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From silent genes to noisy populations – dialogue between the genotype and phenotypes of antigenic variation

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Abstract

African trypanosomes evade humoral immunity through antigenic variation, whereby they switch expression of the *VSG* gene encoding their glycoprotein surface coat. Switching proceeds by duplication from an archive of silent *VSG* genes into a transcriptionally active locus, and precedent suggests silent genes can contribute combinatorially to formation of expressed, functional genes through segmental gene conversion. The genome project has revealed that most of the silent archive consists of hundreds of *VSG* genes in subtelomeric tandem arrays, and that most of these are not functional genes. The aim of this review is to explore links between the uncovered trypanosome genotype and the phenotype of antigenic variation, stretching from the broad phenotype – transmission in the field and the overcoming of herd immunity – to events within single infections. Highlighting in particular the possible impact of phenotype selection on the evolution of the *VSG* archive and the mechanisms for its expression leads to a theoretical framework to further our understanding of this complex immune evasion strategy.

Keywords

Antigenic variation; genome sequence; phenotype; trypanosomes

Mammalian hosts present acquired immune responses that enable them to exert some control over infections with African trypanosomes. On the other hand, these parasites have acquired an antigenic variation system that allows evasion of host immunity and have gained notoriety for the wide range of molecular phenomena and mechanisms that can be shown, at least in the laboratory, to enable antigenic variation. *Trypanosoma brucei* lives in the blood and tissue fluid, coated by a densely packed monolayer of variant surface glycoprotein (VSG), reviewed in (Barry and McCulloch 2001; McCulloch 2004; Pays 2006; Taylor and Rudenko 2006) and <http://www.VSGdb.org/>. The coat protects, at least theoretically, against innate immune mechanisms and denies access to antibodies specific to invariant surface antigens. As the VSG itself is an antigen, and furthermore highly abundant on the parasite, trypanocidal anti-VSG antibodies potentially remove the ongoing trypanosome population. Except, that is, for individuals that have switched clonally to one of many alternative VSGs. These switchers seed the next peak of trypanosomes, and the continuous interplay between VSG switching, trypanosome growth and antibody response allows establishment of a

chronic infection. Different VSGs are antigenically distinct in their variable, N-terminal domain, which generally shares only ~20% homology between variants. The C-terminal domain is more conserved, providing GPI anchorage to the plasma membrane, and is concealed from immune effector mechanisms by the N-terminal domain.

The trypanosome genome sequencing project (Berriman, Ghedin, Hertz-Fowler, Blandin, Renauld, Bartholomeu, Lennard, Caler, Hamlin, Haas, Bohme, Hannick, Aslett, Shallom, Marcello, Hou, Wickstead, Alsmark, Arrowsmith, Atkin, Barron, Bringaud, Brooks, Carrington, Cherevach, Chillingworth, Churcher, Clark, Corton, Cronin, Davies, Doggett, Djikeng, Feldblyum, Field, Fraser, Goodhead, Hance, Harper, Harris, Hauser, Hostetler, Ivens, Jagels, Johnson, Johnson, Jones, Kerhornou, Koo, Larke, Landfear, Larkin, Leech, Line, Lord, MacLeod, Mooney, Moule, Martin, Morgan, Mungall, Norbertczak, Ormond, Pai, Peacock, Peterson, Quail, Rabbinowitsch, Rajandream, Reitter, Salzberg, Sanders, Schobel, Sharp, Simmonds, Simpson, Tallon, Turner, Tait, Tivey, Van Aken, Walker, Wanless, Wang, White, White, Whitehead, Woodward, Wortman, Adams, Embley, Gull, Ullu, Barry, Fairlamb, Opperdoes, Barrell, Donelson, Hall, Fraser, Melville and El Sayed 2005) has at last opened the way towards a more global understanding of the genetic resources available to the antigenic variation system. Combining genome findings with empirical and modelling studies at the level of the parasite population, and building upon findings in the literature, can produce, as discussed in this review, a theoretical framework for antigenic variation that unites the genotype (antigen genes) to the phenotype. The latter can be viewed as a continuum having a “narrow” end (antigens expressed at the cellular level) that broadens to encompass populations of both parasites and hosts, and their interactions. By extending to the “broad” end of the phenotype, the resulting comprehensive framework for antigenic variation provides a set of testable predictions and gives rise to many more questions regarding the balance of forces that shape antigenic variation and factors important for transmission in the field.

