

Antigenic variation in *Giardia lamblia* and the host's immune response

THEODORE E. NASH

Laboratory of Parasitic Diseases, Gastrointestinal Parasites Section, National Institutes of Health, Bldg 4, Rm 126, Bethesda, MD 20892, USA

SUMMARY

Giardia lamblia, a protozoan parasite of the small intestine of humans and other animals, undergoes surface antigenic variation. The antigens involved belong to a family of variant-specific surface proteins (VSPs), which are unique, cysteine-rich zinc finger proteins. The patterns of infection in humans and animals fail to show the expected cyclical waves of increasing and decreasing numbers of parasites expressing unique VSPs. Nevertheless, changes in VSP expression occur within the population *in vivo* owing to selection of VSPs by both immune and non-immune mechanisms. After inoculation of a single *G. lamblia* clone (able to persist in the absence of immune pressure) expressing one VSP ($\geq 90\%$) into mice or humans, the original VSP continues to be expressed until 2 weeks post inoculation (p.i.), when many other VSPs gradually replace it. Selection by immune-mediated processes is suggested because switching occurs at the same time that humoral responses are first detected. In most mouse strains, switching also occurs at about two weeks. Almost all trophozoites are eliminated at three weeks (p.i.), but a barely detectable infection persists over months. In neonatal mice, apparent self-cure is delayed until the sixth or seventh week. Antigenic switching does not occur in adult or neonatal severe combined immunodeficiency disease (SCID) mice, but does occur in neonatal nude mice, thus implicating B-cell-mediated mechanisms in immune switching. Not all VSPs are expressed to the same degree *in vivo*. Some VSPs appear to be preferentially selected whereas others are eliminated on a non-immune basis. In infections in which immunity does not play a role, such as in SCID mice, and during the first week of infection in immunocompetent mice or gerbils, persisting VSPs are preferentially expressed and maintained whereas non-persisting VSPs are replaced within the first week of infection. The purpose of antigenic variation may be presentation of a wide assortment of VSPs to hosts, increasing the chance of a successful initial infection or reinfection. Immune selection of variants comes into play following biological selection.

1. INTRODUCTION

Giardia lamblia (*Giardia duodenalis* or *Giardia intestinalis*) is probably the most common parasitic infection of humans. In developed countries large epidemics are caused by contamination of water supplies by the parasite's cysts (Juraneck 1979), but infections are also common wherever faeco-oral transmission of cysts can occur, such as in day care centres (Black *et al.* 1977) and among travellers (Brodsky *et al.* 1974) and homosexuals (Schmerin *et al.* 1978). In many developing regions where basic sanitation is lacking, *Giardia* infections are almost universal by two years of age (Mata 1978). Therefore, giardiasis is a significant health concern all over the world.

Giardia lamblia is a flagellated protozoan parasite transmitted by ingestion of quadrinucleate cysts, which are passed in the faeces. After ingestion, excystation occurs, liberating two binucleate trophozoites, which multiply within the lumen of the small intestine. This stage of the parasite is responsible for the symptoms associated with giardiasis, which are most commonly

diarrhoea, flatulence, cramping, nausea, vomiting and belching. Malabsorption occurs in the most severe cases. As the trophozoites pass through the intestines, they begin to form cysts, which are excreted in the faeces, sometimes in massive quantities. Cysts in fresh stools are infectious. As few as 10–100 cysts have infected 100% of those experimentally inoculated (Rendtorff 1954*a,b*); and this extreme infectivity coupled with the excretion approaching 10^7 cysts g^{-1} stool are major reasons for the often high prevalence.

Besides being an important pathogen, *Giardia lamblia* is among the most primitive eukaryotes known (Sogin *et al.* 1989) and has become a model to understand how more advanced eukaryotes evolved and also to unravel the more complicated processes of cell differentiation in higher eukaryotes. For example, *Giardia* trophozoites have no morphologically discernible Golgi apparatus but apparently develop one during encystation (Luján *et al.* 1995*a*). This parasite can thus be used to study Golgi morphogenesis and the requirement of a Golgi apparatus for vesicular transport. The process of encystation is perhaps the most primitive developmental

process undergone by eukaryotes; an understanding of this process may yield important information about more complicated developmental processes in other eukaryotes. Recent experiments from this laboratory suggest that the stimulus for encystation is deprivation of cholesterol (Luján *et al.* 1996). Knowledge of the underlying signal transduction and biochemical mechanisms may lead not only to a greater understanding of the parasite and a potential means of inhibiting production of the infectious cyst, but also to insight into the importance of cholesterol in cellular and developmental processes in higher eukaryotes.

