the difference in rates of switching is due to the lab-adapted monomorphic strains having lost a specific pathway involved in the switching event (Barry, 1997), most likely to do with homologous recombination. This has led to a revision of the significance of many of the studies examining antigenic variation and *VSG* switching in these lines. Indeed, recombination events have been shown to be the major mechanism by which *VSG* switching occurs in pleomorphic trypanosomes (Robinson *et al.*, 1999), a finding that differs from that seen in monomorphic trypanosomes, which utilise promoter switching much more frequently (McCulloch and Barry, 1999) (see 1.10 for more details).

1.7 Genome organisation in *T. brucei*

T. brucei has a haploid nuclear genome size of approximately 35 Megabases (Mb), varying between isolates by up to 25% (El-Sayed *et al.*, 2000). There are 11 pairs of megabase chromosomes, several intermediate chromosomes (200-900 kilobases [kb]) and approximately 100 minichromosomes (50-150 kb each). The diploidy of *T. brucei* is unusual in that it has been demonstrated for only the megabase chromosomes, and not for the intermediate or mini- chromosomes. In addition, *VSG* genes and expression sites appear to be haploid, and single copy *VSG* genes can be readily identified (Robinson *et al.*, 1999). Furthermore, homologous chromosomes can differ in size to a large extent, up to fourfold, both within a genome and between strains. This phenomenon possibly arises through subtelomeric recombination events (Gottesdiener *et al.*, 1990; McIville *et al.*, 2000).

The precise number of *VSG* genes in the genome is not known. It was originally proposed to be a repertoire of around 1,000 (Van der Ploeg *et al.*, 1982). The majority of these are thought to be in chromosomal internal locations, although the ratio of complete *VSGs* to pseudogenes has been revised due to insights provided by the genome project, and the proportion of pseudogenes is much higher than previously considered; approximately 95% of 850 *VSG* genes examined (L. Marcello, P. Burton & J.D. Barry, pers. comm.). The remainder of *VSGs* are located subtelomerically, the majority of these on minichromosomes. Indeed, minichromosomes (and possibly intermediate chromosomes) are postulated to act as a reservoir of *VSGs*, as only *VSGs* have been found to reside in the

majority examined thus far (most minichromosomal content consisting of 177-bp ‘stuffer repeats’), and the more primitive *T. vivax* has only one minichromosome per strain (Dickin and Gibson, 1989). The actual repertoire can differ significantly between strains, with only a few genes common to most (Van Meirvenne *et al.*, 1975; Bernards *et al.*, 1986). This is evident in the fact that vaccination by the ‘infect and cure’ method is not cross-protective across strains (Morrison *et al.*, 1985). This difference is thought to occur by gene duplication and deletion, which are integral to the antigenic variation system, and occurring constantly at a high rate in pleomorphic trypanosomes.

1.8 The VSG expression sites

There are 2 separate *VSG* expression systems in *T. brucei*, one specific to the metacyclic life cycle stage, and the other to the bloodstream form. These are sets of expression sites exclusive to each lifecycle stage, termed the metacyclic expression sites (MESs) and bloodstream expression sites (BESs). They are located at subclomeres on megabase chromosomes, and there are estimated to be 20 – 30 BESs (Borst *et al.*, 1990) and possibly as many as 27 MESs (Turner *et al.*, 1988).

Transcription of bloodstream *VSG* genes occurs exclusively from BESs (Johnson *et al.*, 1987; Kooter *et al.*, 1987), one BES being active at any one time. This system of allelic exclusion ensures that only one species of *VSG* is present and exposed to the immune system (Cross, 1984), and is central to the process of antigenic variation in trypanosomes. Transcription occurs via RNA Polymerase I (Gunzl *et al.*, 2003), which has recently been shown to occur in a small nuclear-associated structure, termed the expression site body (ESB) (Navarro and Gull, 2001). Only the active BES was present in the ESB, and this potentially provides the route for the allelic exclusion vital to the *VSG* system. BESs differ in length and composition, although there are components common to all examined so far. They encode polycistronic units, with a variable number of expression site associated genes (*ESAGs*). The *VSG* is located 5–10 kb from the chromosome end, and is surrounded by regions largely devoid of restriction sites. The upstream region extends for 1-2 kb, and contains multiple imperfect 70-bp repeats. Many of the differences in size between BESs can be assigned to different numbers of *ESAGs*. The function of many of the *ESAGs* remain to be elucidated, although *ESAG6* and *ESAG7* encode the two subunits of the surface located heterodimeric transferrin receptor (Steverding *et al.*, 1994), and *ESAG4* is an adenylate cyclase (Alexandre *et al.*, 1996). There have been 11 *ESAGs* identified, with functions identified only for those mentioned above. Only *ESAG6* and *ESAG7* are common

to all BESs looked at so far, and it has been deduced from that these are the only *ESAGs* transcribed from BESs constitutively (Berriman *et al.*, 2002). In *T. brucei rhodesiense*, the serum resistance-associated (*SRA*) gene is an *ESAG* that is necessary to be transcribed to confer infectivity to humans (Xong *et al.*, 1998). In addition, it has been suggested that the existence of different BESs may serve to provide a library of different transferrin receptors, each with differing affinity to transferrin from different host species (Bitter *et al.*, 1998). This hypothesis requires further examination, although *in vitro* studies have shown differences in growth rate when incubated with medium containing transferrin from different species (Gerrits *et al.*, 2002).

A feature of silent BESs is the presence of β -D-Glucosyl-hydroxymethyluracil, or base J, which is a modification of the base thymine that substitutes approximately 0.5-1.0% of thymidine in trypanosomes (Gommers-Ampt *et al.*, 1993). Base J seems to accrue around non-transcribed repetitive sequences and is present in 50-bp repeats found upstream of BESs, and in the 177-bp repeats that make up the majority of the minichromosomes. It is also present in silent BESs (van Leeuwen *et al.*, 1996), but is not found between the telomeric and the 50-bp repeats in the active BES. In addition, base J has not been found in internal *VSGs* (van Leeuwen *et al.*, 1997). Base J has been suggested to play roles in alteration of transcription, preventing recombination between repetitive sequences, or the remodelling of chromatin, which are all potential silencing mechanisms of BESs (van Leeuwen *et al.*, 1998). However, further work is required before any role can be ascribed to base J with regard to the switching on or off of BESs.

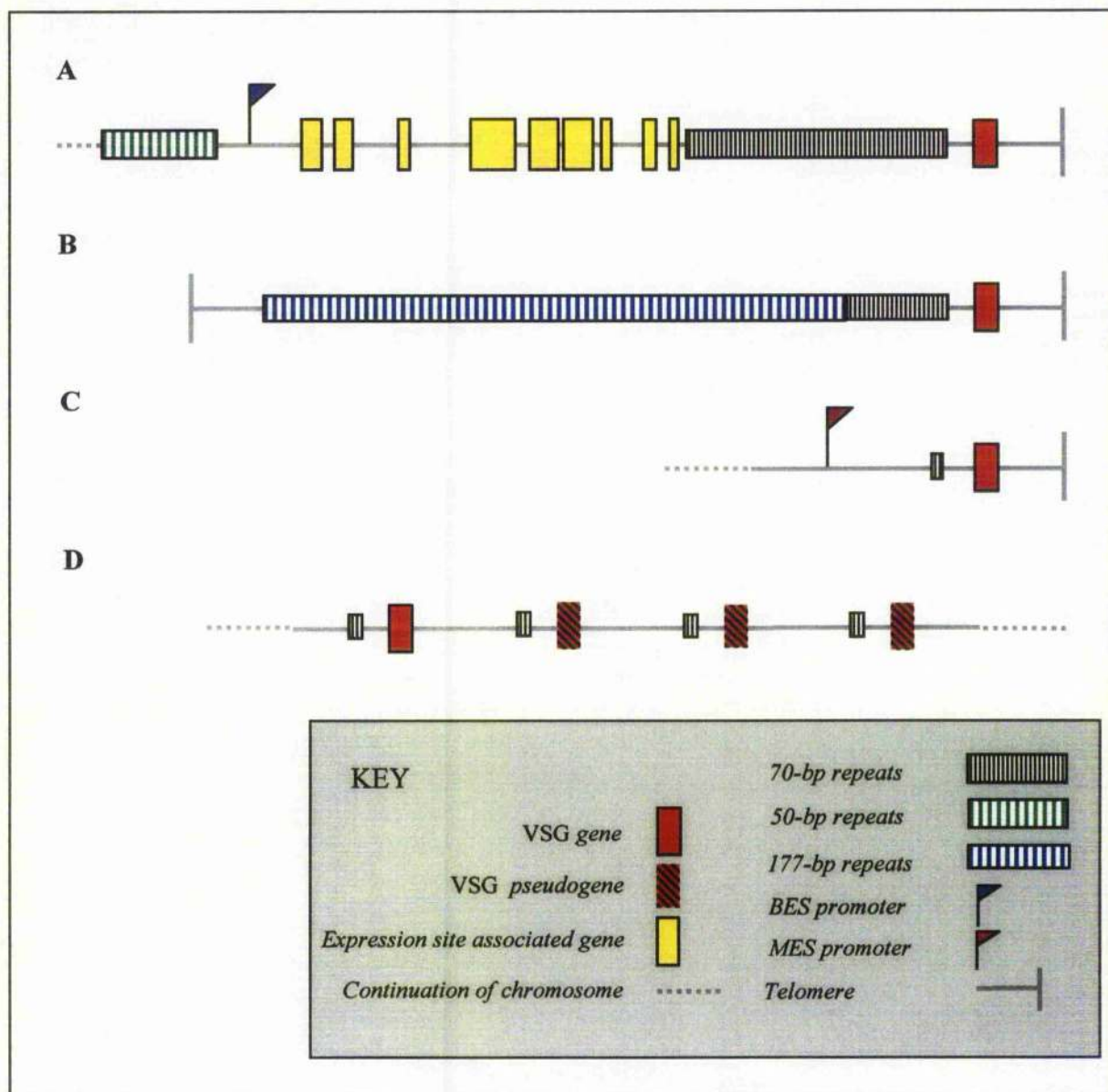


Figure 1.3 Diagram illustrating types of VSG locations within the trypanosome genome, and typical flanking regions. A; bloodstream expression site. B; minichromosome. C; metacyclic expression site. D; internal VSG array. Figures not to scale.

1.9 Genetic mechanisms of antigenic variation

The mechanisms for switching the expressed *VSG* to a new variant can be separated broadly into two categories; *in situ* switching which involves promoters, and duplication mechanisms whereby genes are replaced. As mentioned previously, the *VSG* genes are flanked upstream by a set of imperfect repeats 70-bp long (Liu *et al.*, 1983), the average length being 76-bp. In the BES these take the form of an array, comprising of hundreds of copies. Minichromosomal telomeric genes have fewer repeats than the BESs; 35 repeats in one studied case (Robinson, 1999), and chromosomal internal genes have a small number (1 – 5) (L. Marcello, P. Burton and J.D. Barry pers. comm.). There are short blocks of homology at the opposite end of the gene, in particular a sequence 16 bp long in the 3' untranslated region (3'UTR) (Majumder *et al.*, 1981). This essentially means that each *VSG* occupies a "cassette", with common upstream and downstream regions. These have been suggested as putative functional units for homologous recombination – 'expression cassettes' (Barry, 1997). This has important implications in terms of duplicative transposition.

There are several reported and suggested means by which *T. brucei* replaces the *VSG* in the BES, summarised below and in Figure 1.4, p. 19.

1.9.1 Duplicative transposition of a chromosomal internal gene into an active BES

The basic copy (BC) *VSG* genes are not expressed in their chromosomal location. They have to be copied and transposed into a BES, forming an expression linked copy (ELC), replacing the former ELC (Hoeijmakers *et al.*, 1980; Pays *et al.*, 1983a). Gene replacement occurs through recombination between regions of homology flanking both the BC genes and the BES, such as the 70-bp repeats. Several studies have revealed that the 70-bp repeats are not necessarily utilised in *VSG* switches (Donelson *et al.*, 1983; Pays *et al.*, 1983c; Lee and Van der Ploeg, 1987), although these studies involved monomorphic trypanosomes. Indeed when the 70-bp repeats in monomorphic trypanosomes were either deleted or inverted, there was seen to be no effect upon the incidence of *VSG* duplication into that BES (McCulloch *et al.*, 1997). In studies with pleomorphic trypanosomes, however, every observed activation was associated with the 70-bp repeat sequence

(Delauw *et al.*, 1987; Matthews *et al.*, 1990). In the most detailed study (Burton, 2003), the upstream conversion limit for independent activations of the same gene always mapped to the 70-bp repeats, and indeed occurred at several points along the repeat tract, indicating a homologous recombination reaction rather than a site-specific recombination event. This is in agreement with an experiment in which deletion of RAD51, an enzyme integral to the homologous recombination pathway in trypanosomes, yielded an approximate 10-fold reduction in *VSG* switching (McCulloch and Barry, 1999). Recombinational switching events were not entirely ablated, suggesting the existence of back-up pathways, an example of which has recently been identified (Conway *et al.*, 2002) (see 1.10 for more detail).

Duplicative transposition of silent *VSGs* can be considered the dominant mechanism in trypanosome antigenic variation, as it is the only route to activation for the chromosomal internal repertoire, which constitute the majority of *VSG* genes. It has been shown to be the predominant mechanism in non lab-adapted, pleomorphic trypanosomes in that it is the most frequent route utilised (Robinson *et al.*, 1999).

1.9.2 Duplicative transposition of a silent telomeric *VSG* gene into an active *BES*

This process involves the same principles as internal gene transposition. The duplication, however, can extend beyond the boundary of the expression cassette, resulting in the replacement of large regions of sequence, and possibly the whole telomere (De Lange *et al.*, 1983). This is probably an important mechanism for the array of minichromosomal *VSG* genes, which are located subtelomerically (Barry, 1997; Burton, 2003).

1.9.3 Reciprocal telomere recombination

Reciprocal telomere recombination involves exchange of the active site *VSG* with a *VSG* and other homologous sequences at another subtelomere (Pays *et al.*, 1985a). Neither copy is lost, the two merely exchanging places, with the new *VSG* entering the active expression site. Exchanges could in theory take place over a large region of the telomere, and indeed wide variation has been reported in size of fragments exchanged (Rudenko *et al.*, 1996; McCulloch *et al.*, 1997). This has been suggested to be a significant mechanism of *VSG* switching, although all reports have been in monomorphic trypanosomes. In pleomorphic trypanosomes switching at a much higher rate, this mechanism is likely to play only a minor role, occurring as it does at the rate of random mitotic crossovers.

1.9.4 *in situ switching*

In situ switching involves the switching off of one BES and the independent switching on of another. This mechanism is different from all other models for *VSG* switching in that it does not involve recombination, and is a transcriptional switching event. It was deduced from early studies that in the majority of switching events there was duplication of the BC into the ELC, but in several cases the gene was activated without a detectable duplication occurring (Myler *et al.*, 1984a). It was notable that in these instances the activated gene was subtelomeric (Bernards *et al.*, 1984; Myler *et al.*, 1984b). The molecular basis behind this means of switching is unclear, and although in some of these switches in monomorphic trypanosomes there is evidence of DNA rearrangements upstream of the BES promoter (Gottesdiener *et al.*, 1992; Navarro and Cross, 1996), there was no discernable pattern, and an analysis of nine independent *in situ* switches could not detect any DNA rearrangements (Horn and Cross, 1997). Obviously a synchronous switching on of the silent BES and switching off of the active BES is desirable, first of all to prevent the loss of surface coat, and also to prevent any immune recognition of the 'old' *VSG* due to the presence of a mixed coat (Munoz-Jordan *et al.*, 1996; Chaves *et al.*, 1999). *In vitro* studies have shown the activation of two BESs, and hence expression of 2 *VSG*s on a single trypanosome (Cornelissen *et al.*, 1985; Baltz *et al.*, 1986), suggesting independent activation of each BES. However it has recently been shown that this 'double expressor' state is an unstable one, and when artificially maintained, by the introduction of drug resistance genes and growth under selection, expression seemed to switch rapidly between the BESs (Chaves *et al.*, 1999).

1.9.5 *Mosaic gene formation (segmental gene conversion)*

Mosaic gene formation involves the construction of a novel *VSG* by the recombination of two or more segments from *VSG* genes or pseudogenes. It is thought to occur later in infection, when the chronically infected host has generated effective immune responses to all of the conventionally generated variants (Thon *et al.*, 1989; Kamper and Barbet, 1992). It has been demonstrated that parasites later in infections have assembled functional ELCs from a combination of coding regions, producing an antigenically novel and therefore protective coat (Thon *et al.*, 1990; Kamper and Barbet, 1992). This has been suggested as a mechanism for extending the repertoire and providing an additional source of antigenic variation (Kamper and Barbet, 1992). The extent of recombination can vary from replacement of part of one *VSG* with another (Pays *et al.*, 1983d), leaving a chimaera in

place, to the construction of composite genes from several pseudogenes. The utilisation of pseudogenes to construct new *VSGs* (Roth *et al.*, 1986; Roth *et al.*, 1989) is likely to be more significant. The large pool of pseudogenes, as revealed by analysis of the trypanosome genome project (L. Marcello, P. Burton and J.D. Barry, pers. comm.), is potentially a huge resource for expansion of the antigenic repertoire, especially as none of the pseudogenes will have been expressed, and the only route to activation is recombination into the expression site as part of a composite gene. This has significantly revised upwards the estimates of significance, and frequency, of this method of *VSG* switching.

1.9.6 Point mutations

It has also been proposed that the generation of point mutations within the ELC, giving rise to antigenically distinct epitopes, is an important mechanism (Rice-Ficht *et al.*, 1982; Lu *et al.*, 1994). Studies have shown generation of point mutations altering surface epitopes (Baltz *et al.*, 1991), although this was achieved under drug selection pressure. A more recent study has indicated, however, that point mutations are rarely generated during gene conversion (Graham and Barry, 1996) and that the higher number reported in earlier studies were artefactual, and due to the drug selection pressure imposed. There is also the argument that even many point mutations are unlikely to lead to the changing of all exposed epitopes, and therefore it is doubtful that it is a significant mechanism of antigenic variation.

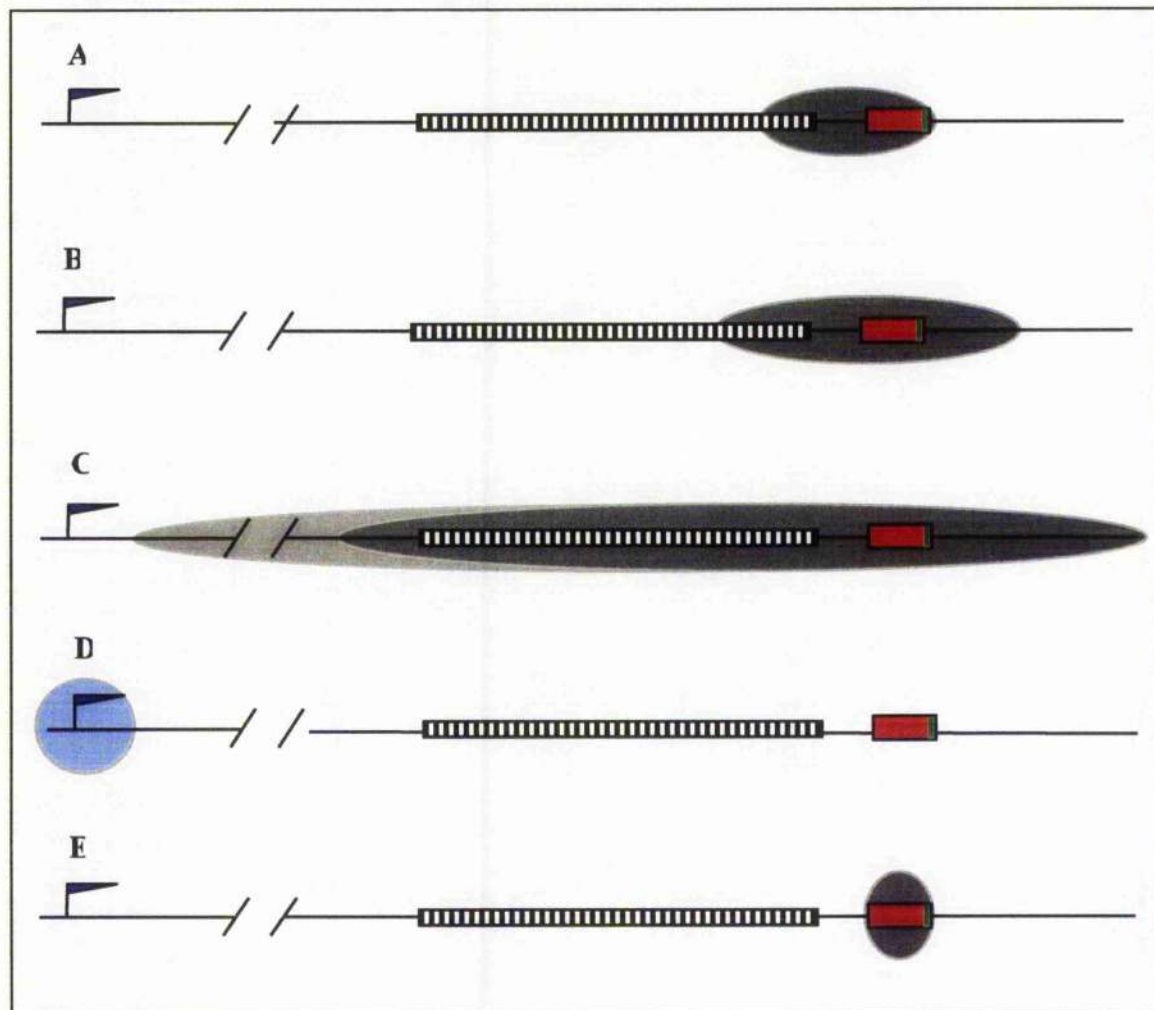


Figure 1.4 Mechanisms of VSG activation. A; Duplicative transposition of VSG involving only the 'expression cassette'. B; Duplicative transposition of telomeric VSG, with boundaries extending beyond the expression cassette. C; Reciprocal telomere exchange, with recombination boundaries varying across the length of the expression site. D; *in situ* promoter switch. E; Mosaic gene formation. The VSG is depicted in red, the 70-bp repeats with vertical hatching, the promoter region as a blue flag. The vertical green bar represents a region of shared homology at the end of VSGs, the 3'UTR. Grey shading indicates the boundaries of potential exchange/recombination. Blue circle indicates promoter switching.

1.10 DNA recombination and antigenic variation in trypanosomes

The fact that the majority of the *VSG* repertoire has the requirement to undergo a recombination reaction in order to be activated indicates the importance of this mechanism in the trypanosome antigenic variation system. Homologous recombination has been demonstrated to be a major driving force. This is evidenced by the reduction in switching observed in *RAD51* knockouts (McCulloch and Barry, 1999). The null mutants were also more sensitive to DNA damaging agents, methylmethane sulfonate and 3-aminobenzamide, indicating impairment in the ability to repair damage to DNA by homologous recombination. In a second study using the same knockout trypanosomes, there was a 10-fold reduction in their ability to integrate transformed DNA constructs *in vitro*, which is consistent with homologous recombination mediating DNA integration (Conway *et al.*, 2002). In addition, in both monomorphic (Liu *et al.*, 1983) and pleomorphic trypanosomes (Burton, 2003) the extent of homologous flanking sequence used in switching reactions varies, which resembles homologous recombination rather than a site-specific reaction.

RAD51 mediated homologous recombination does, however, require large stretches of homology, greater than 100 bp in *Saccharomyces cerevisiae* (Ira and Haber, 2002). The stretches of homology upstream of the *VSGs*, to which all conversion limits map in pleomorphic trypanosomes (Shah *et al.*, 1987; Matthews *et al.*, 1990; Burton, 2003), the 70-bp repeats, are degenerate however, and vary in both their length and extent of homology (Burton, 2003). The efficiency of *RAD51* mediated reactions in *S. cerevisiae* is much reduced by sequence heterology, a 9 bp heterologous insert leading to ablation of the ability of *RAD51* to bypass, or ignore, the heterology and continue the reaction (Holmes *et al.*, 2001), and in fact the presence of *RAD51* has a negative impact upon recombination using only short regions of homology (Ira and Haber, 2002). Therefore, the recent identification of a second pathway of homologous recombination in trypanosomes, which does not involve *RAD51*, and that requires only small stretches of homology – as little as 7-13 bp (Conway *et al.*, 2002), may be highly significant with regards to *VSG* switching. This requirement for only short stretches of homology could mean that this pathway could play an important role in the production of mosaic genes. Characterisation of the pathway

and identification of the components is obviously necessary before any conclusions can be drawn.

1.11 Trypanosomes and disease

1.11.1 African trypanosomiases

Evans in 1880 was the first to report the association between the presence of trypanosomes in the blood of the host, and disease. He noticed trypanosomes in the blood of camels and horses in India, afflicted by a disease known as 'surra'. Soon afterwards Bruce linked trypanosomes with 'nagana' (from the Zulu nakana, or 'tsetse fly disease') in Southern Africa. Since these seminal observations trypanosomiasis has been described in many host species, and the diseases caused and parasites themselves have become the objects of great interest and research.

Trypanosomiasis in humans occurs in two broad categories. A South American illness, Chagas' disease, is caused by *Trypanosoma cruzi*, which is transmitted by reduviid bugs. The African disease, sleeping sickness, is caused by 2 subspecies of *T. brucei* and transmitted by *Glossina* species, or tsetse fly. The 2 subspecies of *T. brucei* are loosely defined by geography and pathology. *Trypanosoma b. rhodesiense* largely occurs in East and Southern Africa, whereas *Trypanosoma b. gambiense* occurs in Western Africa. The course of disease caused by *T. b. rhodesiense* is characteristically short and acute, and *T. b. gambiense* causes a more chronic syndrome.

In Africa, tsetse flies, trypanosomes and wild animals have existed and evolved together for millenia. This evolutionary co-existence has led to a balanced relationship. In contrast, domestic animals are a relatively recent phenomenon on the African landscape. *Bos indicus* breeds – humped cattle e.g. Zebu – are thought to have reached East Africa as recently as 700 AD, along with sheep and goats, with the Arab invasion (Leak, 1999). Therefore the evolutionary relationship has not evolved to an equilibrium state, and disease is frequently the outcome of infection by trypanosomes. Certain African *Bos taurus* breeds, such as N'Dama and West African shorthorn, are thought to be descended from cattle introduced into North-East Africa from 4,500 BC onwards (Leak, 1999). This longer relationship with the trypanosome offers a simplistic explanation for the trypanotolerance exhibited by such breeds.

With reference to cattle in particular, there are 3 species of African trypanosome of concern. *T. vivax* and *Trypanosoma congolense* are the 2 species that cause severe disease among cattle, while *T. brucei* is less significant clinically (it should be noted that these classically ‘bovine’ trypanosomes are also capable of infecting goats, sheep, pigs, and equids – and in the latter all three cause serious disease [see Table 1.1]). The epizootiology of trypanosomiasis caused by these three parasites is largely linked to the distribution and prevalence of the tsetse vector. However, *T. vivax* has the ability to undergo mechanical transmission by Tabanid flies (Desquesnes and Dia, 2003). Mechanical transmission has facilitated the spread of *T. vivax* beyond the African continent to South America and Mauritius after the importation of infected cattle (Jones and Davila, 2001), and within Africa *T. vivax* can cause disease in the absence of tsetse.

As well as the above species causing disease in equids, *T. equiperdum* (very closely related to *T. brucei*; (Isobe *et al.*, 2003)) is the causative agent of ‘dourine’ or ‘slapsiekte’ in horses, mules and donkeys. The parasite is specific to these hosts, and is atypical in that it is venereally transmitted. This fact means that control of the disease is much simpler than those caused by the salivarian trypanosomes, because it does not rely on control of intermediate hosts, and it has been eliminated from many areas.

trypanosome	cattie	sheep	goats	pigs	horses	donkeys
<i>T. brucei</i>	+	++	++	+	+++	++
<i>T. congolense</i>	+++	++	++	+	++	++
<i>T. vivax</i>	+++	++	++	-	++	+
<i>T. evansi</i>	++	+	+	++	+++	++
<i>T. equiperdum</i>	-	-	-	-	+++	++
<i>T. simiae</i>	-	+	+	+++	-	-
<i>T. suis</i>	-	-	-	++	-	-

Table 1.1 Trypanosome species and host range (adapted from (Connor, 1994) key; + = mildly pathogenic, ++ = pathogenic, +++ = severely pathogenic, - = non-infective.

1.11.2 Pathogenesis

Typically, trypanosomiasis in cattle is characterised by chronic infections with relapsing parasitaemias, the major feature of the disease being anaemia. There is a range of conditions, however, extending from death within two weeks to full recovery. The factors influencing the course and outcome of infection are multiple: strain/species of trypanosome, breed of cattle, level of nutrition, and level of challenge (Morrison *et al.*,

1981). The most pathogenic species is *T. vivax*, with *T. congolense* also causing severe disease. *T. brucei* usually causes low grade chronic infections in cattle.

A typical course of disease sees the packed cell volume (PCV) of the cow progressively decrease by around 40 – 50% over the first 4 – 6 weeks. The PCV is a direct indicator of anaemia. During this phase, pallor of the mucous membranes can be evident, and superficial lymph nodes are sometimes palpably enlarged. Intermittent periods of pyrexia occur, particularly with instances of high parasitaemias early in infection. Despite this, animals usually continue to eat throughout the disease, although there is a gradual loss of body condition. After this initial stage, there can be several outcomes. The PCV can continue to fall, leading to death, or it can stabilise and be maintained, leading either to recovery and elimination of parasites, or death due to relapse of parasitaemia and clinical signs. As anaemia becomes more severe, there is a downward spiral, with the animals becoming further weakened and eating less, and in terminal stages some are unable to rise. The cause of death is congestive heart failure, resulting from a combination of anaemia, myocardial damage, and increased vascular permeability, exhibited in latter stages as jugular pulsation, bradycardia and subcutaneous oedema (Morrison *et al.*, 1981).

Anaemia is the major factor in trypanosomiasis, and is common to trypanosome infections in many species. The exact process by which anaemia arises is poorly understood, probably involving a variety of mechanisms and complex pathogenesis. It broadly divides into 2 phases within the context of an infection. The initial phase (3–4 months in cattle) is due to a haemolytic anaemia caused by an increase in red blood cell destruction by the mononuclear phagocytic system (mainly in the spleen, but also in the bone marrow and lungs). This is due to there being a large proportion of damaged red blood cells, again the exact causes being poorly understood, but probably by a combination of the following: possible release of haemolysins by trypanosomes (probably not a specific virulence factor, but more likely to be fortuitously erythrocyte-lytic molecules); attachment of trypanosome antigens - the VSG has been suggested to migrate from the trypanosome to red blood cells (Rifkin and Landsberger, 1990); antigen/antibody complexes; attachment of complement components to the red blood cell surfaces; and the increased fragility of red blood cells during fever (reviewed in Connor, 1994).

1.11.3 Host response

The response of the host to trypanosome infection is critical to the outcome of infection. The majority of experimental work has been done in cattle with *T. congolense*, and mice

with *T. brucei*. There are distinct differences between the immunological responses of the two host species, and indeed the two parasite species behave differently, with *T. congolense* being an obligatory parasite of the intravascular compartment, whereas *T. brucei* exists in extravascular tissue foci as well as within the bloodstream. This makes direct comparison difficult, but there are several factors that are acknowledged to be common to trypanosome infections.

The host immune response can broadly be divided into 2 categories – innate and acquired. A large amount of research has looked into the innate arm of the immune response, particularly with respect to breeds or lines of animal that exhibit trypanotolerant traits. N'Dama cattle and C57BL/6 mice have both been shown to have an ability to control infections better than trypanosusceptible counterparts (Murray *et al.*, 1981; Morrison and Murray, 1985). Indeed, in the case of N'Dama cattle, there is evidence that this phenomenon is not restricted to trypanosomiasis, and the breed has shown to have increased 'tolerance' to tick borne diseases and dermatophilosis (Mattioli *et al.*, 2000). The exact mechanisms behind this are unclear, although it is suspected to be a multigene trait, and mapping is underway to identify candidate genes for further study, in both mice and cattle (Iraqi *et al.*, 2000; Hanotte *et al.*, 2003). Aspects of the innate immune response have been shown to be circumvented by trypanosomes. For example, the alternative pathway of complement activation, which is a first line of defence against microbes, has been shown to be activated only by parasites lacking a VSG coat, indicating the VSG somehow masks the parasite from this innate mechanism (Ferrante and Allison, 1983).

The most important mechanism for the host in controlling trypanosome infection is generally accepted to be humoral, or B-cell directed specific antibody responses, which target the VSG. This has been illustrated by protection being mediated by passive transfer of antibodies (Campbell and Phillips, 1976). The initial parasitaemic peak is mainly controlled by the IgM class of antibody. IgM levels rise rapidly initially (Luckins and Mehlitz, 1976; Musoke *et al.*, 1981), and concentrations seem related to parasite burden (Nielsen *et al.*, 1978). The IgM levels remain high throughout infections when measured. Production of the IgG isotype increases as the infection progresses and affinity maturation occurs, and the specificity and efficiency of this subclass increases with time (Musoke *et al.*, 1981). Experimentally, there is correlation with antibody response and size of inoculation. When cattle were inoculated with a range of irradiated *T. brucei*, from an inoculum size of 1×10^5 to 1×10^9 parasites, above a threshold of 1×10^7 trypanosomes there was complete protection against re-infection by homologous parasites (Morrison *et al.*, 1982a), partial protection with an inoculum of 1×10^6 parasites, leading to a delay in

onset of patent parasitaemia compared with the 1×10^5 inoculum that conferred no protection. In mice injected with a range of irradiated *T. congolense*, from 1×10^3 to 5×10^4 parasites, an inoculum of 1×10^4 parasites induced a detectable IgM response as measured by the single radial immunodiffusion assay – any dose greater than this induced both IgM and IgG antibodies (Morrison and Murray, 1985). The trypanolysis test, however, which is a more direct test of antibody efficacy *in vivo*, was used to assay the same serum responses, and only functioned with an inoculum 1×10^5 or over.

Interestingly, there has been evidence that antibodies are induced only by degenerating parasites, the suggestion being that only pleomorphic parasites that differentiate to the short stumpy life cycle stage can induce a viable specific immune response (Sendashonga and Black, 1982). Mice immunised with purified VSG (*i.e.* not live trypanosomes) produced VSG specific antibodies; the majority of these did not bind to surface-accessible epitopes on live trypanosomes however, but rather reacted with soluble VSG or VSG on acetone fixed smears (Black *et al.*, 1982; Sendashonga and Black, 1982). In contrast, mice infected with pleomorphic trypanosomes produced antibodies that reacted only with surface-accessible VSG epitopes (Sendashonga and Black, 1982). Infections with trypanosomes in mice with monomorphic trypanosomes did not produce detectable antibodies in serum, antibodies could not be detected bound to purified trypanosomes, and collected lymphocytes did not produce VSG-specific antibody-producing hybrids with myeloma cells (Sendashonga and Black, 1982). The obvious major difference between these two forms of parasite is the fact that pleomorphic trypanosomes differentiate to the short stumpy life cycle stage, which has a finite lifespan in the mammal unless taken up by the tsetse vector. These data suggest that B cells in infected animals respond to fragments of trypanosome on which the VSG organisation is intact, probably derived from the senescent short stumpy parasites. However, there has been no subsequent verification of these findings.

Cytokine responses in infected animals are also important, particularly in controlling parasites in extravascular tissues, by the activation of macrophages (Hertz and Mansfield, 1999). The key cytokines involved are interferon γ (IFN γ) and tumour necrosis factor α (TNF α). *T. b. gambiense* induced dose-dependent production of TNF α by macrophages *in vitro*, and there was also a dose-dependent direct lytic effect of TNF α on bloodstream trypanosomes *in vitro*. However, this direct lytic effect was ablated by the addition of red blood cells, suggesting that it plays a role in the extravascular compartment. The addition of anti-TNF α antibodies to culture increased the number and lifespan of trypanosomes

(Daulouede *et al.*, 2001). IFN γ is thought to be the main mediator of TNF α production by macrophages *in vivo*. By analysing *T. b. rhodesiense* infections in IFN γ knockout mice, it was deduced that resistance was associated with the ability to produce IFN γ (Hertz *et al.*, 1998), and it has since been elucidated that the induction of nitric oxide, which is important in combating other microorganisms, is not the pathway via which IFN γ confers resistance in trypanosome infections (Hertz and Mansfield, 1999; Millar *et al.*, 1999). However, the complex interplay and pathways by which cytokines function are not clear with respect to trypanosomiasis, and indeed cytokine profiles differ between mammalian species (see (Defresne, 1998; Goddeeris, 1998)), so the validity of extrapolating between *in vitro*, mouse and cattle models in this case is questionable.

Elimination of trypanosomes from the bloodstream is most likely to be due to antibody-mediated complement lysis. One study has shown the accumulation of radio-labelled trypanosomes in the liver following passive immunisation by injection of hyperimmune serum (MacAskill *et al.*, 1980), suggesting VSG specific antibody opsonisation and phagocytosis by Kupffer cells (a subset of macrophage) in the liver. It is possible, however, that this was due to phagocytosis of 'ghosts', already lysed by antibody mediated lysis. The precise role played by antibody mediated complement lysis in trypanosome removal during *in vivo* infections is unclear. This process would involve the antibody binding to its VSG surface epitope target, and initiating the complement cascade, the end of which leads to pore formation in the trypanosome plasma membrane, perforation and lysis of the parasite. There is circumstantial evidence for this process playing a role, as there is marked reduction in complement component levels during infection, which coincides with parasite peaks (Nielsen *et al.*, 1978). In addition, there are high IgM levels associated with trypanosome infection (Luckins and Mehltz, 1976; Musoke *et al.*, 1981), and cattle IgM binds complement 10 – 20 times as efficiently as IgG (Goddeeris, 1998). Moreover, microscopic analysis of blood collected at remission of parasitaemia reveals many trypanosome ghosts (J.D. Barry, pers. comm.). These observations combine to suggest that antibody mediated complement lysis is the most likely route of trypanosome removal by the host immune system.

1.12 Hierarchical VSG expression

Following inoculation into a new host by tsetse bite, trypanosomes rapidly differentiate to the long slender bloodstream form. At this point, the trypanosomes are expressing VSGs from among the metacyclic subset, and continue to do so for the first few days of infection.

After approximately 5-7 days the MESs are silenced and expression switches to the BESs and the bloodstream variable antigen type (VAT) repertoire (Barry and Emery, 1984). It has been noted that one of the first VATs to appear is the one that was ingested by the fly (Hajduk, 1984). Almost certainly this is due to reactivation of the BES that was active in some of the ingested trypanosomes. The subsequent characteristic infection profile of pleomorphic trypanosomes is of an initial high magnitude and relatively short duration parasitaemic peak, giving rise to intermittent relapse peaks, decreasing in both parasitaemia and timespan as the infection progresses. The interval between peaks also increases with time. Each peak consists of several sub-populations expressing different VATs. This profile is particularly typical of infections in cattle and humans. When the same strain was compared across several host species (*T. vivax* in mice, rats, rabbits, goats and cows), the profile and course of infection differed quite markedly (Barry, 1986). The parasitaemia differed in the timing of emergence, number of parasites, and size of relapse peaks. The progression of infection is undoubtedly a product of many factors, both associated with the host and trypanosome. A major factor is thought to be the semi-predictable progression through the VSG repertoire. This staggered usage of VSGs prevents expression of, and the onset of immune responses against, a majority of the VATs over a short period, and rapid exhaustion of the repertoire. It optimises use of the range of VATs available, and is probably a key to extending the chronicity of an infection. The following paragraphs concentrate on the major studies addressing this phenomenon.

The observation of a loosely fixed hierarchy of expression of variants within the period of replicate infections was made as long ago as 1965 (Gray, 1965). Gray noted the "semi-predictable" order of appearance of different antigen types in tsetse transmitted *T. brucei* infections in rats, with some VATs being expressed more frequently, one in particular being frequently activated and 'predominant', and detected in all infections that were followed. This was termed the "basic strain antigen" and, in the absence of immune pressure, stocks expressing other VATs reverted to this type, thus confirming the "reversibility" of antigenic variation. Although the method of comparison between VATs, by cross-referencing trypanosome samples at infection timepoints with variant-specific antiserum and using agglutination as the discriminatory criterion, does not take into account either the number of VATs the cross-reference antisera was directed against, or the switching rate of the trypanosomes (the latter not feasible at the time), the agglutination test is highly specific, and even if identifying sub-groups of antigens rather than individual VSGs, as was probably the case, this study still illustrated very neatly the repeatability of the hierarchy. It also is quite unusual in using tsetse transmission (in order to address the

question of whether the tsetse cycle affected the observation of hierarchy), as most subsequent studies have used syringe inoculation.

In a similar study (McNeillage *et al.*, 1969) looking at relapse populations in *T. brucei* infections in mice initiated with a clonal trypanosome line, it was also noted that certain antigen types were represented more often than others, and that relapse populations were mixed. In particular it was observed, again by cross agglutination test, but this time against clones grown up from the first relapse peaks, that the same variants appeared in separate infections initiated with two clones, one derived from the other but several passages removed, indicating a maintenance of the hierarchy through generations. In this case it was hypothesised that the reason for some variants to appear more often was in fact differential virulence, and therefore inequality in the ability of the respective variants to grow in mice.

Van Meirvenne (Van Meirvenne *et al.*, 1975) analysed the composition of first relapse peaks in mice and rabbits infected with clonal *T. brucei* stabilates with defined lineages, by cross-referencing specific antisera using immunofluorescence and trypanolysis, and noted that many of the antigens were shared, but only within related trypanosome strains. Clones from two different lineages were examined, and it was seen that there were no shared antigens. Based on this study, the term 'serodeme' was developed to describe a "set of antigenic variants which can all be derived one from the other". It was concluded that the variants isolated from each strain of parasite represented the 'predominant' antigen types of the respective strain, following the lead of Gray (1965).