Before introducing our current understanding of the genotype, it is worth noting that analysis of many aspects of the broad-end phenotype requires epidemiological and ecological tools, and so is far removed from studies that have revealed some molecular detail of antigenic variation. What brings the broad phenotype and genotype together is selection, acting on the phenotype and, in doing so, shaping the genotype and mechanisms for expressing genes singly. In this respect, two concepts are central. Firstly, trypanosomes live, behave and are selected upon as populations, rather than individuals – a view that increasingly is being seen as applying to unicellular organisms (West, Griffin, Gardner and Diggle 2006). The functional importance is that selection operates at this cellular population level. Secondly, transmission between hosts is a major driving force in evolution (Mackinnon and Read 2004). This broadens the selective pressure to the level of the range of available host animals, both within and across species. Again, trypanosome populations provide the testbed on which selection acts. Selection continues down to the molecular level, and at least some of the antigenic variation mechanisms have arisen in consequence, providing a highly streamlined and efficient defensive interface with mammalian hosts. However, some mechanisms, or aspects thereof, might be dead-ends or generally inconsequential.

The genotype

The genetic strategy for antigenic variation is based upon the gradual utilisation of a large archive of silent *VSG* genes, through the sequential expression of alleles that encode antigenically distinct *VSGs* (Barry, Marcello, Morrison, Read, Lythgoe, Jones, Carrington, Blandin, Bohme, Caler, Hertz-Fowler, Renauld, El-Sayed and Berriman 2005; Pays 2005). The archive comprises two main subsets. One includes <200 genes occupying telomere-proximal loci in the ~100 nuclear minichromosomes (which effectively contain only *VSGs* and repetitive sequence DNA), plus ~20 *VSG* transcription loci, known as bloodstream expression sites (BESs). The other consists of genes arranged as tandem arrays in subtelomeres of megabase chromosomes (conventional, gene-containing chromosomes). It is now known that there are ~1500 array *VSGs* in the genome strain of *T. brucei* (and possibly even more in other strains). Having just recently been revealed through genome sequencing, the array subset are of topical interest in the literature (reviewed by (Barry et al. 2005; Taylor and Rudenko 2006)) and do not need detailed description here. Antigen switching involves duplication of archive genes into a transcriptionally active locus known as a bloodstream expression site (BES). Although some other possible switching mechanisms have been recorded, such as transcriptional switching between BESs or other recombinational events, they make little contribution, especially in trypanosome lines switching at normal rates; in such lines duplication is by far the most frequent event, and in any case virtually all silent genes require duplication. The recombinational mechanisms for *VSG* duplication are not understood, but it is known that it involves homologous recombination, albeit with some unusual features (Bell and McCulloch 2003; Proudfoot and McCulloch 2005; Robinson, McCulloch, Conway, Browitt and Barry 2002).

Minichromosomal silent *VSG* genes are duplicated preferentially (Liu, Michels, Bernards and Borst 1985; Morrison, Majiwa, Read and Barry 2005), the copy stretching from a set of imperfect 70-bp repeat units upstream of the coding sequence possibly to the end of the chromosome (de Lange, Kooter, Michels and Borst 1983; Shah, Young, Kimmel, Iams and Williams 1987). The activation of array genes turns out to be more complex, and this set are expressed later in infection (Lee and Van der Ploeg 1987; Thon, Baltz, Giroud and Eisen 1990; Timmers, de Lange, Kooter and Borst 1987). Of nearly 1000 array genes we have fully annotated to date, only 4.5% are intact, the rest being imperfect in several ways (Barry et al. 2005; Berriman et al. 2005). Counterintuitively, the great, apparent wastage of coding sequence in the archive actually may not limit the extent of antigenic variation. It was shown 20 years ago (Roth, Longacre, Raibaud, Baltz and Eisen 1986) that *VSG* pseudogenes can donate segments to create intact, expressed copies, as occurs in several bacterial pathogens (Futse, Brayton, Knowles and Palmer 2005). This process, known as mosaic gene formation, enables pseudogene utilization and overcomes, at least to some extent, archive degeneracy. It relies on the interaction of related *VSG* sequences.

The infrapopulation in the single host

One step up the phenotype (Fig. 1) lies the infrapopulation (i.e. all of the trypanosomes occurring within the body of a single host at a particular time) level, the first layer where questions can be asked as to how selection at the population level affects the *VSG* gene

