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Antigenic variation in its biological context

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The biology of antigenic variation is discussed, and the problems that must be solved to provide a full understanding of antigenic variation are considered. These are (i) the induction of v.s.g. synthesis in the salivary glands of the tsetse fly; (ii) the nature of the restriction on v.s.g. genes that allows only some of them to be expressed in the salivary glands; (iii) the nature of 'predominance' in v.s.g. expression in the mammalian host, and the mechanism by which it operates; (iv) the repression of v.s.g. synthesis in the insect midgut; (v) the anamnestic response that produces expression of the ingested variant in the first patent parasitaemia in the mammalian host; (vi) the mechanism by which only one v.s.g. gene at a time is expressed; (vii) the relationship if any of v.s.g. structure to v.s.g.-associated differences in growth rate and host range; (viii) the role of v.s.g. release within the life cycle and to pathogenesis.

Antigenic variation in African trypanosomes is the mechanism used by the parasite to evade elimination in the mammalian host (reviewed in Vickerman 1974; Gray & Luckins 1976; Vickerman & Barry 1982). The trypanosome is covered by a surface coat, visible as an electron-dense layer some 12–15 nm thick in transmission electron microscopy (Vickerman 1969), which is composed of a matrix of identical molecules known as the variant surface glycoproteins (v.s.g.) (Cross 1975). By regulating the expression of v.s.g. genes from a repertoire of around 1000 in the genome (Van der Ploeg *et al.* 1982), the trypanosome is able to modulate its antigenic profile, for most of the products of the different v.s.g. genes are immunochemically distinct. The rate at which gene switching occurs is very similar to the rate at which the immune system develops an effective antibody response, and the consequence for the host is that infection produces a fluctuating parasitaemia in which production of antibody lytic to the majority of circulating trypanosomes merely serves to select for a minority of trypanosomes expressing a new antigenic type. Normally, this continues until the death of the host unless there is some form of chemotherapeutic intervention. Much has been heard at this meeting about the nature of the v.s.g.s themselves, and of the structure and regulation of the genes encoding them. To put this information into a biological context, the relation between antigenic variation and the full life cycle must be considered.

The life cycle of the African trypanosomes

Four out of seven subgenera of *Trypanosoma* undergo antigenic variation. All of the different species in these subgenera are either transmitted cyclically by the tsetse fly (*Glossina*), or are descendants of such species that are now transmitted mechanically, by other biting diptera, or venereally. The life cycle of the cyclically transmitted species is relatively simple in comparison to some other parasite protozoa, and that of *Trypanosoma brucei brucei*, the species

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which is most popular with laboratory scientists, is illustrated in figure 1. The surface coat is first demonstrable on so-called metacyclic trypomastigotes. This non-dividing form found in the salivary glands of the vector is infectious for the mammalian host, and when the fly takes a blood meal, injected metacyclic forms differentiate into the rapidly dividing long slender bloodstream trypomastigotes which disseminate infection through the bloodstream. In chronic infections, new variants are detectable at roughly seven day intervals within the bloodstream. At around the peak of each wave of parasitaemia, a morphologically distinct form of