Capbern isolated 101 different antigenic types during infection in 11 rabbits with *T. equiperdum*, and analysed the time of appearance of antibodies against each (Capbern *et al.*, 1977). These were arbitrarily separated into 3 categories – a) "early types" which appeared in the first three weeks of infection, b) "semi-late types" appearing slightly later, and c) "late types" which appeared only in rabbits that survived more than a month. The progression was again examined by the agglutination test, in this case with antisera raised against clonal trypanosomes. From this study several important observations were made. The variants appeared with a rapid frequency – new variants could be isolated every two to three days, and several variants could often be isolated at the same time. In addition, when infections were initiated with clones of parasite expressing different variants, which had been previously derived from the original "basic type antigen", the general progression of antigenic types was similar. Interestingly, the basic type antigen, BoTat-1, was isolated from the first relapse peak during all of the 6 infections initiated with descendent clones.

When the expression of several VATs of *T. brucei* was examined in relapse peaks in rats (Miller and Turner, 1981), it was again discovered that the VATs grouped into 3 main groups, designated as – a) “expressed in the majority of relapses”, b) “expressed occasionally” and c) “absent”. This was a more precise and extensive study, where previous experiments had by necessity examined VATs at the population level, Miller and Turner could identify individual trypanosomes, thanks to use of purified antibodies and specific immunofluorescence. This showed significant advantages, in that it allowed the identification of minor types by immunofluorescence, and in addition the ability to immunise with purified VSG minimised the possibility of cross reactive antibodies. From their results, Miller and Turner suggested that there were differences in the likelihood of one VAT switching to another, with the “order of priority” being in effect lineages of VATs, each of which determined the subsequent one. They also stated that this was independent of growth rate, although they did suggest that differential growth rate may be important in determining the major VAT in each parasitaemic peak (see 1.13 for further discussion of this idea).

Myler and colleagues examined the progression of VAT populations in irradiated mice (Myler *et al.*, 1985). In this study the “stability” of each VAT was calculated by immunofluorescence, with clonal lines switching to the “basic type” at different frequencies. It should be noted that in this case, the only VAT switched to from 5 different derived clones was the basic type. Again, VATs were divided into 3 categories – a) the basic type, and one derived clone that did not switch to any other VAT, b) one VAT that after 50 days had 90% of the population expressing the basic type, and c) 3 VATs that were rapidly replaced by the basic type. The growth rates of the different VATs were evaluated and found not to differ. Based on these results, the authors hypothesised that there were different “switch frequencies” determining the probability of one VAT switching to another. This study also emphasised the point made in earlier work, that antigenic variation is independent of the immune response as it occurs in the absence of immune selection, demonstrated by the fact that antigenic variation occurs *in vitro* in *T. brucei* (Doyle *et al.*, 1980).

Robinson and colleagues analysed the emergence of antibodies against several VATs over the course of *T. brucei* infections in four rabbits (Robinson *et al.*, 1999). It was proposed that VATs emerging early in infection have a higher probability, and hence frequency, of expression, than those emerging later. As a result of this, the predictability of the order is higher early in infection than later. This was even seen to be the case within relapse peaks, with VATs activated towards the end of the peak displaying a more irregular expression

pattern across the 4 rabbits than those present at the start. The genomic location and copy number of the *VSGs* isolated were elucidated, and the timing of expression was suggested to be linked with genomic location of the silent, or donor, *VSG* gene. All donor genes analysed in this series of infections were located subtelomerically, with the exception of ILTat 1.71, which was a chromosomal internal *VSG* that appeared relatively late in one infection (isolated on day 17). Studies in monomorphic lines have revealed that early switches predominantly involve subtelomeric genes (Pays *et al.*, 1983b; Aline *et al.*, 1985b; Liu *et al.*, 1985), while chromosomal internal genes were presumed to be expressed later in infection. Due to the internal repertoire representing a large proportion of the *VSGs* available to the trypanosome, it is thought they must play a major role in chronic infections. An earlier study examining the expression of an internal *VSG* (*VSG* 118 in 427 trypanosomes) in rabbit infections, had noted that the *VSG* was isolated at similar time points in 2 infections (days 18 and 20), and when clones were examined, the 118-expressing population had arisen from several independent activations (Timmers *et al.*, 1987). When the expression of *VSG* 118 in a rabbit infection was examined in more detail, it was observed that transcription (measured by Northern analysis) occurred from days 15 through 22, and the parasites were detectable by immunofluorescence during the same period, peaking in numbers at day 20 (Lee and Van der Ploeg, 1987). This subpopulation was also derived from several independent activations. These data combine to suggest that *VSGs* tend to appear within a relatively restricted timeframe, dependent upon their genomic locus, and arise through multiple switching events.

Mosaic gene formation is a rarely detected event, and therefore likely to become of importance once the internal gene repertoire effectively becomes exhausted, due to the presence of antibodies against the gene products (Kamper and Barbet, 1992). However it must be noted that the high predominance of pseudogenes in the internal repertoire of TREU 927 trypanosomes (L. Marcello, P. Burton and J.D. Barry, pers. comm.) suggests mosaic gene formation may be of more import than previously assumed.

1.13 Mathematical models

Theoretical modelling is a tool that has been utilised in order to try and explain the mechanisms that drive the hierarchical expression of *VSGs*. There have been few models that have successfully fused biological knowledge and data with modelling inputs and outcome. This is due to the complexity of interactions involving both host and parasite, and the necessity to simplify the situation in order to model it.

An early hypothesis proposed that different antigenic variants differ in their intrinsic growth rates, leading to the faster growing variants appearing earlier in infection (Seed, 1978). It was concluded from Seed's study that rather than direct outgrowth, there was a level of inhibitive competition, as one clone in particular replaced another at a rate greater than the measured growth rates would have predicted. This progression was semi-predictable. There were, however, several flaws in this model. Two clones with the same serotype, which therefore presumably expressed the same VSG, had differing abilities to compete with 3 other variants, suggesting a phenotype not linked to the VSG. In addition, the possibility of switching events resulting in an increase or decrease in magnitude of VAT-expressing populations was not taken into account. The model proposing differential growth rates has been opposed on theoretical grounds (Kosinski, 1980), where randomly generated differences in growth rates were unable to simulate the *in vivo* picture (all other parameters were assumed to be non-variant). Indeed, it was felt that the differences in growth rates would have to be much greater than those observed to produce a realistic profile. Moreover, Kosinski suggested that "allowing each variant to generate only a limited range of other variants" could simulate the picture in the host. He allowed that significant competition between different VATs could be a mechanism, as could a range of differences in immunogenicity across VATs. Growth rates have been analysed experimentally (Myler *et al.*, 1985; Aslam and Turner, 1992), and no correlation has been found between the expression of different VSGs and differing growth rates. The differential growth of trypanosome clones undoubtedly will occur, but a relationship with the expression of individual VSGs is unlikely. A level of general inhibition has also been observed in *T. brucei* infections, when superinfections were undertaken in batches of mice and sheep, and the inhibition was determined to be independent of the stock of trypanosomes and the immune response, the suggestion being that it was most likely to be due to a decrease of the long slender form mitotic rate (Turner *et al.*, 1996). These findings constitute an aspect of trypanosome infections that deserves further study, as the phenomenon of inhibition could potentially be extremely important, in terms of chronicity of infections, interaction between parasites within the host, and potentially virulence.

Agur and colleagues (1989) suggested a model whereby the hierarchy may be due to differential immune responses to 'double expressor' trypanosomes that were in the transition between different VATs. In this model, switches occur at random, and a proportion of the switching events undergo a phase whereby two species of VSG are expressed on the surface of the trypanosome as one is in the process of being replaced by the other. Only by this being the case, and the double expressors having differential susceptibility to the immune response, could the ordered appearance of variants be

modelled. Different growth rates or switching probabilities were not sufficient. This model has also failed to gain support (see (Barry and Turner, 1991)), although it has not been thoroughly tested. Double expressor trypanosomes have been generated *in vitro* by inserting a second *VSG* into the active BES, and it was demonstrated that the expression of 2 *VSGs* simultaneously is not intrinsically harmful to the cell (Munoz-Jordan *et al.*, 1996). However, in a more pertinent study (Chaves *et al.*, 1999), whereby 2 drug-resistance genes were inserted into two different BESs, *VSG* double expression was maintained only under drug selection, and expression seemed to fluctuate between the two. It seems likely, therefore, that the double expressor is likely to be an unstable and transient intermediate.

Two models have also suggested that chronic infections characteristic of *T. congolense* in cattle were most likely to be explained by a gradual increase in levels of antibody to invariant antigens as the infection progresses (Antia *et al.*, 1996; Agur and Mehr, 1997). This is an unstudied hypothesis. The specific immune response is heavily directed towards the *VSG*, however, although the picture in later stages of chronic infections is not so well understood. Immune responses are generated against invariant antigens (Radwanska *et al.*, 2000b), but the effectiveness or otherwise of these responses has not been examined. It has been suggested that antibody responses are stimulated only by degenerating stumpy parasites (Black *et al.*, 1982), and in this scenario it is feasible that the invariant antigens normally concealed by the *VSGs* would be accessible to the immune system. However, the efficacy or otherwise of these antibodies is not clear, and indeed it is difficult to envisage how the antibodies would be able to surmount the presence of the *VSG* barrier in intact living trypanosomes.

Frank (Frank, 1999) formulated a mathematical model that has been the most satisfactory to date in terms of incorporating current knowledge of trypanosome antigenic variation. Several factors were considered to be independent of the *VSG* – growth, antibody production and effect, and carrying capacity of the host. The one variable was varying the switching rate of one antigen type to another. Randomly varying switch rates did not work; instead only “small variations in the rate at which one antigen type switches to others” can “explain the observation of a loose hierarchy within an infection”, the variations determined by regions of homology between the *VSG* loci and flanking regions. Frank mentions specifically 70-bp repeats as being possibly important. The switching matrix formulated by Frank gives rise to a deterministic process, whereby because a *VSG* cannot switch back to itself, and is predicted to switch predominantly to one or two subsequent variants, which are then subject to similarly directed switching probabilities, the hierarchy is predictable and deterministic, with minor variations. The flaw in Frank’s model is that it

has a lack of reversibility, which should logically be inherent in a system based on sequence homology. For a more detailed examination and discussion of Frank's model see sections 5.2 (p. 109) and 6.5 (p. 143), and for in depth discussion of all models see section 5.2.

1.14 Aims of project

This project aims to dissect further the hierarchical expression of VSGs during *T. brucei* infections, with particular reference to the genomic locus of the silent VSG, and the flanking regions of the silent VSG.

This will be done by examining the timing of expression of a subset of single copy VSGs within replicate chronic infections in mice and cattle, using pleomorphic trypanosomes. Single copy genes are to be used in order that it is known from where the silent gene was duplicated. It is hoped that a representative group of VSGs can be identified, which incorporate all genomic environments that VSGs inhabit, namely bloodstream expression sites, metacyclic expression sites, minichromosomes and chromosomal internal arrays. This will enable the question of the influence of genomic locus on the timing of expression to be further studied. Further to this, characterisation of the flanking regions of the VSGs will be attempted, specifically with respect to the 70-bp repeats, in order to assess the role of flanking regions within the hierarchical expression.

A mathematical model will be formulated, with the assistance of Dr Katrina Lythgoe (University of Edinburgh), to simulate the *in vivo* dynamics of trypanosome infections, incorporating relevant and measured biological parameters, and building upon the valid foundations of the Frank model (1999). This will be used to further examine the findings and conclusions of the experimental work, and to assess the influence of genomic locus and homology upon the kinetics of chronic *T. brucei* infections.

Chapter 2

Materials and Methods

2 Materials and methods

2.1 Reagent abbreviations

BSA bovine serum albumin

CBSS Carter's Balanced Salt Solution (1 x): 0.023 M HEPES, 0.12 M NaCl, 5.41 mM KCl, 0.4 mM MgSO₄, 5.6 mM Na₂HPO₄, 0.035 M glucose, 0.05 mM phenol red, pH to 7.4.

DAPI 4,6-diamidino-2-phenylindole

DEPC Diethyl pyrocarbonate: use at 0.1 % to remove RNAase.

DMSO dimethyl sulphoxide

DNA deoxyribonucleic acid

dNTP deoxynucleoside triphosphate

FITC fluorescein isothiocyanate

HEPES N-[hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid]

PBS phosphate buffered saline (Sigma, Ltd.)

PMSF phenylmethylsulphonyl fluoride

PS phosphate/sodium chloride buffer (1 x) : 0.06 M Na₂HPO₄, 46 mM NaCl.

PSG phosphate/sodium chloride/glucose buffer (1 x) : 0.06 M Na₂HPO₄, 46 mM NaCl, 55 mM glucose, pH to 8.0.

RNA ribonucleic acid

SDS sodium dodecyl sulphate

SOB bacterial medium (per litre): 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl.

SOC SOB bacterial medium plus 20 mM glucose

SSC sodium chloride/sodium citrate solution (1 x): 0.15 M NaCl, 0.015 M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$

TAE Tris/borate/EDTA buffer (1 x): 0.04 M Tris base, 0.04 M glacial acetic acid, 1 mM EDTA

TE 10 mM Tris.Cl, 1 mM EDTA

TLCK $\text{N}\alpha$ -p-tosyl-L-lysine chloromethyl ketone

2.2 Routine handling of trypanosomes

The trypanosome stock used in the chronic mouse infections, EATRO (East African Trypanosomiasis Research Organisation) 795, was a field isolate of *Trypanosoma brucei brucei* collected from a cow in Uhembo, Kenya, in 1964. This was passaged in a mouse for 8 days, and, from this, clonal trypanosomes were grown up in mice for a further 10 days prior to stabilisation. This has resulted in a line of trypanosomes that is genotypically clonal, but phenotypically polyclonal with regard to VSG expression (meroclonal). The trypanosomes switches VSG at approximately 1×10^{-2} switches/cell/generation (Turner, 1997). The ILTat (I.L.R.A.D. [International Laboratory for Research on Animal Diseases] *Trypanozoon* antigen type) 1.2 expressor clone used in the cattle infections was derived from the EATRO 795 line. The ILTat 1.2 trypanosomes switches VSG at about 1×10^{-5} switches/cell/generation (Robinson, 1999). The lower switching rate derives from a number of *in vivo* passages, but the trypanosomes remain pleomorphic.

The mouse infections, undertaken in adult female BALB/c mice, were established by the intraperitoneal injection of approximately 1×10^6 trypanosomes (previously grown in an immunosuppressed BALB/c mouse infected with the EATRO 795 stabilate). The cattle infections were established in adult (approximately 18 months old) Friesian steers by intravenous injection of 1×10^6 trypanosomes (previously grown in an immunosuppressed Harlan MF1 mouse infected with the ILTat 1.2 stabilate). Clonal ILTat lines (ILTats 1.21, 1.22, 1.25, 1.64, 1.67, 1.71 & 1.73), which were used for generation of VAT-specific antisera and *in vitro* complement lysis assays, were grown up in an immunosuppressed ICR mouse from stabilates in liquid nitrogen storage.

Estimation of trypanosome numbers was routinely carried out as described (Herbert and Lumsden, 1976). If an accurate count was necessary, the trypanosomes were counted in the chamber of an improved Neubauer haemocytometer.

2.2.1 Host immunosuppression, trypanosome growth and collection, and stabilate preparation

Trypanosomes were grown from stabilate in ICR or BALB/c mice (Harlan UK) that had been immunosuppressed by cyclophosphamide treatment (250 mg.kg^{-1} body weight, Sigma) 24 h previously. Exsanguination was performed when appropriate by cardiac puncture into 5% sodium citrate anticoagulant in Carter's Balanced Salt Solution (CBSS) (0.15 ml 5% sodium citrate per 0.85 ml blood).

Stabilates were prepared routinely by immediately mixing the blood 2:1 with 22.5% v/v DMSO (in CBSS), and freezing the sample at -70°C over a 24 h period. The stabilate was then transferred to liquid nitrogen for storage. When a stabilate was required for infection, one tube of it was thawed rapidly at room temperature, mixed with 0.15 ml HMI9 medium (Hirumi and Hirumi, 1989) and injected as soon as possible into the immunosuppressed mouse.

For the harvesting of large numbers of parasites, the infection was established in 2 immunosuppressed ICR mice, and the blood was collected at a high parasite titre ($> 1 \times 10^8 \text{ .ml}^{-1}$), and then injected equally, intraperitoneally, into 3 Wistar rats (Harlan UK). The parasites were then harvested at the initial peak, typically 48 – 72 h later, by cardiac puncture exsanguination.

During the cattle experiment, 5 ml blood was taken each day from the left jugular vein into a vacutainer containing EDTA anticoagulant (Beckton Dickinson). 0.2 ml of each daily blood sample was inoculated into an adult Harlan MF1 mouse (ILRI), immunosuppressed 24 h previously by sub-lethal irradiation (600 Rads). If there was parasite growth, the mouse was bled by cardiac puncture when the parasitaemia reached approximately $1 \times 10^8 \text{ parasites.ml}^{-1}$. 0.2ml of this blood was passaged into a second immunosuppressed Harlan MF1 mouse, and this mouse was bled by cardiac puncture when its parasitaemia reached $1 \times 10^8 \text{ trypanosomes.ml}^{-1}$ (2 passages were necessary in order to fulfil legal requirements with regard to the import of cattle derived parasites to the United Kingdom). A stabilate was prepared from the blood of the second mouse immediately, by mixing blood with 20% glycerol in PSG at a ratio of 1:1. The stabilates were suspended for 24 h in the liquid

nitrogen tank above the surface of the liquid, and then transferred to liquid nitrogen for storage.

2.2.2 Trypanosome cloning

Trypanosome clones were isolated from blood as soon as possible after exsanguination of the mouse host, by micromanipulation (McNeillage *et al.*, 1969). A dilution of the trypanosomes was prepared in HMI9, and one-drop samples dispensed into the wells of a Terasaki plate (Greiner Labortechnik). The Terasaki plate was kept moist by lining the edges with wet tissue paper. The wells were examined using a microscope to identify those wells containing only a single trypanosome. 10 μ l of HMI9 was carefully pipetted into that well, and the contents were transferred by micropipette into a 1.5 ml eppendorf tube containing 100 μ l HMI9. The contents were drawn into a syringe containing 0.4 ml of air, and were injected intraperitoneally into an ICR mouse, using the air to push all the liquid from the syringe.

2.2.3 Preparation of blood smears for immunofluorescence and the collection of plasma for in vitro complement lysis

Thin-film blood smears were prepared by placing a drop of blood (approximately 5 μ l) on a glass microscope slide. The blood was spread along the length of the slide using a slide edge, and the smear left to air-dry. The smears were then fixed in acetone for 5 mins, air-dried, and stored at 4° C sealed in polythene bags containing silica gel.

Plasma was prepared from ICR mice injected with the ILTat clone stabilates. At the initial parasitaemic peak the mice were cured with cymelarsen, at 5 mg.kg⁻¹ body weight (Rhône Mérieux), and the blood was collected 72 h later (allowing sufficient time for the antibodies to be raised). The blood was centrifuged at 8,000 x g for 10 mins in a microcentrifuge to pellet the trypanosomes and larger blood components, and the supernatant fluid (= plasma) was collected. The plasma was stored at -20° C.

Plasma was collected every 4 days during the chronic infections in mice. A maximum of 15 μ l blood was taken per mouse every 4 days. Mouse tail blood was collected, by piercing the lateral tail vein with a surgical lancet, and drawing the drop of blood into heparinised capillary tubes (Hawksley & Sons Ltd), which then had one end sealed with Critascale® (Hawksley & Sons Ltd.). The capillaries were centrifuged for 3 mins in a micro-

haematocrit centrifuge (Hawksley & Sons Ltd), and were then broken just above the buffy coat using a diamond, and the plasma was expressed into a 0.5 ml eppendorf tube. The plasma was then stored at -20°C .

Plasma was collected daily during the chronic infections in cattle. Blood was taken into an EDTA vacutainer (Beckton Dickinson), and centrifuged at $8,000 \times g$ in a centrifuge for 10 mins. The supernatant was collected and stored at -20°C .

2.2.4 Purification of trypanosomes from blood

2.2.4.1 DEAE cellulose ion exchange chromatography

This purification process is based on the difference in surface charge between host cells and trypanosomes. Under certain pH and ionic strength conditions, the more negatively charged host cells will bind to the DEAE cellulose (DE52 cellulose, Whatman), and the less negatively charged trypanosomes pass down the column and are collected in the effluent (Lanham and Godfrey, 1970). The cellulose was initially resuspended in PS buffer ($1 \times : 0.06 \text{ M Na}_2\text{HPO}_4$, 46 mM NaCl), and the pH was adjusted to 8.0 by the addition of phosphoric acid (5%). The cellulose was cycled in several volumes of PS buffer until the pH stabilised at 8.0. The cellulose was then added to a column with a sintered glass disc (P1, BDH), and approximately 150 g wet cellulose was used per 10 ml of blood. The cellulose was allowed to settle, and several volumes of PSG buffer ($1 \times : 0.06 \text{ M Na}_2\text{HPO}_4$, 46 mM NaCl , 55 mM glucose , $\text{pH } 8.0$) were run through the column slowly. A disc of 3MM paper (Whatman), cut to the diameter of the column, was carefully placed on the surface of the cellulose. Blood was mixed 1:1 with cellulose, and layered on top of the paper disc using a pipette. A second 3MM disc was then placed on top of the blood/cellulose mixture. The 3MM discs helped maintain the packing of the cellulose column, and allowed the addition of buffer without disturbing the cellulose surface. PSG buffer was then layered on top of the second 3MM disc using a pipette, and was topped up as necessary, care being taken that the column did not dry out. The effluent was collected in a series of 50 ml Falcon tubes (Greiner Labortechnik) until no trypanosomes were detected by light microscopy. The effluents were centrifuged at $1,000 \times g$ for 10 mins, and the pellets were resuspended in 1 ml PSG. The suspensions were then combined, centrifuged for 10 min at $1,000 \times g$, the supernatant was poured off, and the pellet was stored at -70°C .

2.2.4.2 Percoll gradient

Percoll (Sigma, Ltd) permits separation of trypanosomes from blood cells on a density gradient by centrifugation. This method is not as efficient as the DEAE cellulose method in that there is more contamination with red blood cells. In the work described in this thesis, Percoll separation was used only for the isolation of trypanosomes in order to extract genomic DNA. The method involved the mixing of the blood and trypanosomes with a Percoll stock solution (100 ml of 100% Percoll with 8.55 g of sucrose and 2 g of glucose, adjusted to pH 7.4 with HEPES) at a 3 : 5 ratio, and then centrifugation at 1,700 x g for 15 mins at 4° C. Trypanosomes were then collected by pipette from an observable discrete band at the top of the gradient, and washed several times by centrifugation at 1,700 x g, and resuspension of the pellet in PSG buffer.

2.3 VSG purification

Trypanosomes were lysed at a concentration of 1×10^9 parasites.ml⁻¹, in trypanosome lysis buffer (10 mM NaP [93.2% v/v 0.1 M Na₂HPO₄, 6.8% v/v 0.1 M NaH₂PO₄] pH 8.0, 0.1 mM N α -p-tosyl-L-lysine chloromethyl ketone [TLCK] and fresh 1 mM phenylmethylsulphonyl fluoride [PMSF]). The trypanosomes were swirled for 5 mins at 35° C, activating the released endogenous phospholipase C (Cross, 1975). The solution was centrifuged for 15 mins at 15,000 rpm (F1010 rotor, Beckman), and the supernatant containing the released VSG was taken off. The protein was then collected by Fast Performance (DEAE cellulose) Liquid Chromatography (FPLC), using a Biologic LP machine (Biorad). The cellulose had been previously acid/base cycled, by sequentially passing 15 volumes of 0.5 M HCl and 15 volumes of 0.5 M NaOH through it, this process removing the endogenous proteases, before being recalibrated with 10 mM NaP buffer, pH 8.0.

2.4 Protein gel electrophoresis

Protein samples were fractionated and visualised on SDS-polyacrylamide gels. 10% acrylamide (Anachem) gels were made (Sambrook et al., 1989) between two glass plates. The gels were electrophoresed in 1 x SDS running buffer (0.18 M Glycine, 0.023 M Tris, 0.003 M SDS) at 200 V. The gels were washed briefly in water, and then stained by submerging in stain solution (0.25 g Coomassie brilliant blue R [Sigma] in 90 ml of methanol:water [1:1 v/v] and 10 ml glacial acetic acid), for 45 mins to 4 h. Gels were

submerged in destain solution (10% glacial acetic acid, 40% methanol) for 1-3 h, to allow visualisation of bands.

2.5 Serology

2.5.1 *In vitro complement lysis assay*

Trypanosomes were suspended in guinea pig serum (GPS)(Harlan) to 1×10^7 cells.ml⁻¹ (except clonal lines ILTats 1.25 and 1.73, which were suspended to 5×10^6 cells.ml⁻¹). The plasma isolated from the mice was diluted 1:10 and 1:50 in GPS. 5 µl of trypanosome dilution was mixed with 5 µl of serum dilution in a well of a Terasaki plate. After incubation at room temperature for 1 h, the well was examined by phase-contrast microscopy (Robinson *et al.*, 1999). The Terasaki plate was kept moist by lining the edges with agar. The extent of lysis was then determined, by counting 100 trypanosomes, destroyed cells appearing as ruptured "ghosts". The trypanosomes were also incubated for 1 h with GPS alone, as a negative control; with antiserum specific to the clonal line diluted in GPS 1:10 as a positive control; with antiserum specific to a heterologous clonal line diluted in GPS 1:10 as a further negative control. The assay was performed within 1 h of blood collection and the trypanosomes were stored on ice while the dilutions were prepared.

2.5.2 *In vitro agglutination assay*

This method was adapted from the original (Cunningham and Vickerman, 1962). Clonal trypanosomes of a VAT under study were grown up in an immunosuppressed mouse to approximately 1×10^8 parasites.ml⁻¹, and the animal was terminally bled by cardiac puncture. The trypanosomes were diluted to 1×10^7 parasites.ml⁻¹ in PSG 10% FCS (Phosphate saline buffer pH 8.0 with glucose and 10% foetal calf serum). These parasites were then mixed 1:1 with antibody (or plasma) diluted 1:20 in PSG 10% FCS in the wells of a Terasaki plate. The plates were incubated at room temperature for 1 h, and the wells were examined by light microscopy for the presence of agglutinated clumps of trypanosomes.

2.5.3 Immunofluorescence

Indirect immunofluorescence was performed at room temperature on acetone-fixed thin blood smears (Turner and Barry, 1989). Reference antisera, which had been derived in either mouse, rat or rabbit hosts, were used separately as the primary antibody. Prior to the assay the slides were marked with a hydrophobic paint-pen (Mark-Tex corp., BDH) to produce wells for antibody containment. In a humid chamber, the smears were rehydrated for 5 mins by adding PBS to the wells. Wells were drained carefully from one corner using a micropipette, then filled with 1% bovine serum albumin (BSA) in PBS as a non-specific blocking agent, and incubated for 15 mins. The slides were washed 3 times in PBS for 5 mins, drained and dried carefully between wells. Primary antibody diluted appropriately in PBS was added to the wells, and the slides were incubated for 1 h at room temperature. The slide was washed 3 times in PBS, and dried between the wells. Fluorescein isothiocyanate (FITC) conjugated antibody (anti-mouse, -rat, or -rabbit IgG) was then added to the wells, and incubated for 1 h at room temperature. The slides were then washed 3 times in PBS. Two drops of Vectashield® + 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc.) were added per slide, which was then covered with a coverslip, ensuring no air bubbles were present. The edges of the coverslip were sealed using clear nail varnish. Fluorescence was then determined by light microscopy using an arbitrary scoring system: - no fluorescence, (+) faint fluorescence, + slight fluorescence, ++ clear fluorescence, and +++ strong fluorescence. Positive controls were also performed using VAT specific plasma as the primary antibody.

2.5.4 Plate enzyme linked immunosorbent assay (ELISA)

A Maxisorp® 96 C-well plate (Life technologies) was coated with antigen by adding 200 μ l VSG solution (20 μ g.ml⁻¹, made up in 0.05 M carbonate bicarbonate buffer, pH 9.6 in PBS). The plate was left on a shaker overnight at 4° C. Following the overnight incubation, the coating solution was poured off, and the wells were washed 3 times with PBS-T (0.05% Tween 20 [Sigma] in PBS). 1% skimmed milk (Biorad) in PBS was added, and the plate was incubated on a shaker at room temperature 1 h, and the same washing procedure was carried out. The test serum was serially diluted in PBS-T, 200 μ l was added to each well, and the plate was incubated on a shaker for 2 h at room temperature. The plate was washed 3 times again, and 200 μ l of the appropriate peroxidase conjugated secondary antibody (Sigma), diluted in PBS-T, was added to each well. A second 2 h incubation at room temperature was carried out, and the plates were washed as above. One SIGMA

*FAST*TM OPD tablet, and one urea hydrogen peroxide/buffer tablet (Sigma), were then dissolved in 20 ml water (per plate). 200 μ l of this substrate was then added to each well, and the plate was left stationary at room temperature for 30 mins, by which time an orange-yellow colour developed in positive wells. The plates were then read at 450 nm in an automated plate reader (Dynex technologies).

2.5.5 Dot blot ELISA

Dilutions of VSG protein (100, 20 and 2 $\text{ng}\cdot\text{ml}^{-1}$) in PBS were set up, and 1 μ l samples were spotted onto nitrocellulose membrane filters (Hybond) and allowed to air dry. The membranes were placed in blocking solution (PBS 2% skimmed milk 0.05% Tween 20), and incubated overnight on a shaker at 4 $^{\circ}$ C. Following the overnight incubation, the membranes were left in the blocking solution for a further hour at room temperature. The blocking solution was then decanted, and primary antibody diluted in blocking solution was added, and the filters were incubated for 1 h at room temperature. The primary antibody was decanted, and the membranes were rinsed and washed three times in blocking solution, for 15 mins each at room temperature on a shaker. Secondary peroxidase conjugated antibody, diluted in blocking solution, was added, and incubated at room temperature on a shaker for a further 1 h. The membranes were then washed twice in blocking solution, once in PBS-T and finally in PBS, each time for 15 mins at room temperature. Supersignal ECL[®] solutions (Biorad) were mixed 1:1, and left for 5 mins. In a darkroom, the excess PBS was drained from the membranes, which were then placed on Clingfilm (Saran). Supersignal ECL solution was pipetted over the membrane, which was then left for 5-10 mins. The excess solution was drained off, and the membrane was wrapped in fresh Clingfilm. The membrane was then taped into an autoradiograph cassette, and exposed to autoradiograph film (Kodak) for an appropriate time (which is dependent on the degree of luminescence produced by any positive reaction), and the film was developed.

2.6 Cloning of PCR products and transformations of bacteria using TOPO vector (Invitrogen)

PCR products amplified using *Taq* DNA polymerase were suitable for use in the TOPO vector due to the production of a 3' adenosine overhang by this enzyme. Following separation on a 0.7% agarose gel, PCR products were gel purified using the QIAGEN gel extraction kit (following the manufacturer's protocol). 0.5 – 4 μ l of PCR product was

incubated with 1 μl of salt solution (1.2 M NaCl, 0.06 M MgCl_2), made up to a total volume of 6 μl with water. This was incubated for 5 mins at room temperature. 2 μl of the reaction was added to a 25 μl sample of TOP 10F⁺ (Invitrogen) cells, mixed gently, and incubated at 42° C for 45 s, and then immediately transferred to ice for 2 mins. 1 ml of SOC was then added, and the mixture was incubated for 1 h at 37° C. The cells were centrifuged for 1 min at 8,000 x g in a microcentrifuge, the supernatant was poured off, and the pellet was resuspended in 100 μl of SOC. This suspension of cells was then streaked over L-agar plates containing ampicillin at a final concentration of 100 $\mu\text{g}\cdot\text{ml}^{-1}$ (Sigma), and incubated overnight at 37° C. Single colonies were then re-streaked on fresh L-agar plates, with the same concentration of ampicillin. Single colonies from these second plates were then picked and used to inoculate 5 ml L-broth (also containing ampicillin at 100 $\mu\text{g}\cdot\text{ml}^{-1}$), and grown up overnight at 37° C. Plasmids were then prepared from 1.5 ml of the overnight culture using the Qiagen miniprep kit.

2.7 Gel electrophoresis and Southern blotting

2.7.1 Gel electrophoresis

Standard DNA separations were performed on 0.7% agarose gels (Seakem LE, BMA) run at 100V in 1 x TAE (1 x : 0.04 M Tris base, 0.04 M glacial acetic acid, 1 mM EDTA) buffer using a commercial 1 kb ladder as a size marker (Invitrogen). Genomic digests were electrophoresed on a 0.7% agarose gel run at 30 V overnight in 1 x TAE buffer. Gels routinely contained 0.2 $\mu\text{g}\cdot\text{ml}^{-1}$ ethidium bromide (EtBr) to facilitate visualisation of the DNA under UV light.

2.7.2 Genomic digestions

Genomic DNA (usually 1 μg) was digested overnight with the appropriate restriction enzymes, following the manufacturer's protocol (New England Biolabs). The products were then fractionated overnight by gel electrophoresis to ensure a high resolution of the bands, after which the DNA was transferred to a nylon membrane by Southern blotting.

2.7.3 Southern blotting

Prior to blotting, the agarose gels were stained with ethidium bromide, viewed and photographed on a UV transilluminator. The gel was then immersed in 0.25 M HCl for 15

mins (to nick the DNA), rinsed with distilled water, and immersed for a further 30 mins in denaturation solution (0.5 M NaOH, 1.5 M NaCl). The gel was rinsed briefly in distilled water and then placed in neutralising solution (1 M Tris-HCl pH 8.0, 1.5 M NaCl) for 30 mins. The DNA was transferred to a nylon membrane (Hybond-N) by wet blotting using 20 x SSC as the transfer buffer (Sambrook *et al.*, 1989), and blotted for 24 h. After transfer, the DNA was cross-linked to the membrane using an UV spectrolinker (Stratagene).

2.8 Probe manufacture and DNA hybridisation

2.8.1 Radiolabelling

The probes used in this study were manufactured from PCR products, specifically amplifying the N-terminal encoding sequence of the *YSG* from genomic DNA (for list of primers see Table 2.1, p. 49). These were electrophoresed on 0.7% agarose gels, excised and gel purified using the Qiagen gel extraction kit (following the manufacturer's protocol); radiolabelling was performed using the Prime-It II kit (Stratagene). Initially, 50 ng of purified DNA template were mixed with 10 μ l of random-sequence oligonucleotides and sterile, distilled water in a total reaction volume of 37 μ l. This mixture was then heated to 95-100° C for 5 mins, cooled, and centrifuged briefly. Afterwards, 10 μ l 5x primer buffer, 2 μ l α -³²P labelled dCTP and 1 μ l Klenow (5U. μ l⁻¹) were added in order, mixed carefully, and the reaction was incubated at 37° C for 5 mins. the resultant probes were then purified from the unincorporated nucleotides by passing them through NuncTrap columns (following the manufacturer's protocol, Stratagene). Once purified, the probes were denatured at 95° C for 5 mins before use.

2.8.2 Hybridisation

The nylon filters were wetted with distilled water and transferred to a glass hybridisation tube. Approximately 20 ml of Church-Gilbert solution (0.342 M Na₂HPO₄, 0.158 M NaH₂PO₄.2H₂O, 0.257 M SDS and 1 mM EDTA per litre) were added and the filters were prehybridised for a minimum of 1 h at 65° C. the purified, denatured probe was then added, and the hybridisation step was incubated overnight at 65° C. After this hybridisation step, the filters were washed at 65° C, rotating in an oven, progressively with the following series of dilutions: 5 x SSC, 0.1% SDS; 2 x SSC, 0.1% SDS; 0.2 x SSC, 0.1% SDS (50 ml solution used per 15 mins wash). The filters were then rinsed at room temperature in 0.1 x

SSC (without SDS), heat sealed in plastic, and placed next to a phosphorimager screen in an autoradiography cassette at room temperature for 4–48 h, depending on the strength of the signal.

2.8.3 Stripping of nylon filters

The nylon filters were stripped with boiling 0.1% SDS. The solution was poured onto the filters in a heat resistant container and allowed to cool to room temperature. The procedure was repeated again, after which the filter was rinsed in 2 x SSC and was ready for reuse. Following stripping, the filters were placed next to a phosphorimager screen (Fuji) in an autoradiography cassette at room temperature for 24 h for assessment of whether the procedure had been successful.

2.9 Isolation of genomic DNA

Trypanosomes were separated from blood on a Percoll gradient (maximum 1×10^8 parasites. ml^{-1}), and centrifuged at $1,620 \times g$ for 10 mins at room temperature. The trypanosome pellet was then resuspended in 1 ml lysis buffer (50 mM Tris pH 8.0, 1mM EDTA, 100 mM NaCl). 50 μl 10% SDS and 2.5 μl proteinase K (at $20\text{mg}.\text{ml}^{-1}$) were then added, and the preparation was incubated at 37°C overnight in order to lyse the trypanosomes and digest the proteins. The DNA was then subjected to phenol/chloroform extraction and resuspended in 50 mM Tris.Cl (pH 8.0), 1 mM EDTA, and stored at 4°C .

2.10 Phenol/chloroform extraction

The volume of the sample was adjusted minimally to 200 μl by the addition of TE buffer (10 mM Tris.Cl, 1 mM EDTA). An equal volume of phenol/chloroform (1:1 mixture) was then added and mixed thoroughly by inversion. The two phases were separated by centrifugation in a microcentrifuge at maximum speed for 10 mins, after which the aqueous layer was eluted and transferred to a new eppendorf tube. An equal volume of chloroform was then added and the tube contents were mixed by inversion. After centrifugation at maximum speed for 5 mins, the aqueous layer was eluted and added to 1/10 the volume of 3 M sodium acetate and 2 μl of glycogen (Boehringer Mannheim); 2 volumes of 99% ethanol were then added and the tube was mixed thoroughly. The tube was then transferred to -20°C for at least 20 min, after which the DNA was pelleted by

centrifugation at maximum speed for 15 min. The pellet was then washed in 1 ml 70% ethanol, air-dried, and resuspended in an appropriate volume of buffer (usually TE).

2.11 RNA isolation and reverse transcription

2.11.1 RNA isolation from blood

The isolation of RNA from blood containing trypanosomes was carried out in an identical manner for blood from cattle and mice. 200 μ l of blood were added to 1 ml erythrocyte lysis buffer (ELB, Qiagen), and incubated on ice for three periods of 5 mins, vortexing between incubations. The sample was then centrifuged at 320 x g for 10 mins, the supernatant and layer of haemoglobin were pipetted off, and the pellet was resuspended in 400 μ l ELB. A further centrifugation for 10 min at 320 x g was undertaken, the supernatant removed, and the clear pellet was retained. The pellet was resuspended in 350 μ l RLT buffer (Qiagen RNeasy kit) plus 1 % β -mercaptoethanol, and the RNA was isolated using the Qiagen RNeasy mini kit (following the manufacturer's protocol).

2.11.2 Reverse transcription

The RNA was treated initially with DNAase in order to remove any residual DNA that could create false positive results by PCR. 1 μ g RNA was incubated for 15 mins at room temperature with 1 unit of RNAase-free DNAase (Invitrogen) and 1 μ l 10 x DNAase buffer (Invitrogen), made up to a total volume of 10 μ l with DEPC-treated water. After the incubation, the reaction was terminated by the addition of 1 μ l 0.25 M EDTA, and a 20 mins incubation step at 65° C was carried out. cDNAs were generated by reverse transcription using the Superscript II® First-Strand Synthesis System for RT-PCR kit (Invitrogen). 1 μ l of 0.5 μ g.ml⁻¹ oligo dT primer was added to the 11 μ l DNAase-treated RNA solution, and heated to 70° C for 10 mins. The solution was cooled by transferring to ice, and 2 μ l 25 mM MgCl₂, 2 μ l 10 x PCR buffer, 2 μ l 0.1 M DTT, and 1 μ l 10 μ M dNTPs were added, and the solution was mixed and incubated at 42° C for 5 mins. 1 μ l of Superscript II reverse transcriptase (200 U. μ l⁻¹) was then added, and the reaction was incubated at 42° C for 50 mins (a routine negative control included an identical replicate reaction, where 1 μ l water was added in place of the reverse transcriptase, to control whether DNA had been removed by the DNAase step). Next, the tube was incubated at 70° C for 15 mins in order to heat inactivate the enzyme, followed by immediate cooling by

transfer to ice. The final step involved the addition of 1 μl RNase H ($3.8 \text{ U}\cdot\mu\text{l}^{-1}$), and incubation of the reaction for 20 mins at 37°C , to remove the remaining RNA and leave only cDNA, which was then ready for use as a substrate for PCR reactions.

2.12 Polymerase Chain Reaction

50 μl PCRs were routinely set up (except for the reactions using material from cattle infections; see below). This reaction volume comprised 5 μl 10 x PCR buffer, 3 μl 25 mM MgCl_2 , 1 μl 10 mM dNTPs (all components of the Superscript II[®] First-Strand Synthesis System for RT-PCR kit), 2 μl cDNA, 2 μl of both primers at 5 μM , and 0.5 μl (2.5 units) *Taq* polymerase (ABgene), the remaining volume being topped up with water. The thermal cycles used varied depending upon the particular PCR.