system. A key infrapopulation phenotype is ordered expression, where specific variants tend to appear at partially predictable times of infection (Barry 1986; Capbern, Giroud, Baltz and Mattern 1977). This feature, which is common to antigenic variation of different pathogens, is thought to facilitate chronicity of infection, by reliably providing a trickle of variants rather than coexpression of many variants, which could overwhelm the host. Order in trypanosome infections has been ascribed to indirect causes, involving for example cross-reacting immune responses (Recker, Nee, Bull, Kinyanjui, Marsh, Newbold and Gupta 2004) or growth rate differences associated with different VSGs (Seed 1978), or to a trypanosome-inherent property, differential probabilities of activation of VSG genes (Pays 1989). Whereas the former mechanisms require considerable sophistication to encompass the many variants that arise in infection, the latter mechanism has the benefit of simplicity, by linking switching directly to order, and has the distinction of a degree of empirical support (Kamper and Barbet 1992; Laurent, Pays, Van der Werf, Aerts, Magnus, Van Meirvenne and Steinert 1984; Liu et al. 1985; Morrison et al. 2005; Thon et al. 1990). We recently undertook the first quantitative analysis of antigenic variation, which revealed that each examined variant has an unexpectedly predictable (*i.e.* low variance) time for expression in independent infections and that order is highly reproducible (Morrison et al. 2005). It further revealed that which VSG becomes activated at a switch is dictated by the activating VSG, rather than the previously active one. Together, these findings support the theory that order is dictated by the activation probability of a VSG. An additional finding, that VSGs expressed early have similar (although distinct) times for activation, reveals why the infrapopulation at any time comprises a mixture of variants (Fig 1).

To extend this analysis to examine possible roles of other factors in the generation of order, we have turned to mathematical modelling of infection and antigenic variation. Such an approach cannot be expected to reproduce exactly the intricacies and subtleties of a process of this complexity. What it can achieve, however, are clarification of what are the major parameters, and prediction of how different parameters interact. Two important aspects of a model are to include only parameters that will have significant mathematical impact on the outcome, and to include accurately and quantitatively all parameters of that sort. For example, it has been proposed recently, based on *in vitro* experiments, that trypanosomes *in vivo* can remove low levels of antibodies, such as early in an acquired immune response (Pal, Hall, Jeffries and Field 2003). This putative mechanism need not belong as a distinct parameter in a model, because the empirically measured threshold in antibody effects against trypanosomes would include such low level antibody removal. Similar arguments are applicable to indirect trypanosome growth effects arising as infection proceeds, all of which would be included in the measured general inhibition of parasite growth. Including all known, significant parameters, we (KA Lythgoe, L Morrison, AF Read and JDB, unpubl. findings) have developed a model for trypanosome growth and antigenic variation that predicts there are two major determinants of the known pattern of fluctuating parasitaemia and waves of antigenic variants: differentiation to the non-dividing stage present in the bloodstream, and the different activation rates of different VSG genes; antibody responses are merely a function of antigenic variation and growth, acting only to help clear the previous variants. The model therefore predicts that the trypanosome dictates order in antigenic variation, in keeping with the idea that the driver is VSG activation probabilities.

How does order occur? With quantitative evidence now pointing towards intrinsic differences in the probability of activation of *VSGs*, answers might be found through examination of the different types of locus occupied by *VSGs*. Indeed, there has long been evidence that the locus types are involved at different stages of infection. *VSGs* that are the last gene on chromosomes (*i.e.* telomere-proximal genes, most of which are on minichromosomes) routinely are duplicated in the earliest stages of infection, at the first detected switches (Liu et al. 1985; Morrison et al. 2005). Following this, intact array *VSGs* are activated (Lee and Van der Ploeg 1987; Timmers et al. 1987), followed by mosaic genes (Thon et al. 1990). Within each locus type there is also sub-order, which is illustrated graphically through analysis of timing of appearance of variants in different infrapopulations (Fig 2). In the phase when telomere-proximal genes are activated, effectively all infrapopulations express the same *VSG* types, with little variance in their timing. In the phase when array genes, including mosaics, become activated, initially most infrapopulations express ostensibly the same *VSGs*, but there is gradual divergence towards what appear to be infrapopulation-specific variants (Fig. 2). Sequence homology-based ordering of expression can explain this pattern. In our analysis of the *VSG* archive in the genome sequence, array *VSGs* form many subfamilies, and it is likely that sequence homology levels within the subfamilies determine the “assembly” probability of any given mosaic. Mosaics appearing early in that phase of infection (*i.e.* with a relatively high activation probability) would have arisen from recombination between close homologues, but progressively fewer homologous silent partners would be needed for assembly of genes encoding antigenically novel *VSGs*, this being reflected in a gradual lowering in activation probability. With the benefit now of the sequence of (most of) the arrays being available, we can test what proportion of switches in later infection involve mosaic formation, the role of homology in determining which *VSGs* interact, and whether there are temporal patterns of activation.