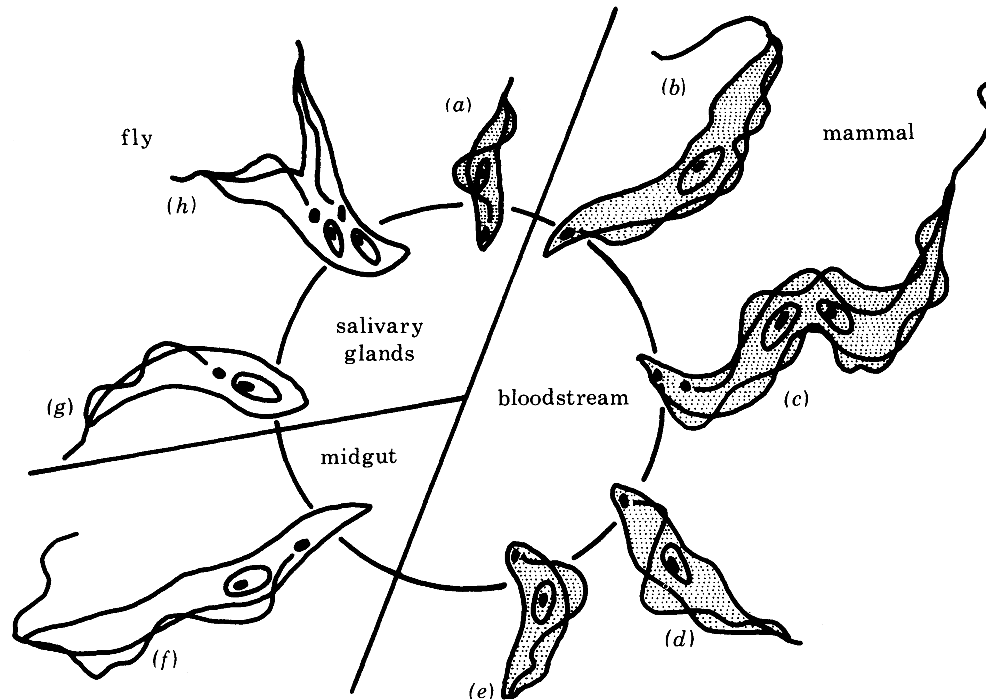

FIGURE 1. Life cycle of *Trypanosoma brucei*. Infection is initiated by injection into the mammalian host of metacyclic trypomastigotes (*a*), which rapidly transform into bloodstream trypomastigotes (*b*). These are present as long, slender forms capable of rapid division (*b*, *c*), which can differentiate into intermediate (*d*) and short, stumpy (*e*) forms. These form a non-dividing population thought to be essential for establishment of infection in the insect midgut by trypomastigotes (*f*). After migrating to the salivary glands these transform to epimastigotes (*g*, *h*) which ultimately differentiate into non-dividing metacyclic trypomastigotes capable of continuing the cycle of infection. The forms carrying a surface coat and therefore capable of undergoing antigenic variation are shaded.

trypanosome, the stumpy form, accumulates. This is generally accepted to represent a differentiation stage within the life cycle (Mancini & Patton 1981), in which some of the long slender dividing trypomastigotes differentiate into the short stumpy form, a terminal stage in the mammal, because these parasites are unable to divide in the mammal or to revert to the long, slender form (Black *et al.* 1984). Short stumpy forms are non-infective to mammals, and have a half-life within a mouse of 24–36 h (Black *et al.* 1984). However, the development of short stumpies is associated with expression of some respiratory enzymes and metabolic adaptations (Flynn & Bowman 1973) that appear to be preparatory to development in the fly. Long slender forms lack cytochromes and have no active Krebs cycle, relying on aerobic glycolysis for ATP generation. Ingestion by the fly of short, stumpy forms is thought to facilitate the development within the insect midgut of dividing trypomastigote forms that have a

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functional respiratory chain. Midgut forms lack the surface coat (Vickerman 1969) and, under culture conditions which mimic this event, they assume a common antigenicity (Seed 1964). Following a complex series of morphological and physiological changes, epimastigotes appear within the salivary glands, and these give rise to the coated but non-dividing metacyclic trypomastigotes that are capable of reinitiating infection.

The biology of antigenic variation

Early experiments on antigenic variation suggested a pleasing symmetry in the expression of different variants. At their most simple, the data suggested that a programmed order of expression was followed. Interruption of this sequence at any point by ingestion by the fly led to the loss of the surface coat, and the assumption of a common surface antigenicity (Gray 1965 *a*, *b*). In the salivary glands, the variant initiating the sequence was re-expressed to prime the cascade in the next cycle of infection (Cunningham 1966). Unforunately the true picture is much more complex, although elements of the original model remain. It is necessary to look much more closely at the different stages in the life cycle which exhibit antigenic variation.

The expression of the surface coat in metacyclic trypanosomes is a key event in that it represents the first stage at which host immunity could be effective in preventing infection since metacyclic v.a.t.s† are introduced into the host by feeding flies. The v.a.t.s present in the metacyclic population seem to be relatively constant within a strain (Jenni 1977 *a*, *b*). This was first shown using polyclonal antibodies which recognized metacyclic populations (Le Ray *et al.* 1978; Barry *et al.* 1978) and it is clear that animals producing such antibodies are resistant to tsetse fly challenge (Nantulya *et al.* 1980). More recently the use of monoclonal antibodies has revealed that the metacyclic population is antigenically heterogenous. The extent of that heterogeneity is a matter of both practical and theoretical interest. Serological experiments have shown that in *T. equiperdum* a single bloodstream trypomastigote can give rise to over 100 serologically distinct v.s.g.s (Capbern *et al.* 1977), and an analysis of v.s.g. genes in *T. brucei* indicated that there may be greater than 1000 v.s.g. genes within the genome (Van der Ploeg *et al.* 1982). If all this potential variation is expressible within the metacyclic population, then the prospects for immunization are bleak indeed. If it is not, then how is v.s.g. activation regulated within the environment of the tsetse salivary glands? The data strongly suggest that the repertoire of v.s.g. genes expressed in metacyclic trypanosomes is restricted. In the most detailed study of its sort to date, a set of 12 different variant specific monoclonal antibodies was prepared which by immunofluorescence accounted for all the metacyclic trypomastigotes present in the saliva of flies infected with a strain of *T. congolense* (Crowe *et al.* 1983). Furthermore, the same set of monoclonal antibodies neutralized up to 10^6 metacyclic trypanosomes produced from the same strain of *T. congolense* in an *in vitro* culture system. The percentage of the population accounted for by each monoclonal antibody varied from 1 to 50%. The situation may be slightly more complex in *T.b. rhodesiense*, where nine different variant specific antibodies accounted for about 80% of the metacyclic population. Whether 1 or 1000 more antibodies would be needed to account for the remaining 20% is unknown (Barry *et al.* 1983). Furthermore, analysis of the repertoire of metacyclic v.s.g.s expressed in stocks isolated from the same region of Kenya over a 20 year period seemed to show that the repertoire was changing (Barry *et al.* 1983). Such a finding, if general, would complicate any attempt to use metacyclic

† Variable antigen types.