The PCRs carried out on the cDNA derived from samples extracted daily during the chronic cattle infections were set up in 10 μl reactions, in order to optimise use of the limited substrate across several PCRs. This reaction volume comprised 1 μl of both primers at 5 μM , 0.1 μl (0.5 units) *Taq* polymerase, 1 μl of custom-made 10 x PCR buffer (which gives final concentrations of 45 mM Tris HCl pH 8.8, 11 mM ammonium sulphate, 4.5 mM MgCl_2 , 6.7 mM β -mercaptoethanol, 4.4 μM EDTA pH 8.0, and 1 mM dNTPs [ABgene]), 1 μl cDNA and 5.9 μl water. Again, the thermal cycles used were dependent on the PCR involved.

The oligonucleotides used in this study are listed in Table 2.1 (overleaf).

oligonucleotide name	sequence (5'-3')
lbsl (splice leader)	GTTTCTGACTATATTG
tb3ut (3' UTR)	GTGTTAAAATATATCA
Tub for 2 (<i>T. brucei</i> beta tubulin)	AGCCAGGTACAATGGACTCC
Tub rev 2 (<i>T. brucei</i> beta tubulin)	CGTTCATGTTGGACTCTGCC
bov act for (<i>Bos taurus</i> actin)	GCGGCATTACGAAACTACC
bov act rev (<i>Bos taurus</i> actin)	TGGGAAGGCAAAGGACTTCC
1.21for	CAAAGGCAGGCGGTTCCGATC
1.21rev	TGTTTGCTTTGCCATCCGCG
1.22for2	TCGAAAACGAAGTTTCCGCG
1.22rev2	ATCGTCTTGACATGGATCCC
1.25for	CACCAGAAGCATGCAAGGCG
1.25rev	TGCTCCATTTGCAACCATCG
1.64for	AAGCGGTAGCAGCAGCTGCG
1.64rev	GCGTCGTCTAGGGTACCGCG
1.67for	GAAGCGGAATATATGCAATC
1.67rev	GCCGTCCTGCTATGCCTGCG
1.71for	GGCAGTGTTTGCCTTAGCCG
1.71rev	TTGCCGGGTTGTTTCCGCG
1.73for	AACAAGCAGCAGCAAGCGGC
1.73rev	CTTGTTGAGGCTTTTCCGCG
1.2for3	AATCGCAAATACCGGCCTGG
1.2rev3	AAGGTTTGCTGTTGCATGCC
221 5'	CCGAATTCGCGATGCCTTCCAATCAGGAGGC
221 3'	CGCGGATCCGCTGTATCGGCGACAACCTGCAG

Table 2.1 List of oligonucleotides used in this study.

Chapter 3

Chronic mouse infections

3 Chronic mouse infections

3.1 Introduction

There have been a large number of studies of antigenic variation in *Trypanosoma brucei*. The majority have concentrated on the molecular mechanisms behind the switching process, and have been performed on monomorphic trypanosomes, which switch at a low rate, several orders of magnitude below that of pleomorphic trypanosomes. In addition, these strains are unable to initiate chronic infections *in vivo*, due to an inability to differentiate to the self-limiting short stumpy form. Monomorphic trypanosomes have a switch rate of between 1×10^{-6} and 1×10^{-7} switches/cell/generation, which is low enough to be attributed to background mutation and mitotic homologous recombination. Pleomorphic trypanosomes by contrast switch at 1×10^{-2} switches/cell/generation (Turner, 1997). This has led to questions about the significance of findings pertaining to antigenic switching in these lines (Barry, 1997).

Studies examining the hierarchical expression of VSGs generally have concentrated on first relapse peaks of parasites in rodents or rabbits (Gray, 1965; Miller and Turner, 1981). By necessity, pleomorphic trypanosomes have been utilised, as these lines have the ability to initiate chronic infections in these laboratory animals. Early studies deduced that there was "semi-ordered" expression of variants (Gray, 1965; Capbern *et al.*, 1977; Miller and Turner, 1981), whereby the same variants were observed to appear at similar timepoints within an infection timeframe. It has since been elucidated that genes activated earlier tend to be subtelomeric, and that duplicative VSG gene processes predominate (Liu *et al.*, 1985; Robinson *et al.*, 1999).

The aim of this investigation was to examine the timing of expression of specific variants in replicate infections in mice, using the high switching line of pleomorphic trypanosome, EATRO 795. No study investigating the dynamics and patterns of VSG switching has thus far been attempted with pleomorphic trypanosomes that switch at this rate (1×10^{-2} switches/cell/generation). The VSGs examined were single copy, so that any detection of a switching event could be positively linked to that particular gene, its genomic locus and surrounding flanking regions.

3.2 The VSG genes under study

An important facet of this study was the identification of single copy *VSG* genes. Preferably, these genes would be from a variety of genetic loci within the trypanosome genome, in order that the linking of the hierarchy with genetic locus of the silent donor *VSG* could be analysed. The genes had to be identified within the EATRO 795/ILTat lineage of trypanosomes. 6 single copy *VSGs* had been identified previously (Robinson *et al.*, 1999). These had been isolated from the first and second relapse peaks of a rabbit infected with ILTat 1.2. This was done by amplifying trypanosomes each day in mice and making stabilates of these 'amplifier' populations. From these, trypanosomes were cloned and grown in immunosuppressed mice. The clonal lines were then analysed; the *VSG* was cloned and sequenced by RT-PCR using *VSG* generic primers (tbs1 and tb3utr, see Table 2.1), and copy number was deduced by Southern blotting analysis. 11 VATs were isolated and analysed, 6 of which were single copy. Of these 6, 5 were subtelomeric - 1 occupied a BES, 2 were in silent MESs, 2 were minichromosomal - and 1 was chromosomal internal (see Table 3.1). It was arbitrarily decided that an ideal number of VATs to study would be a repertoire of 10, with some more examples of internal genes, as these were thought to be more important in the chronic stages of infection.

It was not possible, for logistical reasons, to carry out further chronic infections in rabbits in order to clone out the trypanosomes appearing later in infection. In addition, the ILTat trypanosomes are unable to establish chronic infections in mice. Although EATRO 795 trypanosomes do produce chronic infections, it is not possible to obtain trypanosome clones homogeneously expressing 1 *VSG*, due to the high switching rate. However, the aforementioned rabbit ILTat infection had been followed until midway through the third relapse peak (days 28, 29 and 30 post infection), and amplifier stabilates had been made. In the present study, 23 clones were isolated from these amplifier stabilates, and analysed. From RT-PCR, cloning and sequencing it was established that these clones were of 2 VATs, expressing previously unidentified *VSGs*. These novel *VSGs* were named ILTat 1.73 and ILTat 1.74.

The copy number of each *VSG* was elucidated by generating a PCR fragment from the N-terminal coding regions of the respective genes (for primers see Table 2.1, p. 49), and this was then used to probe Southern blots of ILTat DNA digested with enzymes (specifically *EcoRI*, *HindIII* and *PstI*). From these it was deduced that there were two copies of ILTat 1.74, and one of ILTat 1.73 (for Southern blots of ILTat 1.73, see Figure 3.1, p. 54).

Further to this, the location of the gene could be identified as either telomeric or internal. Genomic DNA from four lines of ILTat trypanosome, each with different passage history, was subjected to the same enzymatic digestion. These were probed with the same gene specific probe (see Fig. 3.1). The bands identified were in identical positions in all three digests in all four ILTat lines, indicating the ILTat 1.73 gene was chromosomal internal. The different passage history, and in effect age, of the three lines would be likely to have resulted in telomeres differing in size due to standard telomere tract dynamics, and if ILTat 1.73 was telomeric, this would have been reflected in different sized bands between the different strains. This was repeated on EATRO 795 procyclic genomic DNA (using procyclic gDNA guaranteed no confusion with expression-linked copies) to ensure there had been no change in copy number or locus position in this high switching line (Fig. 3.2, overleaf).

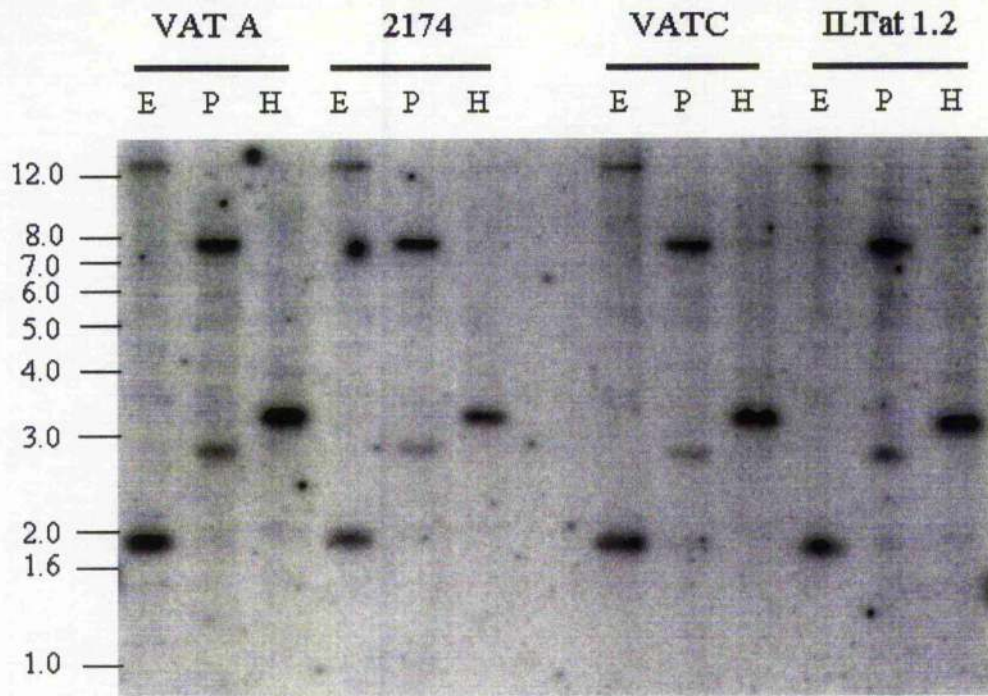


Figure 3.1 Southern blot illustrating single copy number of ILTat 1.73 VSG and possible chromosomal location in gDNA from 4 distinct ILTat populations (digests : E = *EcoRI*, P = *PstI*, H = *HindIII*; marker is 1 kb ladder).

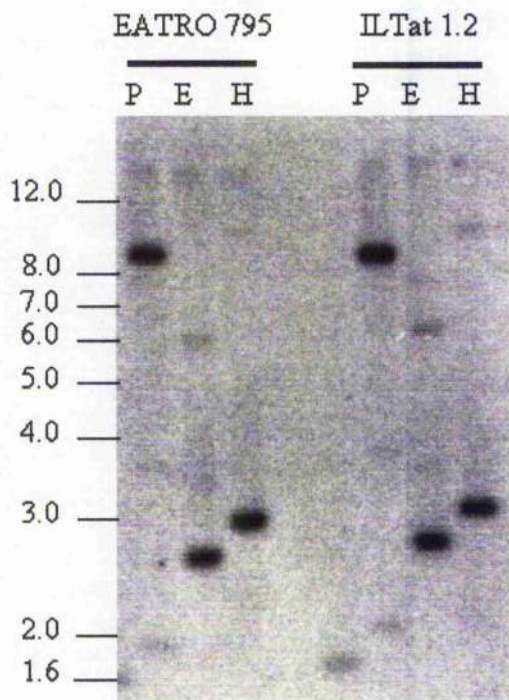


Figure 3.2 Southern blot illustrating single copy number of ILTat 1.73 in EATRO 795 pro-cyclic gDNA, and maintenance of fragment size when compared with ILTat 1.2 BSF gDNA (digests : P = *PstI*, E = *EcoRI*, H = *HindIII*; marker is 1 kb ladder).

Despite many attempts, no further single copy *VSGs* could be identified. Therefore the repertoire available for analysis was 7 single copy *VSGs*, 5 being telomeric and 2 chromosomal internal. However, there was at least 1 example of each type of *VSG* genomic locus represented. The only function of switching that cannot be addressed with the repertoire available is mosaic gene formation, which, as has been previously mentioned, may have significance later in infection. Therefore any conclusions with regard to the hierarchy or otherwise from this study can apply only to full length, or entire *VSGs*.

VSG	Genomic locus	Chromosomal location
ILTat 1.21	minichromosomal	telomeric
ILTat 1.22	MES	telomeric
ILTat 1.25	minichromosomal	telomeric
ILTat 1.64	MES	telomeric
ILTat 1.67	BES	telomeric
ILTat 1.71	internal	internal
ILTat 1.73	internal	internal

Table 3.1 Single copy *VSGs* and location within the trypanosome genome (MES = metacyclic expression site; BES = bloodstream expression site)

3.3 Assessment of methods of analysis

Much effort was spent assessing various methods of following the progression of *VSGs* through an infection, in particular ways of measuring the onset of the specific immune response to the respective VATs. This was important because of the limited amount of material available in chronic mouse infections – approximately 7 μ l of plasma every four days – due to humane considerations regarding continuous blood sampling. This limiting factor in itself was important to ensure no skewing of parameters, by undertaking analysis in an animal where pathology could be exerting an unmeasurable influence. However, the small amount of material meant a sensitive technique was required to elucidate the onset of

immune response to all 7 VATs. The methods examined were plate ELISA, dot-blot ELISA, *in vitro* agglutination assay, and *in vitro* complement lysis assay.

3.3.1 Plate ELISA

The plate ELISA technique was found to be both very sensitive, and very specific. At high dilutions of antibody (1:6561) there was differentiation between VSG species (see Fig. 3.3, p.57). At lower dilution there was cross-reaction, probably because the purified protein used as the immunogen contained the conserved C-terminus, a region that shares up to 40% sequence identity between VSGs (Carrington and Boothroyd, 1996). However there were envisaged disadvantages, in that the test runs were carried out using the hyperimmune sera from rabbits immunised with 1 VSG species – how the ELISA would function when analysing serum from mice infected with trypanosomes expressing many VSGs, in particular if assessing the immune response to minor variants, was not clear. The technique also required the purification of large amounts of VSG for use as substrate, a very time-consuming process. The biggest obstacle for the use of this technique however was the amount of primary antibody required for each test plate, which far exceeded that available from the chronic mouse infections.

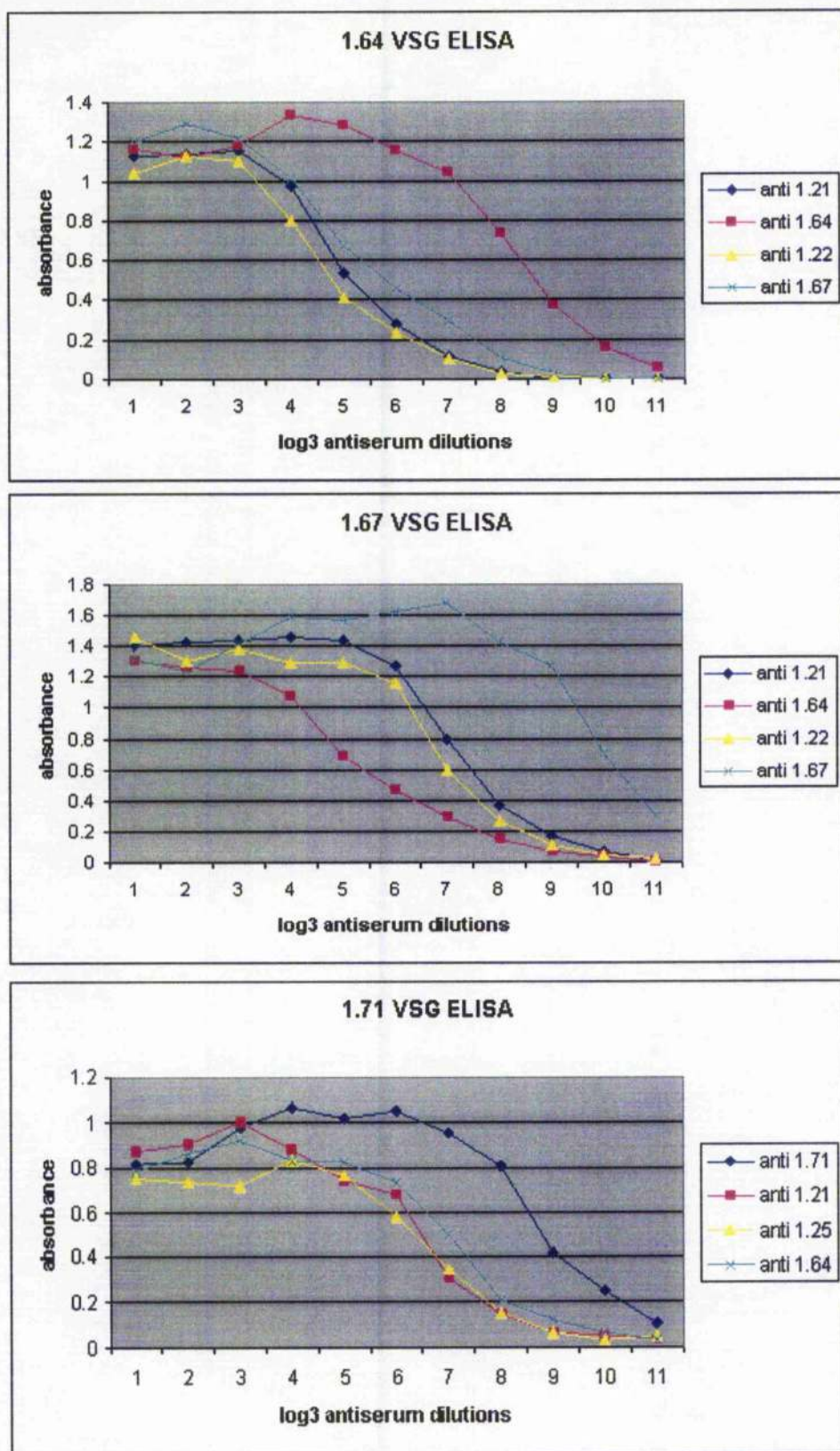


Figure 3.3 Examples of plate ELISA outputs, illustrating specificity, and efficacy at high dilutions of primary antibody ($\log_3 7 = 1/2187$, $\log_3 8 = 1/6561$). Absorbance measurements are arbitrary units. Title indicates VSG used as substrate.

3.3.2 *Dot-blot ELISA*

Dot-blot ELISAs use the same principle as plate ELISAs, but carried out on a smaller scale, and thus the suitability of this method was considered with respect to optimum use of limited amounts of serum. Again this technique was sensitive, the specific antiserum binding detectably at around 2 ng of protein. However in this case, there was a variable amount of cross-reaction, even at low concentrations of VSG (2 ng). Therefore it was concluded that the specificity of this technique was not high enough. Again, as specific immune serum was used, how efficient the technique would be at the beginning of an immune response, or against a minor variant, was not clear.

3.3.3 *In vitro agglutination assay*

Agglutination of cells is one of the properties of specific antibodies, and in particular IgM. Antibodies bind to the surface of a cell, and when two cells with bound antibodies of the correct isotype (IgM) come into contact, there is cross-linking. The clumping of such agglutinated cells provides a quick and visual result. Using the hyperimmune serum as a control, agglutination occurred very rapidly, and there was sensitivity to the level of 1:100 dilution of antiserum. However there was a variable amount of agglutination in the absence of antibody. The reason behind this is not known, but occurred repeatedly. An initial set of chronic infections was run, and the agglutination assay was used to analyse the onset of antibody to the 7 VATs under study. The results using plasma from infected mice were uninterpretable, with agglutination occurring from day 0. Thus the method was discarded as a possibility.

3.3.4 *In vitro complement lysis assay*

The basis of this assay is the main means by which specific antibody leads to the lysis of trypanosomes. The assay involves the antibody binding to its VSG surface epitope target, and initiating the complement cascade, the end of which leads to perforation of the plasma membrane and lysis of the parasite. This technique was very specific, with no cross-reaction observed, even at the high concentrations of antibody used. The specificity is probably due to the fact that the technique utilises live trypanosomes, and the only epitopes exposed are those that induce effective immune responses in *in vivo* infections. The technique also provides optimum use of the limited serum quantities available, and provides the scope to test all VATs plus controls. However, it is a relatively insensitive technique, and is extremely reliant on the quality of the complement – complement is

highly labile, and the method of preparation greatly affects its activity in this assay. A large amount of effort was expended in sourcing a reliable commercial product, the most consistently reliable being guinea pig serum that was frozen at -70°C immediately upon collection (Harlan). Taking everything into consideration, the *in vitro* complement lysis assay was the most favourable method for measuring the onset of the VAT-specific immune responses.

3.4 EATRO 795 trypanosomes

The EATRO 795 pleomorphic line utilised in this study is derived from a population originally isolated in the field from a Kenyan cow (Uhembo, Central Nyanza Province, Kenya; 1964). This was initially passaged for eight days in a mouse and, from that amplified population, single trypanosomes were further grown in mice for 10 days. There have since been a number of syringe passages, but what has resulted is a genotypically clonal but phenotypically polyclonal line with regard to VSG expression. The switching rate of this stabilate is at the high end of the trypanosome range – approximately 1×10^{-2} switches/cell/generation (Turner, 1997). The VATs under study were isolated from the ILTat 1.2 line. This line is itself derived from the EATRO 795 stock, and is isogenic with reference to the VSG repertoire. The ILTat 1.2 trypanosomes switch at a lower rate – approximately 1×10^{-5} switches /cell/generation (Robinson, 1999), which permits preparation of relatively pure VATs for testing the specific immune responses.

Trial infections were initiated in BALB/c mice with differing inoculum sizes; 10 , 10^3 and 10^6 trypanosomes (2 mice per inoculum), and the infections were followed daily until ill effects were observed, at which point the mice were euthanased.

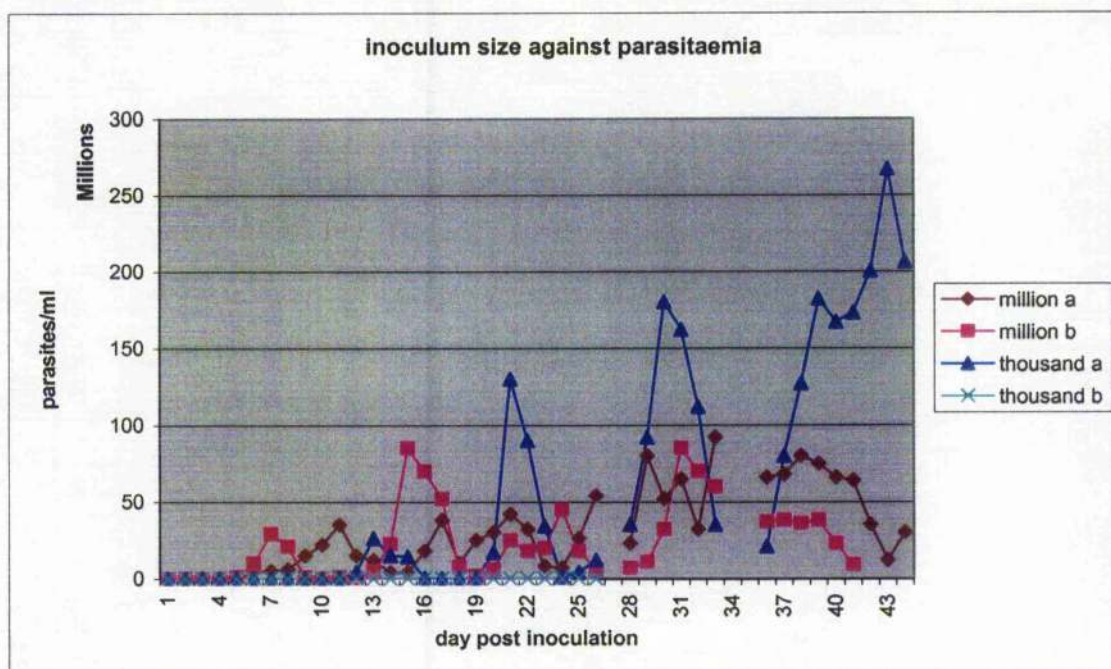


Figure 3.4 Graph illustrating the parasitaemias during infections in BALB/c mice initiated with differing inoculum sizes (10^3 and 10^6 parasites, in two mice, a & b, for each inoculum size) of EATRO 795 trypanosomes. (data for 10^2 inoculum not shown – no parasites detected).

The inoculum of 10 trypanosomes did not produce a patent parasitaemia as detected by haemocytometer (mice were checked daily until day 26 post infection). Only one of the mice infected with 10^3 parasites went on to develop a patent infection. This mouse had a parasitaemia that was first detectable on day 11, with distinct separation between peaks, and the peaks gradually increased in size until there was essentially a fulminant infection from day 38 onwards, and the mouse had to be euthanased. Both mice injected with 10^6 trypanosomes developed patent infections, which were first detectable on days 5 and 6 post inoculation. These had a different profile, with the individual peaks less distinct, and the magnitude being slightly less. In addition, the mice were able to control parasite numbers to a greater degree later in the infection, and indeed the parasite numbers decreased at a timepoint where the 10^3 inoculum infection overcame the mouse. It must be stressed that these were small sample sizes, but the results were convincing enough to draw conclusions. In summary, it was decided to use inocula of 10^6 trypanosomes in the chronic mouse infections, as this gave rise to chronic infections in both mice injected, and did not cause excess pathology that would lead to early termination of the experiment. It was also decided that the end-point of the experimental infections was to be 35 days, as this generally covered the initial peak of parasitaemia and at least 2 relapse peaks, and for

humane purposes, in order to minimise any effects of pathology due to long-term high parasitaemias.

3.5 Initiation and progression of infections

EATRO 795 trypanosomes were amplified from the stabilate WCMP (Wellcome Centre for Molecular Parasitology) 2665 in BALB/c mice that had been immunosuppressed by cyclophosphamide treatment 24 h previously. This stabilate had been prepared in a similar manner, by amplifying in a BALB/c mouse from stabilate WCMP 1041 (derived from ICR mice). Blood was collected by cardiac puncture at the first parasitaemic peak – approximately $1 \times 10^{7.8}$ parasites.ml⁻¹. At this stage of the infection the parasite population was predominantly (at least 80%) of the long slender bloodstream form.

The trypanosomes were incubated with antibody specific to the VSGs under study, and Guinea Pig Serum (GPS) to ensure that any subsequent appearance of these VATs had arisen from *de novo* switching events. To achieve this, the trypanosomes were counted on an improved Neubauer haemocytometer, diluted using GPS and separated into samples of 1×10^7 cells, with antibody concentrations of 10%. These aliquots were incubated for 1 h at 37° C. A second haemocytometer count was undertaken, and 1×10^6 viable cells were then inoculated via the intraperitoneal route into BALB/c mice. BALB/c mice were used because they are inbred, minimising differences due to host variance. In addition, BALB/c mice had been shown to be suitable hosts for chronic infections using EATRO 795 trypanosomes (C.M.R. Turner, pers. comm.). Each experiment used mice of the same batch, and in each case females of approximately the same age were used, to ensure uniformity of age and body size. As a control to test the efficacy of the pre-incubation with antibodies, the inoculum for one set of 8 animals (mice B2-J2; see Table 3.2, p. 65) was not antibody treated. In this case the VATs were detected in the initial parasitaemic peak; when incubated with antibody this was not the case, indicating that the removal of VATs under study from the inoculating population was necessary and was successful.

Parasitaemia was estimated every two days – 1 µl of blood being collected and mixed with 9 µl 0.85% ammonium chloride before being examined by improved Neubauer haemocytometer. The threshold of detection by this method was 1×10^5 parasites.ml⁻¹. Plasma was collected every fourth day.

The parasitaemic profiles of infected mice followed a general pattern. There was an initial peak almost uniformly on days 6-7. The size of this initial peak was however variable,

ranging from 1×10^7 to 2×10^8 parasites.ml⁻¹. Thereafter, there was typically a definable first relapse peak, occurring at around 14 days post infection, followed by a varying number of relapse peaks, which increased in duration and parasite burden, and the separation of which into peaks could become subjective. This period was the most variable between individual mice, in terms of both parasitaemia levels and peak duration. Only one mouse displayed a substantially different profile (mouse B3), there being a gradual increase of parasites, with no definable peak structure, until termination at day 21 post-infection. Otherwise the gradual increase in parasite load generally led to the termination of infection at around day 35 for humane reasons. Mice were also euthanased if the parasitaemia remained above 1×10^8 parasites.ml⁻¹ for two consecutive counts (effectively at least three days). All deaths were due to euthanasia; there were none from fulminant trypanosomiasis. The profiles of parasitaemia are notable in several ways. It was expected that the profiles would be very similar between mice, as seen in previous infections (J.D. Barry, pers. comm.). However, very few large-scale (in terms of number of mice) chronic infections have been carried out with these highly pleomorphic trypanosomes. It is likely the extremely high switching rates lead to variable infection profiles, following a predictable initial period of parasitaemia.

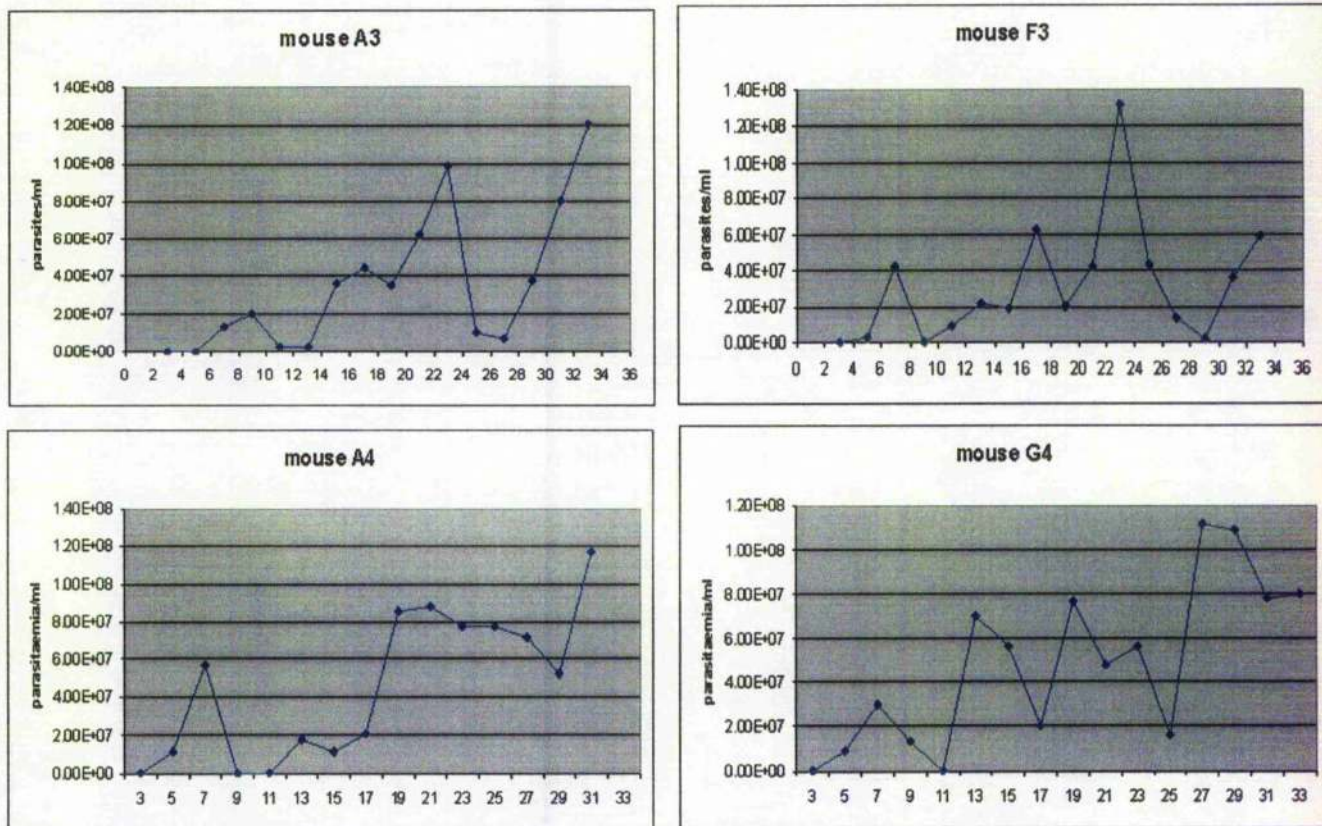


Figure 3.5 Examples of EATRO 795 parasitaemic profiles of selected infections in BALB/c mice. Values on the X axis indicate the number of days post-inoculation.

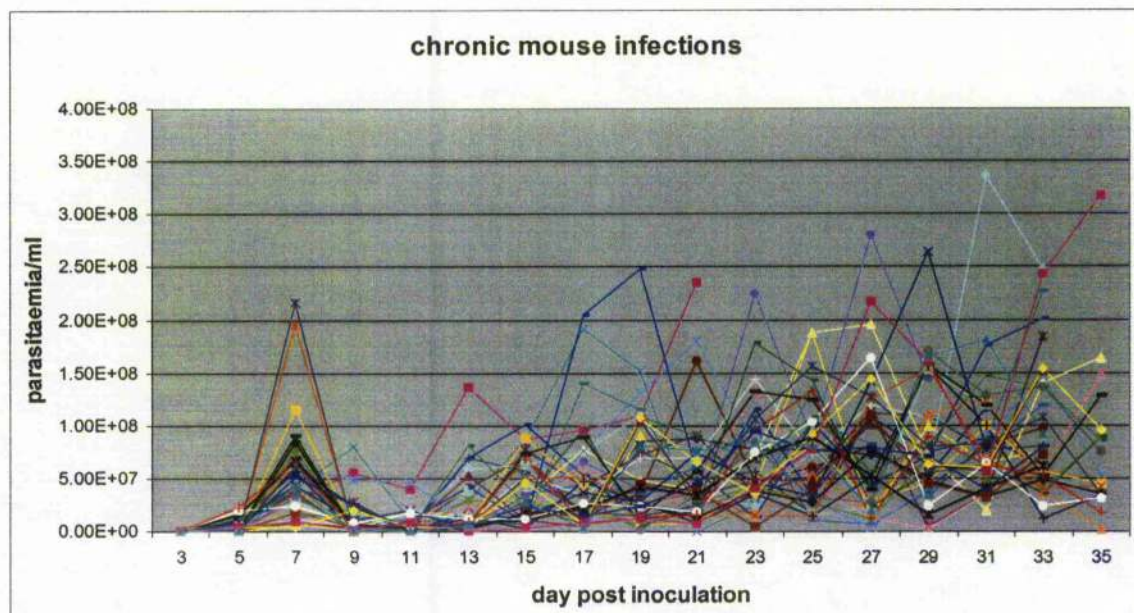


Figure 3.6 EATRO 795 parasitaemic profiles of infections in 50 BALB/c mice. This graph illustrates the pattern of distinguishable initial and first relapse peaks, followed by a more divergent and unpredictable outline.

3.6 The VSG-specific immune responses

3.6.1 Onset and duration of VSG-specific immune responses

The onset of the immune responses was measured by the *in vitro* complement lysis assay. Plasma collected at 4-day intervals was incubated with GPS and clonal trypanosomes expressing a single species of VSG. The onset of the immune response could be measured by the corresponding sample in which lysis of the trypanosomes occurred. Controls were applied in every case. A positive control was included – clonal trypanosomes incubated in GPS with specific antiserum against that VAT raised in mice. 2 negative controls were incorporated, one with the trypanosomes incubated in GPS alone to ensure there was no non-specific trypanosome lysis, the other with the clonal trypanosomes incubated with specific mouse antiserum against one of the other 6 VATs under study. Controls were prepared in duplicate wells on a Terasaki plate. All VATs were incubated with specific

antisera to each of the other respective six VATs under study. No cross-reaction was ever observed.

	MINI	TELO	MINI	TELO	TELO	INT	INT
mouse	1.21	1.22	1.25	1.64	1.67	1.71	1.73
B2	*	7	7	15	7	15	*
C2	*	7	7	15	7	27	*
D2	*	*	7	7	*	11	*
F2	7	7	*	7	*	15	*
G2	7	11	7	15	7	15	*
H2	7	7	7	15	7	15	*
I2	7(31)	7	7(27)	11(27)	7	15(23)	*
J2	7	7	7	15	7	35	*

	MINI	TELO	MINI	TELO	TELO	INT	INT
	1.21	1.22	1.25	1.64	1.67	1.71	1.73
A3	13	17	13	*	*	13	17
B3	9	17	9	*	*	13	13
C3	17	17	13	N	25	17	13
D3	17	17	13	29	9	17	17
E3	17	13	17	17	9	9	21
F3	13	17(25)	13	17	9	13	13
G3	13	17(25)	13	17	13	17	17
H3	17	17(25)	13	17	17	17	13
I3	13	17	13	17	9	N	*
J3	17	17	13	17	9	25(33)	*

A4	15	15	15(33)	19(23)	15	31	19
B4	19	N	15	19	15	N	19
C4	15	19	11	19	15	19	15
D4	19	23	19	19	11	19	19
E4	15	19	15	19(27)	15	15	15
F4	19	19	15	19(23)	15	19	15
G4	15	19	15	19(23)	7	27	11(19)
H4	15	19	15	19(31)	15	23	15

A5	15	19	15	19(23)	15	19	15
B5	15	19	15	19(27)	15	19	19
C5	15	19	11	19	11	23	19
D5	15	15	15	19	15	15	15

Table 3.2 Day of onset of specific immune response to VATs as measured by *in vitro* complement lysis assay for 4 sets of chronic infections. Seven VATs under study are ILTats 1.21, 1.22, 1.25, 1.64, 1.64, 1.71 & 1.73. MINI = minichromosomal, TELO = telomeric, INT = chromosomal internal. Numbers = day post inoculation that immune response was first detected. Numbers in brackets = day on which immune response became undetectable, if this occurred. N = no immune response detected. * = analysis not done due to exhaustion of mouse plasma sample. Mice B2–J2 were a control batch, where VATs under study were not eliminated from inoculum. Mice A3–J3, A4–H4 & A5–D5 represent 3 separate sets of infections, carried out in an identical manner, with VATs under study removed from the inoculum by incubation *in vitro* with specific antibodies.

A total of 30 chronic mouse infections were analysed (Table 3.2). Of these, the first batch of 8 mice (B2-J2) represented a control where the VATs under study were not removed from the inoculum by incubation with specific antibody. The other 3 batches (A3- J3, A4-H4 and A5-D5) represented the 22 chronic infections in which the VATs were removed by this treatment of the inoculum. By examining the difference between these 2 sets of data, it can be deduced that the incubation of the inoculum with the specific antibodies did remove at least the majority of any trypanosomes expressing the VATs. The day of onset of immune response against ILTats 1.21, 1.22 and 1.25 was delayed by an average of 6 – 10 days in the experimental mice. The difference was 2 days for ILTat 1.67, and 2-4 days for ILTat 1.64. There was not a great degree of difference for ILTat 1.71, and ILTat 1.73 was not analysed in the control group due to exhaustion of mouse sera through refining the technique. It can be argued from these data, that ILTats 1.21, 1.22, 1.25, 1.64 and 1.67 were present in varying, probably very small, numbers in the initial inoculum, whereas ILTat 1.71 was probably not present. Those that had been present were removed by the specific antibody incubation step in the experimental mice, on the basis of the differing times of onset of specific antibody between the control and experimental groups.

There are several notable features of the immune response analysis. Firstly, there are only 4 instances when no immune response is detected. This occurs in mouse B4 for ILTat 1.22, mouse C3 for ILTat 1.64, and mice I3 and B4 for ILTat 1.71. These VSGs are located in MESs for ILTats 1.22 and 1.64, and chromosomally internal for ILTat 1.71. In these cases it appears that there was no detectable switching to these VATs during the chronic infections. Overall, these data indicate that this repertoire of 7 VATs is utilised to a large degree in the 35-40 day time period of the chronic mice infections. Additionally, in the majority of cases, the VAT-specific immune response remained detectable to the termination point of the infections. This indicated there was no waning of the immune response, at least below the threshold detected by the assay, and meant that there could be no reappearance of sub-populations expressing the respective VAT at a later timepoint in the infection. There were, however, occasional instances of the immune response dropping below the threshold detectable by the *in vitro* complement lysis assay. This phenomenon was never observed for ILTats 1.21 or 1.67, encoded respectively by minichromosomal and BES silent genes. It was seen during one infection for ILTat 1.25 (4.5% of infections analysed), which is a minichromosomal gene, once for ILTat 1.71 (4.5%), a chromosomal internal gene, and once for ILTat 1.73 (5%), also chromosomal internal. However, the VAT-specific immune response dropped below detectable levels on 7 occasions for ILTat

1.64 (35%) and 3 (13.6%) for ILTat 1.22, both MES genes. Collectively, these instances occurred over a number of mice, and therefore weak immune response of an individual mouse could be discounted; although the likelihood of this occurring should have been decreased in any case by using inbred mice. Nevertheless, if the results for control mouse I2 are examined, one can see that there was loss of the detectable immune response against 4 of 6 VATs examined, and in this case the loss was likely due to generalised weakening of the specific immune response, probably due to stress or illness in the mouse. This situation does not apply to the experimental mice, however.

The drop in detectable immune response can be explained in several ways. There would be, as has already been mentioned, dampening of the immune response generally, although this would have occurred for responses against all the VATs. Additionally, there could have been a fault in the assay, such as degradation of complement, or antibody. This can be discounted, as the positive and negative controls will have ensured that the complement functioned, and the same serum sample was used across several VATs, so any degradation would appear consistent as in mouse I2. Thirdly, there could conceivably have been quantitatively fewer trypanosomes expressing the respective VATs in the particular mice. This assumes that the titre of the VAT-specific immune response is uniformly directly proportional across the different VSGs up to a threshold level. If the relevant sub-population drops below that threshold, and there is not continuous, or relatively much less, further switching back to that VSG, then the immune response could wane. In the subset of single copy *VSG* genes under study, the metacyclic *VSG* genes, and in particular ILTat 1.64, were the genes for which this drop in immune response seemed to occur. The MVSGs are the proteins expressed by the trypanosome injected initially by the tsetse, and it is presumably preferable for the animal not to have been exposed to them previously in order to initiate infection. Therefore it could be hypothesised that limitation of switching to these genes, as a by-product of their genomic environment, could help to optimise transmission in an endemic situation.