2. ANTIGENIC VARIATION AND SELF-CURE OF THE *GIARDIA* INFECTION

Giardia lamblia undergoes surface antigenic variation (Nash *et al.* 1988; Adam *et al.* 1988). In contrast to other organisms in which surface antigenic variation was suspected because of the presence of cyclical waves of parasite markers in the blood, surface antigenic variation in *Giardia lamblia* was unsuspected. The process was discovered and characterized as a phenomenon *in vitro*. Its presence *in vivo* was unrecognized and its role in the infection and disease unknown.

Our understanding of the importance of the immune response in the control of *Giardia* infections comes mainly from three lines of evidence: (i) experimental *G. muris* infections in mice (Roberts-Thompson *et al.* 1976; Nash 1993); (ii) experimental *G. lamblia* infections in gerbils (Belovic 1983; Aggarwal & Nash 1987), neonatal mice (Gottstein *et al.* 1990) and adult mice (Byrd *et al.* 1994); and (iii) clinical observations in humans. In infections in mice and gerbils, most trophozoites are apparently eliminated by immune-mediated mechanisms (Nash 1993). A large body of evidence indicates that both T- and B-cell responses are required for elimination of infections in *G. muris*-infected mice (Nash 1993). In humans, patients with hypogammaglobulinaemia have prolonged infections, which are difficult to cure (Hermans *et al.* 1966; Ament *et al.* 1973); this difficulty suggests that the effector mechanisms are dependent upon antibodies, most probably intestinal IgA responses. *G. lamblia*-infected immunocompetent adult mice either cure themselves or eliminate most trophozoites (Byrd *et al.* 1994), but severe combined immunodeficiency disease (SCID) mice are unable to control the infection (Byrd *et al.* 1994). However, elimination of the parasite is often not complete. Naturally and experimentally infected humans may remain infected for long periods of time (Rendtorff 1954); in some strains of *G. lamblia*-infected mice (Byrd *et al.* 1994) and in *G. lamblia*-infected gerbils (Lewis *et al.* 1987) more sensitive examinations reveal barely detectable infections to be present in apparent 'cures'.

Except in the recent studies detailed below, the role of antigenic variation in prolonging infections has not been studied. Although *G. muris* is a good model in the mouse, it cannot be grown in culture; the nature of its surface antigens as well as much of its biology therefore remain largely unknown.

Table 1. *Characteristics of antigenic variation in vitro*

1. Present in all isolates; VSPs shed into medium
2. Only one VSP per trophozoite
3. VSPs change spontaneously <i>in vitro</i>
4. Rate of change is VSP- and isolate-dependent
5. Rates vary from about 1:100 to about 1:3000–5000
6. VSPs differ in their resistance to trypsin and chymotrypsin
7. Monoclonal antibodies to VSPs can show complement-independent cytotoxicity

3. ANTIGENIC VARIATION *IN VITRO*

To understand how antigenic variation is important in host–parasite interactions, a brief review is necessary of the nature of the process *in vitro* and the proteins involved. Surface antigenic variation in *G. lamblia* is defined as the replacement of one member of a family of surface proteins with another. The VSP (variant-specific surface protein) covers the entire parasite, including the flagella, as a coat 18 nm deep (Pimenta *et al.* 1991).

What knowledge is available to date about antigenic variation in *Giardia* is summarized in table 1. Antigenic variation has been found to occur in all isolates where reagents have been available to allow adequate assessment. Only one VSP is normally detected on each trophozoite (Nash *et al.* 1990b) but this assessment was performed with a small number of anti-VSP-specific monoclonal antibodies (mAbs). A larger sampling employing more VSPs is necessary to confirm this finding. As far as can be determined, antigenic variation occurs spontaneously in culture; no specific signal that induces antigen switching has been identified (Nash *et al.* 1988). However, negative selective factors have been identified and include specific anti-VSP antibodies (Nash *et al.* 1988) and intestinal proteases (Nash *et al.* 1991). The rate of change from one VSP to another is variable and is isolate- and VSP-dependent. For the small number of VSPs studied, the rates of change range from about 1:90 to about 1:3000–8000 (Nash *et al.* 1990a). Lastly, monoclonal antibodies to VSPs commonly show complement-independent cytotoxicity (Nash & Aggarwal 1986; Nash 1992).