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v.s.g.s as the basis for a vaccine. If the repertoire is limited does this imply that there is a pool of v.s.g. genes expressed *only* in the salivary glands? Apparently not. Analysis of trypanosomes present in the mammal following fly bite shows that the metacyclic v.s.g.s continue to be expressed within the lymph and the chancre (the characteristic erythematous lesion which appears two or three days after, and at the site of, the infecting bite), and then within the bloodstream in the first patent parasitaemia (Barry & Emery 1984). It is possible that some v.s.g. genes are incapable of expression within the salivary glands. If so, what are the differences between these two pools of genes, and what external stimuli or selections govern expression?

It should be clear from the above discussion that the expression of metacyclic v.s.g.s is a non-stochastic event and must be accommodated within any model for the control of gene expression within the salivary glands. Similar problems are encountered when considering the expression of different v.s.g. genes within bloodstream populations. Gray first noted that certain antigenic types always tended to appear early in the course of chronic infections, and that there seemed to be a very rough ranking within such variants (Gray 1965*a*). Furthermore, following cyclical passage of trypanosomes after expression of these variants, the same antigenic types tended to occur again early in the course of the next cycle of infection (Gray 1965*b*). The same phenomenon was observed in syringe-passaged populations: reversion to a similar set of variants tended to occur in a non-immune animal (Gray 1965*a*). Gray termed such variants 'predominant', and it was his observations that led to the concept of some ordering within the progression of antigenic variation. A similar phenomenon has been observed in acute infections in so-called 'relapse experiments', in which small numbers of an homogeneous cloned population of trypanosomes (10–1000) are injected into a rat or mouse (Van Meirvenne *et al.* 1975; Miller & Turner 1981). The host responds by the production of antibody to this variant and, after a crisis, enters remission until the appearance of the first recrudescent population, which is analysed for its antigenic composition. Because small numbers of cloned trypanosomes were used in the original inoculum, all the members of the relapse population can be assumed to be clonally derived. Again, it transpires that expression of different antigenic types in such relapse populations is non-random, there being a 'statistically definable' order of priority of expression (Miller & Turner 1981). This concept has been extended into many species of trypanosomes. In *T. equiperdum*, in which by far the most extensive serological analysis has been carried out (Capbern 1977), variants have been classified as 'early', 'middle' or 'late' (Baltz *et al.* 1977). Here again, an explanation is needed at the molecular level. One possibility is that genes encoding 'predominant' v.s.g.s have a higher rate of occupancy of an expression site, either by reason of their location (for example, telomeric), or because homologies in flanking sequences around the gene and at the expression locus favour a higher rate of gene conversion, or because a multiplicity of pathways to expression exists for such variants, or any combination of these. There could be other explanations. For example, it has been argued that predominance could simply be a reflection of differential growth rates (McNeillage & Herbert 1968). The most convincing argument against this comes from a computer analysis of published data on the emergence of 'predominant' v.s.g.s, which concluded that random generation of variants followed by selection operating through growth rates alone would not be capable of producing the degree of variant ordering that has been reported in the literature (Kosinski 1980). Unknown environmental factors could have a major influence on selection of the 'fittest' variants. This would imply the existence of physiological characteristics which correlate with antigenic type. There are published precedents for this, including growth rates (Van Meirvenne

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et al. 1975; McNeillage & Herbert 1968), and infectivity to particular hosts (Van Meirvenne *et al.* 1976; de Gee *et al.* 1979). More recent studies have shown that the rate of differentiation from long slender to short stumpy trypomastigotes is under *host* control, and also that parasite growth in an *in vitro* tissue culture system is regulated by short-lived factors produced in fibroblast monolayers (Black *et al.* 1984). Variant-specific responses to either of these stimuli could affect the composition of relapse populations.