Field populations

Probably the most striking outcome of the sequencing project so far is the realization that the mosaic phenomenon could enable trypanosomes to overcome herd immunity, a major component of the broad-end phenotype (Barry et al. 2005). In the absence of any studies directly addressing this theory, it has little empirical basis. It is known that different trypanosome isolates, even from the same location, have largely distinct *VSG* repertoires (Van Meirvenne, Magnus and Vervoort 1977), and this has also been apparent from analysis of a few *VSG* sequences (Frasch, Borst and van den 1982; Pays, Deckerck, Van Assel, Babiker, Le Ray, Van Meirvenne and Steinert 1983). Trypanosomes have a broad host range and evidence is, at least for *T. vivax*, that the same set of variants are expressed in the same order in different host species (Barry 1986). It is to be expected, therefore, that trypanosomes with different genotypes compete only minimally with respect to *VSGs* and that transmission will not be compromised by the antigenic variation system *per se*. In contrast, those of the same genotype might be expected to compete with each other strongly, due to the presence of anti-*VSG* antibodies, meaning that superinfection is at best difficult. However, this may be a source of strong selection for maintenance and expansion of the large set of damaged genes in the *VSG* archive. In the presence of anti-*VSG* antibodies from

foregoing infection, each new infrapopulation could survive if it embarked on a stochastically different “string” of variants (Barry et al. 2005). It is an interesting possibility that progressive development of infrapopulation-specific strings might explain the tendency, in Figure 2, towards infrapopulation-specific variants. A critical element of superinfection is the transmission, from tsetse, of the metacyclic trypanosome population, and it has been argued that a distinct strategy for VSG coat diversity enhances the probability of superinfection (Barry et al. 2005).

Shaping the genome

Looking downwards from the broad-end phenotype, it can be argued that the requirements of antigenic variation have shaped the genome quite radically. Use of a strategy of mosaic strings in the face of herd immunity would select for pseudogenes rather than intact genes, as there is a requirement for the sequences of expressed genes to be created *de novo* each time, such as by combinatorial assembly from a pool of genes that cannot individually be expressed and thus cannot induce immunity against the epitopes they encode. The benefits of having ordered antigenic variation at the infrapopulation level have led to selection of different locus types for *VSG* genes, as well as, in case of the pseudogenes, the existence of sequence subfamilies. The trypanosome’s genome is expanded and modified to a unique extent to accommodate these requirements. Besides the well recorded set of minichromosomes that are thought to have evolved as a pool of the telomere-proximal locus type (Van der Ploeg, Schwartz, Cantor and Borst 1984), the subtelomeres of the main chromosomes are now being recognized as disproportionately large (Berriman et al. 2005). It has even been shown recently that, on one chromosome, a *VSG* array-bearing subtelomere is twice the length of the chromosome core (Callejas, Leech, Reitter and Melville 2006)! Many of the features of the *VSG* gene archive add up to a conspicuous example of the implicit genome, where information is cryptically encoded (Barry 2006).

It is a major challenge to test empirically whether these proposed selective pressures do operate and have led to evolution of the trypanosome genome. Several factors could have prominence in the field, such as the density and species range of hosts present in a given trypanosomiasis focus, the incidence of infection in these host populations, the severity of disease, the level of transmission, and the number (and mixing) of parasite genotypes present in a given area. Combination of these factors would exert pressure for optimal length of infection to maximize transmission, and this would be reflected in an archive of corresponding size and diversity.

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Fig. 1. Model framework for antigenic variation, from broad phenotype in the field to molecular information and mechanisms responsible for switching.

The model links all levels of antigenic variation, from molecular mechanisms to what happens in the field. Evolutionary pressure is exerted at the field level and results in selection for mechanisms and features lying lower within the system. Biological levels are displayed in the left column, and their elements subject to selection are shown in the right column. Features responsible for these elements, and proposed to link the levels, are displayed in the central column. MVAT: metacyclic variable antigen type (VSG type expressed by a metacyclic stage trypanosome).

Fig. 2. Sequential expression of variants in multiple infections.

Spreadsheet analysis of the data of (Capbern et al. 1977), who analyzed the appearance of 74 variants of *Trypanosoma equiperdum* in 11 rabbit infections. The curve with diamond symbols (left y-axis) shows the mean (± 1 SEM) of the time of appearance of each variant in the rabbits, as measured by antibody responses against each variant. There is a gradual increase in these times, and neighbouring variants have closely similar times of appearance. These data concur with those of (Morrison et al. 2005), in that there is very little variance in the time of appearance of each variant. The data also resemble the continuum in sequential appearance of variants reported for *T. vivax* (Barry 1986), and do not support the conclusion of (Capbern et al. 1977) that variants fall into 3 timing groups; the gap between days 15 and 19, and between days 30 and 32, probably represent the remission phases between parasitaemia peaks.

The curve with square symbols (right y-axis) represents the proportion of infections in which each variant appeared, and the two arrows show when two mosaic-encoded *VSGs* (Thon et al. 1990) were expressed. It is clear that, following the initial phase, there is a tendency towards infection-specific variants, and this begins when the first mosaic gene-encoded variant was detected. This pattern is consistent with the theory that distinct 'strings' of mosaics arise in distinct infections.

The data have been corrected to compensate for the removal of some rabbits from the experiment at later stages of infection (Capbern et al. 1977).