4. THE NATURE OF THE VARIABLE ANTIGENS

Perhaps the most intriguing feature of antigenic variation is the unique nature of the variable surface proteins (table 2). They are cysteine-rich, usually containing 11–12% cysteine, which commonly appears as CXXC motifs (Adam *et al.* 1988; Nash 1992; Gillin *et al.* 1990; Mowatt *et al.* 1991). They have a conserved carboxy-terminal region which, except for the last 5 amino acids, is hydrophobic and may serve as a transmembrane domain. There is also an amino-terminal signal peptide. Most interestingly, most VSPs have one or more zinc finger domains, which resemble a combination of LIM (condensed from lin-11, Isl-1 and mec-3 proteins) and ring finger types of zinc fingers; these bind Zn as well as other metals (Nash 1992; Nash & Mowatt 1993; Zhang *et al.* 1993). Analysis of

Table 2. Characterization of variant-specific surface proteins

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1. Family of related cysteine-rich proteins
 2. Relative molecular mass ranges from about 50 kDa to over 200 kDa
 3. Conserved carboxy terminus contains a hydrophobic putative membrane-spanning region
 4. Cysteine-rich (11–12% cysteine) commonly as CXXC. No free-SH moieties
 5. Contain Zn finger motifs. Zn and Fe are found in native VSP
 6. The only surface Zn finger proteins known
 7. Lack identifiable associated carbohydrate
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one purified native VSP showed the presence of both Fe and Zn (Luján *et al.* 1995b); no associated carbohydrates were identified (Luján *et al.* 1995b). Despite the high cysteine content, free sulfhydryl groups have not been detected on the surfaces of viable organisms (Aley & Gillin 1993). These are the only proven zinc finger proteins known to be located on the surface of any organism.

It is not known in what way these proteins benefit the parasite or interact with the host. They may simply act to protect the parasite from digestion by intestinal proteases (Nash *et al.* 1991). Cysteine moieties may protect these microaerophilic organisms from oxidative attack or from the effects of nitric oxide. Zinc finger proteins have an increasing number of identified functions. They interact with DNA and thereby control transcription (Berg & Shi 1996), and bind HIV-1 RNA and control HIV-1 RNA packaging (Poon *et al.* 1996). They are important proteins in developmental processes (Galcheva-Gargova *et al.* 1996; Pieler & Bellefroid 1994), are active in signal transduction pathways (Pieler & Bellefroid 1994), and also play a role in the interactions between structural proteins (Ancsin & Kisilevsky 1996). Intriguingly, Fe-containing proteins such as lactoferrin (He & Furmanski 1995), the Zn-containing proteins of HTLV-1 (Lindholm *et al.* 1990) and the tat protein of HIV-1 (Frentrel & Pabo 1998) can traverse the cytoplasm and end up in the nucleus by an undefined pathway. Lactoferrin has been clearly shown to be able to control transcription directly (He & Furmanski 1995). The potential therefore exists for VSPs to affect the function of the host cell in novel and unexpected ways.

5. THE SIGNIFICANCE OF ANTIGENIC VARIATION IN PARASITIC AND NON-PARASITIC MICROORGANISMS

Antigenic variation is commonly, if not universally, mentioned as a mechanism developed to escape the immune responses of the host (Borst & Greaves 1987). This explanation may be a simplification of a much more complicated situation. First, antigenic variation occurs in some free-living protozoa. *Paramecium* (Caron & Meyer 1989) and *Tetrahymena* species (Allen & Gibson 1973) undergo surface antigenic variation in response to environmental changes. Both contain

cysteine-rich surface antigens, but the structure of these surface antigens differs from that of those in *G. lamblia* and also differs among the ciliates themselves. The surface antigens of *Paramecium* species have a higher molecular mass than do the *Giardia* VSPs, contain multiple repeating units, and lack Zn finger motifs (Caron & Meyer 1989). *Tetrahymena thermophila* has multiple CXXC motifs similar to those of *G. lamblia* (Tondravi *et al.* 1990), but does not possess classic histidine-containing Zn fingers. The biological relevance of antigenic variation in these protozoa is unknown; however, because they are not parasitic, escape from the host's immune response cannot be the adaptive purpose of such variation.