A complication is the evidence that growth rates of individual clones of trypanosomes may not correlate with the growth rates observed in mixtures (Seed 1978; Miller & Turner 1981), that is, that there are competition effects conceivably for host-derived growth factors of the kind described above. Another point to bear in mind is that trypanosomes are not simply confined to the vasculature. Their occurrence in cerebrospinal fluid and in deep brain sites is associated with the onset of the coma from which the name sleeping sickness is derived, but they can also be found in lymph and in other tissue sites many of which are far removed from the vasculature or lymph bed. Furthermore, the major serotype isolated at any one time from the blood can be quite different from the major serotype present in, for example, the lymph or the brain (Seed & Effron 1973). In other words, an animal may be expressing multiple 'predominant' antigenic types at any one time, of which that found in the blood is only one example. Predominance may therefore be one aspect of a very complicated example of population dynamics which may be beyond the scope of the molecular biologist alone to describe. This problem is discussed in more detail elsewhere (Seed 1984).

It is at least agreed that on ingestion by the tsetse fly, the surface coat is lost, and all trypomastigotes assume a common surface antigenicity. Early work suggested that whatever variant was ingested, the same 'basic' antigen was reacquired in the salivary glands and indeed expressed in the first patent parasitaemia (Cunningham 1966). As we have seen, this is an oversimplification, but the assumption has been that the ingested variant is 'lost'. Again, more recent studies refute this as a generalization. In a study in which flies were infected with five different antigenic types, or with mixtures from these five, then regardless of the type used to infect the flies, the first antigenic types detectable in the bloodstream (on days 1–4) were those previously identified as metacyclic antigenic types. From days 4–10 however, up to 18 different predominant types were identified in the blood of the mouse, one of which was always the ingested variant (Hajduk & Vickerman 1981). Several points should be made in connection with this observation. First, even in immunosuppressed animals, the metacyclic types switch in the bloodstream to new types. In other words, selective removal by antibody is not necessary: metacyclic variants must switch to express new variants with high frequency. This conclusion is supported by other studies (Barry & Emery 1984). Second, as already discussed, the expression of the same repertoire of metacyclic types in the salivary glands and in the bloodstream regardless of the variant ingested, suggests that these genes may be under separate genetic or environmental control or both. Third, the occurrence of the ingested type, with other 'predominant' types, in the first patent parasitaemia, suggests some form of anamnestic response.

In summary, then, a complete description of antigenic variation must include an explanation for the following features of the system. First, induction of v.s.g. synthesis in the salivary glands. Second, the apparently selective activation of a subset of the available gene repertoire in the metacyclic population. Third, the phenomenon of predominance or loose ordering of appearance of different antigenic types within the chronically or acutely infected mammalian host.

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Fourth, the repression of v.s.g. synthesis within the insect vector. Fifth, the anamnestic switch in the first patent parasitaemia in the bloodstream of the mammalian host, following cyclic transmission. Sixth, and fundamentally, there must be an explanation for expression of only one v.s.g. gene at any one time. As it is generally accepted now that trypanosomes are diploid (Tait 1980), there must at least be some form of allelic exclusion operating.

It is fair to say that although an immense amount of invaluable information has been generated on the structure and organization of both v.s.g.s and their genes, none of the five features of the system outlined above have been satisfactorily explained. The difficulties of obtaining sufficient numbers of metacyclic trypanosomes have precluded any study of the biochemistry of their v.s.g.s. It is an article of faith, probably justified, that they are no different from their counterparts in the bloodstream. Analysis of the genes encoding metacyclic v.s.g. has been restricted to those variants that are also commonly found in bloodstream populations. At the time of writing, there is no evidence to suggest that these genes are strikingly different from any other v.s.g. genes. It is extremely difficult to look at the activation of v.s.g. genes within the environment of the tsetse salivary glands. Advances in the *in vitro* cultivation of trypanosomes, particularly with *T. congolense* (Gray *et al.* 1981), should permit study at the gene level of the differentiation from uncoated epimastigotes to coated metacyclics, and will also provide sufficient metacyclic trypanosomes to allow at least a rudimentary analysis of the biochemistry of metacyclic v.s.g.s. There is no satisfactory explanation for the 'ordering' of appearance of different variants in the bloodstream population. There has been much discussion about the environment of v.s.g. genes as determinants of expression, particularly with respect to those genes that are telomeric. Since the first analyses of v.s.g. gene rearrangements and their relation to expression (Williams *et al.* 1979; Hoeijmakers *et al.* 1980), there has been much debate as to whether there are two classes of v.s.g. gene, one of which is activated by the generation of the so-called expression-linked copy (e.l.c.), and the other that can be activated without recourse to the gene duplication event necessary for e.l.c. formation. This latter class is invariably telomeric whereas e.l.c. genes usually have a non-telomeric 'basic copy' (b.c.) which serves as the template for the formation of the e.l.c., which is always telomeric (there are also examples of telomeric b.c.s). It could be argued that such data imply the existence of two different activation pathways which could have significance for the biology of antigenic variation. Recent experiments show that this division is an artificial one, and that the same gene can display e.l.c. or non-e.l.c. behaviour depending on the variant that is the precursor to its expression and on the chance selection of clones from the relapse population (Young *et al.* 1983). Both the generation of e.l.c.s and the production of telomeric basic copies probably involve analogous duplicative transpositions. A number of such duplicated genes can then coexist in the genome while only one of them is expressed. Control of antigenic variation therefore lies not in the duplication event, which gives rise to telomeric copies, but in the manipulation of telomeric v.s.g. genes into and out of an environment allowing expression (Young *et al.* 1983). This then makes it very hard to base selective gene activation in, for example, metacyclic populations on the presence of a telomeric basic copy.