3.6.2 Analysis of VAT-specific immune responses

The analysis of the VAT-specific immune responses was undertaken in several ways. The simplest approach was to analyse whether or not there was a significant difference between the times of onset of responses against the different VATs. The second approach was to rank the timing of onsets within individual infections, and then carry out comparisons of the ranking between infections. This reduced the possibility of extremes (statistical outliers) impacting on the mean outcome. The linking of timing of onset of VAT-specific

immune responses to peaks of parasitaemia was also considered. This approach however was discarded, as in many cases the interpretation of separation of peaks was ambiguous. Moreover, one could not be certain that the actual VAT growth correlated with the parasitaemia peak associated with the immune response. The last approach was to combine data for the different genomic locus types, and investigate whether any difference in timing of onset of immune responses corresponded with different types of genomic loci inhabited by the silent donor *VSG*, by testing in the order of prediction.

3.6.2.1 Time of onset of VAT-specific immune response.

Time data were normalised by transforming them as $\log_{10}(\text{time}+)$. “Time+” indicated that occasions where no immune response was observed were scored as 36 days *i.e.* endpoint of experiment + 1 day. General Linear Models were used to analyse the data, with $\log_{10}\text{time}+$ as the response variable, and VAT code and mouse batch as factors. Parametric assumptions were checked by analysing normal distribution, and testing for equal variances. It was vital to analyse any effect of mouse batch on the outcome of the analysis for several reasons. Firstly, sampling of plasma samples commenced on day 1 for batch A3-J3, and day 3 for batches A4-H4 and A5-D5. This led to an automatic difference of 2 days in the time of onset between the two groups, which had to be factored. Moreover, any difference between batches would invalidate the results, as the findings from the data would not be consistent across three different experiments, due to either lack of experimental rigour, or differences between the mice.

Time of onset of immune response differed significantly between the VATs ($F_{6,106}=7.49$, $p < 0.0001$) (see Fig. 3.7, overleaf). There was also a significant difference between the two batches ($F_{1,106}=5.82$, $p=0.018$), as was expected due to the difference in sampling times. Importantly, however, there was no interaction between mouse batch and variances between VAT groups ($F_{6,106}=1.39$, $p=0.225$), so the relative time of appearance of the VATs was not different between batches.

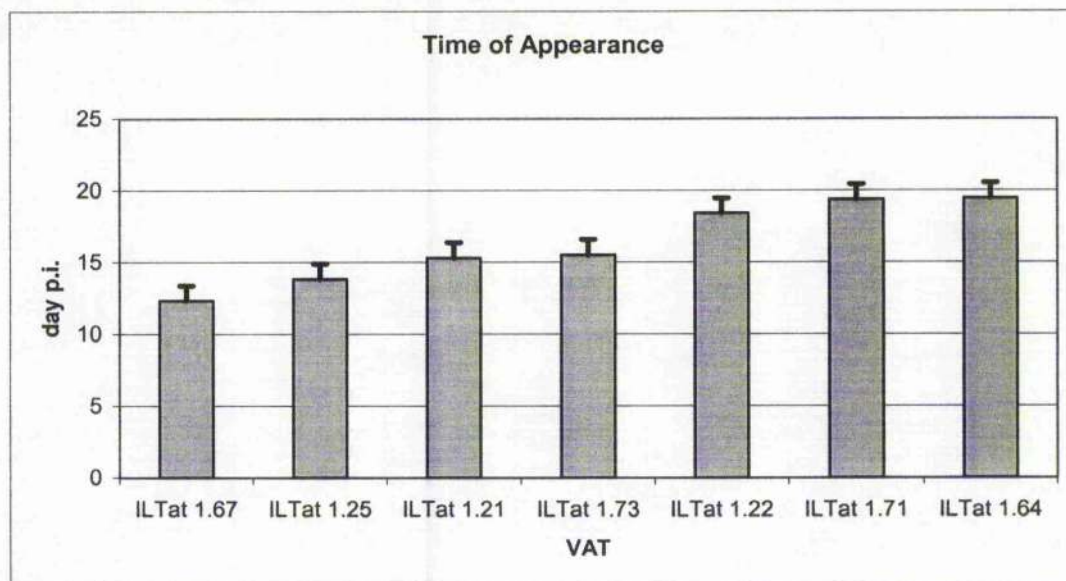


Figure 3.7 Least square mean of 'time+' for onset of immune response against VATs. "Time+" indicated that occasions where no immune response was observed were scored as 36 days *i.e.* endpoint of experiment + 1 day. Day p.i. = day post inoculation. The error bars represent +/- SEM, n = 22.

3.6.2.2 Ranking of the appearance of VAT-specific immune responses.

The onset of VAT-specific immune responses was ranked according to their order of appearance within individual mice infections. Therefore, the first VAT immune response was assigned a value of 1, the second 2, and so on. If immune responses to different VATs appeared at the same time, they were assigned the same value, *i.e.* VATs A and B appeared first, they were assigned a value of 1, VATs C and D next, these were both ranked as 2. If no immune response was observed, the VAT was assigned a ranking one greater than the highest within that infection. "Rank" data were normalised by transformation as reciprocal of rank. General Linear Models were used to test the hypothesis, with reciprocal of rank as the response variable, and VAT code and mouse batch as factors. Parametric assumptions were checked by analysing normal distribution and testing for equal variances.

The ranking differed significantly between the VATs ($F_{6,112}=8.03$, $p<0.0001$). There was no significant mouse batch effect ($F_{1,112}=1.68$, $p=0.197$), and therefore no interaction between batch and variances between VAT groups ($F_{6,106}=0.83$, $p=0.550$). In this case, the difference of 2 days between the sampling timings should not have had an effect as the ranking system was designed to remove any, as the difference in day number would not

have affected the ranking position. Again, the lack of batch effect validates experimental consistency.

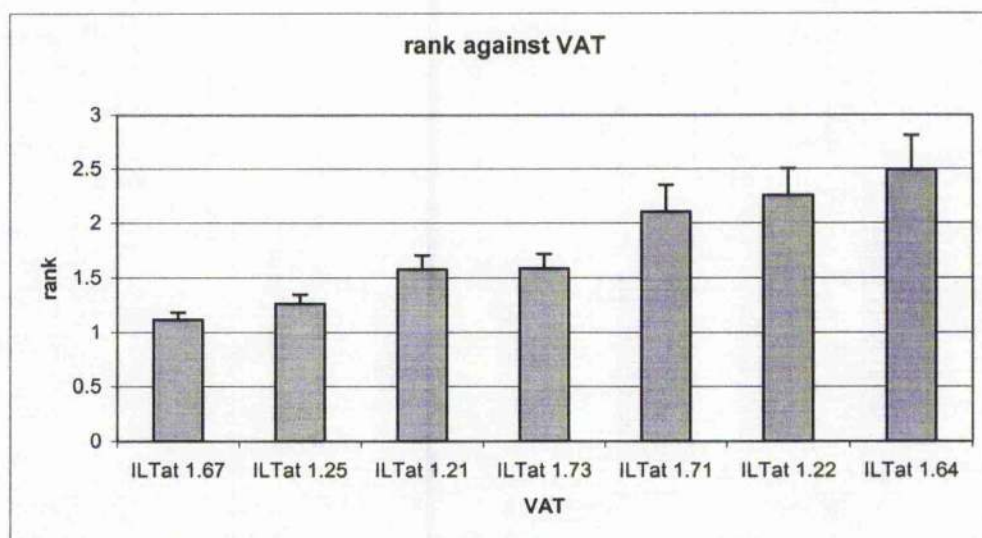


Figure 3.8 Least square mean of rank against VATs ("rank" refers to the ranking of the onsets of the VAT-specific immune response within individual mice infections). The error bars represent +/- SEM, n = 22.

3.6.2.3 Comparison of time of onset, and ranking of immune responses.

When the results for the "rank" and "time+" data are compared, they give very similar results (see Table 3.3, overleaf). In both sets of analysis, the immune response against ILTat 1.67 was first to appear, followed by ILTat 1.25, ILTat 1.21, ILTat 1.73, ILTat 1.71, ILTat 1.22, and finally ILTat 1.64. When time of onset is analysed, the fifth out of the seven is ILTat 1.22, then ILTat 1.71 and finally ILTat 1.64. There is general agreement between the two sets of analysis, with only ILTats 1.71 and 1.22 differing. This is likely due to the immune response against ILTat 1.71 appearing relatively very late in the infection in a few cases (mice J3, G4 and C5), and therefore skewing the data.

Rank	Time+
ILTat 1.67	ILTat 1.67
ILTat 1.25	ILTat 1.25
ILTat 1.21	ILTat 1.21
ILTat 1.73	ILTat 1.73
ILTat 1.71	ILTat 1.22
ILTat 1.22	ILTat 1.71
ILTat 1.64	ILTat 1.64

Table 3.3 Comparison of "rank" and "time+" as measurements of hierarchy ("rank" refers to the ranking of the onsets of the VAT-specific immune response within individual mice infections; "time+" indicated that occasions where no immune response was observed were scored as 36 days *i.e.* endpoint of experiment + 1 day). Top of table : earliest onset of immune response, bottom : latest.

3.6.2.4 Testing in the direction of prediction.

Predictions for the order of appearance of the onset of immune responses were based on the results of the General Linear Model "time+" (see 3.6.2.1), equivalent to 'observed' data, and also on the location of the individual genes within the genome, equivalent to the 'expected' data. The 'expected' data were based also on previous work involving 6 of the genes under study in rabbits (Robinson *et al*, 1999). Thus, VATs encoded by *VSG* genes located in bloodstream expression sites were expected earliest, followed by those encoded by minichromosomal genes, then those encoded by metacyclic expression site genes, and finally those encoded by genes situated internally in megabase chromosomes. Data for genes belonging to one of these groups were pooled; ILTats 1.21 and 1.25 are both

minichromosomal genes, ILTats 1.22 and 1.64 are both MES genes, and ILTats 1.71 and 1.73 are both chromosomal internal. This gave 4 groups of data.

Using a directional heterogeneity test (Rice and Gaines, 1994), the order of appearance between these four groups was seen to be highly significant ($p < 0.0001$) (Fig. 3.9, below).

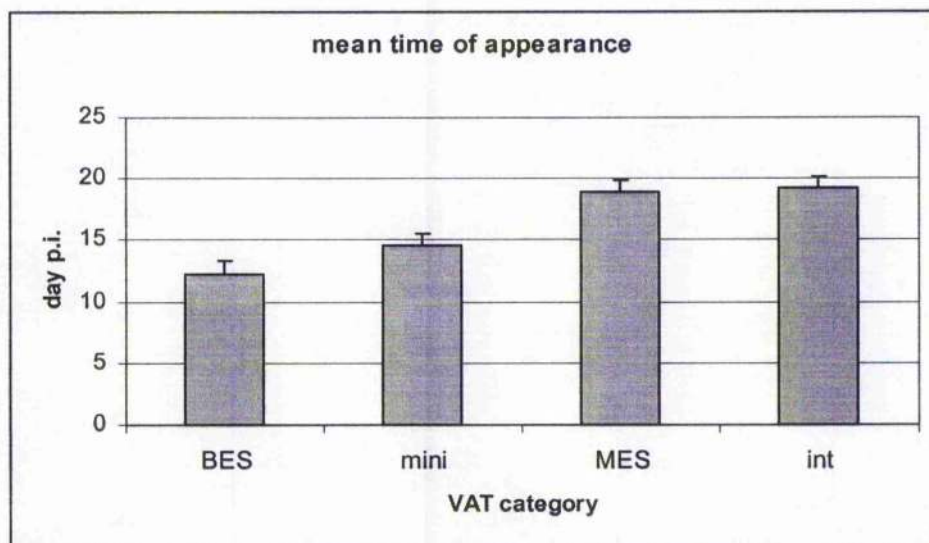


Figure 3.9 Least square mean of "time+" for onset of immune response against category of VAT (BES = bloodstream expression site, mini = minichromosomal, MES = metacyclic expression site, int = chromosomal internal). "Time+" indicated that occasions where no immune response was observed were scored as 36 days *i.e.* endpoint of experiment + 1 day.

3.7 Summary

The results of the chronic mouse infections indicate that there is a statistically significant difference in the onset of VAT-specific immune responses across 3 separate batches of chronic infections, comprising a total of 22 mice. The general progression of infections in the mice was itself notable, with a predictable early course of parasitaemia, including very similar magnitude and timing of the initial parasitaemic peak. Thereafter, however, the progression and parasitaemic profile were very variable, probably a reflection of the high switching rate and pleomorphism of the EATRO 795 trypanosomes. The VAT-specific immune responses also had characteristic features. The immune responses generally lasted the duration of the infections, which would ensure no reappearance of sub-populations

expressing the respective VAT later in the infection. In addition, there were very few instances where there was no immune response detected to a particular VAT, indicating that within the period of these infections in mice, the subset of VATs under study were all usually expressed. When the onset of the VAT-specific immune responses was analysed, by comparing the average times of onset, and by ranking arbitrarily depending on the sequence of appearance, it was found in both cases that there were significant differences between the different VATs. This indicates a definite hierarchy of expression within this subset of VATs. It is also possible to say that, for this subset, the hierarchical differences can be significantly grouped within data from different genetic loci types. These findings help to increase the resolution of examining the hierarchy, which had previously been limited to the difference between subtelomeric and chromosomal internal genes. It must be stressed that there are limitations in the study. The analysis was limited to the immune response, and therefore was not directly measuring the switching event at the level of the trypanosome, or the development of VAT subpopulations. In addition, assumptions were made regarding proportionality of immune response to number of trypanosomes, and the fact that there was not any qualitative difference between the immune response against different VATs. However, these were reasonable assumptions, as there is no published work in disagreement. Moreover, this is a very small subset of the VSG repertoire, seven out of possible hundreds. In addition, only 2 of each genetic locus type for *MVSGs*, minichromosomal *VSGs* and chromosomal internal *VSGs*, and 1 BES *VSG* were analysed. Also, findings from this subset can pertain to only switching events involving full length *VSGs*, and not events such as mosaic gene formation. These experimental restrictions were insurmountable, however, and despite these limitations, the significant differences between the onset of the VAT-specific responses does give an hitherto obscured insight into the hierarchical nature of VSG switching. Furthermore, these results were obtained from a substantial number of infections, using a highly pleomorphic trypanosome, and provide a basis to explore further the nature of hierarchical VSG switching, both in cattle hosts and by mathematical modelling.

Chapter 4

Chronic cattle infections

4 Chronic cattle infections

4.1 Introduction

As mentioned in the previous chapter, most work on African trypanosomes has concentrated on molecular mechanisms in laboratory-adapted monomorphic *T. brucei*. Very few *in vivo* studies have examined the dynamics of the parasite within the context of a chronic infection. Even fewer have concentrated on the situation in what is considered a 'natural' host of the parasites, cattle. Work on cattle trypanosomes has been concerned mainly with *T. congolense* and *T. vivax*, as they are considered the major pathogens of those hosts, whereas *T. brucei* causes little disease. Due to this, most studies have concentrated on pathological or immunological aspects of infection (Dargie *et al.*, 1979; Morrison *et al.*, 1982a; Williams *et al.*, 1996), and very few have looked at antigenic variation (an exception being (Barry, 1986)). It has been shown that the kinetics of trypanosome infections differ significantly between different hosts, in terms of both duration and parasitaemic profile (Barry, 1986), specifically *T. vivax* infections. How these differential progressions of infections could affect, or be affected by, trypanosome dynamics with respect to antigenic variation *in vivo* is intriguing and is one of the questions this investigation addresses.

The aim of this study is to examine the hierarchical expression of VSGs in replicate cattle infections. The emergence of a set of 7 variants within the time course of an infection are analysed, importantly by direct examination of the parasite population itself, but also by investigation of the development of the host's specific immune response to each VAT. The VSGs involved are all single copy, in order that any linkage between genomic locus and hierarchy could be analysed. Cattle are used for several reasons. Firstly, as mentioned above, they are primary hosts for *T. brucei*. Secondly, chronic infections are a natural outcome of trypanosome infections in cattle, which therefore are a more valid host for the study of this disease state. Lastly, a relatively large amount of material can be sampled from a cow on a daily basis, removing the restrictions inherent to the mouse model. The aim was to gain information on the dynamics of an *in vivo* trypanosome infection, and also to test whether hierarchical switching of VSGs is related to the genomic locus of the silent donor gene.

4.2 Rationale behind the choice of host breed and parasite strain

As it was desirable to extract the maximum information from these infections, conditions had to be optimised. The breed of cattle was important, as it has an effect on parasite numbers. The majority of previous experiments looking at antigenic variation had utilised European *Bos taurus* breeds of cattle, or crosses thereof; Charolais cross steers (Barry, 1986) being a notable example. *Bos indicus* breeds, such as boran and zebu, have been shown to exhibit varying degrees of trypanotolerance (Njogu *et al.*, 1985; Welde *et al.*, 1989), while African taurine breeds such as N'Dama are well known for this trait (Murray *et al.*, 1981). There has also been a large amount of anecdotal evidence that the level of parasitaemia tends to be greater in European susceptible breeds (J.D. Barry, M. Murray, P.A.O. Majiwa, pers. comm.). For this reason it was decided to use Friesian steers, as this was the European breed most easily obtained in Kenya, and steers are much less expensive than adult females. The cattle came from an area of Kenya (Naivasha, Rift Valley Province) free from trypanosomiasis, and the experiment was carried out on the premises of the International Livestock Research Institute (ILRI) in Nairobi, Kenya, also an area with no trypanosomiasis or vectors.

A pool of 13 available cattle was screened for previous exposure to *T. brucei*, *T. vivax* or *T. congolense*. The screening was done by antibody ELISA, the respective antigen coated plates being provided by the ILRI serology department. 1 cow was positive for exposure to *T. vivax*, and 1 cow was borderline positive for *T. brucei* exposure - these cattle were discounted for use immediately - and none was positive for *T. congolense*. The *T. vivax* exposure must have come about via mechanical transmission by biting flies other than tsetse, and the *T. brucei* result was possibly a false positive, as the result of the ELISA reaction was not sufficiently positive to indicate definite exposure. 2 cattle were chosen that were negative for exposure to all trypanosome species, and on the basis of size similarity (the exact age of each individual in the cohort was not known - age was approximated at 18 months by weight, information from the farmer of origin, and dental eruption); these were cattle BW49 and BV154. These candidate cattle were screened in a similar manner for exposure to *Theileria parva*, *Theileria mutans*, *Anaplasma marginale*, and *Babesia bigemina*. These tick-borne parasites cause serious disease in cattle and therefore it was necessary to ensure the candidate cattle were not infected, in order to guarantee any pathology observed was due to the experimental trypanosome infection. In addition, *A. marginale* can cause changes in immunoglobulin levels (E. Authie, pers.

comm.). Cow BW49 was positive for exposure to *T. parva*, *T. mutans*, and *A. marginale*, while BV154 was positive for *A. marginale* and *B. bigemina*. As it was vital to eliminate these parasites and in particular *A. marginale*, a chemosterilisation regime was undertaken (Rogers and Dunster, 1984). The cattle were housed in tick-free accommodation, and blood smears examined weekly to check for any re-infection by *Anaplasma*. Cattle had *ad libitum* access to hay, water and mineral supplements throughout the study.

	BW49	BV154
<i>Trypanosoma brucei</i>	NEG	NEG
<i>T. congolense</i>	NEG	NEG
<i>T. vivax</i>	NEG	NEG
<i>Anaplasma marginale</i>	POS	POS
<i>Theileria parva</i>	POS	NEG
<i>T. mutans</i>	POS	NEG
<i>Babesia bigemina</i>	NEG	POS

Table 4.1 ELISA results for candidate Friesian cattle BW49 and BV154 (POS = positive, NEG = negative).

The trypanosome line used in this study was the ILTat 1.2 expresser clone. This line is derived from the EATRO 795 line, and so is isogenic with reference to the VSG repertoire. The ILTat 1.2 trypanosomes switch at a lower rate – approximately 1×10^{-5} switches/cell/generation (Robinson, 1999). It was decided to use this line because of the lower switching rate (the EATRO 795 line switches at approximately 1×10^{-2} switches/cell/generation, (Turner, 1997)). It was felt this would lead to a more easily examinable progression of the switching hierarchy, given that with the projected parasitaemias of 1×10^7 parasites.ml⁻¹ in a 400 kg cow with an approximate blood volume of 25 l there would be an enormous number of parasites (an estimated 2.5×10^{10}) in total at the peak. If these parasites were switching at the rate of the EATRO 795 line, all of the VATs under study could feasibly appear very early in infection, which although biologically accurate, would make elucidation of the hierarchy difficult. In addition, the ILTat trypanosomes are more monomorphic than the EATRO 795 line, as they grow exponentially in mice and do not yield chronic infections. However they undergo the transition to short stumpy in larger hosts (rabbits; (Robinson *et al.*, 1999)), and it was hoped this more monomorphic trait would give rise to higher parasitaemias. There was also a long-term aim to clone parasites from stabilates made later in the infection, with the hope of identifying VATs from the chronic stage. EATRO 795 trypanosomes switch at a rate that precludes obtention of phenotypically ‘pure’ clones, as during the time it takes to grow

a clone in amplifier mice, a large number of switching events have occurred and the population is no longer clonal with respect to VSG expression. Therefore the lower switching line was selected.

4.3 Initiation and progression of infection

Before initiation of infection, temperature and weight measurements were taken. Blood was sampled, pre-infection haematological parameters were measured, and pre-infection plasma samples were stored at -20°C . ILTat 1.2 trypanosomes were grown up from frozen stabilate in irradiated Harlan MF1 mice to a level of approximately $1 \times 10^{7.5}$ parasites. ml^{-1} and the mice were terminally exsanguinated by cardiac puncture. The trypanosomes were incubated with specific antibodies to the 7 VATs under study, along with guinea pig serum, for 1 h at room temperature, in order to eliminate the possibility of inoculating parasites that had already switched during the growth in mice. 1×10^6 viable parasites were then diluted into 5 ml of PSG, and injected via the intravenous route into each cow.

Each day for a total of 70 days, progression of infection was followed. 5 ml of blood was taken from the left jugular vein into a vacutainer containing EDTA. Rectal temperature was measured using a mercury thermometer. The cattle were weighed on a weekly basis using a weighing crush. Peripheral lymph nodes were palpated to check for any enlargement, mucous membranes examined for signs of anaemia, and general demeanour and appetite were checked regularly.

Daily samples and measurements were made using the 5ml of blood from each cow. Parasitaemia was measured by haemocytometer (threshold 1×10^5 trypanosomes), and if none were detected then the examination of the buffy coat was undertaken (threshold 1×10^2 parasites). RNA was extracted from 0.2ml blood and stored at -70°C for later use in RT-PCR. Haematological parameters were measured using an automated haematology machine, and in addition a manual measurement of PCV was done, by centrifuging blood contained in a sealed capillary tube in a microcentrifuge and measuring the PCV using a manual PCV measurement chart (Harlan). 0.2 ml blood was injected via the intraperitoneal route into an irradiated Harlan MF1 mouse in order to amplify the parasite population if present, both as a method of detecting low levels of trypanosome and also in order to make representative stabilates of the daily populations. In order to transport the trypanosomes back to the United Kingdom, it was necessary to passage the parasites through 2 mice as a filter against bovine pathogens, in particular foot and mouth disease virus. Plasma was

extracted from the remainder of the blood, intended for use in ELISA and *in vitro* complement lysis.

4.3.1 Parasitaemia

Parasitaemia was measured by three different means; haemocytometer, buffy coat examination, and inoculation into immunosuppressed mice. The haemocytometer counts were undertaken by incubating 10 μ l of blood with 90 μ l of 0.85% ammonium chloride. This allowed accurate counting by lysing the blood cells first, and leaving only the trypanosomes. The detection threshold of this method is 1×10^5 trypanosomes. ml^{-1} . Using the haemocytometer, however, the ability to follow the parasitaemia was very limited. The initial peak was detectable in both cattle, on days 12, 13 and 14 post-infection in cow BW49 reaching a peak of 2.8×10^6 trypanosomes. ml^{-1} , and on days 13 and 14 in BV154 peaking at 3×10^5 trypanosomes. ml^{-1} . Thereafter no parasites were detectable by haemocytometer during the remainder of the 70 day infection in BW49, and only intermittently on days 19, 20, 24, 31, 65 and 66 for BV154, each incidence not exceeding 10^5 parasites. ml^{-1} . The buffy coat technique (Murray *et al.*, 1977) involved centrifuging 50 μ l of blood in a sealed capillary tube in a microcentrifuge, cutting the tube above the buffy coat using a diamond pen, and expressing the buffy coat onto a glass slide. The area under the buffy coat was then thoroughly examined under a coverslip. The threshold of detection is approximately 1×10^2 parasites. ml^{-1} . Utilising this method, detection was more sensitive (see Figure 4.2, p. 81), although inaccurate.

The most sensitive detection was inoculation of the cattle blood into immunosuppressed mice. An inoculum of 0.2 ml cow blood was used daily, and passage to the second mouse occurred when the parasitaemia reached $1 \times 10^{8.1}$ parasites. ml^{-1} . This gave a method of crudely assessing the parasitaemic profile in the cattle, by assuming that the time to passage was directly proportional to the number of parasites inoculated. Therefore by inversely plotting the number of days to passage, an impression of the parasitaemic profile was obtained (see Figure 4.1). It was assumed that if no parasites could be detected in the mice by 10 days post-inoculation, then there had been no parasites in the inoculum. The comparative sensitivity of these techniques has been examined previously (Paris *et al.*, 1982) and this study also found that amplification in mice was the most sensitive.

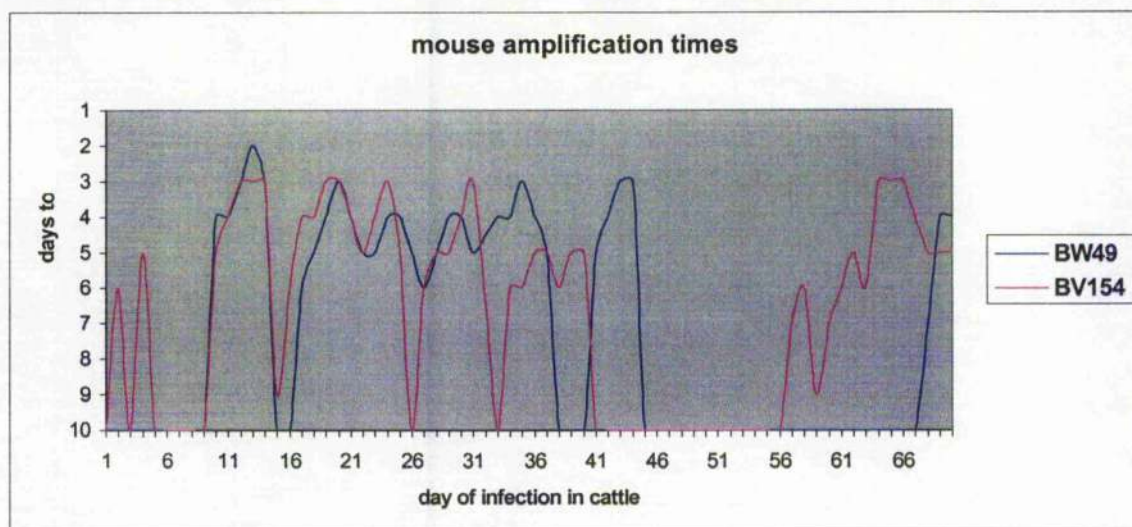
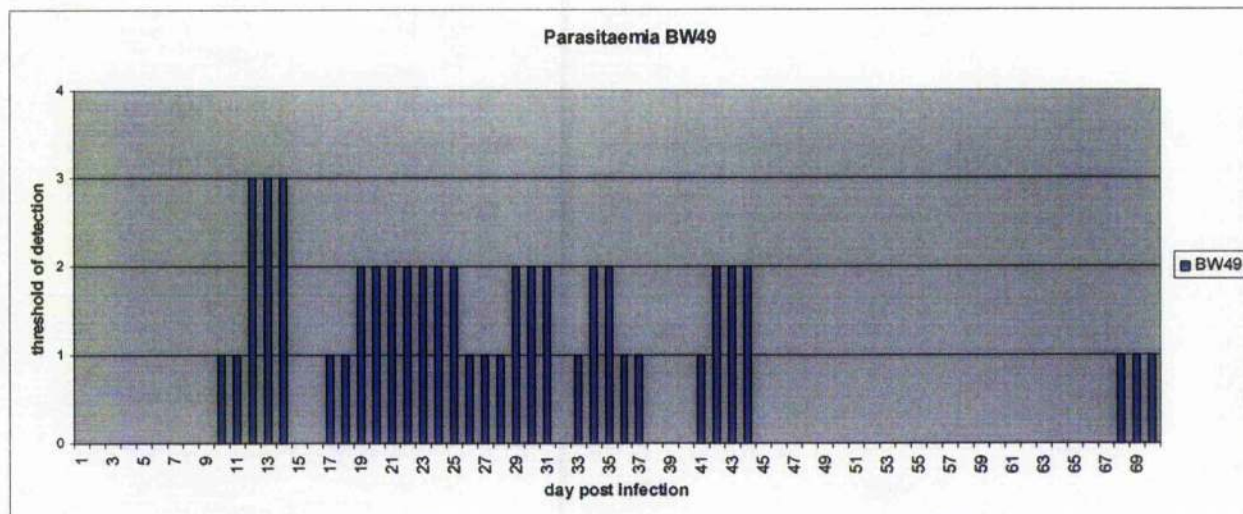


Figure 4.1 Parasitaemic profiles of cattle BW49 and BV154 as measured by inversely plotting the time in days from inoculating 0.2 ml cattle blood into an immunosuppressed mouse to achievement of a parasitaemia of 1×10^8 trypanosomes.ml⁻¹.

The parasite levels in the cattle were lower than anticipated. This caused problems with the amount of parasite RNA that it was possible to extract each day (see 4.6.3, p. 97). The levels of parasitaemia anticipated, however, were based largely on infections initiated with *T. vivax* (Barry, 1986), *T. congolense* (Morrison *et al.*, 1982b) and *T. evansi* (Payne *et al.*, 1992; Thammasart *et al.*, 2001), all of which are more pathogenic in cattle than *T. brucei*. The total number of parasites however is still substantial; a 320 kg cow such as BW49 will have a blood volume of approximately 20 litres, and at the maximum observed parasitaemia of 2.8×10^6 parasites.ml⁻¹, there would be at least 5.6×10^{10} parasites within the circulation of the animal.

A



B

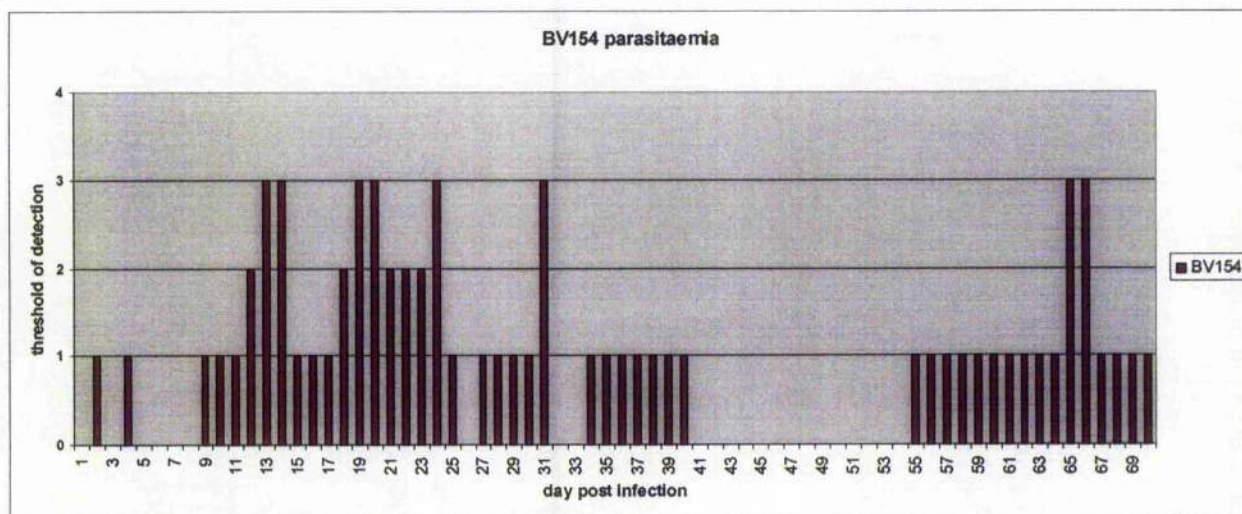


Figure 4.2 Graph illustrating detection thresholds of parasitaemia during infections in cattle BW49 (graph A) and BV154 (B). Y-axis; 0 = parasites undetectable by any method, 1 = parasites only detectable by amplification in irradiated mice, 2 = parasites detectable by examination of buffy coat, 3 = parasites detectable by haemocytometer. (NB. No measurements taken on day 32 post infection)

It was not possible to elucidate the minimum number of parasites that could be amplified in mice, but this would be the method of choice for any further *T. brucei* infections in cattle. It could be deduced that the more pleomorphic EATRO 795 strain would have resulted in even lower levels of parasitaemia. A parallel infection was carried out using the highly pleomorphic line TREU 927 (see Appendix), and in this case parasites were never detected by haemocytometer, very rarely by buffy coat, but did amplify every day (with 1 exception) in mice.

4.3.2 Health and haematological parameters

The health of the cattle was measured in several ways. Rectal temperature was monitored daily as an indicator of pyrexia incidents, a pathological feature of trypanosome infections. Body weight was measured weekly, as weight loss is a direct indicator of suppression of diet due to lethargy, arising from pyrexia and anaemia. The cattle were examined directly for any external signs of trypanosomiasis; superficial lymph nodes (prescapular, prefemoral and submandibular) were palpated to check for the generalised lymphadenopathy characteristic of the disease, and mucous membranes were examined for pallor, indicating anaemia, the second cardinal pathological sign of trypanosomiasis.

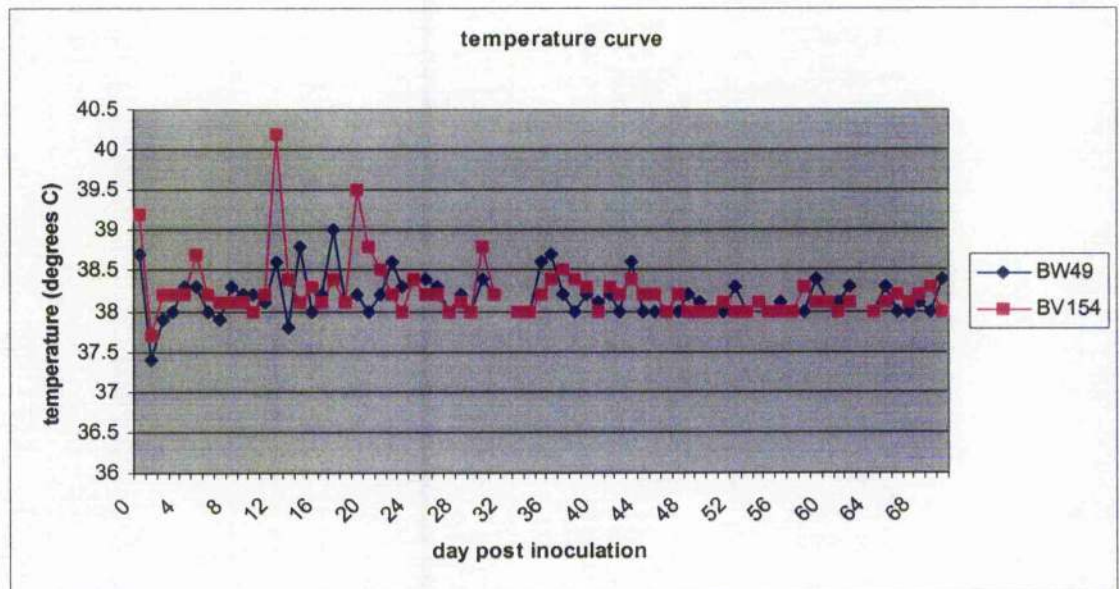


Figure 4.3 Graph of daily rectal temperatures of cattle BW49 and BV154 during the 70 day infection.

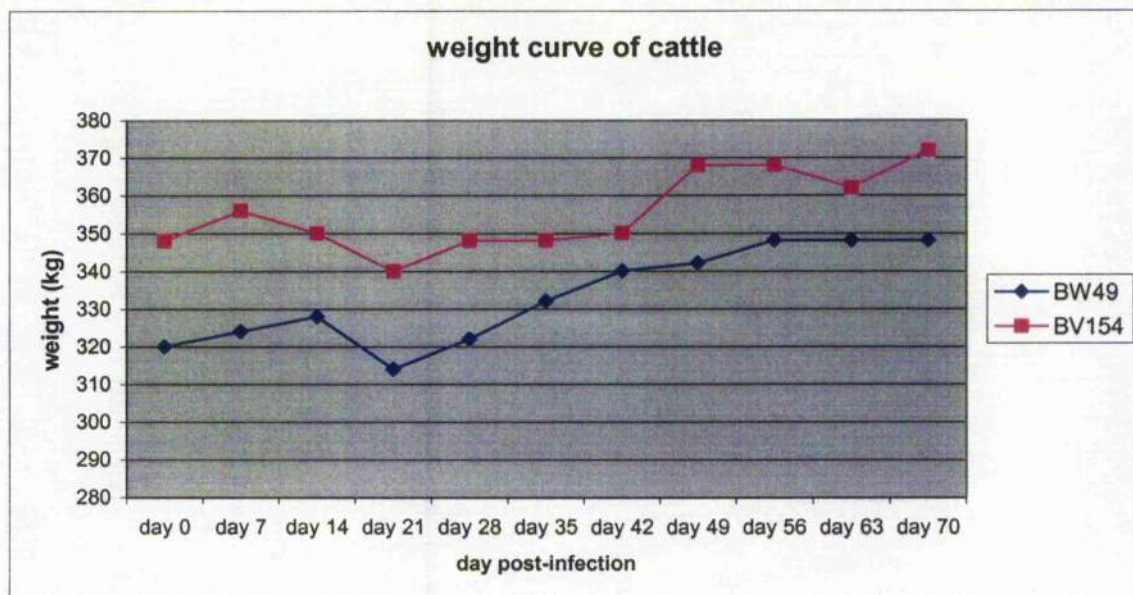


Figure 4.4 Graph of weekly weight measurements of cattle BW49 and BV154 during the 70 day infection.

There were very few overt signs of pathology in the cattle during the course of the infections. No gross pathology was observed; there was not any measurable lymphadenopathy or visible pallor of the mucous membranes. There were peaks of pyrexia, particularly in cow BV154, early in infection. These coincided with peaks of parasitaemia on days 12-13 and day 19, corresponding with the initial and first relapse peaks. The temperatures measured on these days were at the high end of normal for cattle (normal value range 37.5 – 39.5° C (Blood, 2000)), but did not last for extended periods. There were smaller spikes of pyrexia during the BW49 infection, again early in infection between days 12 and 20, corresponding to the initial peak and the first relapse peak. Thereafter, there were no periods of raised temperature in either cow. Body weight dropped initially, falling to the lowest point in both on day 21, falling from 328 to 314 kg in cow BW49 (losing 4.2 % of body weight), and 356 to 340 kg in cow BV154 (4.4 %). This period of loss was in the initial period of infection, specifically during the initial parasitaemia peak and the first relapse peak. Thereafter in both cattle there was a gradual increase over the remaining seven weeks. In the case of BW49, the cow's weight increased from 314 to 348 kg, a weight gain of 0.69 kg.day⁻¹. BV154 increased from 340 to 372 kg, growing at a rate of 0.65 kg.day⁻¹. This rate of weight gain is within the normal range for adult Friesian cattle fed an *ad libitum* diet of hay and concentrates, recommended as 0.50 – 0.75 kg.day⁻¹ for *Bos taurus* cattle (Radostits and Blood, 1985). These measurements combine to indicate that the acute phase of the infections associated with higher levels of

parasitaemia, particularly the first sets of parasitaemic peaks, produced both a level of pyrexia and weight loss. After these initial peaks in temperature and drop in body weight however, the rate of weight gain and temperature measurements returned to normal levels.

Haematological parameters were measured using an automated haematology machine (Vettest, Idexx) for the first 58 days. Thereafter reagents were unobtainable due to logistical problems. As a back-up, PCV was also measured daily using a haematocrit centrifuge. PCV is a direct measurement of anaemia, as it is the percentage volume of the blood taken up by red blood cells. Haemoglobin concentration and red blood cell count were also measured. Both are more indirect measurements of anaemia. In addition, the whole white blood cell concentration was measured. White blood cells rise as a result of the onset of immune responses during microbial infections, and in trypanosomiasis, are an indirect measurement of general immunosuppression, another feature of trypanosome infections (Urquhart *et al.*, 1973).