Second, evasion of the host's immune response is probably not the only biological function of variant-specific surface proteins in some organisms. These proteins may have other important biological functions, which alter or change the course of infection. One example is the pilus of *Neisseria gonorrhoeae*, which is responsible for adherence to epithelial cells (Heckels 1986). Antigenic variation also occurs in *Plasmodium falciparum*; it appears likely that the varying antigens bear differing affinities for endothelial cell receptors (Baruch *et al.* 1995; Su *et al.* 1995; Smith *et al.* 1995).

6. ANTIGENIC VARIATION OF *G. LAMBLIA* IN HUMAN INFECTIONS

After the description of antigenic variation as a phenomenon *in vitro* (Nash *et al.* 1988; Adam *et al.* 1988), it was important to show whether it occurred in human and animal infections and its importance in the pathophysiology of infection and disease. Antigenic variation has been studied in a number of experimental models, including humans (Nash *et al.* 1990c) and gerbils (Aggarwal & Nash 1988), as well as in neonatal (Gottstein *et al.* 1990) and adult mice (Byrd *et al.* 1994). Interestingly, the patterns of infection have not been characterized by alternating waves of parasites expressing unique VSPs. The results of human experimental infections are perhaps the most informative, although they are somewhat limited.

A number of experimental human infections were made with a clone derived from isolate GS (Nash *et al.* 1985). Immediately after axenization, surface-labelling experiments showed a predominant VSP of molecular mass approximately 57 kDa (Nash & Keister 1985; Aggarwal *et al.* 1989; Nash & Mowatt 1992). Subsequently, mAb G10/4 (Aggarwal *et al.* 1989) was produced to this VSP and the encoding gene was isolated. One clone, GS/H7 (Aggarwal *et al.* 1989), which expressed this VSP, was used for experimental infections, as was the uncloned parental isolate, GS (Nash *et al.* 1985).

Four volunteers were inoculated with 50 000 trophozoites of the clone GS/H7 by intestinal intubation and the changes in VSPs analysed over time by means of the VSP-specific mAb in immunofluorescence reactions (Nash *et al.* 1990) (see figure 2). The original VSP H7 was predominantly expressed at 6 days p.i. in all four patients, but was replaced by day 22 as assessed by direct analysis of the intestinal trophozoites or after

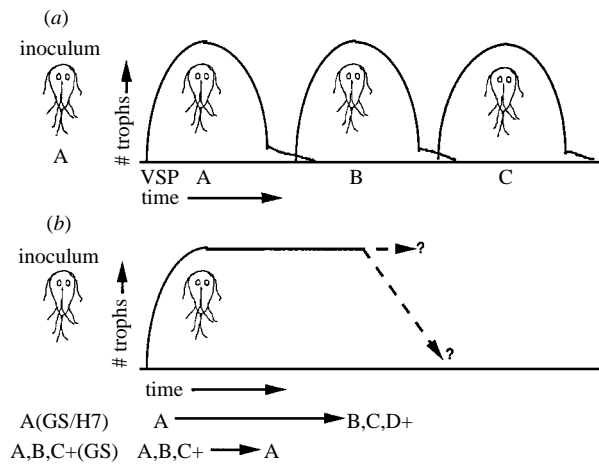


Figure 1. (a) A schematic representation of the predicted pattern of *Giardia lamblia* infections if the infection resembles other organisms that undergo antigenic variation. (b) A schematic drawing of the course of infections in humans inoculated with a single clone (A, VSP H7) or a mixed population of the original isolate containing A (adapted from Nash *et al.* (1990c)). The vertical axis represents the relative number of trophozoites in the small intestine and the horizontal axis represents time. The horizontal lines below the graph show the pattern of VSP expression over time and approximately when antigenic switching occurs (see text).

excystation and axenization from stool samples. Sequential analyses in one patient showed a gradual decrease in VSP H7 trophozoites beginning about 2 weeks p.i. (at the time of the initiation of a humoral response); clones derived from the day 17 isolate showed a mixture of VSPs. Antibody responses were detected to the VSP in the original inoculum by day 14 and to the surfaces of the mixed VSP-expressing population isolated on day 21 and at later dates. Therefore, the initially expressed VSP was replaced not by one VSP, as might have been anticipated, but by many, all present at the same time. Analysis of the population for more than 21 days and quantification of trophozoites in the bowel was not possible.