Similar arguments apply to explanations of predominance based on the assumption that generation of telomeric copies is the rate-determining step in an activation pathway, and that telomeric v.s.g. genes therefore have a higher frequency of expression. This could be part of the answer, but really there are no data at all on the rate of generation of telomeric v.s.g.s, nor the frequency with which they are expressed. It is not even established that generation of

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e.l.c.s or rearrangement of telomeric v.s.g.s always occurs in association with gene expression. Antigenic variation is a low frequency event detectable in 1 in 10^4 to 1 in 10^5 of the population, and the switched population can be isolated through its selective advantage in an immunocompetent animal (Doyle *et al.* 1980). Gene switching events that do not lead to expression could be occurring with equal, or even very much higher frequency, but there is no way of selecting for trypanosomes that have undergone such 'non-productive' switching events, other than through the chance selection of clones.

In the absence of any quantitative information on the dynamics of these gene rearrangements and the relation to expression, it will be impossible to correlate gene location with probability of expression, in any environment. It should also be pointed out that analyses of this type have only been carried out on 'predominant' variants isolated from the bloodstream. If, as suggested elsewhere (Seed & Effron 1973; Seed 1984) multiple 'predominant' variants coexist within the host in different environments, are all these variants expressing v.s.g.s that are the product of genes having telomeric basic copies? Also, although early work on predominance suggested that the same trypanosome stocks produced the same 'order' in different host species, there is at least one documented example where this is not the case. Experiments with *T. vivax* suggest that this species may show selective growth of different antigenic types in different hosts (de Gee *et al.* 1979). Goats and mice were inoculated with identical samples of parasites and the antigenic types expressed compared. Certain types seemed to be expressed in goats that were not expressed in normal mice, though they did appear in infections of lethally irradiated mice. While differences in growth rates of antigenically different cloned populations of *T. brucei* in the same host have been described, little is known about possible variable antigen associated differences in host susceptibility. However, as it is known that even *T. brucei* and *T. congolense*, which are nominally infective to mice, can be very difficult to establish from field isolates (Gray 1970; Goedblad *et al.* 1973) the possibility that there is selection for particular variants that are capable of infecting laboratory rodents should be considered, as this clearly imposes a bias on all subsequent experiments.

A last point to make about telomeric v.s.g. genes is that they exist in a hazardous environment. Genes transposed into such an environment are frequently 'lost' when they are no longer expressed, as in the classic e.l.c. phenomenon, while others persist (Van der Ploeg & Cornelissen, this symposium). Loss of a telomeric v.s.g. is a consequence of a gene conversion event in which it is replaced by another v.s.g. gene. Sometimes, the gene conversion event is incomplete, and gives rise to a new v.s.g. gene, which is a hybrid of the donor and acceptor gene (Pays *et al.* 1983*a*, *b*). Telomeric v.s.g. genes seem to be particularly susceptible to gene conversion so, if predominance *is* related to the existence of telomeric basic copies, how are some telomeric v.s.g. genes selectively protected from gene conversion into oblivion?

Unlike induction of v.s.g. synthesis, there is a good *in vitro* system for looking at repression of v.s.g. synthesis. Bloodstream trypomastigotes, when placed in the appropriate culture system at 28 °C, differentiate into a form indistinguishable from that found in the insect midgut, termed the procyclic trypomastigote. This process is accompanied by the loss of the surface coat within 48 h (Barry & Vickerman 1979), and the exposure of common underlying antigenic determinants (Seed 1964). A recent study has shown that v.s.g.-specific mRNA synthesis is terminated within 2 h of the shift to new growth conditions, and that repression of v.s.g. synthesis takes place with a half-life of 30 min (Overath *et al.* 1983). Although mRNA synthesis was abolished, the e.l.c. was retained in inactive form. An attractive corollary to this is that

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the anamnestic response observed in the first patent parasitaemia following cyclical transmission is a function of the retention of this inactivated e.l.c. Again, there is insufficient evidence to support or refute this hypothesis. Does ingestion of a variant with a telomeric basic copy produce an anamnestic response? Why are these genes not activated in the salivary glands? There is also a documented example of a transformation to procyclic trypomastigotes in which an e.l.c. was lost (Pays *et al.* 1981).