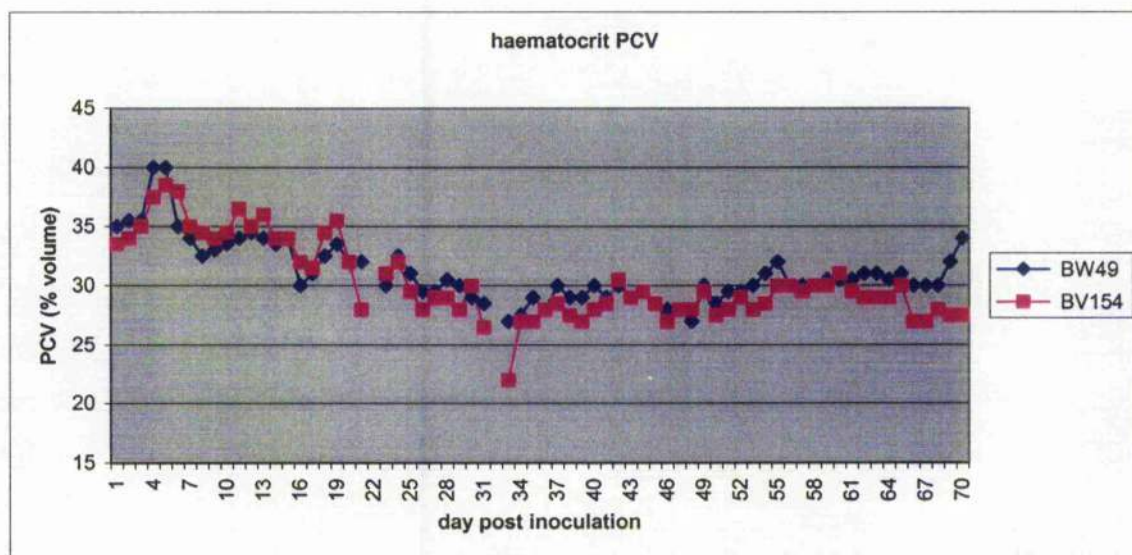


Figure 4.5 Graph of daily manual PCV measurements of cattle BW49 and BV154 during the 70 day infection.

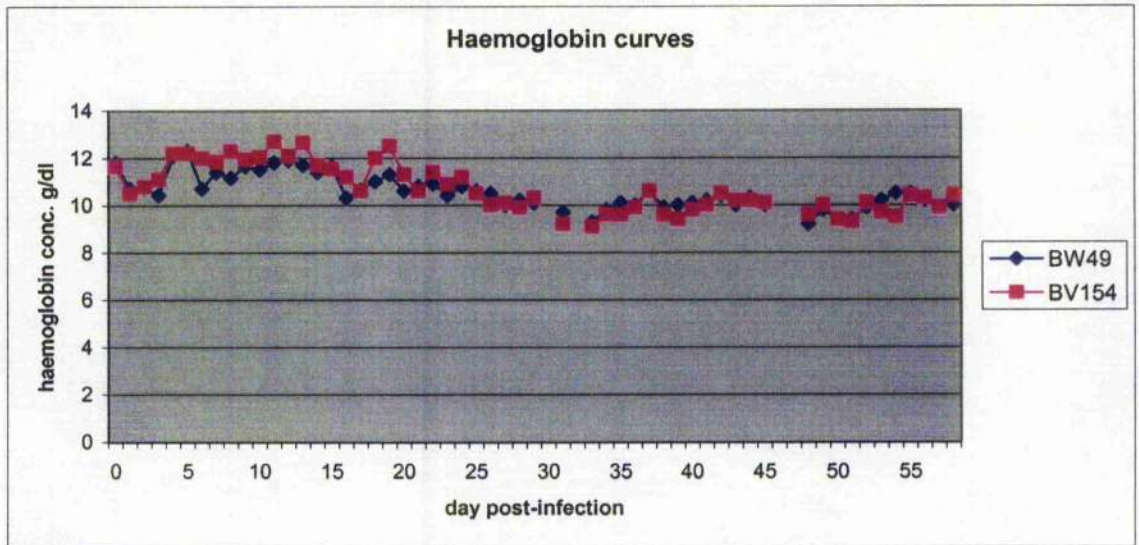


Figure 4.6 Graph of daily haemoglobin concentration measurements of cattle BW49 and BV154 for the first 58 days of the 70 day infection.

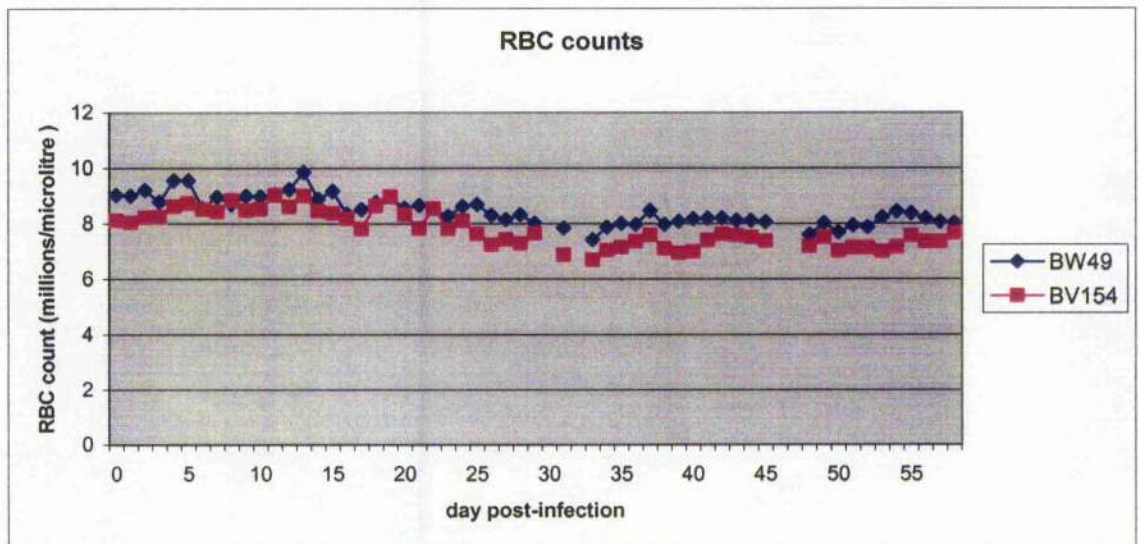


Figure 4.7 Graph of daily red blood cell size measurements of cattle BW49 and BV154 for the first 58 days of the 70 day infection.

The measurements of PCV, haemoglobin concentration, and red blood cell count indicate that there was a moderate drop in red blood cell numbers and haemoglobin concentration, following a similar profile in the two cattle. PCV gradually dropped from a starting point of approximately 35 % to approximately 27 % by day 35 post-infection. Thereafter it remained stable, even slightly increasing to approximately 29 % by the end of the 70 day observation period. Haemoglobin concentration dropped by approximately 1 g.dl⁻¹ across

the 70 days. These parameters, while indicating that the trypanosome infection did produce a very moderate anaemia, did not in either case go below the bottom threshold of the considered normal ranges for cattle (PCV: 24 – 46 %, haemoglobin concentration: 8 – 15 g.dl⁻¹, RBC: 5 - 10 μm (Jain, 1986)). The PCV did not ever approach the level, 15%, at which the experiment would have been terminated, for humane reasons.

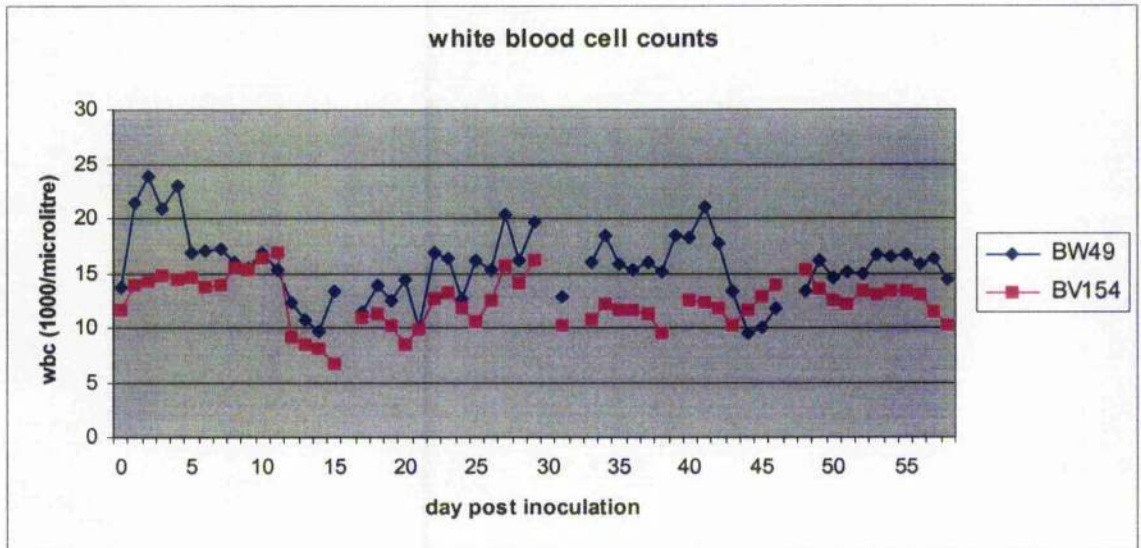


Figure 4.8 Graph of daily white blood cell counts of cattle BW49 and BV154 for the first 58 days of the 70 day infection.

The leukocyte counts represented a count of total white blood cells, therefore taking into account effector cells of both non-specific and specific immune response. The levels were higher than the accepted upper normal threshold level in cattle ($4 - 12 \times 10^3 \text{ cells.}\mu\text{l}^{-1}$ (Jain, 1986) during most of the 58 days measured. Nevertheless, this high level was considered normal for Nairobi (Majiwa, pers. comm.), as the accepted levels are based on averages under conditions in European/North American agriculture, whereas the cattle in Kenya, and in particular exotic cattle such as Friesians, are under challenge from a wider array of microbes. There was a drop in white blood cell numbers in both cattle from days 5 – 15 post-infection. Attributing this drop to any host or parasite effect, however, would be speculative. Thereafter the numbers stayed relatively stable, with minor fluctuations.

As a summary of the pathological and haematological measurements taken over the progression of the 70 day infections, it can be said that in the initial stages there was intermittent pyrexia and slight weight loss up to 21 days post-infection, and also a slight drop in the PCV. However both the cattle then proceeded to gain weight for the remainder

of the period of observation, pyrexia incidents ceased, and haematological parameters stabilised. In combination with the parasitaemia data, it can be deduced that the initial period of infection, corresponding with the highest levels of parasitaemia, led to pyrexia and a progression of non-clinical anaemia, combining to contribute to a weight loss of approximately 4% body weight over the first 3 weeks. Thereafter, as parasite numbers reduced, conditions stabilised. No gross pathological signs were observed throughout the infections, and it can be concluded that the ILTat line of trypanosomes are non-pathogenic to Friesian cattle. As a caveat, it must be stressed that these infections were followed for only 70 days, and it cannot be concluded that if the infections were left to progress, there would have been no subsequent disease.

4.4 Results of the *in vitro* complement lysis assay

The measurement of the onset of the immune response to each of the seven VATs under study was attempted in two ways, by ELISA, and by the *in vitro* complement lysis assay. The methodology of the *in vitro* complement lysis assay was identical to that used in the chronic mouse study, apart from a few minor modifications: plasma collected from the cattle on a daily basis was incubated with GPS and clonal trypanosomes homogeneously expressing a single species of one of the VSG under study (the GPS used in Nairobi was collected from guinea pigs on site, and frozen immediately at -70° C upon collection). The onset of the immune response was measured; lysed trypanosomes appearing as ruptured 'ghosts' under phase/contrast microscopy. Controls were applied in every case. A positive control was included – clonal trypanosomes incubated in GPS with specific antiserum against that VAT raised in mice. 2 negative controls were incorporated, 1 with the trypanosomes incubated in GPS alone to ensure there was no non-specific trypanosome lysis, the other with the clonal trypanosomes incubated with specific mouse antiserum against 1 of the other 6 VATs under study. Controls were prepared in duplicate wells on a Terasaki plate. No cross-reaction was ever observed.

Locus	VSG	Cow BW49	Cow BV154
mini	<i>ILTat 1.21</i>	25	22
MES	<i>ILTat 1.22</i>	26	26
mini	<i>ILTat 1.25</i>	23	22
MES	<i>ILTat 1.64</i>	25	27
BES	<i>ILTat 1.67</i>	18	27
int	<i>ILTat 1.71</i>	64	66
int	<i>ILTat 1.73</i>	40	28

Table 4.2 Day of onset of VSG-specific immune response in each cow to the seven VSGs under study (BES = bloodstream expression site, MES = metacyclic expression site, mini = minichromosomal, int = chromosomal internal).

The order of appearance of immune response to the 7 VATs under study was similar to that in the chronic mice, with the immune responses against the VATs encoded by the BES gene (*ILTat 1.67*) and the minichromosomal genes (1.21 and 1.25) appearing earlier, between days 18 and 27, and those against VATs encoded by the MES genes (1.22 and 1.64) and chromosomal internal genes (1.71 and 1.73) appearing later, between days 25 and 66. Indeed in the two cattle, the immune response against VATs encoded by internal genes tended to appear markedly later, on days 28 and 40 for *ILTat 1.73*, and days 64 and 66 for *ILTat 1.71*. In addition, in the case of *ILTat 1.67*, the immune response was regularly detected earliest in the mice infections, and in cow BW49 appeared as early as day 18 post-infection. Obviously the conclusions drawn are limited by the sample size of 2 cattle. The onset of the immune responses to the seven VATs under study was much later in terms of day post-infection compared with the mice. Most likely this was due to the lower switching rate in the *ILTat* trypanosomes, which switch at 1×10^{-5} switches/cell/generation compared with 1×10^{-2} switches/cell/generation in the case of the EATRO 795 line, the assumption being that the generation of populations expressing novel variants would take longer in the lower switching trypanosome. In addition, the time to patency of infection was much longer in the cattle, 9 and 10 days, compared with an

average of day 5 for the mice, this probably being a product of body volume and growth rate of the parasites.

In almost every instance, the immune response to each variant remained detectable by this method until the end of the 70 day monitoring period. This suggested no waning of the immune response during this period. However, the immune responses to ILTats 1.21 and 1.64 in cow BV154 did drop below the detectable threshold. In the case of ILTat 1.21, the immune response was detectable from days 22 to 66 post-infection. The immune response to ILTat 1.64 was first detected on day 27, and became undetectable on day 60. This could be due to the lower parasitaemias detected in BV154 leading to a lesser magnitude of immune response in this cow. It could also be due to individual variation between the immune responses of the two cattle – they were not related, and comparison is not at the same direct level with the inbred BALB/c mice.

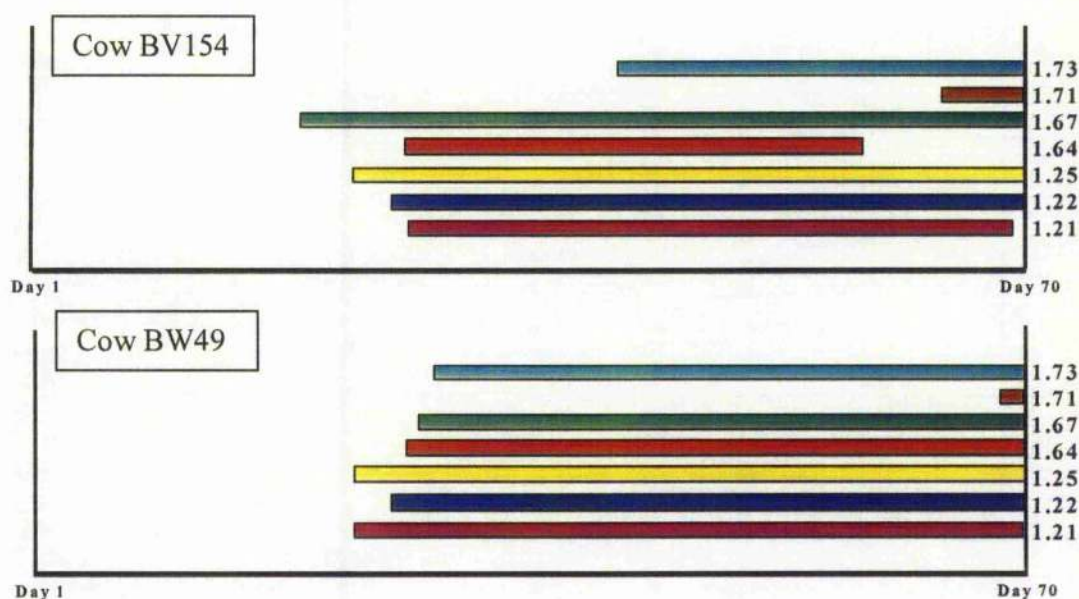


Figure 4.9 A schematic representation of the immune response onset and duration against the seven VSGs (ILTats 1.21, 1.22, 1.25, 1.64, 1.67, 1.71 & 1.73) in cows BW49 and BV154.

4.5 ELISA results

The second technique used to detect the host immune response was ELISA. In theory this technique should provide a method for differentiating between the levels of the different isotypes of specific immunoglobulin, in particular IgG and IgM, as well as defining more sensitively the onset of the specific immune response. The different isotypes are thought to play different roles in control of parasite numbers within the host, IgM featuring earlier in infection although maintaining high levels throughout (Luckins and Mehlitz, 1976; Musoke *et al.*, 1981), and the role of IgG increasing as the infection progresses and affinity maturation strengthens. IgM is involved in complement mediated lysis *in vivo*, and IgG more in opsonisation (Goddeeris, 1998).

The ELISA substrate was VSG purified from clonal trypanosomes grown up in rats, and purified as in section 2.2.5.1 (p. 41). This was whole soluble form VSG, including both the conserved C-terminus, and the variable N-terminus. The ELISA protocol was performed as in 2.5.4 (p. 42). Plates were tested using plasma sampled at 5-day intervals for the first 30 days, and at weekly intervals thereafter, the rationale being to concentrate on the onset of the specific immune response. Positive controls used serum from a rabbit immunised on 4 occasions at 3-weekly intervals with the same purified VSG protein. The rabbit was euthanased following the final immunisation, providing hyperimmune serum rich in polyclonal antibody versus the relevant VSG. Negative controls used serum prepared in an identical manner to a heterologous VSG, pre-infection serum from the appropriate cow, and dilution buffer alone. 2 plates were prepared for each VSG per cow. The first was used to measure the IgG1 level, as this is the subtype that predominates during trypanosome infections (Musoke *et al.*, 1981). The second plate measured the IgM response.

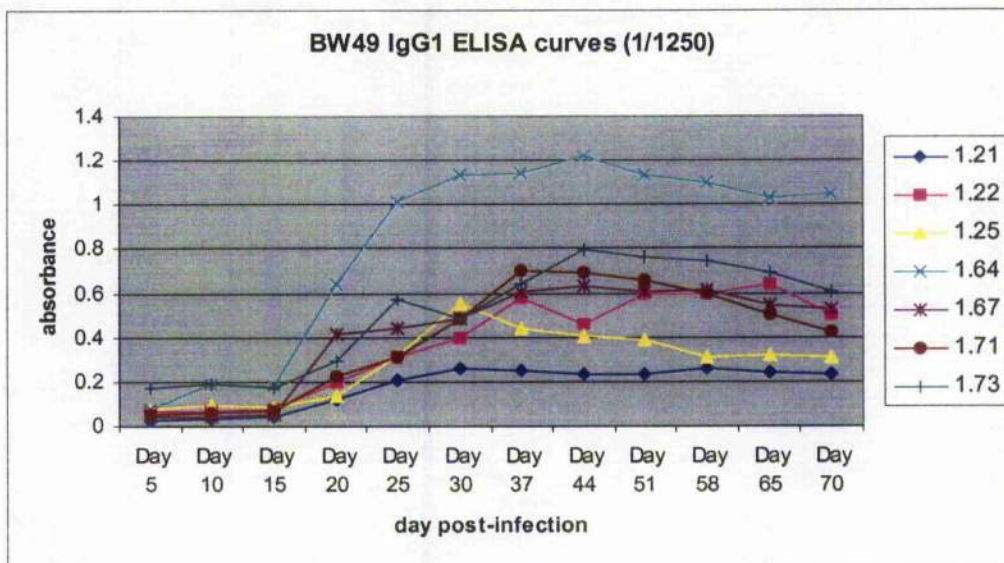


Figure 4.10 Curves of absorbance levels at timepoints throughout the 70 day infection, measuring the IgG1 levels to the seven VSGs under study in cow BW49. (Plasma samples were tested diluted 1/1250)

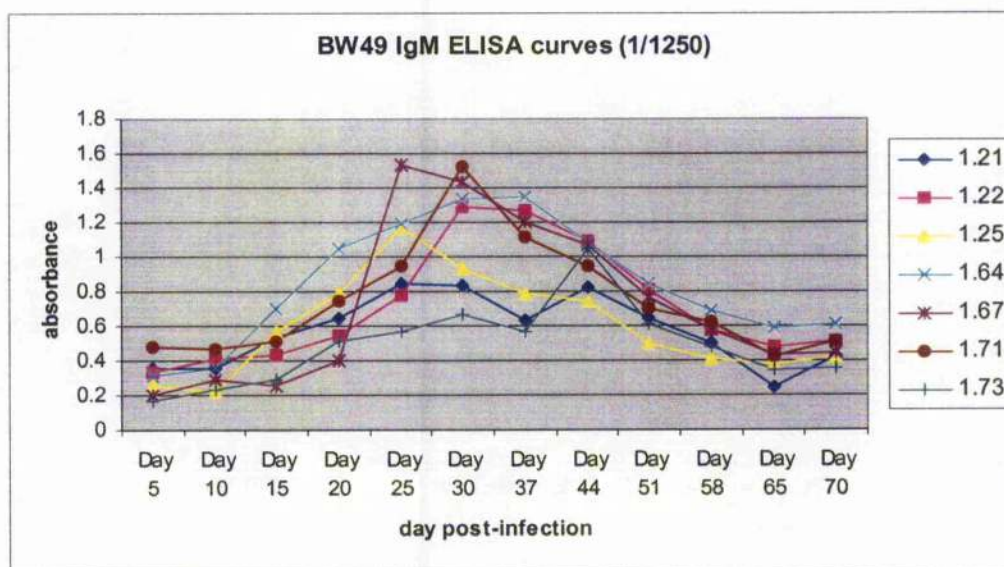


Figure 4.11 Curves of absorbance levels at timepoints throughout the 70 day infection, measuring the IgM levels to the seven VSGs under study in cow BW49. (Plasma samples were tested diluted 1/1250)

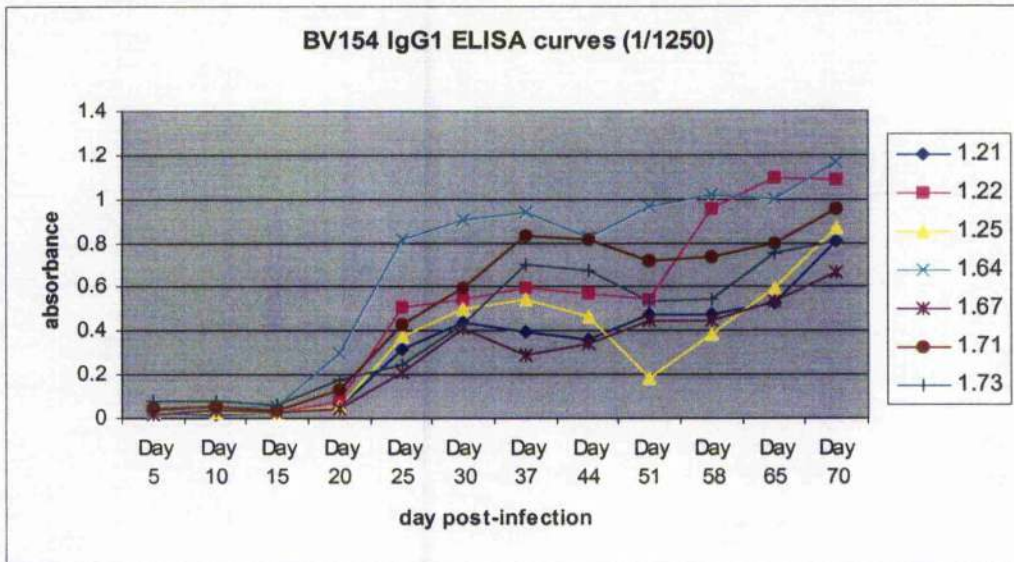


Figure 4.12 Curves of absorbance levels at timepoints throughout the 70 day infection, measuring the IgG1 levels to the seven VSGs under study in cow BV154. (Plasma samples were tested diluted 1/1250)

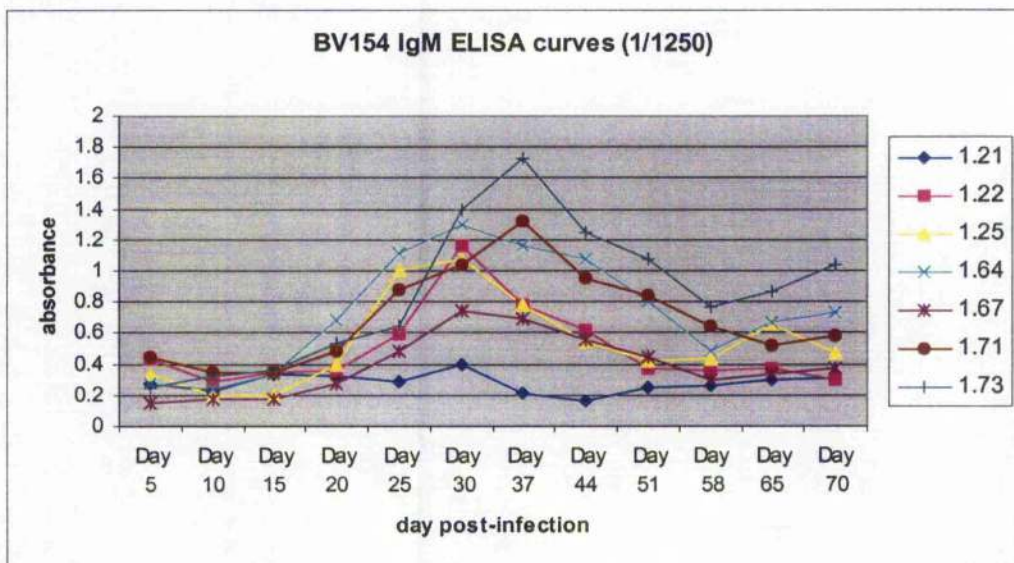


Figure 4.13 Curves of absorbance levels at timepoints throughout the 70 day infection, measuring the IgM levels to the seven VSGs under study in cow BV154. (Plasma samples were tested diluted 1/1250)

The results of the ELISA analyses were not conclusive, preventing discerning of a discrete onset of the specific immune responses. There was an observable difference between the progression of the IgG1 and IgM levels, and indeed between the profiles of the 2 cattle. There was very little similarity between the results obtained by the *in vitro* complement lysis and ELISA. The levels of immunoglobulin measured for the 7 VSGs under study, and VAT-specific antibody profile over the timecourse of the infections, were very similar to

each other within each cow. The IgM levels became detectable on day 15 post-infection, and rose to a peak between days 30 and 37 in both cattle, and then decreased again, dropping to a level slightly above that at the start of infection. The peak does correlate with the peaks of parasitaemia in both cattle, and in addition the drop corresponds with the period where no parasites were detectable in the cattle. This does not agree with observations made previously, where the IgM levels were maintained throughout infection (Luckins and Mehltz, 1976; Musoke *et al.*, 1981), although does concur with one observation that IgM levels are proportional to parasite burden (Nielsen *et al.*, 1978). With respect to the IgG1 levels, they also followed similar profiles across the infection for all of the VSGs. In cow BW49 the levels rose slowly, beginning to increase on day 20 to a peak between days 37 and 44, and thereafter remained at a slightly decreasing plane. In cow BV154, interestingly, there was a similar initial peak at day 37, which decreased to day 51, and then there was a second, greater peak in antibody levels, rising steadily until the end of the infection. These two profiles agree with the parasitaemic profile in the cattle, the second increase in BV154 corresponding with the second relapse peak of parasitaemia, which commenced on day 57. In contrast, the second relapse peak in cow BW49 was detected on day 68. A likely explanation for the similarity of the levels of antibody between the immune responses to the different VSGs is that the substrate was not specific enough. As it was whole VSG protein utilised, the cross-reactive, conserved regions of the protein were available for binding. The control plasma was able to differentiate between the VSG species, but this was hyperimmune plasma raised in rabbits by serial immunisation. The plasma extracted from the cattle was polyclonal, the antibodies against the VSGs were not purified in any way, and undoubtedly there will have been antibodies against conserved epitopes, raised against dead trypanosomes, or via fragments of parasites killed by the specific immune response. Therefore the ELISA has inadvertently provided a method of assessing the general IgM and IgG1 anti-VSG response, and agrees with the general observation that the IgM response is detectable earlier than that of the IgG1 response, but unfortunately has not enabled the distinction between the specific immune responses raised against the 7 VSGs under study.

4.6 Analysis of VSG expression by RT-PCR

RNA was extracted each day from the cattle blood collected during the infection. Specific oligonucleotide primers were designed from within the N-terminus coding region of each VSG, and the PCR conditions were optimised (for primer sequences see Table 2.1, p. 49). Products from each set of primers, produced by RT-PCR from RNA extracted from clonal

trypanosomes grown in an immunosuppressed mouse, were sequenced to ensure the correct product was amplified. The aim was to utilise the *VSG*-specific primers to detect the populations deriving from a switching event to the relevant *VSG*, and follow the longevity of a population expressing a particular *VSG*. This method, unlike following the specific immune responses, theoretically is detecting directly the products of a switching event at the soonest possible time point, and should give a clearer indication of any hierarchical switching.

4.6.1 Controls

Several positive control primers were also designed (for primer sequences, see Table 2.1, p.49). Primers were produced for bovine actin, to ensure RNA extraction proceeded as expected. In addition, primers were designed against trypanosome β -tubulin, in order that the presence of trypanosome RNA could be ascertained in those instances where other *VSG*s were being expressed. It was also decided to use primers to detect the RNA of the *VSG* expressed by the ILTat 1.2 trypanosomes that were inoculated, as a further positive control. A reaction combining cDNA from ILTat 1.2 trypanosomes and primers against the ILTat 1.2 *VSG* were included in each panel as a positive PCR control. As all primers designed and used were complementary to sequences within the coding sequence, it was essential in each case that DNAase treatment was carried out thoroughly, and RT positive and RT negative reactions were included. Additionally a reaction was included without any template DNA in order to ensure no contamination of PCR reagents.

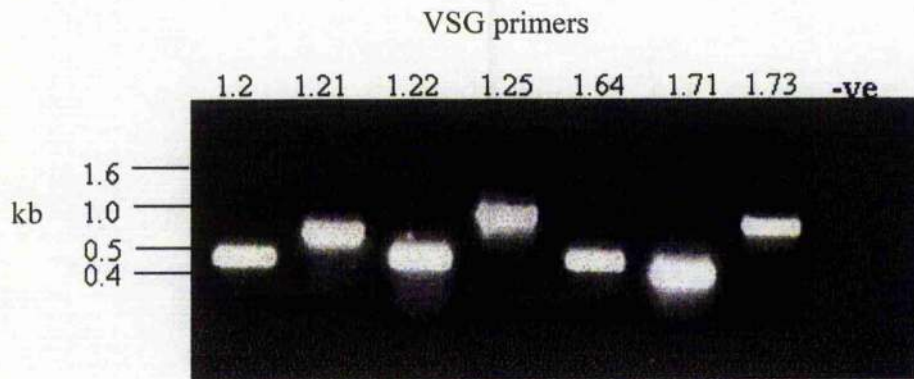


Figure 4.14 Size of expected PCR products. PCR reactions performed using ILTat 1.2 gDNA (y axis = 1 kb size standard ladder). -ve = no template negative control. The PCR products were run on a 0.7% EtBr stained gel.

As a means of quantifying the lower detection limit of the RNA extraction and RT-PCR methods used in the cattle, an *in vitro* control was undertaken. This was a necessary trial, as, although the peaks of parasitaemia in the cattle regularly exceed 1×10^6 trypanosomes.ml⁻¹, for much of the rest of the time, the levels would be much below this. In addition, the VAT-specific sub-populations would be only a fraction of this. In order to follow the populations of trypanosome expressing the VATs under study, a sufficient level of RT-PCR sensitivity was required. Lister 427 trypanosomes (a monomorphic line much utilised and characterised in *in vitro* studies) were grown *in vitro* in HMI-9 medium at 37° C to a concentration greater than 1×10^6 trypanosomes.ml⁻¹. An accurate count was determined using a haemocytometer, and serial dilutions were performed in horse blood, from 1×10^5 to 10 parasites.ml⁻¹. At each titre point, RNA extraction and RT-PCR were undertaken in an identical manner to that in the cattle. The primers used in the PCR reactions were designed and directed against the N-terminus of the 221 VSG, which is the predominantly expressed VSG in 427 trypanosomes.

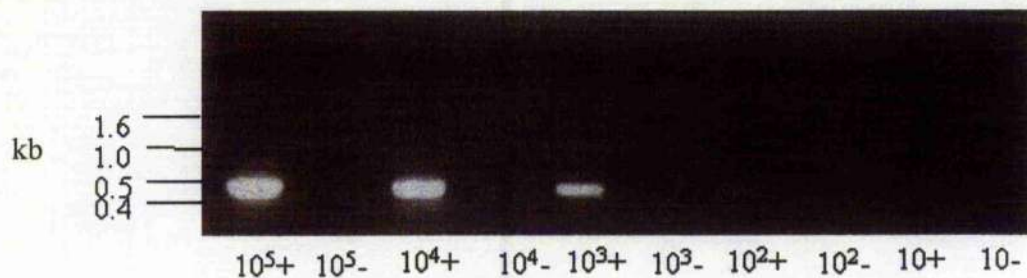


Figure 4.15 Agarose gel illustrating lowest concentration of *Lister 427* trypanosomes in horse blood detectable by 221 VSG-specific RT-PCR (numbers on x-axis refer to concentration of trypanosomes/ml, + = RT+ve, - = Rt -ve; y-axis 1kb ladder). The PCR products were run on a 0.7% EtBr stained gel.

The lowest detectable concentration of trypanosomes using this method was 1×10^2 trypanosomes. ml^{-1} , although the band on the agarose gel was very faint. The probable lowest reliable threshold was 1×10^3 trypanosomes. ml^{-1} . Southern blotting of the agarose gels and probing using PCR products was not attempted. There were insurmountable differences in this control experiment from the cattle samples, such as the use of horse blood instead of bovine. In addition, it cannot be guaranteed that all the 427 trypanosomes expressed the 221 VSG, although it was highly likely > 99% did (J.D. Barry, pers. comm.), therefore extrapolation of counted trypanosomes to detection of the 221 VSG must be cautious. However, it was concluded that this experiment provided an insight into problems that may have been encountered with low levels of parasitaemia. Additionally, the observed threshold level was concluded to be sufficiently sensitive for the expected parasitaemic profiles.

4.6.2 Possible use of mouse stabilates derived from trypanosome populations in cattle for VSG-specific RT-PCR.

Trypanosome stabilates were derived from the cattle blood by amplifying any parasite population present in an irradiated mouse, which was then passaged further through a second irradiated mouse. The possibility was considered of growing these stabilates in a mouse host, and then extracting RNA for use as a substrate for RT-PCR. The number of trypanosomes, and therefore the quantity of trypanosome RNA, would be much greater than that extracted from the cattle blood. To test this, an RT-PCR reaction was carried out with RNA extracted from ILTat 1.21 trypanosomes grown in an immunosuppressed

mouse. This was achieved with a stabilate 1 passage from the original cloned stabilate, a scenario identical to the mice stabilates that were derived from the cattle.

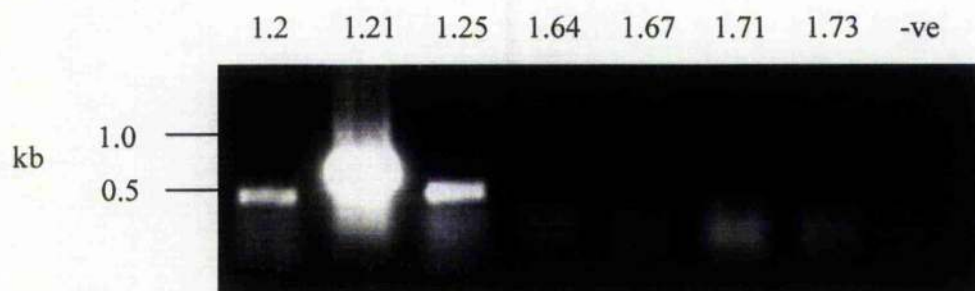


Figure 4.16 RT-PCR panel illustrating products obtained from RNA extracted from mouse blood. The amplified stabilate (ILTat 1.21) had a similar passage history to the stabilates derived from the cattle blood (Figures at side indicate size in kb of ladder fragment; RT negative panel not shown). The PCR products were run on a 0.7% EtBr stained gel. Primers used were complementary to sequences within the respective VSG N-terminus coding region (see Table 2.1).

It can be seen from the RT-PCR panel (Fig. 4.16) that, although the ILTat 1.21 product is the most abundant product, there have been switching events detectable by PCR, namely to ILTat 1.2, ILTat 1.22, and less so to ILTat 1.64 and ILTat 1.71. As the starting material was three passages from the original clone stabilate, and that would be the same period elapsed by the cattle derived trypanosomes, it was concluded that accurate RT-PCR detection of VATs required application directly to the RNA extracted from the infected cattle.

4.6.3 Levels of RNA extracted from daily blood samples during cattle infections

The quantity of RNA extracted on a daily basis from the cattle was measured by UV-spectrophotometer. As this source of RNA was the only suitable option for the RT-PCR approach, the amount could be a significant limiting factor in determining the presence or otherwise of VSG transcripts. Measurements were taken for the first 45 days of infection in both cattle in the first instance, and these were to be examined for correlation with success or otherwise of the RT-PCR analysis.

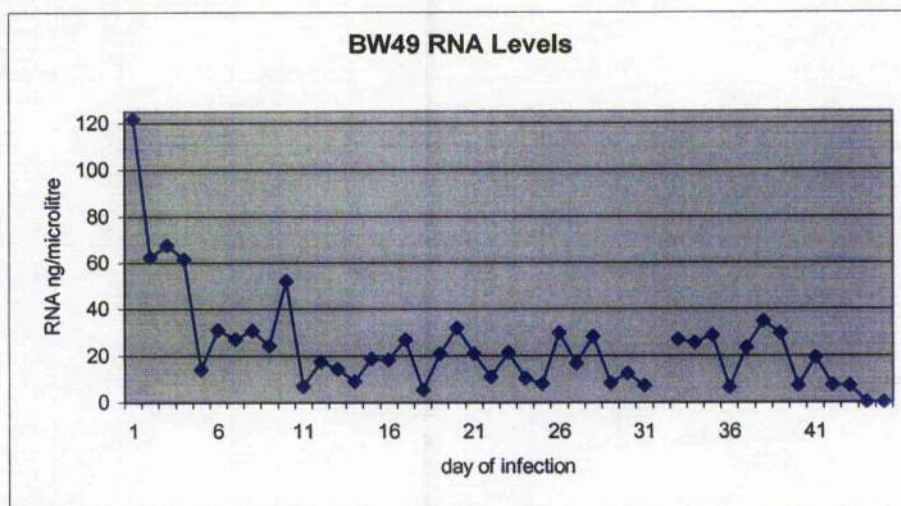


Figure 4.17 RNA levels ($\text{ng}\cdot\mu\text{l}^{-1}$) per daily sample for cow BW49 for the first 45 days of infection.

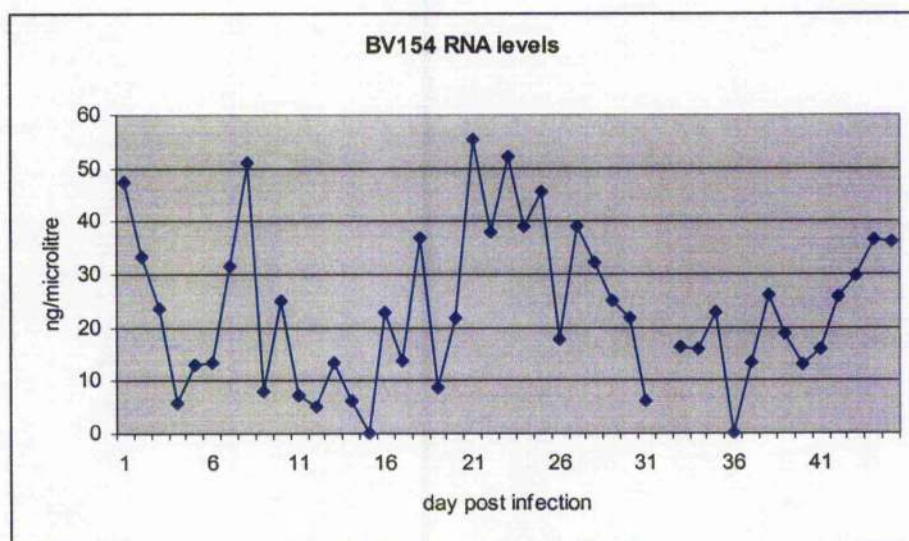


Figure 4.18 RNA levels ($\text{ng}\cdot\mu\text{l}^{-1}$) per daily sample for cow BV154 for the first 45 days of infection.

The levels of RNA indicated by UV-spectrophotometer are low, almost invariably under $50 \text{ ng}\cdot\mu\text{l}^{-1}$. The recommended optimum amount for first-strand cDNA synthesis (Invitrogen) is between 1 ng and 5 μg of total RNA. The levels obtained from the cattle fell within these parameters, but were close to the minimum recommended threshold. The fluctuating levels did not correlate either with the measured parasitaemia, or with the white blood cell counts observed in the 2 cattle. This low quantity is due to the extraction of RNA from only 200 μl of blood. This relatively small volume of blood, and concomitant

low number of trypanosomes will inevitably result in variation in RNA yield. No further measurements of RNA levels were taken, as it was deemed the positive control PCR reactions for bovine actin and trypanosome β -tubulin would provide information on the ability to amplify from the RNA substrate. As these are both constitutively expressed proteins, the absence of any detectable PCR product would indicate the isolation of insufficient RNA.