In three other experiments (Nash *et al.* 1987, 1990c), six volunteers were inoculated with an uncloned GS isolate (therefore a mixed VSP population), which no longer expressed the H7 VSP in significant amounts (0.1–1.2%) (Nash *et al.* 1990c) (figure 1). After 12 weeks, the H7 VSP was found on 40–91% of the isolated trophozoites. Another VSP also present in the inoculum in low proportions (1.0–2.6%) (Nash *et al.* 1990c) remained unchanged. Therefore, after inoculation one VSP increased in numbers whereas another, initially present at about the same frequency, did not.

These studies show that antigenic variation occurs in *Giardia* infections in humans. The VSPs of the original inocula had been replaced by 3 weeks p.i. The H7 VSP appeared to be favoured because, from an initial mixture of VSPs, small populations expressing this VSP increased many times compared with those expressing other VSPs. Humoral responses occurred to the initial VSP and then to the undefined surface VSPs of the 3-week isolate in some patients (Nash *et al.* 1990c).

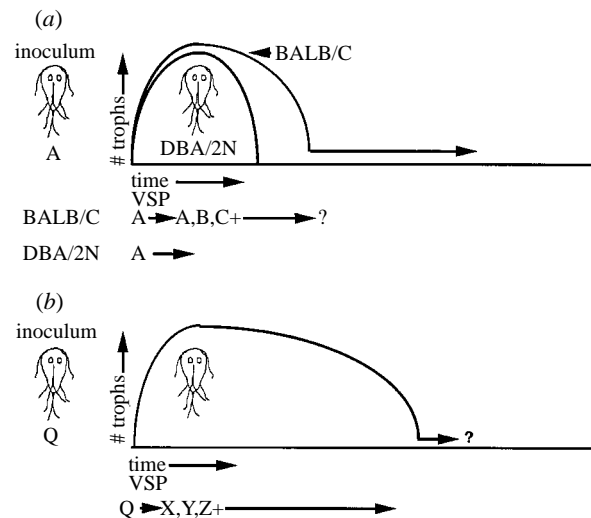


Figure 2. (a) A schematic representation of the course of infections and the VSP expression of *Giardia lamblia* infections in adult BALB/c and DBA/2N mice (adapted from Byrd *et al.* (1994)). (b) A schematic representation of the course of infection in adult gerbils (adapted from Aggarwal *et al.* (1989)). A, H7 VSP; Q, VSP expressed in clone WB/A6; see figure 1 and text for further details.

The loss of the VSP occurred at about the time the humoral responses were first detected, suggesting that humoral responses were important in their elimination.

7. *GIARDIA LAMBLIA* INFECTIONS IN RODENT MODELS

The pattern of infection and timing of antigenic variation in neonatal mice resembles that found in humans (Gottstein *et al.* 1990). However, neonatal mice are immunologically immature and are difficult to manipulate.

Because experimental human and neonatal mouse infections had obvious limitations, an adult mouse model of *Giardia lamblia* was developed (Byrd *et al.* 1994). Earlier studies, based on a single isolate, suggested that only neonatal mice could be infected (Hill *et al.* 1983). Subsequently, it was shown that there was a large degree of biochemical and biological diversity among different *Giardia lamblia* isolates (Nash 1992) so it was of interest to determine whether some of these could successfully infect adult mice. Of the nine isolates tested, only the GS isolate could reliably infect adult mice, so the model was established using the GS/H7 clone. In BALB/c mice, as well as in a number of other mouse strains, the infection peaked at about 1 week and the parasites were usually undetectable by microscopic examination at about 3 weeks p.i. (figure 2). However, if the small intestinal contents were cultured, trophozoites could readily be detected. In contrast, DBA/2-infected mice were self-cured at 2 weeks p.i. (figure 2). When the GS/H7 clone was employed, the same clone used in human infections, the proportion of trophozoites expressing the initial VSP decreased to between 0 and 20%, 2 weeks p.i. It has been impossible to determine the expressed VSPs in the small number of trophozoites surviving after

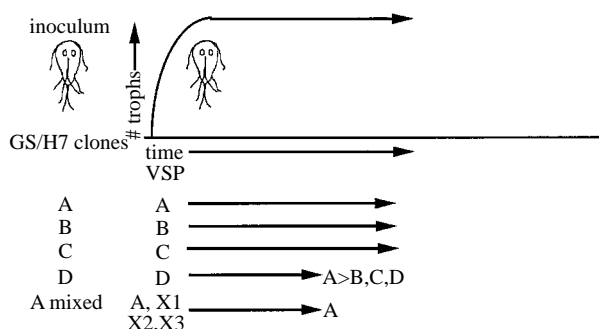


Figure 3. Schematic representation of the course of infections in *Giardia lamblia* infections and VSP expression in adult SCID mice (adapted from Byrd *et al.* (1994) and S. M. Singer and T. E. Nash, unpublished data). A, H7 VSP; see figure 1 and text for further details.