Lastly, there is still no satisfactory explanation for the expression of only one v.s.g. gene at a time. The existence of only one expression site would offer the simplest control system, but recent experiments are consistent with the presence of at least two different expressions sites (Van der Ploeg, this symposium). It has been suggested that there may be a unique v.s.g.-specific promoter sequence that could be within a tandem repeat known to contain a 35 base element present at the 5′ end of all v.s.g. mRNAs (De Lange *et al.* 1983). This element is separated from the remainder of the coding sequence by a minimum of 40 kilobases, suggesting that either there is an enormous precursor mRNA, or that this element is actually added post-transcriptionally. Not all the copies of the 35mer are found in the main repeat. Some copies are present as 'orphons' elsewhere in the genome (Nelson *et al.* 1983). The 35mer is also found associated in non-v.s.g. mRNAs, and even in other members of the order Kinetoplastida (Nelson *et al.* 1984). Its relation to the control of v.s.g. gene expression remains to be established.

It is clear from all the above that although we have learned much about v.s.g.s and their genes, a great deal remains to be done before we can fully understand what is undoubtedly a very complex system. One area remains virtually untouched; the interaction of v.s.g.s with their environment.

V.s.g.s and their environment

Several examples have been cited of apparently v.s.g.-associated characteristics. There are three reports in which different growth rates have been associated with different variants (McNeillage & Herbert 1968; Van Meirvenne *et al.* 1975; Miller & Turner 1981), and two examples of competition when mixtures of different variants are grown together (Seed 1978; Miller & Turner 1981). There are two reports of variant antigen-associated differences in host susceptibility (Van Meirvenne *et al.* 1976; de Gee *et al.* 1979), the first of which involves susceptibility of man to infection with *Trypanosoma brucei*. *Trypanosoma brucei brucei* is not infective for humans because it is lysed by the action of the high density lipoprotein (h.d.l.) fraction of normal human serum (Rifkin 1978*a*). Both *T.b. rhodesiense* and *T.b. gambiense* are resistant to lysis by normal human serum, but the three members of the *brucei* subgroup are morphologically indistinguishable. A laboratory accident resulted in the infection of an individual and led to the analysis of 11 antigenically distinct clones from the infecting serodeme, which showed that all were serum-sensitive except one, which was serum-resistant, and this was the clone that produced the infection (Van Meirvenne *et al.* 1976). The accident provided an example of variant antigen-associated host range specificity, as well as highlighting the specious nature of the sub-specific classification of *T.b. brucei* and *T.b. rhodesiense*. The second example is provided by the differential infectivity of variants of *T. vivax* for goats and mice, discussed above (de Gee *et al.* 1979). The expression of a restricted pool of variants in the metacyclic population provides another example of variant antigen-associated host range specificity. Could the structure of the v.s.g. actually be responsible for some of these restrictions? If so, how could it operate? No function has ever been ascribed to the surface coat other than

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a purely protective one. Protection operates at two levels, the first being the physical masking of underlying common antigenic determinants (Barry 1979) and structures that activate complement by the alternate pathway (Tetley *et al.* 1981), and the second being the ability to vary this protective mask. The only structural constraint would therefore seem to be the ability to form a cohesive layer having both lateral stability and stable attachment. If active transport or facilitated diffusion of growth factors or metabolites through the plasma membrane is essential, it is conceivable, given the diversity of v.s.g. sequences, that some v.s.g.s facilitate the movement of such molecules more than others.

Much is known about the composition, sequence and biosynthesis of v.s.g.s (reviewed in Borst & Cross, 1982; Englund *et al.* 1982; Turner 1982*a*; Cross, this symposium) but until very recently nothing was known about their tertiary structure. For the past two years, members of the Molteno Institute Antigen Group have been collaborating with Professor Don Wiley and his colleagues at Harvard University on an X-ray crystallographic analysis of v.s.g.s. Of ten soluble v.s.g.s supplied to date, five have been crystallized. Chance observations revealed that the best resolution is obtained on crystals prepared from the N-terminal hypervariable domain, which can be isolated following limited proteolysis (Johnson & Cross 1979). This domain has been crystallized from two variants, MITat 1.2 and ILTat 1.25, producing crystals which diffract to at least 0.29 and 0.25 nm resolution, respectively. A single isomorphous derivative has been solved for MITat 1.2 which has allowed the construction of a three-dimensional electron density map at 0.6 nm resolution, which even at this level reveals some quite remarkable features. It shows a clear, dimeric, rod-shaped molecule composed of a bundle of α-helices approximately 8 nm long. The α-helical rods are symmetrically disposed around a molecular twofold axis such that at one end of the molecule six α-helices are visible, four are apparent in the central section, and possibly two groups of three can be seen at the other end (D. Freymann, P. Metcalf, M. Turner & D. Wiley, unpublished). It is extremely unusual to see such detail at this level of resolution, and may be an indication of a very highly ordered structure. Whether the same or a similar structure is conserved in other v.s.g.s is unknown, and this highlights the importance of solving the structure of more than one v.s.g. Although protein sequences in this domain are hypervariable, the distribution of cysteines appears to be highly conserved (Rice-Ficht *et al.* 1981; Cross, this symposium) and some sequences around a cysteine at position 15 show significant homologies which are probably indicative of a common structure (Olafson *et al.* 1984). The primary test for a newly evolved v.s.g. sequence generated perhaps by partial gene conversion (Pays *et al.* 1983*a*, *b*) may therefore be not whether it represents an immunochemically distinct variant but whether it can form this structure. Whether all v.s.g.s have such a structure, and whether the structure forms a secondary function (for example, permeability barrier) therefore are crucial questions awaiting answers.

