based on a quaternary structure-dependent tilt of the proximal histidine with respect to the heme. Karplus and coworkers (*26*) pointed out the potential significance of this tilt on the basis of theoretical analysis of the x-ray crystallographic studies of Baldwin and Chothea (*28*).

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Expression of Two Variant Surface Glycoproteins on Individual African Trypanosomes During Antigen Switching

Abstract. *Individual* Trypanosoma brucei rhodesiense *organisms were observed in the process of switching variant surface glycoproteins (VSG's). During this switch, trypanosomes simultaneously expressed both pre- and postswitch VSG's uniformly over their surface as detected with monoclonal antibodies. Analysis of this switching event showed that trypanosomes expressing any one of three distinct preswitch VSG's could switch to expression of from one to three different postswitch VSG's. Up to 2.7 percent of the trypanosome population was in the process of switching at one time.*

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African trypanosomes undergo frequent gene switching events that result in major phenotypic changes observable as antigenic variation (*1–4*). This antigenic variation is manifested by expression of a series of immunologically distinct variant surface glycoprotein (VSG) coats on trypanosomes during the course of infection in mammalian hosts. Each of these VSG's is produced through selective expression of one of several hundred VSG genes within the genome of individual trypanosomes (*5*). The precise mechanism whereby one VSG gene is selected for transcription in a trypanosome at a given time is unknown, although genomic DNA rearrangements are apparently involved (*6*).

Studies of differential gene expression in trypanosomes are hampered by a major drawback of the system. It has not been possible to observe actual switching events or to determine precise pre- and postswitch trypanosome phenotypes. We have, however, found a time during trypanosome infection when the switch from expression of one VSG gene to another occurs naturally in most of the parasites. This high switch frequency, together with a relatively high parasitemia in experimental animals at the time of the switch, has permitted us to examine specific events of antigenic variation.

Variant antigen type (VAT) switching occurs at a high frequency during the initial parasitemia in mice inoculated with *Trypanosoma brucei rhodesiense* in the metacycle stage (obtained from the salivary glands of laboratory-reared tsetse flies) (Fig. 1). Trypanosomes in the early blood stage initially continue to express all metacyclic-variant antigen

Table 1. Percentage of MVAT-expressing trypanosomes that also expressed the indicated BVAT's in a BALB/cJ mouse inoculated with metacyclic trypanosomes from tsetse flies infected with WRATat 1.10. Direct immunofluorescence reactions on live trypanosomes were carried out as described in the legend of Fig. 2 for trypanosomes collected from one mouse on days 4 through 7 of infection. Similar results were obtained in other mice inoculated with metacyclic trypanosomes derived from WRATat 1.1 or 1.14 (as described in the legend of Fig. 1), although the highest percentage of double-labeled trypanosomes occurred in mice inoculated with metacyclic trypanosomes derived from WRATat 1.10 or 1.14. No double-labeled trypanosomes were observed before day 4 or after day 6 of infection in any of the mice.

MVAT number*	Percentage of MVAT's†	BVAT specificity (percent)‡		
		WRATat 1.1	WRATat 1.14	WRATat 1.10
		Day 4 of infection		
4	10	0	0	0
6	49	0.15	0.23	0
7	30	0.4	0.7	1.2
		Day 5 of infection		
4	5	0	3.7	0
6	33	1.5	1.7	0
7	16	3.9	5.0	0
		Day 6 of infection		
4	1	0	0	0
6	1	0	0	0
7	4	1.5	10.5	3.8

*All VAT's are mutually exclusive (*7*, *15*, *16*, *18*), and each is defined by a monoclonal antibody: MVAT's 4, 6, and 7 by antibodies 3.2C5.2, 3.2C2.2, and 3.103.1, respectively; and BVAT's 1.1, 1.14, and 1.10 by antibodies 12.4F3.1, 21-14-146D, and 59-10-92J, respectively. †Values indicate the percentage of trypanosomes expressing each MVAT at the time of assay for dual expression. ‡Values are percentages of trypanosomes reactive with monoclonal antibodies specific for the indicated MVAT's that also reacted with a monoclonal antibody specific for a BVAT. Percentages were calculated on the basis of single counts of 200 to 2000 trypanosomes. Values of zero indicate that the particular switch from MVAT to BVAT was not observed in samples of at least 2000 trypanosomes.

types (MVAT's) (*7*). However, the fraction of parasites expressing MVAT's decreased from 93 percent on day 4 to 15 percent on day 6 after mice were infected. This switch from expression of MVAT's to blood-stage VAT's (BVAT's) apparently occurs without specific immune clearance of MVAT's (*4*). The switch from MVAT's to BVAT's was also observed during trypanosome infection in mice rendered incapable of producing antibody by irradiation. This observation suggests that trypanosomes expressing MVAT's are not selectively eliminated and subsequently replaced by trypanosomes expressing BVAT's. Rather, individual trypanosomes appear to replace MVAT VSG's with BVAT VSG's.