4.6.4 RT-PCR results

RT-PCRs were undertaken for each cow on every sample taken on a day where parasites were detectable, by haemocytometer, buffy coat examination, or amplification in irradiated mice. This meant an initial pool of 33 samples for cow BW49 and 46 for BV154. In the instance of *VSG* transcripts being detected readily in these sets of samples, examination of the remainder would be undertaken. Reactions were directed towards detection of the constitutively expressed bovine actin and trypanosome β -tubulin. In the event of successful amplification of their transcripts, PCRs subsequently were aimed at the seven *VSGs* under study. The samples collected from cow BV154 were independently "blinded", by assigning a code to each sample, which was translated only upon completion of the PCRs. This was done to ensure lack of bias during interpretation of gel results, and to provide validation of the pattern of results obtained for cow BW49.

Bovine actin gene products were obtained in the majority of samples analysed; 78.8% from BW49 (26 samples of 33 analysed), and 80.4% from BV154 (37/46). This indicated that the blood sampling, RNA extraction, DNAase treatment and reverse transcriptase steps all functioned in these samples. It is likely that in the cases where no bovine actin detectable, one or more of these steps had failed. However, there was a disappointingly much lower demonstrable level of trypanosome β -tubulin transcripts in samples from both cattle. 39.4% (13/33) of samples from BW49 and 41.3% (19/46) of samples from BV154 had detectable trypanosome RT-PCR products. This was, respectively, 50% and 51.3% of samples positive for bovine actin. These figures alone indicate that the amount of trypanosome material available in the samples from the cattle was indeed a limiting factor. However the similarity in the number of trypanosome positive samples from the two cattle does indicate uniformity in experimental technique.

The samples that were positive for trypanosome β -tubulin were then examined for the presence of specific *VSG* transcripts. Products for all 7 *VSGs* were identified in both cattle.

VSGs were identified in 53.8% (7/13) of the samples from BW49 and 36.8% (7/19) from BV154. The period within each infection during which *VSGs* were detectable is remarkably similar. In cow BW 49, *VSG* transcripts were detectable on days 12 and 13 post infection, corresponding with the initial parasitaemic peak, on days 19, 20, and 23, which relate to the first relapse peak(s), and on day 43, which matches with a small independent peak after the first relapse. For BV154, *VSGs* were first detected on day 12 of the infection, again matching the initial parasitaemic peak, and thereafter on days 17, 18, 20, 21, 23 and 24, during the first relapse peak(s) (for parasitaemias refer to Fig. 4.1, p. 80).

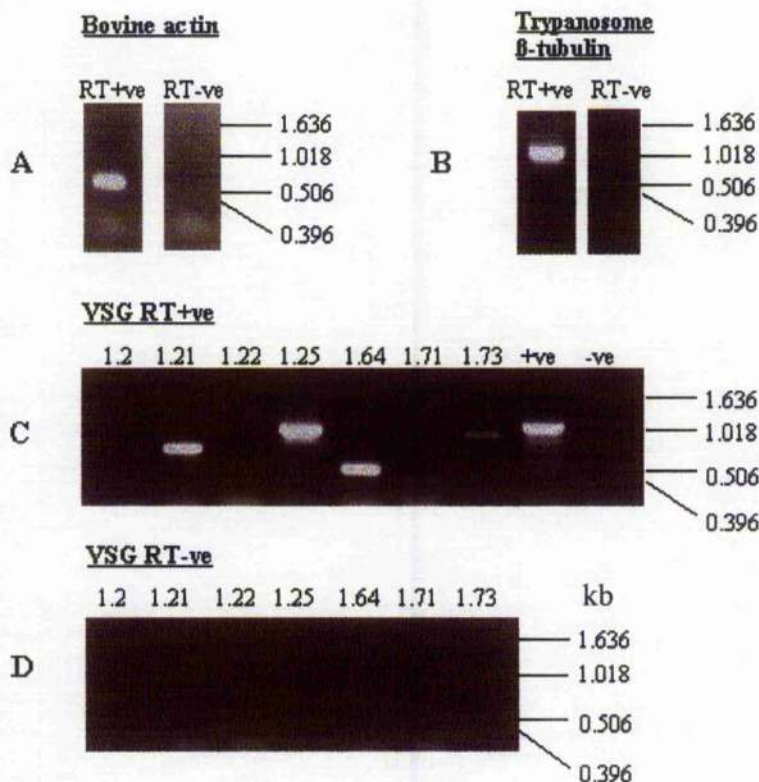


Figure 4.19 Example panel of RT-PCR products. Agarose gels illustrating products obtained from cow BV154, day 20. Panel A; bovine actin, Reverse Transcriptase (RT) positive (+ve) and negative (-ve). B; trypanosome β -tubulin, RT +ve and -ve. C; VSGs, RT+ve. Positive control (ILTat 1.25 primers against ILTat 1.2 gDNA). Negative control, no template. D; VSGs, RT -ve. The PCR products were run on a 0.7% EtBr stained gel.

As can be seen in Figures 4.20 and 4.21, some of the PCR products were extremely faint, presumably due to the low amounts of RNA in the original sample. Ideally further confirmation by a method such as nested PCR would have been undertaken, but unfortunately this was not possible due to time constraints. For the same reason, sequences

of all PCR products obtained were not determined, although several products were shown to have the expected sequence.

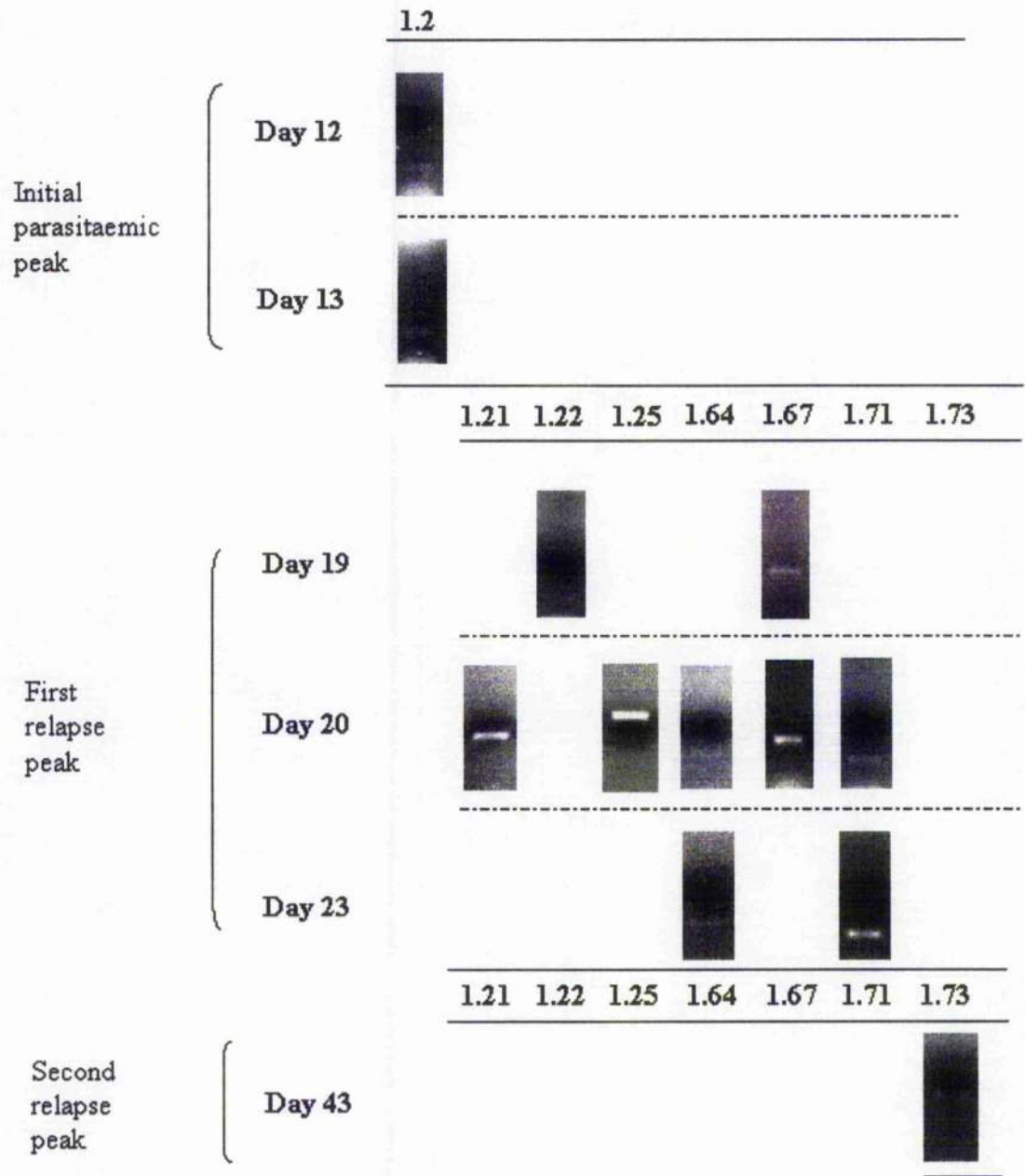


Figure 4.20 Schematic illustrating positive products on agarose gels, obtained from samples from cow BW49 for the 8 VSGs examined, and the days post-infection when detected.

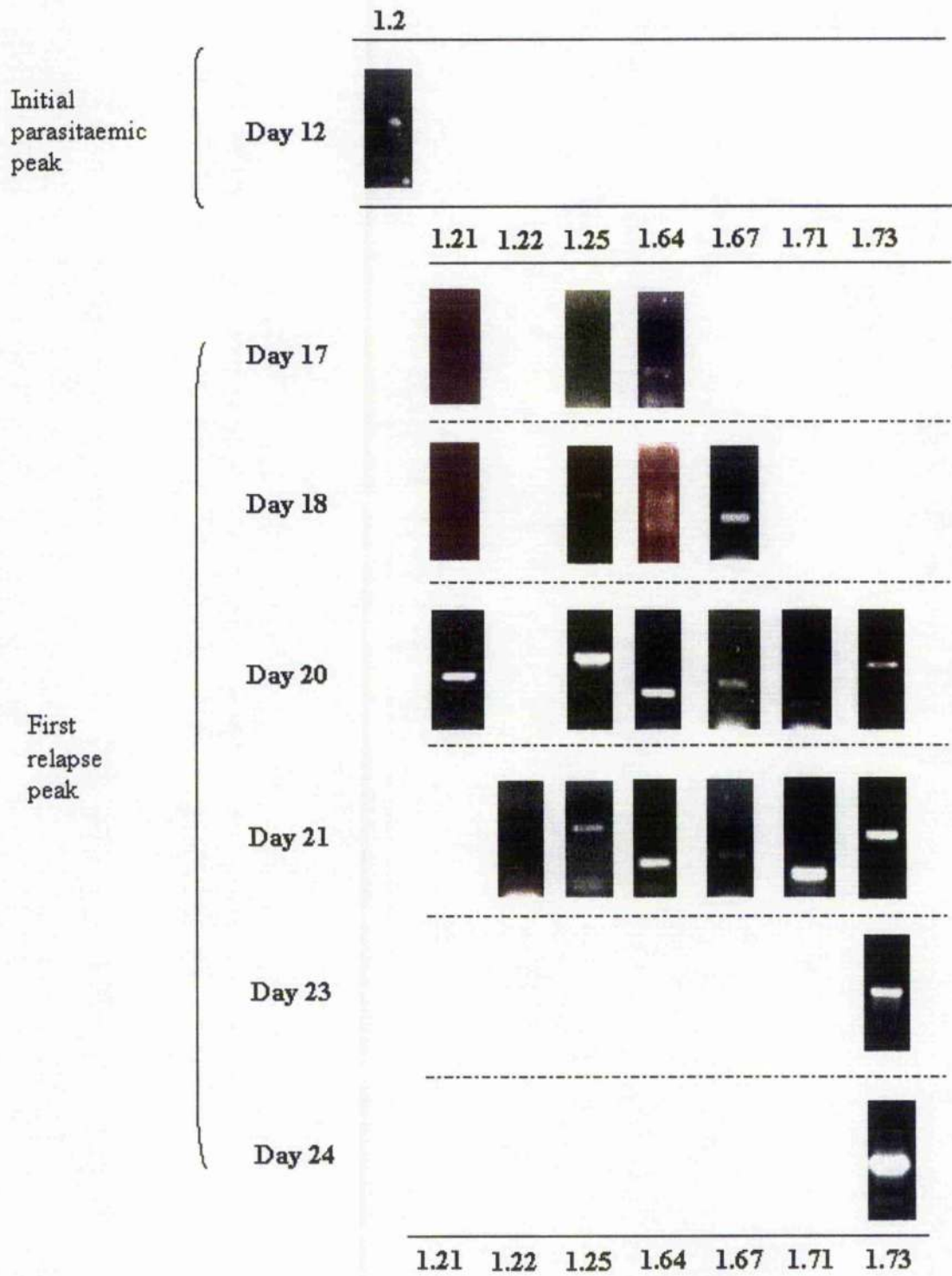


Figure 4.21 Schematic illustrating positive products on agarose gels, obtained from samples from cow BW49 for the 8 VSGs examined, and the days post-infection when detected.

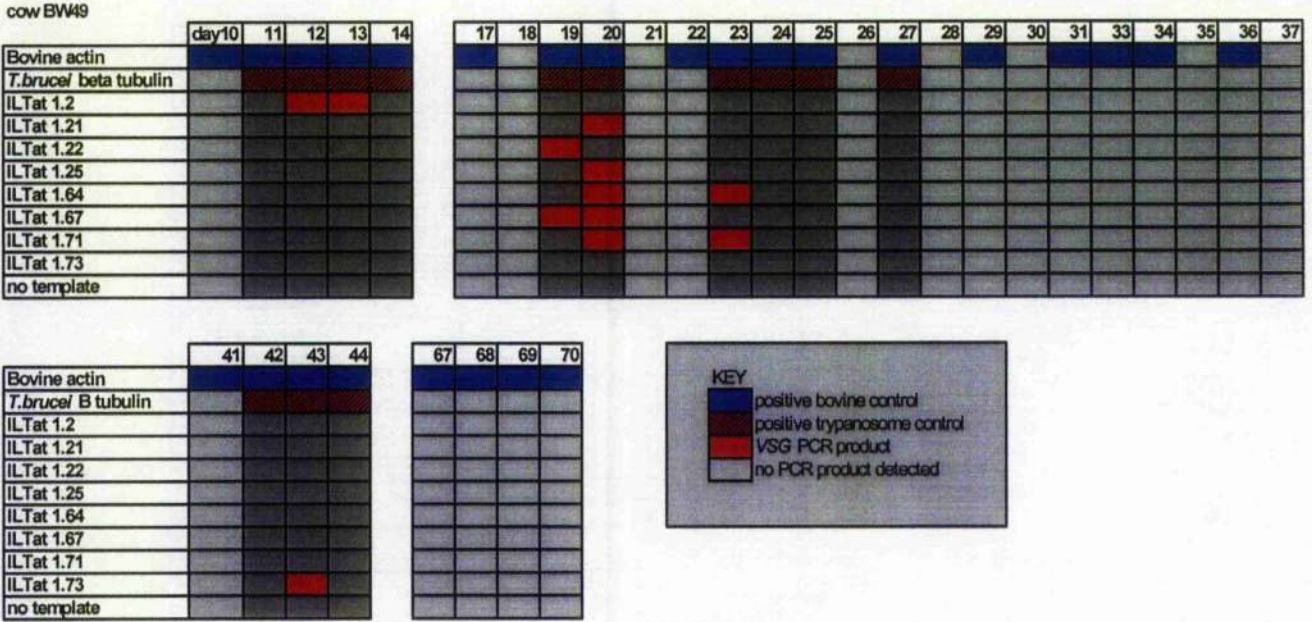


Table 4.3 Summary of PCR results for all samples analysed by RT-PCR during chronic infection of cow BW49. Separate blocks represent peaks of parasitaemia.

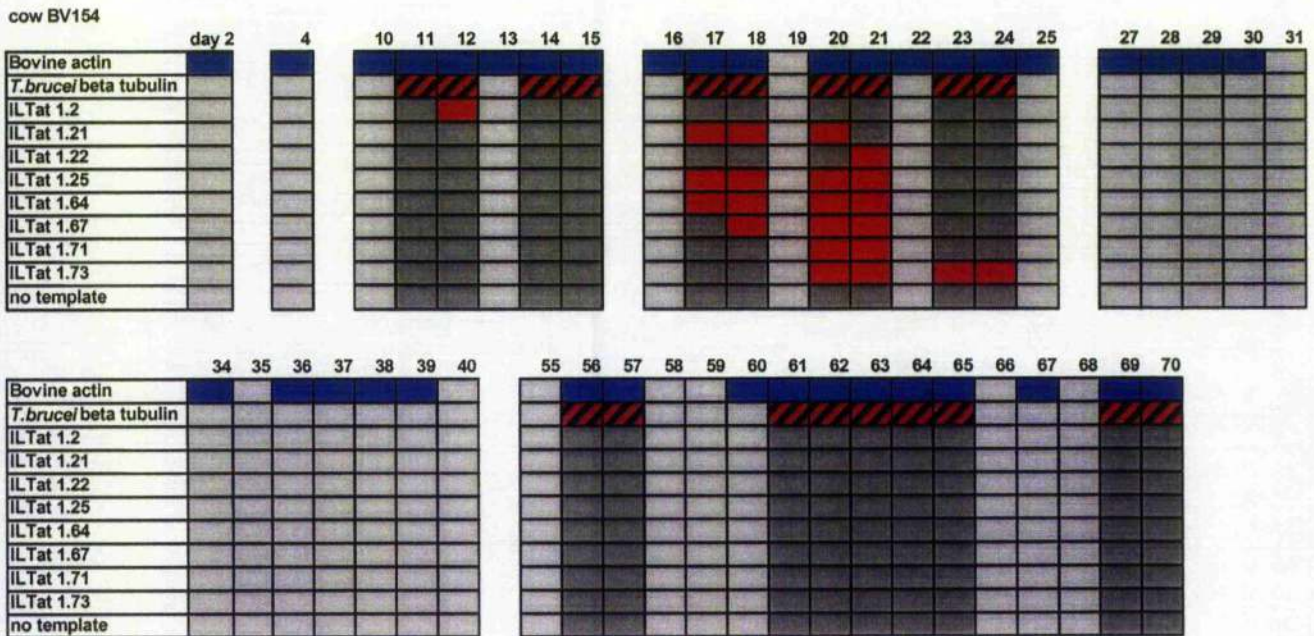


Table 4.4 Summary of PCR results for all samples analysed by RT-PCR during chronic infection of cow BV154. Separate blocks represent peaks of parasitaemia. Key as for Table 4.3.

The pattern of appearance of the specific VSGs is somewhat obscured by interruptions in the data where it was not possible to detect trypanosome RNA (see Tables 4.3 and 4.4, p. 103). Of the 8 VSGs examined, only ILTat 1.2 was detectable in initial parasitaemic peaks. ILTat 1.2 was the VSG expressed by the inoculating clonal line, and thus was expected to appear early in infection. The results indicate that the initial parasitaemic peak did not contain any of the 7 relapse VSGs under study.

In both cattle, the majority of PCR products for the VSGs under study were detected during the first relapse peak. Indeed all 7 appeared in this peak in BV154, and the same occurred in BW49, with only 1 exception. The order of appearance can be distinguished only with uncertainty. In BW49, ILTats 1.22 and 1.67 were detected first, on day 19, and there were transcripts of ILTats 1.21, 1.25, 1.64, 1.67 and 1.71 on day 20. Unfortunately on days 17, 18, 21 and 22 it was not possible to amplify any trypanosome products. By day 23, only ILTats 1.64 and 1.71 were detectable, and for the remainder of the first relapse peak no specific transcripts were detected. ILTat 1.73, however, was detected on day 43, during a peak of short duration (4 days) that occurred 4 days following the end of the first relapse peak. Thereafter there were no PCR products detectable for the remainder of the infection. It was disappointing not to obtain information for the days 17, 18, 21 and 22, as these possibly would have given more information on the dynamics of this subset of VSGs.

For the BV154 infection, the picture is slightly clearer, although there were still gaps in the information. ILTats 1.21, 1.25 and 1.64 were detected on day 17, and these were joined by ILTat 1.67 on day 18. Day 19 did not have any detectable bovine or trypanosome material. On day 20, it was possible to amplify products for all 7 VSGs apart from ILTat 1.22. On day 21 there was no longer an ILTat 1.21 transcript, but ILTat 1.22 appeared for the first time, along with ILTats 1.25, 1.64, 1.67, 1.71 and 1.73. By days 23 and 24 only ILTat 1.73 was present. Thereafter there were no PCR products detectable for the remainder of the infection. Again, the overall picture is frustrated by the lack of information available on days 16, 19 and 25. However, in this infection the appearance of the 7 candidate VSGs did span the course of one distinct parasitaemic peak, indicating that this peak is made up of several sub-populations, each deriving from switching events and expressing the VSGs under study. In the case of BW49, the VATs other than ILTat 1.73 did occur over a time period similar to that of their counterparts in the BV154 infection, but seemed to form a subpeak at the beginning of a large period of continuous parasitaemia.

ILTat VSG	BW49	BV154	VSG locus
1.2	12,13	12	*
1.21	20	17,18,20	mini
1.22	19	21	MES
1.25	20	17,18,20,21	mini
1.64	20,23	17,18,20,21	MES
1.67	19,20	18,20,21	BES
1.71	20,23	20,21	int
1.73	43	20,21,23,24	int

Table 4.5 Table illustrating days on which VSGs were detectable by RT-PCR in cattle BW49 and BV154 (mini = minichromosomal, MES = metacyclic expression site, BES = bloodstream expression site, int = chromosomal internal, * = inoculating VAT)

4.7 Summary

When the results of the PCR are combined with those of the *in vitro* complement lysis assays, a broader picture of the infection in the two cattle can be outlined (see Figure 4.22, overleaf). The 2 sets of results correlate, with detection of PCR product slightly preceding that of the respective immune response (with the exception of ILTat 1.73 in cow BW49). In addition, there was very little overlap between the PCR products and the immune response, indicating the removal of trypanosomes expressing the respective VSG by the specific immune response. The mRNA that gave rise to PCR products appeared only within a restricted timeframe, and seemed not to reappear, indicating 1 continuous subpopulation existing until the emergence of the antibody response. The restriction of the subset of VSGs to a narrow timeframe, early in terms of the infection, is similar to the previous findings in rabbit infections (Robinson, 1999). These observations indicate that the subset of VSGs examined, are activated relatively early in a truly chronic, long-term infection, and leads to intriguing questions as to what is occurring later. Unfortunately, there is discontinuity in the PCR data, due to the low amounts of trypanosome RNA extracted in the cattle samples and occasional failure of RNA extraction. However, the complement lysis results, combined with those from PCR, provide a hitherto unseen insight into the dynamics of a long term infection with regard to both parasite and host.

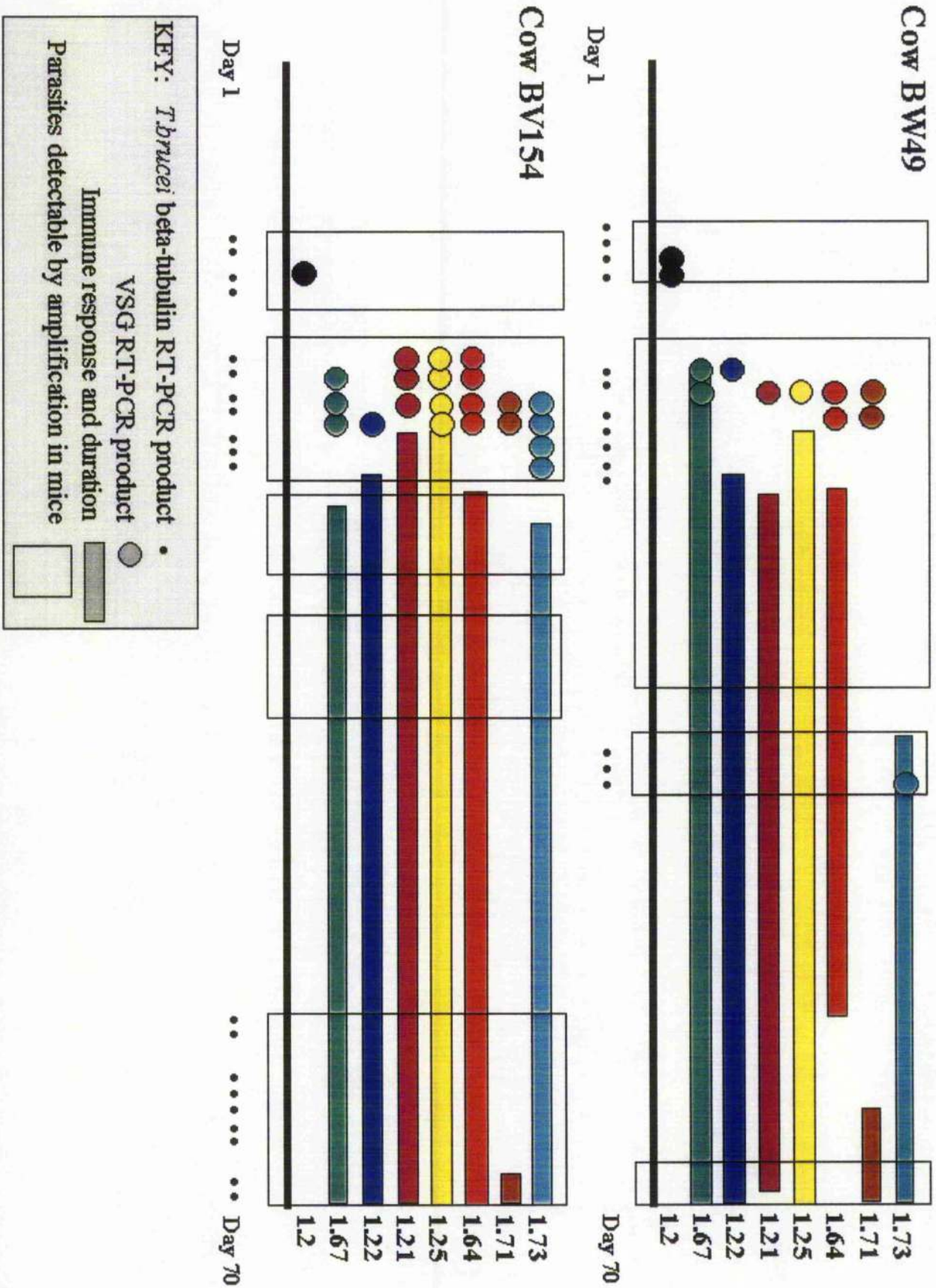


Figure 4.22 Graphical representation of VSG expression and specific immune responses during trypanosome infections in cattle BW49 and BV154.

Chapter 5

Mathematical model

5 Mathematical Model

5.1 Introduction

The population dynamics of a parasite population within a host are a consequence of parasite effects, host effects, and the interaction between the two factors. In trypanosome infections, there is a characteristic profile of a chronic undulating parasitaemia, with peaks getting smaller and wider apart. It is thought that these kinetics result from the system of antigenic variation, a mechanism by which trypanosomes produce sequential novel VSG coats, thereby inherently pre-empting the host's specific immune responses, the functional target of which is the VSG. A typical trypanosome infection has a number of characteristics. The appearance of variants is hierarchical to an extent, possibly leading to optimal use of the available repertoire of *VSG* genes, and extending the chronicity of the infection. Each peak of parasitaemia consists of multiple sub-peaks, each a population expressing a different VSG. There is never a patent re-expression of any variant, as the specific immune response to that variant seems to remain throughout the timecourse of an infection.

A variety of mechanisms have been proposed to be the basis of the "semi-predictable" ordering of expression of VSGs, as has been observed experimentally (Gray, 1965; Capbern *et al.*, 1977; Miller and Turner, 1981). Much of this speculation has not been satisfactory in terms of withstanding either theoretical or experimental scrutiny. For example, it has been suggested that differential growth rates conferred upon the trypanosome by the expression of different VSGs was a possible system (Seed, 1978), but this has been discounted both theoretically, where the observed growth differences were not sufficient to produce the profile seen in trypanosomes (Kosinski, 1980), and experimentally, where again the growth rates observed between clones expressing different VSGs were not markedly different (Aslam and Turner, 1992).

Modelling has been used as a tool for examining the dynamics of interactions between parasites and hosts (Lythgoe, 2000; West *et al.*, 2000). Modelling allows manipulation and examination of the effects of certain aspects of the relationship. The technique by definition is population based, and the output is phenotype driven. Attempts at modelling the dynamics of trypanosome infections thus far have had mixed results. Several models have arisen from hypotheses that do not have biological or experimental support, such as differential immunity towards trypanosomes expressing two VSGs during a switching

event (Agur *et al.*, 1989), and the influence of the immune response against invariant antigens (Agur and Mehr, 1997). There are examples of models that produce viable outputs, and successfully incorporate established biological features of the parasite (Frank, 1999). The aim of this study was to produce a model that could build on the positive aspects of the published theoretical models, include as many as possible known biological parameters, and simulate the *in vivo* kinetics and dynamics of a trypanosome infection, with the key variable being the semi-ordered expression of VSGs.

5.2 Background

The credibility of any proposed idea depends on the soundness of its foundations. Modelling is no exception, and for this reason it is worthwhile critically reviewing previous work. In addition, certain aspects of previous models will be incorporated into this study. A number of mechanisms have been proposed to be behind the semi-predictable expression of the VSG repertoire. Frank (Frank, 2002) grouped the various hypotheses broadly under 3 headings; natural selection, antigen cross-reactivity and variable switching rates. I will combine the first 2 under the broad heading of 'natural selection', and use the heading 'molecular switching' for the latter category.

5.2.1 *Natural selection*

The ability of different VSGs to confer different growth rates upon the trypanosomes has long been proposed as a mechanism for producing the sequential peaks of parasitaemia (Seed, 1978). This would arise due to the first variants being those that more efficiently made use of the resources available, and once these are removed by the specific immune response, those with slightly less of a competitive advantage would predominate, and so on. There are, however, fundamental flaws with this hypothesis. Firstly, the range of differences in growth rates would need to be significant to produce such a progression of infection (Kosinski, 1980). Indeed experimental analysis has suggested that any observed difference in growth rates is not sufficient to support this theory (Myler *et al.*, 1985; Aslam and Turner, 1992). In addition, although individual clones derived from trypanosome populations can exhibit different growth rates, the linking of this phenotype to the expression of particular VSGs has not been proven by study of large numbers of clones. However, in Seed's study there were two clones, expressing the same VSG, with different growth rates, indicating that growth is not a function of VSG expression.

A switching intermediate, a trypanosome that expresses 2 VSGs at the same time before protein turnover results in the removal of the previously expressed VSG, has been suggested as a fundamental aspect in determining the ordered appearance. Agur *et al.* (1989) produced a model whereby these double expressor trypanosomes had differential susceptibility to the host's immune response. Using this model, a range of susceptibility of double expressors produces a hierarchical progression of VAT expressing sub-populations, even with all switches having similar probabilities. Although simulations in the same paper support this hypothesis, it has no biological support. Double expressor trypanosomes have been generated *in vitro*, demonstrating that the expression of 2 VSGs at once is not in itself intrinsically harmful (Chaves *et al.*, 1999). A second study suggested that expression fluctuates between 2 VSGs when double expression was maintained by insertion of drug resistance genes into 2 different BESs, and subsequent maintenance under drug selection (Munoz-Jordan *et al.*, 1996). This indicates the double expressor is likely to be an unstable, transitory intermediate.

A similar possibility not proposed before, which would in effect function in the same manner as the double expressor theory, is where the immune response specific to each VSG differs in the qualitative ability to either recognise or kill that variant. In effect, this translates as a range of immunogenicity across the VSGs. There have not been any studies done to corroborate this hypothesis. It also suggests that the VSGs are present concurrently, and it is the differential immune response that determines the longevity of any particular VSG-expressing sub-population. However, data suggest that this is not the case, and there are discrete separate VAT populations, certainly if compared between parasitaemic peaks (Robinson, 1999).

The prospect of an increasing role of non-variant antigens as an infection progresses has been suggested as significant in shaping the profile of trypanosome infections (Antia *et al.*, 1996; Agur and Mehr, 1997). The suggestion is that after the first few peaks of parasitaemia, invariant antigens become a significant target of the host's immune response, particularly as a proportion of these proteins are invariant for functional reasons. Certainly Agur & Mehr suggest that only by including this parameter in their model was it possible to mimic the phenomenon of parasitaemic peaks becoming wider apart and lessening in magnitude. There is limited experimental evidence of immune responses being detected against invariant antigens during *T. congolense* infections in cattle (Authie *et al.*, 1993), and *T. brucei* infections in mice (Radwanska *et al.*, 2000b). However there is no experimental substantiation of the effectiveness of these responses. Indeed, it can be suggested that the increase in antibodies against invariant antigens will inevitably occur in

chronic infections, as there will be exposure via cells lysed by the specific immune response, and from senescent short stumpy trypanosomes. However, the efficacy of these antibodies is questionable, as these antigens are not exposed on living trypanosomes due to the VSG barrier, and they are therefore probably not relevant in terms of protective responses.

5.2.2 *Molecular VSG switching and homology*

The proposed variable in our model that affects the profile of *in vivo* infections, and in particular the semi-predictable order of expression of VSGs, is the different activation rates of different (categories of) VSGs. This aspect of trypanosome molecular biology has been used to produce the most compelling model to date (Frank, 1999). The molecular switching of VSGs is a well studied process, although not fully understood. Our model proposes to input two partially overlapping aspects that influence VSG switching; the position of the *VSG* within the genome, and sequence homology between silent and active *VSG* genes.

The influence of different genetic loci on the position of the silent *VSG* gene within the hierarchy has been well studied. In both monomorphic (Liu *et al.*, 1985) and pleomorphic (Robinson *et al.*, 1999) trypanosomes it has been established that subtelomeric *VSGs* tend to be activated before *VSGs* located internally in chromosomes. Other events such as mosaic gene formation (Thon *et al.*, 1990; Kamper and Barbet, 1992) seem to be rare occurrences, probably occurring later in the infection period (Barry, 1997). A secondary, but related phenomenon is the predominance of duplicative switching processes involving the replacement of the active gene with a silent gene by a recombinatorial mechanism. The other mechanism is *in situ* switching, whereby 1 bloodstream expression site's promoter is switched off, and another turned on. The ratio of these mechanisms has been measured as at least 9:1 respectively (Robinson, 1999) in pleomorphic trypanosomes. The earlier appearance of subtelomeric *VSGs* is likely to be a reflection of this, as the association and consequent recombination between two subtelomeric genes, probably is easier than between an interstitial and a subtelomeric locus. The predominance of duplicative switching events means that sequence homology must play a role in switching. It has been demonstrated that proteins involved in homologous recombination (in particular RAD51) exert influence in antigenic variation (McCulloch and Barry, 1999). Homologous recombination requires relatively long stretches of sequence homology. This is most likely provided by the array of 70-bp repeats which are upstream of the *VSG* genes in varying numbers. However, the formation of mosaic genes later on in infection, if mediated by

homologous recombination, has to involve homology within the *VSG* coding sequence. This particular aspect has not been examined thus far, due to the limited number of confirmed mosaic *VSGs* isolated (Thon *et al.*, 1989; Kamper and Barbet, 1992).

Frank (1999) concluded that only “minor modifications of switch rates by natural selection are required to develop a sequence of ordered parasitaemias”, the modifications being a function of the different probability of particular *VSGs* switching to others, producing a range of switching rates. He does not attribute this to specific mechanisms, but the model provides a matrix of switching frequencies that affect the loosely ordered profile, the variations determined by regions of homology between the *VSG* loci and flanking regions, with the 70-bp repeats specifically being mentioned as possibly being important. Growth, antibody production and effect, and carrying capacity of the host were considered to be independent of the *VSG*. The model is essentially deterministic, whereby variant A will switch to variant B, which will then switch to variant C and so on. However, this produces a lack of reversibility in the system. If homology was a significant factor, then variant B is presumably as likely to switch to A, as it is to C.

A modification of the Agur model (1989) inadvertently agrees with the significance of differential switching. It is mentioned in passing that when the role of the double expressor intermediate is much reduced, and “preferential activation of telomeric genes” or “DNA nucleotide sequence controlled preferential activation” are introduced, this leads to a hierarchical progression of *VSGs*.

In our model we consider a refinement of Frank’s mechanism for switching. In this enhancement, there are variants that are more likely to be switched to than others, and this can be dependent or independent of the variant that is switched from, dependent respectively upon the relative significance of genomic position and sequence homology. This results in a system that is less deterministic than Frank’s model, which is important, as it reduces artificial rigidity, and should reflect with greater accuracy the picture *in vivo*.

5.3 Model input

The model begins from the point of inoculation of 4000 trypanosomes, with a repertoire of 30 *VSGs*. The population of trypanosomes will grow logarithmically, until reaching a level where the density-dependent formation of short stumpy form trypanosomes causes the parasitaemia to plateau (Reuner *et al.*, 1997). This differentiation stage is also essential in shaping the profile of infection, and incorporating aspects of a biologically valid model of

in vivo stumpy stage formation (Tyler *et al.*, 2001) was important in order to produce a more holistic simulation.

The population of trypanosomes within the host consists of 2 morphological forms, which vary in their proportions as infections progresses; the total population of long slender trypanosomes, V , and short stumpy form trypanosomes, M . Maximum stumpy production occurs at the concentration of cells K . Within this greater framework there also exist several sub-populations, each expressing an antigenically unique VSG. For a given variant i , there is a slender population v_i , and a stumpy population m_i . Slender cells have an intrinsic growth rate $r_{i,t}$ and there is a probability f that when a cell divides one of the daughter cells will differentiate to a terminal short stumpy cell. This probability increases as the total number of parasites increases, as this is a density dependent phenomenon (Reuner *et al.*, 1997). All variants switch at the same rate, which is measured as the global switching rate; 1×10^{-2} switches/cell/generation (Turner, 1997). There are differences in the rates at which particular variants are switched to, but this is independent of the variant that it is switched from. The rate at which a particular variant i is switched to from variant j is defined as s_{ij} .

The immune response to variant i is the second aspect that will determine the fate of populations v_i and m_i . The killing rate depends on the strength of the acquired immune response a_i , and the maximum rates at which the immune system eliminates slender cells, d_i , and stumpy cells, δ_i . There is also a general killing function. At the beginning of infection the growth rate is at a maximum, r , but as the duration of the infection increases there is a generalised inhibition of growth rate independent of the acquired immune response to each of the variants. For all of our simulations we chose a value of ρ of 2500, meaning it takes 26 days for growth to be inhibited by 50% as determined experimentally (Turner *et al.*, 1996).

Symbol	Parameter
V	total number of long slender cells
M	total number of short stumpy cells
K	number of cells at which maximum stumpy production occurs
v_i	slender cells of variant i
m_i	stumpy cells of variant i
r_i	growth rate of variant at time t
f	probability of one daughter cell differentiating to a short stumpy cell
r	maximum growth rate of variant
$s_{i,j}$	rate at which variant i is switched to from variant j
a_i	acquired immune response
d_i	maximum rate of killing of slender cells of variant i
δ_i	maximum rate of killing of stumpy cells of variant i
ρ	general killing function
τ	time lag for onset of acquired immune response
c_i	Scaled constant of the acquired immune response against variant i
C	Scaled constant of the acquired immune response
x	rate of the intrinsic growth of the immune response
p_i	rate at which i gets switched to independent of variant j it is replacing
$h_{i,j}$	measure of readiness of variant i to replace variant j based on homology

Table 5.1 Summary and explanation of model parameters.

Following this the dynamics of slender and stumpy cells are given by;

$$\frac{dv_i}{dt} = v_i r_i (1 - f) - v_i d a_i + \sum_{j=1}^n (v_j s_{i,j} - v_i s_{j,i})$$

$$\frac{dm_i}{dt} = v_i r_i f - m_i \delta a_i$$

where

$$f = 1 - e^{-(V+M)/K}$$

and

$$r_t = r e^{-t/\rho}$$

The modelling of the acquired immune response to each variant, a_i , was that used by Tyler *et al* (2001), using a single equation;

$$\frac{da_i}{dt} = c_i (1 - a_i) \left(\frac{v_i' + m_i'}{C} \right)^x$$

where c_i , C and x are constants, and the prime (v_i' and m_i') indicates the number of cells at time $t-\tau$ where τ is the time lag that it takes the immune system to respond. The growth of the immune response against variant i has an intrinsic rate which is dependent on the number of cells in populations v_i and m_i . The parameter x was used as a variable, determining how the intrinsic growth rate of the immune response varies with the increasing population.

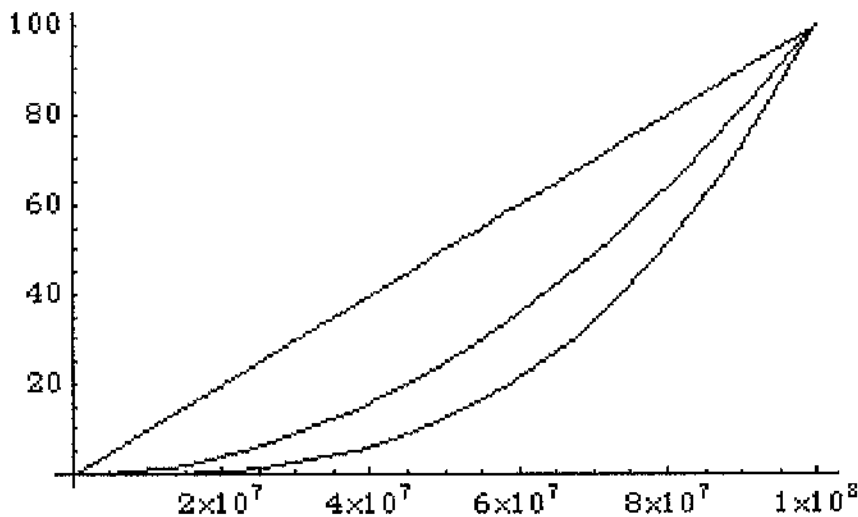


Figure 5.1. The intrinsic rate of growth of the immune response (y-axis) for a given number of cells in the host. $c_i=100$, $C=10^8$. Lines from top to bottom, $x=1, x=2, x=3$.