A second area in which v.s.g. structure could influence trypanosome viability is in the structure of the C-terminus of the molecule. V.s.g. release is a puzzling and intriguing feature of trypanosome biology. The ease with which v.s.g.s may be released from the trypanosome has greatly facilitated study of v.s.g. biochemistry, but it is not the behaviour to be expected of an intrinsic membrane protein. This paradox has recently been resolved by the discovery that v.s.g.s are actually released from trypanosomes through the action of an enzyme, which acts on the hydrophobic anchor that holds the v.s.g. in place on the plasma membrane (Cardoso de Almeida & Turner 1983). Unusually for a membrane protein, this anchor seems to be a glycolipid which is attached to the C-terminal amino acid (Holder & Cross 1981; Holder 1983),

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and the enzyme involved is some sort of lipase which releases a fatty acid, allowing the v.s.g. to float off into solution. The fatty acid in a variant of *T.b. brucei* has been characterized as myristic acid (Ferguson & Cross 1984). Is it possible that changes in this moiety could influence host range? Coated bloodstream trypomastigotes are lysed by h.d.l. in serum, whereas uncoated procyclic trypomastigotes are not (Rifkin 1978*b*) suggesting that the coat is a determinant in the lytic event. Since the h.d.l. fraction is a source of both lipolytic enzymes and lipid binding proteins it is possible that interactions with the glycolipid portion of the v.s.g. molecule are determinants in the effector mechanism, which has yet to be elucidated.

V.s.g. release apparently involves a fascinating and unusual mechanism of great interest to biochemists, but does it have any relevance to the natural history of a v.s.g. molecule? Disruption of trypanosomes in a Waring blender cannot be said to resemble physiological conditions, and activation of the enzyme could be an adventitious consequence of rupture of the lysosomal membrane, for example. This seems unlikely for a number of reasons. First, the enzyme is qualitatively absent from procyclic trypomastigotes, which lack the surface coat, suggesting a link between antigen expression and enzyme activity (Cardoso de Almeida *et al.* 1984*b*). Second, the enzyme is itself membrane-bound (Cardoso de Almeida *et al.* 1984*b*). More recently we have shown that it seems to be associated with the plasma membrane (A. Gurnett, J. Raper, M. L. Cardoso de Almeida, M. J. Turner & F. Opperdoes, unpublished data). Again this suggests a role for the enzyme in v.s.g. metabolism.

At what stages in the life cycle could v.s.g. release occur, and what could be the significance? At least three possibilities can be considered. First, v.s.g. release could be occurring continuously from rapidly dividing bloodstream trypomastigotes as a consequence of turnover. There is one literature report stating that long slender trypomastigotes do not release v.s.g. or continually turn over v.s.g., but that short stumpy forms do shed v.s.g. (Black *et al.* 1982). There has been some controversy in the past about the presence or absence of v.s.g. in the plasma of infected animals, and whether or not it is an artefact produced by allowing infected blood to stand before collecting plasma (reviewed in Turner 1982) but the more recent measurements of plasma concentrations would seem to satisfy some objections (Diffley *et al.* 1980). Parasitaemias in rats of between 4×10^8 and 2×10^9 trypanosomes per millilitre of blood produced 28–320 μg v.s.g. per millilitre of plasma. These are, of course, very high parasitaemias, and the assay used was not sufficiently sensitive to monitor v.s.g. levels in chronic low level parasitaemias. No information is presented about the presence or otherwise of short stumpy forms, but the stock used is described as pleomorphic, which could accord with the idea that release is mediated by short stumpy forms. It is not clear whether in these circumstances v.s.g. release occurs only from dying trypanosomes. Could there be a role for such released v.s.g. in the pathogenesis of the disease, such as in induction of immune complex disease (Tizard *et al.* 1978) or immunosuppression (Jayawardena & Waksman 1977)? V.s.g.s bound to the plasma membrane display only a fraction of the variant specific antigenic determinants present on the intact molecule (Pearson *et al.* 1980; Black *et al.* 1982; E. N. Miller, L. M. Allan & M. J. Turner, unpublished data) and it has been suggested that the release of v.s.g. and exposure of many 'irrelevant' antigenic determinants could provide a form of immunological diversionary tactic (Turner 1982*b*). An intriguing possibility is that released v.s.g. may influence qualitatively and quantitatively, the spectrum of variants found in succeeding waves of parasitaemia (Cardoso de Almeida 1983). A second possibility is that release is essential during antigenic switching. This would imply both that v.s.g. release can be uncoupled from cell death