To detect antigen switching events, we used monoclonal antibodies specific for preswitch MVAT's and postswitch BVAT's in immunofluorescence analyses of blood-stage trypanosomes. Monoclonal antibodies specific for MVAT surface epitopes were conjugated with rhodamine, and those specific for BVAT surface epitopes were conjugated with fluorescein. Because these two fluorochromes emit light in the red and green range, respectively, pairs of rhodamine- and fluorescein-conjugated antibodies were used with the appropriate fluorescence filter system to detect the expression of both MVAT (preswitch) and BVAT (postswitch) VSG's on individual trypanosomes.

On day 5 after mice were infected with metacyclic trypanosomes, some blood-stage trypanosomes were reactive with both an antibody to MVAT and an antibody to BVAT, indicating the presence of two different VSG's (Fig. 2). This dual expression was manifested on the trypanosome surface because assays were carried out with live trypanosomes. Further evidence for the surface VSG specificity of the monoclonal antibody markers is that these antibodies neutralize infectivity of homologous VAT trypanosomes (*7*). Only antibodies reactive with surface epitopes of VSG molecules have been shown to neutralize trypanosome infectivity (*8*).

Dual expression of VSG's is apparently related to antigenic switching by trypanosomes from MVAT's to BVAT's because dual expression was only detected on days 4 through 6 of infection, a period during which MVAT's were being replaced by BVAT's. Dual expression of VSG was not observed with metacyclic-stage trypanosomes (from tsetse flies) used to initiate infections in mice or with blood-stage trypanosomes after day 6 of infection.

Table 2. Percentage of blood-stage trypanosomes double-labeled during the first parasitemic wave in mice infected with metacyclic trypanosomes. Each of three BALB/cJ mice was infected by intraperitoneal inoculation with 5×10^3 metacyclic trypanosomes obtained from tsetse flies infected with one of the WRATat's indicated. Each value was calculated by summing the percentage of all blood-stage trypanosomes double-labeled for all nine combinations of antibodies to MVAT's and to BVAT's on the days indicated. Separate determinations were made for each mouse. Monoclonal antibodies used were as described in Table 1.

Source of metacyclic trypanosomes	Percentage of double-labeled trypanosomes on day of infection		
	4	5	6
WRATat 1.1	N.D.*	1.3	0.36
WRATat 1.10	1.0	2.7	0.63
WRATat 1.14†	0.6	1.6	0.34

*Not determined because of low parasitemia. †WRATat 1.14 is a clone from the first parasitemia in a C57 BL/6J mouse infected with metacyclic trypanosomes from tsetse flies infected by feeding on WRATat 1.1 organisms.

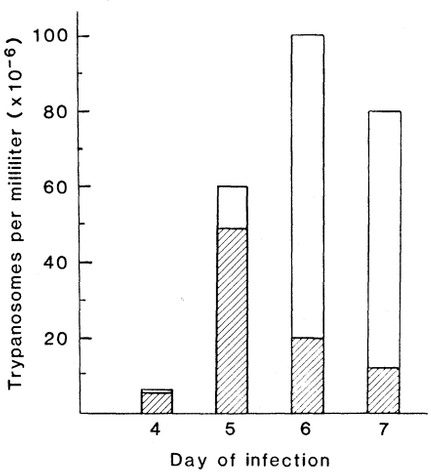

Fig. 1. Time course of parasitemia and MVAT expression in a BALB/cJ mouse inoculated with 5×10^3 metacyclic trypanosomes derived from WRATat 1.10. Parasitemia is indicated by bars, and the fraction of trypanosomes that expressed MVAT's is indicated by shaded areas. The fraction expressing MVAT's was determined by an immunofluorescence assay of trypanosomes in acetone-fixed blood smears (*7*). A pool of 16 monoclonal antibodies that identify all WRATat 1.1–derived MVAT's was used as the trypanosome-specific antibody. Trypanosomes were all derived from a human isolate of *T. b. rhodesiense* (LVH/75/USAMRU-K/18) (*15*). WRATat 1.10 is a clone from the second parasitemic wave in a C57 BL/6J mouse inoculated with WRATat 1.1; WRATat 1.1 is a trypanosome clone obtained directly from the human isolate. These two WRATat clones (in addition to WRATat 1.14 in Table 2) are distinct VAT's that do not cross-react serologically (*15, 16*). The results are typical for BALB/cJ and C57 BL/6J mice inoculated with from 10^3 to 2×10^4 metacyclic trypanosomes derived from WRATat 1.1, 1.10, or 1.14. Similar results were obtained in C57 BL/6J mice immunosuppressed by irradiation immediately before inoculation.