When $x=1$, the growth rate of the immune response is directly proportional to the increase in the number of trypanosomes. For higher values of x , the rate of growth of the immune response is relatively slow when the number of trypanosomes is low, and relatively fast as the number of parasites increases. Thus as x increases, a threshold like effect is seen, where for small numbers of trypanosomes, the immune response does not respond, but beyond the threshold, the immune response increases rapidly to its maximum. The latter scenario is similar to what occurs *in vivo* with trypanosomes, and with immunogens in general. This threshold-like effect has been seen when immunising cattle with different size inoculi of *T. brucei*; no protective effect was seen below 1×10^7 trypanosomes, but above this level complete protection was observed (Morrison *et al.*, 1982a). When mice were infected with different titres of *T. congolense*, it was not possible to detect any antibody at all (by single radial immunodiffusion assay) below 1×10^5 parasites, but above this threshold antibody was detected in all mice (Morrison and Murray, 1985).

There is also the built-in assumption that the immune response does not wane against specific VSGs during the lifetime of the infection. Although in both the cattle and mice chronic infections examined in this thesis there have been examples of the VSG-specific immune response dropping below the detection threshold by *in vitro* complement lysis assay, in the majority of cases the immune response has been present, after first detected, as long as measurements were taken. Other studies have agreed with this trend (Robinson, 1999). It could be argued that in all of these cases the infections are not truly chronic (35 days infection in mice; 70 days infection in cattle; 35 days infection in rabbits), as certainly cattle infections have been followed for hundreds of days (Luckins and Mehlitz, 1976). However in no study has there been a reliable instance of reappearance of a VSG, indicating that the VSG-specific immune response remains patent. If we consider one of the aspects of the model, the switching to variant i determined by the genomic locus of i , this parameter is independent of the gene it is replacing. Therefore in this case all VSGs are likely to be switched to from the incumbent VSG at their inherent switching rate, regardless of whether they have already appeared. This scenario, which is inherent in our model, causes constant boosting of the VSG-specific immune responses throughout the period of the infection.

5.4 Model output

5.4.1 *Effect of genomic locus of VSG*

Several sets of simulations were carried out in order to test the influence of manipulations upon VSG switching. The first range of switching was drawn from one or more uniform log distributions, where the fastest switching variants will be switched to nearly 100,000 times more frequently than the slowest switching variant. The rate at which a variant is switched to is independent of the variant that it is replacing. This is different from Frank's model, where each variant has a unique probability of switching to another variant, depending on the incumbent variant. All switching rates were then rescaled so that all slender cells have an inherent probability of 0.01 of switching to a new (or the same) variant at each division. These scenarios are equivalent to taking into account only the genomic position of each gene. Therefore those with a high probability of being switched to correspond to VSGs situated at subtelomeres, those with medium probability interstitial, and low switchers that arise from rare events such as mosaic gene formation.

$$\begin{pmatrix} a & b & c & d & e \\ a & b & c & d & e \\ a & b & c & d & e \\ a & b & c & d & e \\ a & b & c & d & e \end{pmatrix}$$

Figure 5.2 The switching matrix S , where each entry to $s_{i,j}$ is independent of the VSG incumbent in the expression site. (Compare with Figure 5.11, where the switching is homology based). Letters in each column represent switching probabilities, and if entry into the matrix is represented by the rows, the probability of switching to a particular VSG in each entry is the same, regardless of the VSG being switched from.

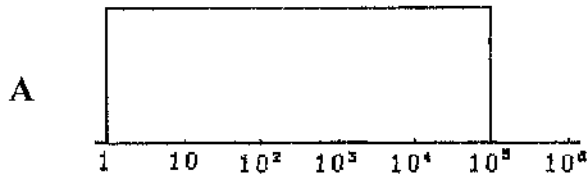


Figure 5.3 Switching distribution A. Distribution from which the switching rates were chosen for initial simulations. Only effects of genomic position considered. 30 switch rates were drawn from the distribution shown. Switch rates were then in all cases rescaled so that all slender cells have a probability of 0.01 of switching to a different, or the same, variant per population doubling.

The first set of simulations specifically examined the effect of the intrinsic growth rate of the VSG-specific immune response on shaping the outcome, where the rate of growth $x = 1, 2$ or 3 (see Fig. 5.1). Constant parameters used in all simulations are $n=30$, $r=0.1$ per hour, $\rho=2500$, $d=0.5$ per hour, $\delta=0.1$ per hr, $K=1 \times 10^8$, $c=100$, $C=1 \times 10^8$, $\tau=100$ (for definitions see table 5-1). The graphical output allocates different VSG-expressing sub-populations a colour, which also designates the respective VAT-specific immune response. The total parasite population is represented by a black line.

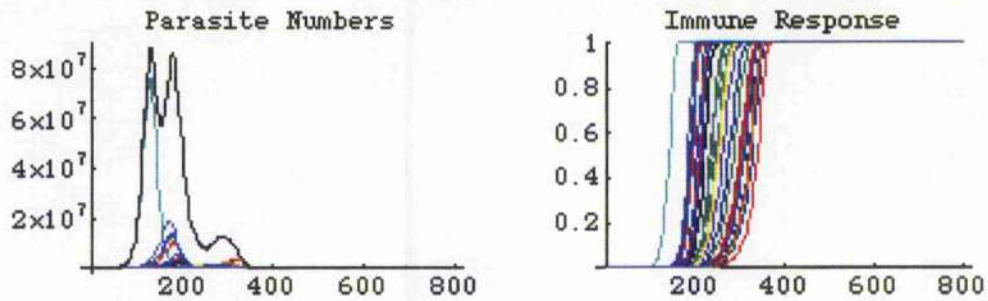


Figure 5.4 Time series of parasite dynamics and the immune response where the rate that a variant is switched to is independent of which variant it is replacing. Switching rates were chosen from distribution A (Fig 5.3). X-axis; hours post inoculation. Y-axis left graph; parasites.ml⁻¹. Y-axis right graph; strength of immune response where 1 = maximum. The intrinsic rate of growth of the immune response $x = 1$.

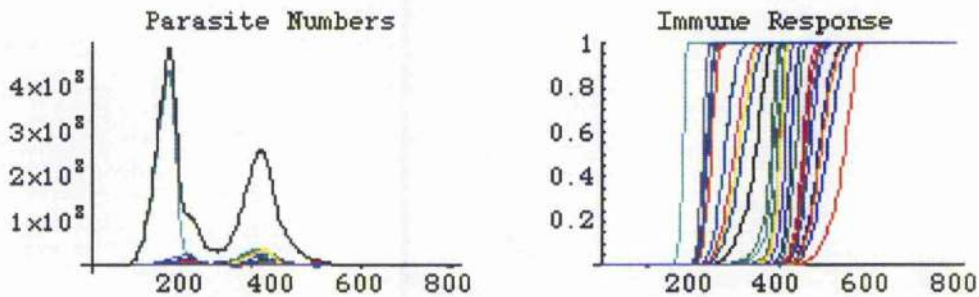


Figure 5.5 Time series of parasite dynamics and the immune response where the rate that a variant is switched to is independent of which variant it is replacing. Switching rates were chosen from distribution A (Fig 5.3). X-axis; hours post inoculation. Y-axis left graph; parasites.ml⁻¹. Y-axis right graph; strength of immune response where 1 = maximum. The intrinsic rate of growth of the immune response $x = 2$.

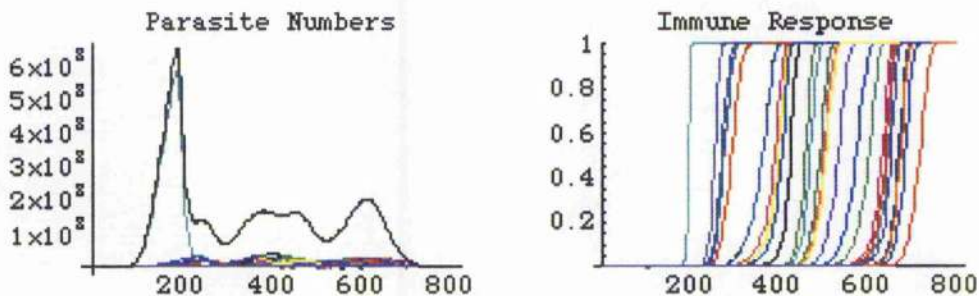


Figure 5.6 Time series of parasite dynamics and the immune response where the rate that a variant is switched to is independent of which variant it is replacing. Switching rates were chosen from distribution A (Fig 5.3). X-axis; hours post inoculation. Y-axis left graph; parasites.ml⁻¹. Y-axis right graph; strength of immune response where 1 = maximum. The intrinsic rate of growth of the immune response $x = 3$.

The time series of parasite dynamics in Figures 5.4, 5.5 and 5.6 examined the outcome when all variables, including switching parameters, remained constant, apart from the intrinsic rate of growth of the immune response. Common to all outputs were several key observations; each peak of parasitaemia is made up of several sub-peaks expressing a different VSG, except for the initial peak, which is predominately a single VAT-expressing population. None of the variants reappear later in the infection. The immune response against variants with a higher switch rate occurs earlier than those with lower switch rates.

The simulations illustrated that a linear rate of growth of immune response ($x=1$) leads to a relatively truncated parasitaemic profile, with the appearance of one major peak. The immune responses to all VSGs rapidly rose to their maximum effectual value, and the infection was no longer patent by approximately 350 hours (14.5 days). By comparison, when the value of x increases, the timecourse concomitantly increases, so that when $x=2$ the infection lasts around 500 hours (20.8 days), and when $x=3$ the infection persists until 700 hours (29.1 days). The number of peaks also increases, and when $x=2$ there are 2 distinct peaks, with the second peak approximately 200 hours (8.3 days) later and around half the magnitude. The outcome resulting from x being at its maximum value, 3, gives rise to three parasitaemic peaks, each separated by around 200 hours (8 days), but the 2 relapse peaks being roughly a third of the magnitude of the initial parasitaemic peak. The immune responses for increasing values of x take a longer time to become fully patent, and more spread out, corresponding to the respective parasitaemic peaks within which the relevant VAT-expressing sub-population occurred. The value of x that produces an output most similar to that seen *in vivo* in our time series is $x=3$. This value gives infections that are more chronic, with more parasitaemic peaks that decrease in magnitude after the initial parasitaemic peak, and an immune response profile that mirrors the more chronic type of infection produced.

These primary simulations, while carried out in order to examine the role of the intrinsic rate of growth of the immune response, themselves throw up some intriguing conclusions. They suggest that a repertoire of only 30 VSGs can result in an infection period of 4 weeks, when the relevant effects of stumpy formation and the immune response are taken into consideration. They also indicate that when the rate of growth of the immune response is relatively slow when the number of trypanosomes is low, and faster as the number of parasites increases, this produces an infection outline similar to that seen *in vivo*. Indeed, the time series emphasise the importance of this factor in shaping the duration and profile of the infection, and altering the intrinsic rate of growth of the immune response can double the effective lifetime of the parasite within the host in our simulations.

The effect of the distribution of switching rates was then examined. In the first case, 2 separate distributions of switching rate were modelled. The first, larger range of switching rates is analogous to all subtelomeric genes, which includes all minichromosomal *VSGs*, and those inhabiting bloodstream expression sites. If homology between all *VSGs* is considered equal, as duplication involves only the conserved flanks outside the coding regions, then these genes can be inserted into the active expression site by a minimum of one recombination event, which may be break induced replication (Barry and McCulloch, 2001). The second group of *VSGs* with a more narrow and lower switching rate, is analogous to internal *VSGs*, which each require at least 2 homologous recombination events, or mosaic gene formation, which require at least 3.

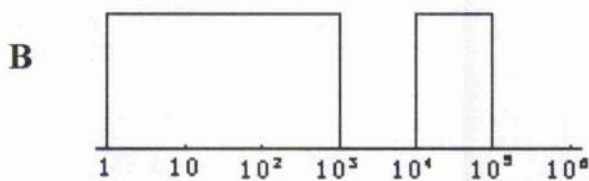


Figure 5.7 Distribution B. Distribution from which the switching rates were chosen for second simulations. Only effects of genomic position considered. 20 switch rates were drawn from the distribution on the left, and 10 from the right-hand distribution. Switch rates were then in all cases rescaled so that all slender cells have a probability of 0.01 of switching to a different, or the same, variant per population doubling.

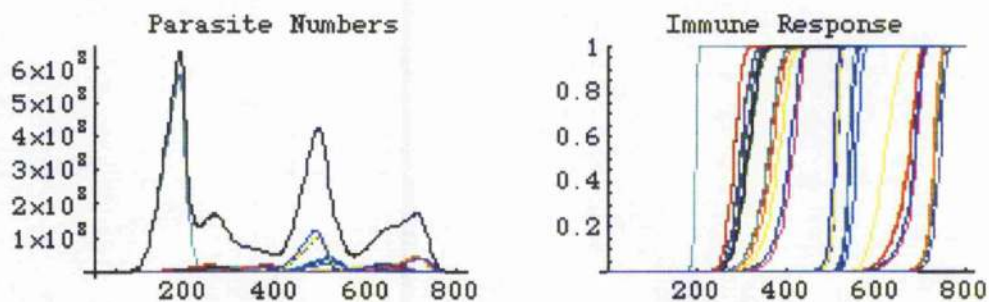


Figure 5.8 Time series of parasite dynamics and the immune response where the rate that a variant is switched to is independent of which variant it is replacing. Switching rates were chosen from distribution B (Fig 5.7). X-axis; hours post inoculation. Y-axis left graph; parasites. ml^{-1} . Y-axis right graph; strength of immune response where 1 = maximum. The intrinsic rate of growth of the immune response $\lambda = 3$.

The second range of switching rates involved 3 sets of *VSGs*, each with a different likelihood of being switched to. From the repertoire of 30, 10 *VSGs* were assigned as

having a high probability of being switched to (high switchers), ten with a moderate probability (moderate switchers) and ten with a low probability (low switchers). This is similar to the experimental observations, grouping VSGs into early, medium and late appearing variants (Capbern *et al.*, 1977; Miller and Turner, 1981) The switching rates of the high switchers were taken randomly from a normal distribution with a mean of 100 and a variance of 50. Medium switchers were drawn from a similar distribution one magnitude lower, and the low switchers from a magnitude lower still. This scenario is equivalent to only taking into account the genomic position of each variant. Therefore those with a high probability of being switched to correspond to VSGs situated at telomeres, those with medium probability intact genes that are interstitial, and low switchers with interstitial incomplete genes associated with rarer events such as mosaic gene formation.

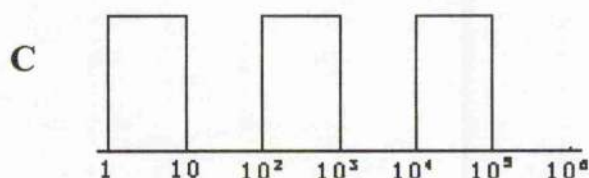


Figure 5.9 Distribution C. Distribution from which the switching rates were chosen for third simulations. Only effects of genomic position considered. 10 switch rates were drawn from the distribution on the left, 10 from the middle, and 10 from the right-hand distribution. Switch rates were then in all cases rescaled so that all slender cells have a probability of 0.01 of switching to a different, or the same, variant per population doubling.

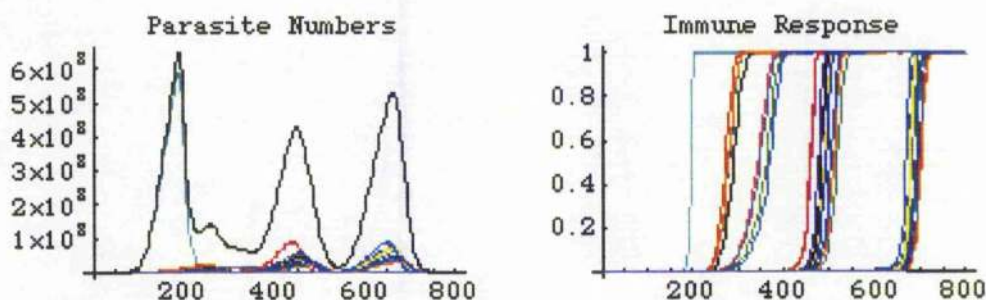


Figure 5.10 Time series of parasite dynamics and the immune response where the rate that a variant is switched to is independent of which variant it is replacing. Switching rates were chosen from distribution C (Fig 5.9). X-axis; hours post inoculation. Y-axis left graph; parasites.ml⁻¹. Y-axis right graph; strength of immune response where 1 = maximum. The intrinsic rate of growth of the immune response $\lambda = 3$.

The switching distributions B and C alter the dynamics of the time series significantly. Again, the same key observations hold true; each peak of parasitaemia is made up of several sub-peaks expressing a different VSG, except for the initial peak, which is predominately a single VAT-expressing population. None of the variants reappear later in the infection. The immune response against variants with a higher switch rate occurs earlier than those with lower switch rates. There is however more of a delay between the first peaks; there being approximately 300 hours (12.5 days) between the initial and first relapse peaks for distribution B, and around 250 hours (10.4 days) for distribution C. The peaks are also much more distinct in the time series for distributions B & C. The main difference in the parasitaemic profile between the two simulations is the amplitude of the second relapse peak, which is larger for distribution C. The magnitude of the VAT-expressing subpopulations is also noticeably greater in the outputs produced by the discontinuous distributions B & C.

The pattern of the VAT-specific immune responses becomes progressively more clustered with the parasitaemic peaks as one compares the profiles from distribution A to C. The time of onset, and time to full patency, are not altered, and the length of the infection is not different, merely the profile. Thus the switching distribution directly affects the pattern of VAT-specific immune response onset, which in turn shapes the parasitaemic profile.

The simulations based on position alone have given rise to some intriguing outputs, suggesting that the rate of growth of VAT-specific immune response would be an important aspect to study *in vivo*. The outputs indicate that the rate of growth of the VAT-specific immune response is relatively slow when the number of trypanosomes is low, and faster as the number of parasites increases. The pattern of VAT growth, and respective VAT-specific immune response, is then further influenced by the distribution patterns of the VSG switching rates. A continuous distribution gives rise to an infection with a large initial parasitaemic peak, followed by two indistinct relapse peaks, and the emergence and maturation to full patency of the VAT-specific immune responses are spread evenly throughout the infection. In contrast, a discontinuous switching distribution, while not affecting the duration of the infection, gives larger VAT-specific subpopulations, therefore more distinct relapse peaks, and clustering of the VAT-specific immune responses with the respective parasitaemic peaks. These simulations neatly provide an insight into the interplay of various factors, and illustrate that the shaping of the infection profile cannot simply be explained by the influence of a main factor alone, which has been the assumption of previous models (Seed, 1978; Agur *et al.*, 1989; Agur and Mehr, 1997).

5.4.2 Effect of homology only.

A second set of simulations was done to examine the influence on the dynamics exerted by the extent of homology shared between the variants, the assumption being that the greater the homology between VSGs, the greater the probability of recombinational switching occurring. The key difference from Frank's model (1999) is that we assumed $s_{ij}=s_{ji}$, and therefore the probability of variant i switching to variant j was the same as variant j switching to variant i . The assumption was also made that variant A was most homologous to variant B, variant B was most and equally homologous to A and C, etcetera. This reversibility is not present in Frank's model, and is a necessary aspect if homology is considered as a factor.

$$\begin{pmatrix}
 v & w & x & y & z \\
 w & v & x & y & z \\
 x & w & v & y & z \\
 y & x & w & v & z \\
 z & y & x & w & v
 \end{pmatrix}$$

Figure 5.11 The switching matrix S , where $v > w > x > y > z$. Letters in each column represent switching probabilities, and if entry into the matrix is represented by the rows, the probability of switching to a particular VSG in each entry is determined by homology, whereby the likelihood of switching back to the same VSG (v) is highest, and switching to a different VSG with the highest degree of homology (w) is the next most likely outcome, and so on.

The first simulation considered that the switching rate between variants is reliant upon the extent of homology shared. Variants were assigned a number, from 1 to 30, and this ranking corresponded to the homology hierarchy; for example variants 1 and 2 would have the highest amount of shared homology, and variants 1 and 30 the lowest. The 30 switching rates were chosen from the distribution shown (Figure 5.9). The highest switching rate corresponded to the rate that a variant will switch to itself, $s_{i,i}$. The next highest rate was then determined by the closeness of homology, therefore $s_{i,i} (+ \text{ or } -) 1$. The global switching rate was then rescaled to 0.01 per population doubling. As with previous simulations, constant parameters used in simulations were $n=30$, $r=0.1$ per hour, $\rho=2500$, $d=0.5$ per hour, $\delta=0.1$ per hr, $K=1 \times 10^8$, $c=100$, $C=1 \times 10^8$, $\tau=100$ (for definitions see Table 5.1), and the rate of intrinsic growth of the specific immune response was $x=3$.

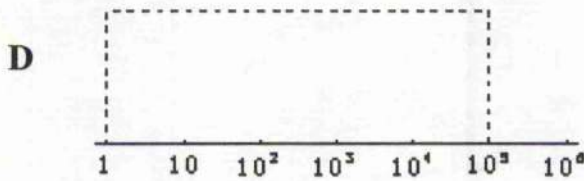


Figure 5.12 Distribution D. Distribution from which the switching rates were chosen for simulations based on homology only. 30 switch rates were drawn from the distribution shown. Switch rates were then in all cases rescaled so that all slender cells have a probability of 0.01 of switching to a different, or the same, variant per population doubling.

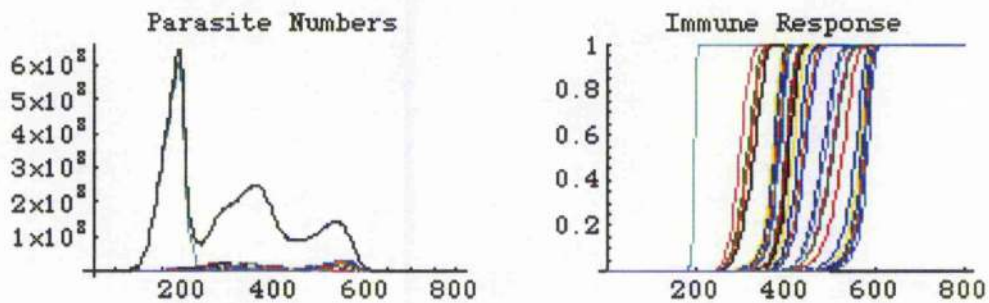


Figure 5.13 Time series of parasite dynamics and the immune response where the rate that a variant is switched to is dependent on homology. Switching rates were chosen from distribution D (Fig 5.12). X-axis; hours post inoculation. Y-axis left graph; parasites.ml⁻¹. Y-axis right graph; strength of immune response where 1 = maximum. The intrinsic rate of growth of the immune response $\alpha = 3$.

The time series produced by the homology-dependent scenario yet again is different. As with previous simulations, each peak of parasitaemia is made up of several sub-peaks expressing a different VSG, except for the initial peak, which is predominantly a single VAT-expressing population; none of the variants reappear later in the infection; and the immune response against variants with a higher switch rate occurs earlier than those with lower switch rates.

The large initial peak is followed by two indistinct relapse peaks. The relapse peaks are lesser in magnitude and closer together than in the position-dependent ($\alpha=3$) simulations. The infection also terminates earlier, at approximately 600 hours (25 days), compared with 800 hours (33.3 days) for the position-dependent simulations. This is a reflection of the immune response profile, which reaches full effectiveness against all VSGs earlier than during the position-dependent ($\alpha=3$) simulations. The pattern of VSG-specific response

onset is also different, and the VSG-specific responses do not group clearly with the parasitaemic peaks.

5.4.3 *Effect of a mixture of homology and position.*

It is highly likely that the VSG switching system is not based on homology alone, or genomic position alone. A degree of both aspects is probably involved, with the proportional significance of each important. Working from this theory, the final simulation involves two classes of variant. The first class includes those whose switch rate is independent of the variant they are replacing, and is analogous to position-dependent switching. The second class encompasses switching whereby the shared homology is the driving force. The homology of the 30 variants was determined by randomly drawing switch rates from the left hand distribution in Figure 5.14 (dashed). 15 of these variants were then randomly allocated to the class of variants whose switch rate is independent of what variant they are replacing. Their switch rates were randomly chosen from the right hand distribution (solid line). Constant parameters used in simulations were $n=30$, $r=0.1$ per hour, $\rho=2500$, $d=0.5$ per hour, $\delta=0.1$ per hr, $K=1 \times 10^8$, $c=100$, $C=1 \times 10^8$, $\tau=100$ (for definitions see table 5-1), and the rate of intrinsic growth of the specific immune response was $x=3$.

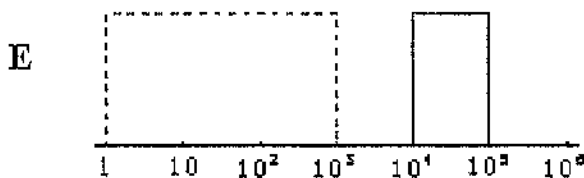


Figure 5.14 Distribution E. Distribution from which the switching rates were chosen for simulations based on a mixture of genomic position and homology. The homology of the 30 variants was determined by randomly drawing switch rates from the left hand distribution (dashed). 15 of these variants were then randomly allocated to the class of variants whose switch rate is independent of what variant they are replacing. Their switch rates were randomly chosen from the right hand distribution (solid line). Switch rates were then in all cases rescaled so that all slender cells have a probability of 0.01 of switching to a different, or the same, variant per population doubling.

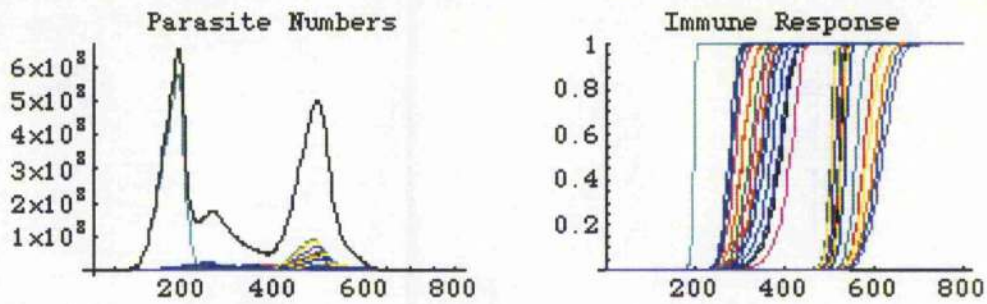


Figure 5.15 Time series of parasite dynamics and the immune response where the rate that a variant is switched to is dependent on a mixture of genomic position and homology. Switching rates were chosen from distribution E (Fig 5.14). X-axis; hours post inoculation. Y-axis left graph; parasites.ml⁻¹. Y-axis right graph; strength of immune response where 1 = maximum. The intrinsic rate of growth of the immune response $x = 3$.

The simulations incorporating aspects of both homology and genomic position have the same key findings as the previous time series; each peak of parasitaemia is made up of several sub-peaks expressing a different VSG, except for the initial peak, which is almost entirely composed of a single VAT-expressing population; none of the variants reappears; and the immune response against variants with a higher switch rate occurs earlier than those with lower switch rates. The duration of the infection is similar to that of the homology-dependent simulation; approximately 600 hours (25 days). The profile is different, there being the initial parasitaemic peak and one distinct relapse peak, and the individual VAT-expressing subpopulations are greater in magnitude. The immune response clusters with the parasitaemic peaks in a similar manner to that seen in the position-dependent simulations.

5.5 Summary

The application of a mathematical modelling approach to the hierarchical switching of VSGs has allowed the examination of several aspects of the system, and their subsequent effects on the dynamics of an infection. At the outset it must be stressed there are certain limitations; the system examined has a total of 30 variants, which is obviously less than that seen *in vivo*; the analysis of the output is subjective by nature in terms of interpretation, and therefore any conclusions drawn must be made with caution. However, as the model has been created upon the basis of the known biology of the parasite, and assuming these limitations are taken into consideration, tentative conclusions can be drawn.

The most apparent effect was exerted by the intrinsic rate of growth of the immune response. When the rate of growth of the immune response is relatively slow when the number of trypanosomes is low, and faster as the number of parasites increases, this 'threshold'-type effect can significantly affect the infection dynamics. Indeed, the simulations suggested that this aspect is highly significant to the chronicity of the infection, and changing the intrinsic rate of growth of the immune response from a directly proportional one to a threshold type one, can in fact double the effective lifetime of the organism within the host. This indicates that the details of the VSG-specific immune response, and its kinetics, would be an interesting avenue of further examination *in vivo*, as to how it determines the dynamics of a trypanosome infection. Differential antibody kinetics in different hosts, for example, could be a contributing factor to the diverse infection profiles seen with the same parasite in different hosts (Barry, 1986).

The type of switching distribution also has a significant impact upon the profile of the infection. While not affecting the period of infections, discontinuous distributions of switching ranges lead to clustering of the VAT-specific immune responses with the parasitaemic peaks, and an increase in the magnitude of the individual VSG-expressing sub-populations, with a concomitant increase in the size of parasitaemic peaks in general. With continuous distributions, the opposite situation occurs, and the VAT-specific immune responses are spread evenly throughout the infection, and the relapse peaks are lower in magnitude and less distinct. The profile produced by the discontinuous distributions is more similar to that seen *in vivo*, and suggests that there are distinct subsets of VSGs with differing ability to switch to one another, within the global switching rate of 0.01 switches per population doubling.

The relative roles of genomic position and homology driven switching are less clear. From the simulations, either the discontinuous distributions of genomic position-driven switching, or where there was a mixture of the influence of homology and genomic position, produced profiles that were consistent with that seen *in vivo*. This suggests that the system is not solely homology driven, which agrees with earlier experimental work that suggested genomic position was important (Liu *et al.*, 1985; Robinson *et al.*, 1999). Although simulations involving genomic position alone produce viable outputs, it must be remembered that in these *in silico* infections the repertoire is limited to 30 VSGs, which in effect would represent the very beginning of a true infection, where genomic position effect may well dominate. The output produced by the combined aspects of homology and genomic position is valid however, and perhaps raises the most pertinent question to arise from these simulations with respect to the switching system. It is the relative significance,

and proportional influence of, these 2 driving forces behind the individual switching rate of each VSG that shape the eventual infection profile, and it is this ratio that needs to be elucidated in order to fully understand the impact of hierarchical switching on infection dynamics.

Chapter 6

Discussion

6 Discussion

6.1 Introduction

This thesis set out to examine the hierarchical expression of *VSG* genes during chronic infections of *Trypanosoma brucei*. Specifically, the role of the genomic context of the *VSGs* was studied, with respect to the timing of appearance within replicate *in vivo* infections in cattle and mice using pleomorphic trypanosomes. A subset of single copy *VSGs* was used, and their pattern of appearance was elucidated. A mathematical model was constructed (with the collaboration of Katrina Lythgoe, University of Edinburgh), incorporating all relevant and measured biological parameters of an *in vivo* infection, with the main variable examined being the differential switching of *VSGs* within the repertoire. Utilising these simulations, the effect of both genomic position of the *VSG*, and shared homology between *VSG* genes, was investigated.

6.2 The timing of appearance of *VSGs* during *in vivo* infections

The chronic mouse infections indicated that there is a statistically significant difference in the timing of onset of VAT-specific immune responses across replicate batches of infections with two separate analyses; one by comparing the average time of onset (General Linear Model [GLM]; $F_{6,106}=7.49$, $p < 0.0001$), and the second by ranking the onset by sequence of appearance (GLM; $F_{6,112}=8.03$, $p < 0.0001$). When the results for the ranking and average time of onset data are compared, they give very similar results with respect to the *VSG* hierarchy within the subset examined. In both sets of analysis, the immune response against ILTat 1.67, the VAT encoded by a BES gene, appears first, followed by ILTat 1.25, then ILTat 1.21 (both encoded by minichromosomal genes) and in turn ILTat 1.73 (chromosomal internal). When examining ranking the next in order is ILTat 1.71 (chromosomal internal), then ILTat 1.22 and finally ILTat 1.64 (both MES). When the average time of onset is analysed, the fifth out of the seven is ILTat 1.22 (MES), then ILTat 1.71 (internal) and finally ILTat 1.64 (MES). There is general agreement between the two sets of analysis, with only ILTats 1.71 and 1.22 changing relative positions in the respective hierarchies. In terms of the influence of genomic locus, and assuming the timing of onset of the immune response is directly proportional to the relevant switching event, the results allow further dissection of the previous findings of

earlier expression of subtelomeric genes (Liu *et al.*, 1985; Robinson, 1999), and indicate that there is further substructuring of the temporal expression patterns within the broad heading of 'telomeric genes'. ILTats 1.67, 1.21 and 1.25 (encoded by BES, and minichromosomal genes respectively) do tend to appear earlier in infection in this study, in agreement with the earlier publications. It would have been interesting to isolate clones of the ILTat 1.67 expressor trypanosomes in order to elucidate whether the activation of this BES gene was due to *in situ* switching, duplicative activation, or a combination of the two mechanisms. The cloning was unsuccessful, presumably because the population at that time was very polyclonal as indicated by RT-PCR, reducing the probability of isolating trypanosomes from the relevant sub-population.

The results from the chronic cattle infections agree with the mice data in several aspects. The order of appearance is generally similar, in terms of both the detection of the VAT-specific immune responses, and the identification of VSG-specific transcripts by RT-PCR. There were, however, several differences between the data. The actual timing was later in the 2 cattle, but this can be attributed to host differentials in terms of the immune system, and the fact that the ILTat line of trypanosomes used in the cattle switches at a much lower rate, 1×10^{-5} switches/cell/generation, compared with 1×10^{-2} switches/cell/generation for the EATRO 795 trypanosomes used in the mouse studies. The immune response against ILTat 1.71, encoded by an interstitial gene, was detected much later than the transcripts, 41 days later in the case of cow BW49 and 44 days later in the case of BV154 (see Figure 4.23). Assuming PCR amplification yielded the correct product, and that there was no degradation of plasma, both factors that were controlled for, this raises somewhat of a conundrum. No ILTat 1.71 transcripts were detectable after days 23 in cow BW49 and day 21 in BV154. The low parasitaemias, however, throughout the infections, led to difficulty in continuously obtaining sufficient quantities of RNA for RT-PCR, resulting in interruptions in the VSG data when no trypanosome products were detectable by RT-PCR. Therefore the possibility remains that there were low numbers of parasites expressing this VSG for a longer period of time. In addition, there could have been variability in the efficiency of the primer pairs used to detect VSG products, and perhaps this should have been tested initially. The *in vitro* complement lysis assay is an effective and unequivocal test in terms of its interpretation, but the lysis effect very quickly dilutes out. The immune response against ILTat 1.71 may have been present and effective much earlier than detected, but below the level of sensitivity of this test, and only become patent due to stimulation by regular subsequent redundant switches. The use of ELISA was an attempt to address this, but unfortunately was unsuccessful.

The early appearance of VSGs encoded by minichromosomal genes is consistent with previous studies examining both a high switching trypanosome, the SUSB 48 line (isogenic to the ILTat trypanosomes), and the lower switching ILTat line (Robinson *et al.*, 1999). It has been suggested that this subtelomeric class of VSG has evolved as a method of increasing the pool of subtelomeric VSGs available (Borst *et al.*, 1993), beyond the 20 or so BESs. Certainly VSGs are the by far the most abundant protein encoding genes on minichromosomes. It has also been demonstrated that there are large arrays of 70-bp repeats upstream of minichromosomal VSGs (Shah *et al.*, 1987), 35 repeats in the case of ILTat 1.21 (Robinson, 1999). These expanses of repeats provide a large degree of homology, possibly enhancing the likelihood of switching to these genes. Therefore the minichromosomes probably serve as a library of VSGs that are readily available for comparatively easy, and therefore frequent, recombination into BESs, due to both their telomeric location, and the large stretches of sequence homology shared between the two types of chromosome.

ILTats 1.22 and 1.64, both encoded by MES genes, tend to appear almost as late, if not later according to analysis by rank, than the chromosomal internal genes in the subset of VSGs under study. The metacyclic genes are the subset of VSGs that are expressed upon inoculation by the tsetse fly in order to establish the infection. They reside in dedicated monocistronic expression sites containing very few or no 70-bp repeats (Alarcon *et al.*, 1994; Graham and Barry, 1995; Burton, 2003), and their expression usually is superseded by BESs after approximately 7 days (Barry and Emery, 1984). Duplicative reactivation of the metacyclic VSGs (MVSGs) in the BESs has been observed (Matthews *et al.*, 1990; Robinson *et al.*, 1999), as has one instance of *in situ* MES activation in bloodstream forms (Donelson *et al.*, 1998), which is thought to be a rare event arising through the very strong selection imposed to find this variant (J.D. Barry, pers. comm.). The possibility of *in situ* MES activations in this study was not examined, although the inability to isolate a clone expressing ILTat 1.67 from the polyclonal cattle stabilates indicated this would have been very difficult in any case. It could be hypothesised that limitation of expression of MVSGs during infections would be advantageous to the trypanosome population in an endemic situation, as a high proportion of endemic immunity against the infection-initiating VSG sub-population would have a strong deleterious effect upon transmission. A decreased likelihood of duplicative transposition, possibly a by-product of the low number of 70-bp repeats, could explain this scenario. ILTats 1.22 and 1.64 possess 1.5 and 0.5 70-bp repeats respectively (Matthews *et al.*, 1990; Burton, 2003). The observation of several instances of waning of the immune responses to both MVSGs (35% of ILTat 1.64 and 13.6% of ILTat 1.22 infections, compared with a range of 0-5% for the five other genes under study),

possibly suggesting that the immune responses to these VSGs are not being boosted as consistently. If the supposition is accepted that the switching based upon genomic position occurs at consistent rates and relative proportions throughout the infection, it can be hypothesised that, as an infection progresses and the VAT-specific immune responses become patent to VATs that have already appeared, boosting intensity of VAT-specific immune responses will mirror the switching hierarchy itself, and the immune response against those rarely switched to will be concomitantly less stimulated. This is possibly what is occurring with the MVSGs.

As already mentioned, the VSGs encoded by the chromosomal internal genes ILTats 1.71 and 1.73 also appeared late in this set of analyses. The late expression of VSGs from the internal arrays has been demonstrated previously in pleomorphic trypanosomes (Robinson *et al.*, 1999), and has long been postulated to become of import once exhaustion of telomeric genes has occurred. This is likely to be due to the difference in the relative ease, or frequency, of interactions between the BES subtelomere and subtelomeric genes, and chromosome internal genes. It has been demonstrated that, when a VSG normally expressed later in infection was translocated into a telomeric region, which presumably will share more regions of homology with the expression site and surrounding stretches, earlier activation resulted (Laurent *et al.*, 1984). Recent examination of the TREU 927 genome database has indicated that the internal VSG arrays are actually largely composed of pseudogenes, with only 5% of over 850 VSG sequences being intact (L. Marcello, P. Burton and J.D. Barry, pers. comm.). Barring this being a strain specific phenomenon, which is unlikely, it suggests the recombination of the VSG pseudogenes, either to replace parts of already existing VSGs, or to create novel composite genes, may in due course be determined to be a major driving force behind antigenic variation. It also raises the possibility that the intact internal genes are members of a subset that are more likely to be switched to, due to the requirement of a single recombination reaction, than the pseudogene repertoire, which will presumably necessitate at the very least two recombination events. Certainly in the most thorough examination of hierarchical VSG expression in trypanosomes (Robinson, 1999), all silent VSGs that were found to be expressed early in the hierarchy were intact.

The labelling of many VAT or VAT-specific antibody appearances as 'late' in several studies (Capbern *et al.*, 1977; Myler *et al.*, 1985; Robinson *et al.*, 1999), including this one, is really a misnomer in the context of the true level of chronicity that is seen in 'natural' trypanosome infections. Most of the *in vivo* studies, including this one, have concentrated on the first couple of relapse peaks at the most, which is in reality the beginning of the

infection. This is due to the use of rodent or rabbit models, which in the case of rodents at least are unable to harbour truly long term infections. In all probability, the experimental examination of antigenic variation thus far has merely scratched the surface. Certainly, trypanosome infections in cattle have been followed for hundreds of days (Luckins and Mehrlitz, 1976), and the question of what occurs with respect to antigenic variation in the truly chronic stages of an infection is intriguing. From this perspective, the subset of internal *VSGs* examined have appeared relatively early, although generally after most of the subtelomeric genes examined (barring *MVSGs*). Therefore the intact subset of internal *VSGs* putatively forms a semi-compartmentalised group that appear after the more frequently activated subtelomeric *VSGs*, but before the, at present time theoretical, mosaic gene repertoire. A notable feature of the cattle and mice infections in the present work is the relatively restricted period within which the studied subset of *VSGs* became activated. In the cattle infections, all 7 *VSG* transcripts, with the exception of ILTat 1.73 in cow BV154, were detected by RT-PCR in the same first relapse peak within three days of each other. The VAT-specific immune responses detected in the mice infections, with more exceptions due to the larger sample size, also were quite clustered, generally appearing within 8 days of each other. This lends credence to the idea that intact *VSG* genes in general are more likely to be switched to, and the hierarchy observed within this study, and in others (Robinson, 1999), is in fact the hierarchy within a subset of genes that comprises the intact *VSG* genes, and a second tier of hierarchical control exists for the pseudogene repertoire.