3 weeks. The timing of the loss of the H7 VSP was similar to that found in humans.

Humoral responses are implicated in elimination of VSPs *in vivo*. In neonatal nude mice, VSP switching does occur and animals fail to self-cure. However, in adult (Byrd *et al.* 1994) and neonatal (Gottstein & Nash 1991) SCID mice inoculated with the GS/H7 clone, switching did not occur; this result suggests the operation of B-cell-mediated mechanisms (figure 3), most probably antibodies, in elimination of this VSP. Therefore, humoral mechanisms could be a major selective force in antigenic variation. In addition, when SCID mice were inoculated with a mixed population containing GS/H7, the resulting population expressed VSP H7 almost exclusively *in vivo* (figure 3) (Byrd *et al.* 1994; Gottstein & Nash 1991). In contrast, axenic cultures seeded with the identical inoculum continued to show the same or decreased numbers of VSP H7-expressing trophozoites. These results were similar to the findings of this laboratory in immunocompetent human infections and suggested a selective non-immune-related biological advantage *in vivo* of VSP H7 over other VSPs.

Experimental infections of gerbils also implicated non-immune selection of VSPs (Aggarwal & Nash 1988). In these studies, gerbils were inoculated with various clones of the WB isolate (Smith *et al.* 1982). By 3 days p.i., incipient loss of the original VSP was evident; by 7 days p.i. practically none were detected. It is unlikely that effective immune responses occurred so early during the course of the infection and so non-immune selection factors were probably responsible for the elimination and replacement of the initially expressed VSP.

The SCID mouse model offered a way to eliminate immunological selective pressures to detect non-immune selection (figure 3). Four GS/H7-derived clones and monoclonal antibodies specific to their predominantly expressed VSPs were produced (T. E. Nash and J. T. Conrad, unpublished data). With these reagents, the loss or gain of these expressed VSP could be quantified in SCID mice. In preliminary experiments (S. M. Singer and T. E. Nash, unpublished observations), one of the four VSPs predominantly expressed by those clones was no longer expressed at 2 weeks and the VSP from another clone was expressed

at decreased frequencies. Expression of the two other VSPs remained unchanged. In immunologically competent mice, the clone that had been eliminated in SCID mice showed 20% expression at 1 week. These results most clearly demonstrate that in the absence of immunological selection, in either SCID or naive immunocompetent mice during the first week of infection, some VSPs (derived from the same clone) have a survival advantage over others.

8. CONCLUDING REMARKS

No single selection factor adequately explains the range and variable course of *G. lamblia* infection seen *in vivo* among various hosts. The data best fit a combination of immune and non-immune selection processes. There is no doubt that antigenic variation occurs in *Giardia lamblia* infections, but cyclical waves of increasing and decreasing numbers of parasites of single phenotypes seem not to occur. In permissive host-parasite infections, the VSP initially expressed may survive in the host and continue to be expressed until the immune response of the host eliminates the population expressing that VSP. If the initially expressed VSP is not suited to the host, then another VSP population is selected, some members of which can survive, but these are eliminated when an effective immune response comes into play. Why and how humans and animals can self-cure or develop long-term chronic infections is unclear, but antigenic variation remains a reasonable explanation for persistence. It may be that the host is presented with the entire repertoire of VSPs within a short time and is able to recognize and eliminate all trophozoites. Or on the other hand, isolates may be able to express some VSPs at a lower rate, which would allow expression of some VSPs later during the course of the infection. In this way they would escape earlier immune responses and, if suited to the host, survive. Alternatively, some hosts may not be able to mount a fully successful response, or other non-VSP surface antigens may also be important.

Antigenic variation is a mechanism to create diversity within the constraints of a particular molecular structure or conformation. This diversity is not created solely to circumvent the immune response of the host. The first priority of a parasite is to survive its host.

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