The pattern and intensity of fluorescence on double-labeled trypanosomes suggests that antigen switching involves dilution of old VSG with new VSG. Double-labeled trypanosomes showed less intense fluorescence with each label than single-labeled trypanosomes, although individual trypanosomes were always uniformly stained over the entire cell surface. The intensity of reactivity varied, with an apparent reciprocal relation between the MVAT (preswitch) and BVAT (postswitch) specificities. Some trypanosomes reacted strongly with the rhodamine label (antibody to MVAT) and weakly with the fluorescein label (antibody to BVAT), whereas other trypanosomes showed the reverse reactivity. This observation suggests that each of the two antigens on the double-labeled trypanosome is present in a lower concentration than on single-labeled trypanosomes. The fluorescence pattern of double-labeled trypanosomes further suggests that new VSG is rapidly and uniformly distributed over the entire cell surface, diluting old VSG in a manner consistent with the fluid membrane model of higher eukaryotic cells (*9*).

The time during the cell cycle when VSG switching occurs is not clear. This process of VSG dilution could occur over several cell divisions, with new VSG added to the trypanosome surface as new membrane is formed. Alternatively, new VSG could be expressed without cell division if there is normally a constant turnover of VSG (*10*). Also, it is not clear whether antigenic switching is solely achieved by dilution of old VSG with new VSG. At some point during this dilution process there may be a mechanism for specific elimination of residual old VSG, perhaps by enzymatic cleavage (*11*) or capping (*12*), as has been suggested. Some mechanism for elimination of old VSG would seem important for trypanosome survival, so that both a stimulus and a target for host immunity could be removed.

Because precise analysis of the order in which different VAT's occur will aid in understanding the genetic events responsible for antigenic variation, we examined individual switching events of trypanosomes (at the phenotypic level). The results of double-label studies indicated that one MVAT can switch to at least three different BVAT's. On day 6 after mice were infected with metacyclic trypanosomes derived from clone WRATat 1.10, MVAT 7 trypanosomes were switching to WRATat 1.1, 1.14, and 1.10 BVAT's (Table 1). This indicates that trypanosomes expressing a single MVAT are capable of directly

generating a heterogeneous blood-stage population, a capability that would increase the chance of parasite survival in immunocompetent hosts. The degree of BVAT heterogeneity that each MVAT could produce is probably extensive. In this study, monoclonal antibody markers for only three BVAT's were used, and these BVAT's made up 16 percent at most of the trypanosome population at one time. Antigen switching of MVAT's to other BVAT's was probably also occurring. Additionally, in other studies we found that trypanosomes of one MVAT could also switch to other MVAT's.