If we consider the intact *VSG* repertoire, a common feature is the 70-bp repeat array. Within the subset of *VSGs* included in this study, the minichromosomal ILTat 1.21 gene is known to have an array 35 repeats long (Robinson, 1999), and the *MVSGs* ILTats 1.22 and 1.64, have 0.5 and 1.5 repeats respectively (Matthews *et al.*, 1990; Burton, 2003). The 70-bp repeats of the remaining genes have not been sequenced, despite several attempts using *SspI* digestion, and MVR (Multiple Variable Repeat) mapping. The different types of genomic locus have been found, however, to have similar numbers of repeats. BESs tend to have hundreds of repeats running to several kb (Aline *et al.*, 1985a; Berriman *et al.*, 2002), although the exact number can vary (Berriman *et al.*, 2002). Minichromosomes also have a substantial number of repeats, but the number is a magnitude lower than that of the BESs (Shah *et al.*, 1987; Robinson, 1999; Burton, 2003) Internal genes seem to have a low number of repeats, many having as few as 1-3 (L. Marcello and J.D. Barry, pers. comm.). Although it has been demonstrated that switching events do not require the presence of 70-bp repeats (McCulloch *et al.*, 1997), that analysis was carried out using monomorphic trypanosomes. Every conversion limit mapped in pleomorphic trypanosomes has utilised

the 70-bp repeat region (Delauw *et al.*, 1987; Matthews *et al.*, 1990; Burton, 2003). The use of the 70-bp repeats and downstream conserved sequences such as the 3'UTR 16-mer or telomeric repeats as a form of 'expression cassette' has been suggested (Barry, 1997). If the distribution of 70-bp repeats was even partially consistent dependent upon genomic locus, then this study gives a cautious amount of credence to the level of sequence homology between *VSG* loci, possibly the number of 70-bp repeats, influencing the timing of expression of *VSGs*. In this putative system there is no requirement for homology between *VSGs* themselves, and supports the idea that, in the case of intact *VSG* genes at least, the position within the hierarchy can be dependent on genomic location.

6.3 The influence of telomeres, and the maintenance of *VSG* diversity

Preferential activation of subtelomeric genes in *T. brucei* has been observed several times, in both monomorphic (Young *et al.*, 1983; Myler *et al.*, 1984b; Liu *et al.*, 1985) and pleomorphic (Matthews *et al.*, 1990; Robinson *et al.*, 1999) trypanosomes. This favoured activation is likely to be due to the extensive shared homology, in terms of 70-bp repeat arrays common to the mini- and megachromosomes, and also large stretches of sequences common to most of the megachromosomes, which consist of expression site sequence, *ESAGs* and sub-telomeric hexanucleotide (GGGTTA_n) repeats. This sequence homology can certainly facilitate homologous recombination, a main enzyme of which, RAD51, has been demonstrated as a driving force behind antigenic variation in monomorphic trypanosomes (McCulloch and Barry, 1999). In addition, the putative large degree of interaction between telomeres in general, possibly arising from common positioning in the nucleus as has been demonstrated in *P. falciparum* (Freitas-Junior *et al.*, 2000), enhance the likelihood of telomeric *VSG* switching.

Subtelomeres are regions of chromosomes that have been proposed to be ideal reservoirs of divergent gene families (Barry *et al.*, 2003). The occurrence of these gene families in subtelomeric regions probably derives from the tendency of telomeres to be regions of high ectopic recombination, due to the high level of shared repeat sequences, as has already been alluded to in the case of trypanosomes. The fact that telomeres cluster together at the nuclear periphery, as has been elegantly illustrated in *Plasmodium falciparum* (Freitas-Junior *et al.*, 2000), which would facilitate recombination reactions. General examples of gene families that occur subtelomerically include the olfactory receptor (OR) genes in humans, the largest gene family that occurs in *Homo sapiens*, the variation within which

allows humans to recognise over 10,000 different smells (Mefford and Trask, 2002), and several multigene families in *Saccharomyces cerevisiae*, the SUC (β -fructofuranosidase), MAL (α -glucosidase/maltose permease), and MEL (α -galactosidase) genes (Zakian, 1996), which are speculated to broaden the range of utilisable carbon substrates .

In parasites other than trypanosomes, there are several examples of subtelomeric gene families. *Giardia lamblia*, *Theileria parva* and *Plasmodium vivax* all contain subtelomeric gene families (Barry *et al.*, 2003). *Plasmodium falciparum* contains at least 2 sets of antigens that undergo antigenic variation at the surface of the infected red blood cell, which have members at sub-telomeres. These are the *rif* and *var* genes, which respectively encode rifins and *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) respectively (Kyes *et al.*, 2001). The *var* genes are more extensively studied. Within an individual parasite, there are approximately 60 *var* loci, and only one is expressed at a time and is transcribed *in situ*. When the laboratory strain 3D7 was examined, the 59 *var* genes partitioned into 23 chromosomal internal genes, and 36 subtelomeric genes (Kraemer and Smith, 2003). The subtelomeric genes were then further differentiated into two groups, dependent on direction of transcription, towards the centromere, or the telomere. Diversification of *var* genes is hypothesised to be due to frequent recombination events, and it has been suggested, based on direct evidence of probably meiotic recombination, that the subtelomeric genes are a subset that undergo a large degree of ectopic recombination in order to generate a continuous pool of changing variants (Freitas-Junior *et al.*, 2000; Scherf *et al.*, 2001). Examination of *var* gene sequences indicated the sharing of motifs between different *P. falciparum* strains (Taylor *et al.*, 2000), suggesting frequent exchange. It has been suggested that one of the subgroups of sub-telomeric genes is resistant to frequent recombination, due to different flanking regions and orientation (Kraemer and Smith, 2003), explaining the conservation of a few *var* genes across *P. falciparum* strains. The effect of gene orientation has been illustrated in *T. brucei* (Van der Werf *et al.*, 1990), whereby the telomeric AnTat 1.1 VSG became activated late in infections when in its native, reverse orientation, but if correctly positioned was expressed readily early in infection. This observation of reverse orientation of a telomeric VSG has only been seen once, however, and its importance to general hierarchical expression of VSGs may be negligible. It does seem in *P. falciparum* therefore that there is also a certain degree of substructuring in terms of recombination, and the subtelomeric genes are key to the maintenance of diversity. There are important differences, however, when compared with trypanosomes. The trypanosome is exposed to the immune system for the entirety of its time within the mammal, employing a single class of protein to efficiently pre-empt the

host. *Plasmodium* undergoes a multi-stage life cycle, and each antigen is only temporarily, if regularly, exposed. Thus, the selection pressure is much more intense in the case of the trypanosome VSG, and the antigenic variation system has the necessity to function constantly. In addition, PfEMP-1 has a functional role, displaying variable cytoadherent properties that enable sequestration, and avoidance of the screening of the blood by the mononuclear phagocytic system in the spleen. Therefore gene conservation in *P. falciparum*, although possibly dependent on chromosomal location (Kraemer and Smith, 2003), presumably occurs at least partially for this functional reason.

There is a paradox here, in the sense that divergent gene families exist in a region where homogenisation logically would tend to be the case, due to the large number of conversion events that replace one sequence with another. For example, the possibility in trypanosomes of continuous background conversion events occurring in the expression sites, could lead to homogenisation across the BESs. Gene conversion events occurring in the absence of an antigenic switch have been demonstrated (Myler *et al.*, 1988), indicating conversion events taking place in inactive BES. ILTat 1.67, however, one of the subset of single copy genes utilised in this study, is a single copy VSG inhabiting a BES. The conservation of this VSG is evidenced by its presence in the EATRO 795 line and the ILTat 1.2 trypanosomes derived from it. These trypanosome lineages are separated by a significant number of *in vivo* passages, and if there were a large degree of VSG flux within the BES subset of VSGs, ILTat 1.67 would probably be lost, as it has no 'basic copy' elsewhere in the genome. These findings taken together indicate that although there is generation of diversity by gene conversion, there is at least a degree of conservation over time. The lack of homogenisation can be explained by the presence of different VSGs in the different BESs, meaning that any homology driven gene conversion would then progress dependent upon the sequence of the individual VSG. The minichromosomes also seem to remain relatively stable, certainly when examined experimentally over approximately 100 generations (Wickstead *et al.*, 2003), and this paradoxically is also a method of maintaining diversity, by partially compartmentalising the minichromosomes as a pool of intact telomeric VSG genes, and preventing homogenisation.

Whether VSG genes are copied back from active, or inactive, expression sites into the internal arrays, other inactive expression sites (BES or MES), or minichromosomes is not clear. The fact that there is evolutionary divergence of VSG repertoires, leading to the lack of cross-protective immunity between strains, suggests that there must be a dynamic process of VSG conversion events, with a relative degree of flux. The evolution and

plasticity of a VSG repertoire within a population of trypanosomes, and how it would progress, is an aspect that would be interesting to study.

The generation of mosaic genes, which has been much alluded to in this discussion, may be the most important factor in the generation of *VSG* diversity. Although not examined extensively in trypanosomes, several examples have been identified as being constructed from various different *VSGs* (Roth *et al.*, 1986; Kamper and Barbet, 1992), and 1 study in particular noted that the donor *VSGs* were incomplete (Thon *et al.*, 1989), and therefore unable to be expressed themselves. This therefore provides a route for novel epitopes to be expressed, and in light of the large number of pseudogenes that have been found in the sequenced genome of TREU 927 (95% of over 850 *VSGs*; L. Marcello, P. Burton and J.D. Barry, pers. comm.) may be much more significant than previously assumed. Other parasites use the production of novel genes by recombination as a main method of generating antigenic variation. The rickettsia *Anaplasma marginale* has 2 immunodominant surface proteins, major surface proteins (MSPs) 2 and 3. Each is expressed from a single locus, and antigenic variation proceeds by the recombination of pseudogene sequence into the central hypervariable region of the expressed variant (Meeus *et al.*, 2003). The gene conversion reactions involved with MSP2 either involve the whole length of the pseudogene (Brayton *et al.*, 2002), or short segments from the hypervariable region (Brayton *et al.*, 2001). In the case of MSP2, the repertoire of only 9 or 10 pseudogenes is suggested to be sufficient to generate the approximately 10,000 variants necessary to cause the lifelong persistent infections in cattle associated with *A. marginale* (Meeus *et al.*, 2003). MSP2 also has a large stretch of 5' flanking region, approximately 600bp, conserved between the pseudogenes and expressed gene (Brayton *et al.*, 2001), an arrangement conducive to recombinational mechanisms. In *T. brucei* no recombination hotspot has been identified that would be comparable to the hypervariable region, but the 3' region of *VSGs* is relatively conserved, and conversion limits have been mapped to this area (De Lange *et al.*, 1983; Donelson *et al.*, 1983; Pays *et al.*, 1985b). It is feasible however that there is a hierarchy within this possibly very large subset of trypanosome antigenic variation. Recombination reactions involving *VSG* genes or pseudogenes are more likely to occur between *VSGs* or pseudogenes that share greater sequence homology, and a range of probabilities of gene conversion events probably exists across the silent *VSG* repertoire for each BES *VSG*. It has been shown that, in trypanosomes, recombination reactions can be initiated by as little as 7-13 bp homology (Conway *et al.*, 2002), so a range of probability of recombination is very likely to exist over the *VSG* repertoire. Obviously the resulting *VSG* will need to be functionally expressable, as well as antigenically novel. The examination of hierarchical progression of *VSGs* in infections

utilising the genome strain TREU 927 would be the ideal way to answer the possibly critical question of the role of the *VSG* pseudogene repertoire.

6.4 The chronic stages of trypanosome infections

One of the most perplexing aspects of trypanosome chronic infections is the long duration of subpatent periods that occur during the later stages of the infection. Indeed, during the two cattle infections in this study there were gaps of 22 and 14 days between the initial and first relapse parasitaemic peaks. As it is considered that switching occurs as regularly during these periods as at any point in the infection, why any new variants do not grow up to create patent peaks is intriguing. Presumably the immune response would preclude the population consisting of previously exposed variants. There are several possibilities that could possibly explain this phenomenon, which is central to the development of chronicity.

A build up of trypanosome-derived factors could feasibly lead to the suppression of trypanosome growth. For example in cattle BW49 and BV154 the total number of parasites feasibly reached 2.5×10^{10} trypanosomes at the zenith of parasitaemia, and there was maintenance of a broad collective series of subpeaks for approximately 30 days in each case. Considering these numbers, there could be a significant build up of stumpy induction factor (SIF), or toxic metabolites, from the cumulatively enormous trypanosome population, which may then take some period of time to be removed. In the case of the cattle BW49 and BV154, the gaps can potentially be explained by significant suppression due to build up of SIF or metabolites leading to a low parasite population, the growth of which is then retarded by the immune response removing a significant proportion of the switched variants. The problem with this hypothesis is that later on in the very chronic stages of infection, the gaps between the peaks becomes larger, and the peaks themselves smaller (Barry, 1986), which argues against this being the case. The possibility of there being an increased sensitivity of the trypanosomes to the as-yet undefined SIF as the infection progresses, in a positive-feedback type mechanism similar to that seen with immune cells when under constant stimulation (Adler *et al.*, 2003), also exists, although there is no evidence to suggest this. It would require either that the SIF is persistent, and it has been shown to be stable, at least under *in vitro* conditions (Vassella *et al.*, 1997), or that the putative SIF receptor(s) either increase in number with consistent stimulation, or increase in sensitivity. This latter scenario is feasible, as it has been demonstrated that the Lister 427 line of trypanosomes, which has been utilised *in vitro* for many years, and is considered monomorphic, does still respond to the 'stumpification' triggers, but the

threshold is much higher, presumably due to the lack of stimulation over time (Breidbach *et al.*, 2002). However there has been no demonstration that either of these situations occurs *in vivo*, or that this sort of adaptation can occur within the timeframe of an infection. The examination would require long term chronic infections in hosts that can maintain them for many trypanosome generations, such as cattle.

Another observed influence, that is presumably trypanosome dependent, is the observation of growth inhibition as an infection progresses (Turner *et al.*, 1996). The inhibition was not immune response-related, and seemed to be due a lowering of the replication rate of the long slender trypanosomes. Importantly in that study, the inhibition was not strain dependent, as has been seen in superinfections using *T. congolense* (Morrison *et al.*, 1982b), and there was therefore no indication of the competition-type inhibition that has been examined in *Plasmodium chabaudi* (Taylor *et al.*, 1997). This finding, therefore, constitutes the only evidence produced thus far for non-specific growth inhibition, and deserves to be pursued, as this phenomenon is potentially an extremely important factor in the chronic stages of an infection.

It has been suggested that host-derived factors could also influence the parasitaemia. At least two models have included the accumulation of antibodies against invariant antigens during the infection as a key influence on the characteristic profile of trypanosome chronic infections (Antia *et al.*, 1996; Agur and Mehr, 1997). Many of the conserved antigens on the trypanosome surface are functional, and the increasing exposure via whole, or fragments of, lysed trypanosomes and possibly also senescent stumpy forms is suggested to have an effect on the viability of the population as a whole. Although antibodies have certainly been detected that are directed against conserved proteins (Authie *et al.*, 1993; Radwanska *et al.*, 2000a; Radwanska *et al.*, 2000b), the effectiveness or otherwise of these responses has not been examined. It is difficult to envisage how, in an intact living trypanosome, the antibodies would be able either to reach their target on the trypanosome surface with the VSG barrier present, or target flagellar pocket antigens. A recent study showed it is possible to target cryptic epitopes on the VSG, but only by the use of camel-derived monomeric immunoglobulin (Stijlemans *et al.*, 2003). In addition, it has been demonstrated that there is efficient clathrin mediated endocytosis of antibody bound to GPI-anchored protein in the flagellar pocket, which is a possible route of removal of antibody-targeting of flagellar pocket antigens (Grunfelder *et al.*, 2003). As yet, therefore, there is no evidence for this suggested influence being significant in terms of shaping the course of trypanosome infections.

A further possibility is that the parasites are still present, but the greater proportion is no longer in the haematic system during the periods of low parasitaemia. All methods routinely used to detect living trypanosomes rely on their presence in peripheral blood (Paris *et al.*, 1982), and certainly these were the only methods utilised in this study. It is known that *T. brucei* is also an extravascular parasite (Seed and Effron, 1973), although the proportion of parasites that are in which compartment at various points of the infection is not known. Active invasion has been suggested, specifically across the blood brain barrier, the trypanosomes entering between the endothelial cells with the aid of surface associated acid phosphatases and released proteases (Lonsdale-Eccles and Grab, 2002). Adhesion to bovine (atrial) endothelial cells *in vitro* has also been described in the case of *T. congolense* (Hemphill and Ross, 1995). Although the sequestration of parasites in sites such as the brain (Abolarin *et al.*, 1982) and the eye (Whitelaw *et al.*, 1988) has been demonstrated, this has generally been after fulminant infections, when the parasites are likely to be in almost every organ, and may not be of particular relevance to the chronic *T. brucei* type infection we have modelled. The resulting 'sequestration' is therefore likely to be a by-product of a severe infection, and the consequent 're-emergence' of the sequestered parasites after drug treatment due a by-product of being in immunologically and pharmacologically privileged sites such as the brain or eye. The evidence therefore for active sequestration of trypanosomes is very tenuous, but the possibility of the extravascular foci being important in the kinetics of *T. brucei* infections cannot be dismissed, and deserves further attention.

A final putative reason for the long periods of quiescence in terms of parasitaemia is decreased viability of the individual parasites. As has been mentioned, there is an increased likely relevance of mosaic switching and role of the large *VSG* pseudogene repertoire. If the intact *VSG* genes are considered a subset that are more likely to be switched to, they will generally appear earlier in an infection. Very little is known about mosaic *VSG* formation, aside from a few instances of their identification (Roth *et al.*, 1986; Kamper and Barbet, 1992), and even less is known about the components or efficiency of the recombination machinery involved in the process. This raises the possibility of non-functional, or semi-functional, switches occurring. This 'non-functionality' could of course result from the formation of a mosaic gene with epitopes that have already been exposed to the immune response. Additionally, however, a proportion of switches may result in aberrant *VSGs* that cannot be functionally expressed, or are expressed in a manner that will affect viability of the cell. *VSG* mutation studies *in vitro* illustrated that directed mutations reduced their expression significantly, the impact on expression being dependent on which region was mutated (Wang *et al.*, 2003). Most *VSGs* were still GPI-anchored however, and

cells were viable. Whether this alteration of VSG expression dynamics, which in most cases resulted in five- to hundred-fold reduction in VSG steady-state levels (although 1% of normal VSG expression levels is still around 100,000 molecules per cell), is comparable to what could be produced by the mis-generation of mosaic genes is not clear. In addition, the question of the *in vivo* viability of any of the VSG mutants produced was not addressed. At this stage, therefore, an increasing role of greater numbers of dysfunctional switches as the infection progresses remains purely hypothetical.

6.5 Implications of the mathematical model

The mathematical model described in Chapter 5 is an attempt to examine specific aspects that are proposed to affect the dynamics and kinetics of trypanosome infections.

Specifically, the differential switching of *VSGs* within the repertoire was assessed, in terms of genomic locus and shared homology influencing the hierarchical progression of *VSGs*. An additional possible impact on the profile of infections was studied, namely the intrinsic rate of growth of the VAT-specific immune responses. The attraction of mathematical modelling is the ability to manipulate particular inputs and inspect the impact upon the system. Caution obviously has to be implemented in interpretation, as it will never be possible to include all aspects of *in vivo* infections *in silico*. However, with this caveat taken into consideration, tentative conclusions can be drawn, particularly with regard to features that warrant further examination.

The most valid model to date with regard to *VSG* switching (Frank, 1999) concluded that it was only by a range of variations in the rate at which each *VSG* switches to the other can the phenomenon of the characteristic trypanosome infection profile be produced (for critique of other models, see 5.2, p. 109). This essentially gives rise to a probability cascade as the infection progresses, and a quite deterministic progression through the repertoire, although there is some variation between different time-series. This infection profile cannot be generated by a randomly selected range of switching probabilities. Changing the orders of magnitude over which the switch rates varied (S) affected the duration of the infection *in silico*, such that when S was low (3), the variants all appeared relatively early, whereas at higher values ($S=6$) the same number of variants forms a greater number of discrete peaks, and the infection lasts longer. The basis of this system is that variants essentially get a 'head start' over each other due to the differential switch rates, and then temporarily predominate until removed by the immune response, the order being determined by probability of switching between variants. This probability range,

suggested Frank, is likely to be due to ranges of homology between *VSGs*, in terms of *VSGs* themselves, 70-bp repeats, and BESs.

There are, however, several flaws in Frank's model. The limitation of growth of any particular variant is entirely due to the generation of VAT-specific immune responses, whereas in reality stumpy formation will play a highly significant role, and in turn alter the dynamics of the immune response. In addition, it is assumed that *in situ* switches also are dependent on homology between the BESs. Although rearrangements have been observed in active (Navarro and Cross, 1996) and inactive BESs (Gottesdiener *et al.*, 1992), the pattern has not suggested specific rearrangements. The analysis of 9 independent *in situ* switches *in vitro* could not detect any DNA rearrangements (Horn and Cross, 1997), suggesting no reliance on sequence homology or interaction. Whether either of these factors influences the central thesis of Frank's argument is questionable. However, if homology is the basis of the switching matrix, the lack of reversibility in Frank's model becomes highly influential, and a potentially serious flaw. Frank's switching matrix is deterministic, meaning when one VAT switches to another, the subsequent VAT is then more likely to switch to another, and so on. This means the progression through the hierarchy within a single infection is forced down a directed route, which however may differ slightly between infections in the actual order of appearance. In a system based largely on homology, we propose that a *VSG* is as likely to switch back to its predecessor, or itself, as to another equally homologous, but novel, gene. As an infection progresses this both lowers the effective switching rate, meaning that the proportion of switches that will result in an antigenically new *VSG* and viable population will decrease (Turner, 1999), and also acts to boost the immune system against previously expressed VATs.

In our model we have examined 2 levels of switching, based on genomic locus and on homology between *VSGs*. The hierarchy within the genomic locus influenced switching is independent of homology between the *VSGs* themselves. Therefore this order of switching will occur irrespective of the *VSGs* within the loci. The proposed second tier of switching, however, relies entirely on stretches of homology shared between *VSGs* themselves, a possibly important feature, particularly in the formation of mosaic genes. This 2-level hierarchy means that although the progression via the route of shared homology between *VSGs* is semi-deterministic, but with a level of inbuilt 'short range' reversibility, the influence of genomic locus on switching will remain constant throughout the infection. It is this latter class within which the effective switching rate will decrease most therefore as the infection progresses, as it is less likely to produce novel variants.

Our model also incorporates aspects of trypanosome biology affecting the *in vivo* growth of the parasite, that have been studied experimentally; the acquired immune response and short stumpy formation (Tyler *et al.*, 2001), and a non-specific general inhibitory function (Turner *et al.*, 1996). These factors are essential, as they will each affect the dynamics of an infection, and any model not taking them into account is ignoring fundamental influences. It has been illustrated that modelling only 1 aspect of the dynamics, the stumpy formation, gave profiles that were similar to that seen *in vivo*, but the model of best fit incorporated the effect of the acquired immune response (Tyler *et al.*, 2001). The modelling of these inputs is by necessity done in a relatively simple way, in order not to introduce a confusing and unnecessary multiplicity of parameters that will also confound the interpretation of the output. Therefore, although not every aspect of every influence on infection dynamics can be included, the model presented in this thesis does represent the most complete and satisfactory simulation to date of *in vivo* trypanosome infections.

The model outputs generated 2 main levels for discussion. The most apparent effect seen was when the intrinsic rate of growth of the VAT-specific immune responses was modified. The simulated time-series suggest that when the rate of growth is relatively slow at low numbers of trypanosomes, and faster as the number of parasites increases, leading to a threshold-type effect, it in effect increases the duration of the infection and the number of parasitaemia peaks (where rate of growth of the immune response $x=3$; see Figs. 5.4, 5.5 and 5.6, p. 119). This is interesting because all other aspects, such as VSG switching rate, remain constant. Therefore, when the growth rate of the immune response is exponential, the variants are quickly removed by an extremely efficiently responding immune response (Fig. 5.4, p. 119). As the rate of growth of the immune response is decreased, it takes longer for the immune responses to become fully patent. This means that the immune responses to the main VAT-expressing subpopulation(s) in each peak will be generated relatively quickly, but the small subpopulations that arise later in the peak as a result of switching events, and are then subjected to short stumpy formation and reduction in numbers, will not give rise to a patent VAT-specific immune response, allowing them to grow into the subsequent parasitaemic peak following the removal or exhaustion of SIF (see Fig. 5.6, p. 119). This threshold-type immune response is potentially an extremely influential aspect of trypanosome infection kinetics, and allows for a degree of persistence of small subpopulations, such as those present between peaks. Obviously, caution has to be exercised in the interpretation of a model, and there is the need to see if this situation occurs during *in vivo* infections. The possibility of this aspect being highly influential in shaping the differential kinetics seen in infections with the same trypanosome strain in different hosts (Barry, 1986), and also in the same species of host with different genetic

backgrounds (Tyler *et al.*, 2001), should be considered. A different intrinsic rate of growth of the VAT-specific immune responses between the host species, or different immunogenetic backgrounds, could quite feasibly be one of the factors involved.

The second facet to the model was the examination of the influence of the VSG switching hierarchy on the dynamics of an infection. The simulations incorporated a repertoire of 30 VSGs, the switching distribution and therefore linkage within the switching hierarchy of which was manipulated in several ways. The distributions were either continuous or split into discontinuous groups. Initially the switching was dependent upon the genomic locus of the VSG. There was then introduced a second level of switching dependent upon the extent of homology between VSGs. The discontinuous distributions can be seen to be analogous to the idea of early, medium and late switchers (Capbern *et al.*, 1977), fast, medium and slow (Miller and Turner, 1981), or telomeric, internal and mosaic gene formation (Robinson, 1999), depending on the distributions. In our simulations they were postulated to be representations of discontinuity within the switching probabilities due to genomic locus alone (Figs. 5.8 and 5.10, p. 121 and 122), or between switching due to genomic locus and homology (Fig. 5.15, p. 127). The first notable feature of the time-series was that the discontinuous distributions gave outputs that were more consistent with the picture seen *in vivo*. The parasitaemic peaks, initial and relapse, were more distinct, and the VAT-specific immune responses clustered with the respective parasitaemic peaks. The switching distribution did not affect the duration of the infections, rather the makeup of the peaks. The clustering of the VAT-specific immune responses with the parasitaemic peaks agrees with what was observed with the experimental cattle in this thesis, where the VAT-specific immune responses grouped very closely with the relevant detected RT-PCR products (except for, in both instances, ILTat 1.71; see 6.2, p. 131). With continuous switching distributions, the opposite is the case, the relapse peaks being smaller and not very distinct, and the VAT-specific immune responses were spread evenly through the infection. The continuous sets of distribution were analogous to either genomic locus only (Fig. 5.3, p. 118), or homology only (Fig. 5.12, p. 125). If we consider the outputs most similar to those seen *in vivo*, it suggests that there are distinct subsets of VSGs, with differing probabilities of activation. This gives a discontinuous range of switching probabilities, within the overall switching rate.

The comparison of the simulations examining the roles of switching due to genomic position or homology did not produce such clear evidence, although several important observations can be drawn from them. The outputs that were closest to those seen *in vivo* were either the discontinuous switching distributions involving genomic position-driven

switching, or where there was a combination of position and homology influences. Therefore the simulations suggest that the system is not purely driven by homology between *VSGs*, which agrees with the one of the starting hypotheses of this thesis regarding the importance of genomic position, and with previously published work (Liu *et al.*, 1985; Robinson *et al.*, 1999). The fact that simulations involving the effect of genomic position alone produced viable outputs is perhaps not surprising. The restricted repertoire of *VSGs*, at 30 genes, could easily represent a proportion of the genes utilised early in infection, which putatively are intact genes upon which the influence of genomic position is the greatest. In addition, this number of genes allows us to model only a truncated time-series when compared with *in vivo* infections. It is the time series incorporating both tiers of influence however, that is the most pertinent to the *VSG* switching system as a whole. The proportional relevance of the influences of genomic position and *VSG* homology driven switching is perhaps the vital question to ask, particularly with reference to the recent discovery regarding the large number of pseudogenes. The examination of this ratio, and how it would evolve as an infection progresses, in terms of actual and perhaps more importantly effective switching rates, is likely to be critical in order to fully understand the role of hierarchical *VSG* switching in determining the dynamics of a chronic trypanosome infection.

It must be stressed that conclusions drawn from an *in silico* model can only suggest the direction in which experimental endeavour should proceed. However the simulations presented in this thesis do provide several avenues for further examination, in particular what seems to be a highly influential role played by the intrinsic rate of growth of the VAT-specific immune responses, and the proportional influence of genomic locus- and homology-driven *VSG* switching as an infection progresses.

6.6 Future work

One of the key elements, mentioned regularly in this thesis, is the potential role of the pseudogene repertoire. The unexpected discovery that the great majority of the internal *VSG* repertoire is composed of pseudogenes (95% of over 850 *VSGs*), has led to the speculation that mosaic gene formation, previously considered to be probably a rare event, and minor player, in antigenic variation (Barry, 1997; Robinson, 1999), may well be of great significance. The hypotheses presented regarding the significance of homology between *VSGs* in terms of influence on *VSG* switching hinge on this idea. With the resource of the TREU 927 genome, the possibility now exists to investigate the

pseudogenes. This would also allow examination of the hierarchy as a whole (aside from the minichromosomal genes, which are not included in the genome project), in much further detail than has been possible. Chronic infections using TREU 927 could be set up and followed, and the progression through the repertoire examined. The regular cloning and sequencing of VSG-specific RT-PCR products throughout an infection is possible using the splice leader primer and a primer specific to the 3'UTR region. It is not possible to clone the TREU 927 trypanosome with regard to VSG expression, as within one passage of a mouse the phenotype will have switched. In addition, the infections may be restricted to rodents or rabbits, as in a parallel infection run in one cow with TREU 927, the trypanosome levels were not sufficient for RT-PCR (see Appendix). Microarray technology could also be utilised.

The examination of the effect of the threshold-type VAT-specific immune responses, observed in the mathematical model, would also be very interesting. The purification of VSGs would allow the examination of the progression of the VAT-specific immune responses by ELISA, with adequate controls to negate the cross-reactive C-determinant, and accurate measurement of immunoglobulin levels. If effort was concentrated upon the VSGs that tend to appear early, such as ILTats 1.67 (BES), 1.21 and 1.25 (minichromosomal), then it would not be necessary to initiate long term chronic infections. A relatively large volume of serum is required, however, which may involve the use of hosts such as rabbits. It would be ideal to compare this effect between hosts, or between hosts of differing immunogenetic backgrounds, to see if this is an aspect that determines the differential kinetics observed in previous studies (Barry, 1986; Tyler *et al.*, 2001).

Appendix

7 Appendix

7.1 TREU 927 infection

An 18 month-old Friesian steer (BW42) was inoculated with 1×10^6 TREU 927 trypanosomes (supplied by C.M.R. Turner, University of Glasgow), and the infection was followed in parallel with the ILTat infections described in Chapter 4, although it was started one week later, and was therefore followed for 63 days. Parameters were measured in the same manner.

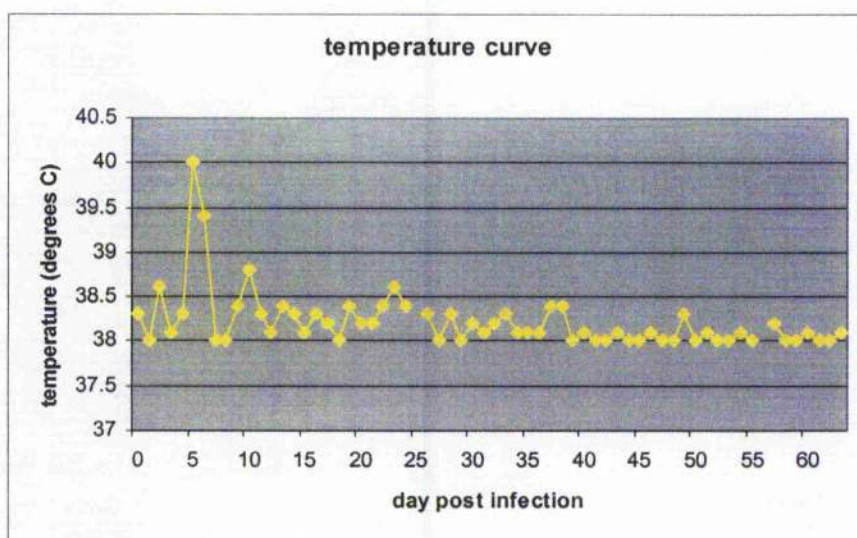


Figure 7.1 Measurements of daily rectal temperatures of cow BW42.

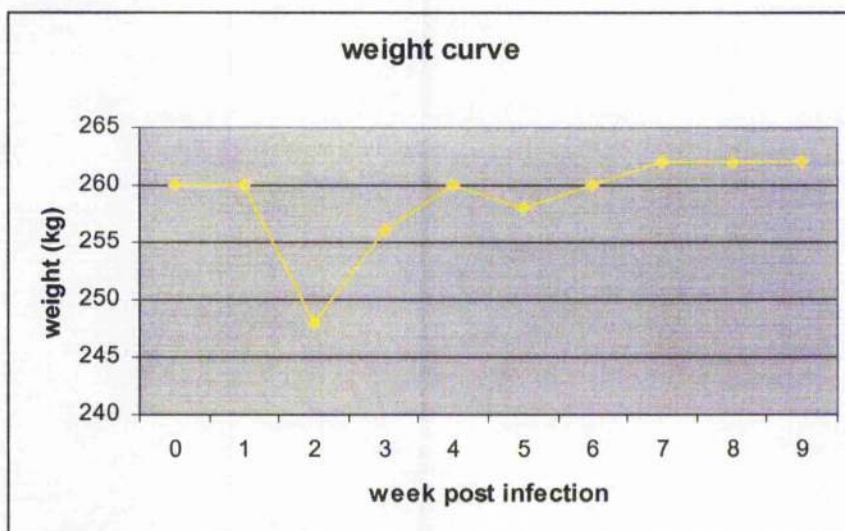


Figure 7.2 Weekly weight measurements of cow BW42.

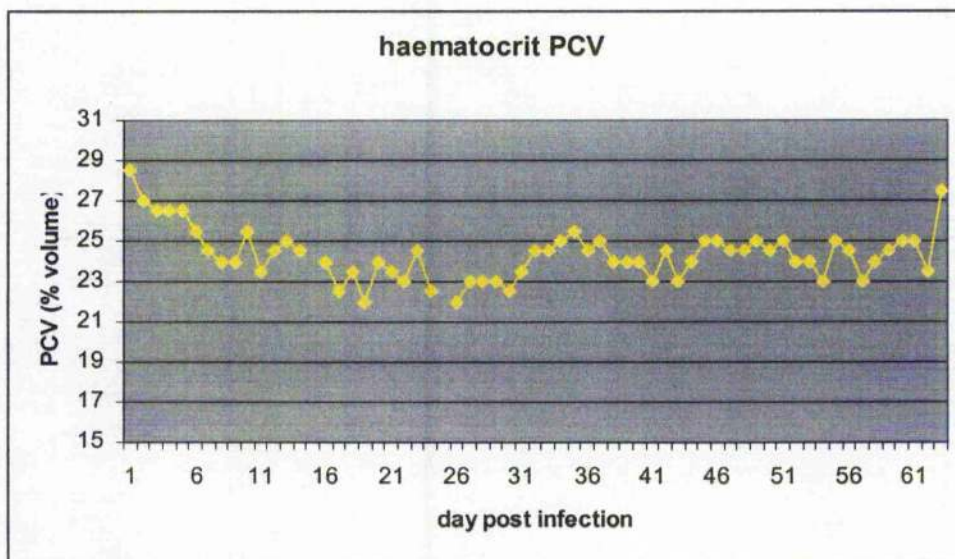


Figure 7.3 Daily haematocrit PCV measurements of cow BW42

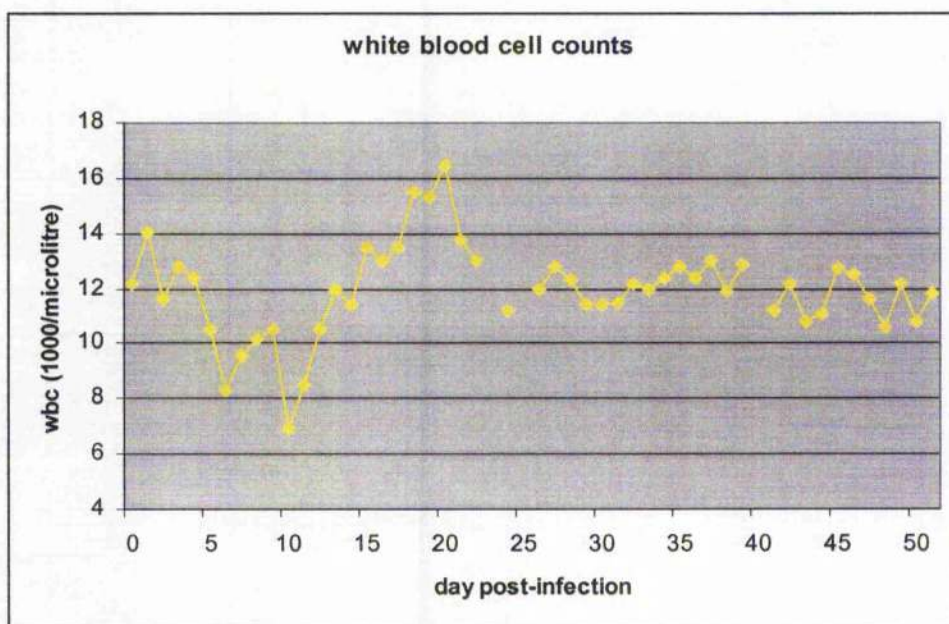


Figure 7.4 Daily white blood cell counts of cow BW42 (up to day 51).

The haematological parameters examined in cow BW42 followed a similar pattern to that seen in cattle BV154 and BW49, and described in Chapter 4. There was, however, retarded growth in cow BW42. Whereas cattle BV154 and BW49 maintained growth rates within those expected of uninfected cattle kept in similar conditions, cow BW42 saw a significant loss of weight between weeks 1 and 2, losing 12 kg (4.5% of bodyweight). Thereafter, weight was steadily regained, reaching the initial weight of 260 kg by week 4 post-

infection. For the remainder of the infection there was a very small amount of weight gain (2 kg, representing a growth rate of $0.05 \text{ kg}\cdot\text{day}^{-1}$). These data indicate that there was reduced performance in terms of weight growth for the cow infected with TREU 927. Although overall no weight was lost, during the period of the infection a Friesian steer would be expected to gain at least $0.5 \text{ kg}\cdot\text{day}^{-1}$ (Radostits and Blood, 1985), which over the 63 days represents 31.5 kg. The impact on growth rate is a significant effect, and can possibly be attributed to the TREU 927 trypanosomes, as the cattle infected with the ILTat 1.2 clone maintained the expected growth rate. Caution must be exercised, however, as only 1 cow was infected.

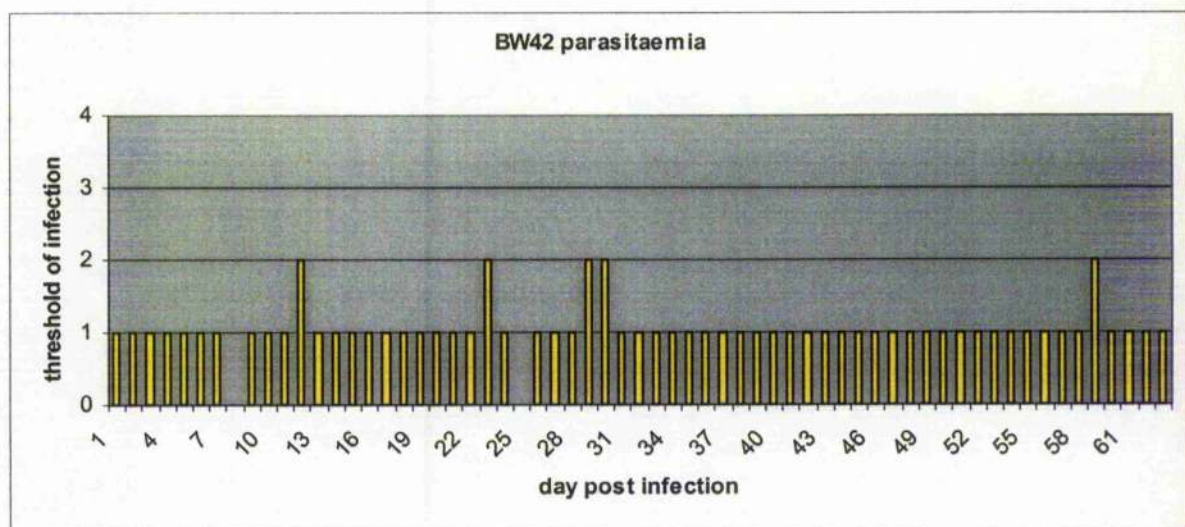


Figure 7.5 Graph illustrating detection thresholds of parasitaemia during infections in cow BW42. Y-axis; 0 = parasites undetectable by any method, 1 = parasites only detectable by amplification in irradiated mice, 2 = parasites detectable by examination of buffy coat, 3 = parasites detectable by haemocytometer. (NB. No measurements taken on day 25 post infection)

When the progression of parasitaemia is examined, the profile is markedly different to that seen with the ILTat 1.2 clone in cattle BV154 and BW49. There were no instances where the parasites were detectable by haemocytometer, and only 5 days out of 63 (7.9% of the infection period) when parasites were detectable by the buffy coat method. In addition, it was possible to amplify parasites by inoculation of cattle blood into mice every day, apart from one (day 8). These data suggest that the parasite population is maintained at a low level throughout the infection, and there are not peaks of high parasitaemia or long periods of quiescence. Obviously, it must be stressed this is information from 1 infection only, and speculation is impossible on how the infection would have progressed if followed further.

However, if the profile was repeated in subsequent infections, it would have implications with regard to following infections in cattle with TREU 927 trypanosomes, particularly considering that TREU 927 is the reference strain for the genome project.

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