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and that release can be controlled in such a way that no uncoated surface is exposed in the bloodstream, as this would result in lysis by complement activation through the alternate pathway. This is an exciting hypothesis, but as antigenic variation occurs only at low frequency it is difficult to test, and there are no data bearing on this point. A third possibility is that the enzyme is activated to bring about v.s.g. release during transformation to the uncoated form found in the insect midgut. As described above, this event can be brought about *in vitro*, and both v.s.g. synthesis and release have been monitored in a recent study (Overath *et al.* 1983). This showed that whereas repression of v.s.g. synthesis occurred with a half life of 30 min, v.s.g. release was detectable between 6 h and 60 h after induction of transformation, and v.s.g. was found in the culture supernatants as a mixture of the soluble form, and an N-terminal proteolytic fragment. Because the ratio of these two forms remained unchanged, it was suggested that release involved both conversion to s.v.s.g. and proteolysis.

A common theme to all these possibilities is that the enzyme must be selectively activated to perform its function. It is clearly inactive most of the time. As a minimum, it must be activated on cell death, and at a maximum it must be activated in viable populations. How is this brought about? It has been said that uptake of calcium ions brings about v.s.g. release (Bowles & Voorheis 1982). However, we have observed no stimulation of calcium or other divalent cations on enzyme activity in partly purified preparations, nor do chelating agents have any effect, either stimulating or inhibitory (unpublished observations). We have described the inhibitory effects of zinc, cadmium and aluminium ions on the enzyme, but ascribe the effects to non-specific protein fixation (Cardoso de Almeida *et al.* 1984*a*). The occurrence of a v.s.g. release pathway may have the most far reaching consequences, if it is induced by transmembrane signalling by some environmental molecule. Activation of this pathway may also repress v.s.g. mRNA synthesis in the nucleus in procyclic trypomastigotes, and, if v.s.g. release *is* coupled to induction of antigenic variation it is therefore clear that more investigations into the biology of antigenic variation are of the most profound importance for our goal of controlling this most elusive parasite.

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Discussion

W. E. Ormerod (*London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K.*). All speakers, hitherto, have implied that a basic assumption that they have adopted, is that *Trypanosoma brucei* divides solely by binary fission in the blood. This is not an assumption that appeals to a parasitologist because of the pleomorphism that this species exhibits both in the field and in early passages in the laboratory. The evidence, reviewed by Ormerod (1979) suggests that the slender form develops from a stage outside the blood, increases in number by binary fission and finally turns into the senescent stumpy form which is then removed from the blood. Although strains become selected on passage for rapid reproduction in the blood, the existence of an extravascular stage can never wholly be dismissed. Recently work in my laboratory (Abolarin *et al.* 1982; Hussein & Ormerod 1984) has revealed an intracellular stage of *T. brucei*, in the ependymal cell, present in all three of the major subspecies, which fulfills the criteria of an extravascular phase form which the succession of pleomorphic forms might be considered to develop. It is too early to state with any certainty exactly what is the role of this intracellular stage, but its existence underlines the importance of extravascular reproduction and the necessity that its essential pleomorphism should be taken into account in any theory on the nature of antigenic variation of *T. brucei*.

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M. J. Turner. Dr Ormerod is correct in stating that I implied that trypanosomes divide solely by binary fission in the blood, and I accept that this could be an over-simplification – whether or not antigenic variation occurs in an intracellular population would then be another of the long list of 'don't knows' I have put forward in my talk. At this stage in our understanding I would suggest that it does not matter where antigenic variation takes place, since antigenic variation is not like mating type switching in yeast in which it is possible to look at the immediate progeny of any switch. Many generations pass before antigenically switched populations even reach the stage where they can be cloned, and numbers then have to be further amplified before clones can be analysed immunochemically, biochemically or molecular genetically. We are therefore looking at the footprints left by an event that happened in prehistoric times, so far as the trypanosomes are concerned. We do it that way because we have to – at this stage we cannot look at these rare events in single cells, although a time will come when we may be able to. What is important, I think, is a recognition of the limitations imposed on our understanding by the methods we use: what I call the biological application of Heisenberg's Uncertainty principle! I have tried to put across in my talk where I see that those limitations apply at present. Certainly, analysis of extravascular events of the kind Dr Ormerod proposes is something which may have to be contemplated in the long term.

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