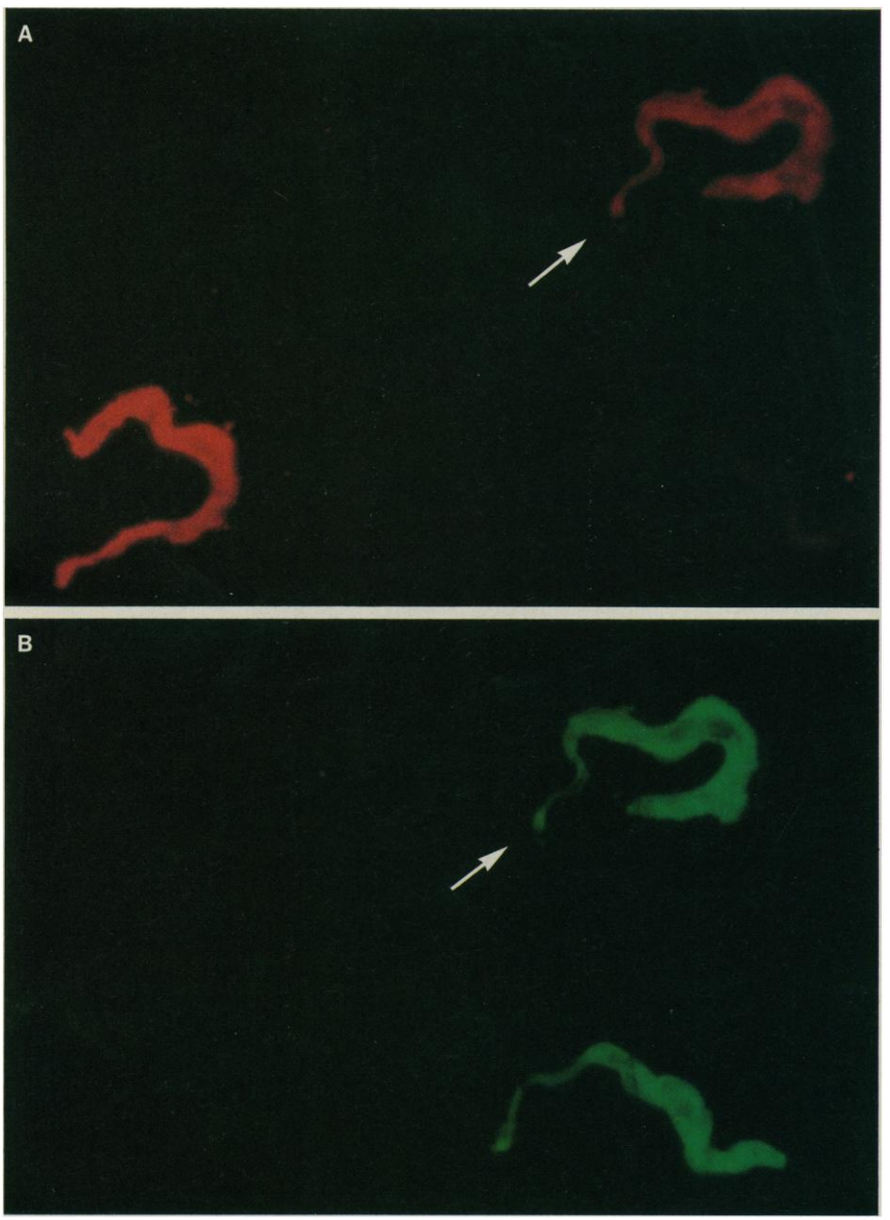

Fig. 2. Reaction of individual trypanosomes with two monoclonal antibodies, one MVAT-specific (rhodamine-conjugated) and one BVAT-specific (fluorescein-conjugated). The panels are photographs of the same microscopic field taken through two different filter systems, one that detects rhodamine (A) and one that detects fluorescein (B). The trypanosome that appears only in (A) is an MVAT and fluoresces red (with MVAT-specific antibody 3.2C2.3); the trypanosome that appears only in (B) is a BVAT and fluoresces green (with BVAT-specific antibody 12.4F3.1). One trypanosome (marked by an arrow) in the process of switching from MVAT to BVAT is labeled with both antibodies and appears in both panels. Trypanosomes were isolated from blood collected from the tail vein of a mouse infected with metacyclic trypanosomes from WRATat 1.10–infected tsetse flies. Live trypanosomes obtained on day 5 of infection were diluted in phosphate-buffered saline (*p*H 7.2) containing 10 percent fetal bovine serum. Pairs of conjugated antibodies, one MVAT-specific and one BVAT-specific, were mixed with trypanosomes (on ice). After 5 minutes of incubation, wet mounts of this parasite-antibody mixture were examined for red or green fluorescence without prior washing. Dilutions of fluorochrome-conjugated monoclonal antibodies were chosen that gave sufficiently low background fluorescence to allow detection of specific surface fluorescence on trypanosomes. Trypanosomes were still motile at this stage. All assays were performed with live organisms; however, acetone-fixed trypanosomes were used to obtain these micrographs. Adequate exposure of the film was achieved through use of *n*-propyl galate specimen mounting medium to reduce photobleaching of fluorochromes (*17*).

In addition to the ability of each MVAT to switch to more than one BVAT, each BVAT could arise from more than one MVAT (Table 1). For example, WRATat 1.14 trypanosomes were derived from MVAT's 4, 6, and 7. Together with the observation that each MVAT can switch to at least three BVAT's, this indicates that there is no strict linear sequence of VAT expression when individual switching events are analyzed.

Estimates of the percentage of trypanosomes undergoing antigen switching were made by determining the percentage of the entire trypanosome population that was double-labeled with all nine monoclonal antibody combinations used on each day of infection (Table 2). Antigen switching was measured on days 4 through 6 in mice inoculated with metacyclic trypanosomes from tsetse flies infected with one of three different BVAT's. In all mice, maximum switching was observed on day 5, a time during infection when a major shift from MVAT's to BVAT's was occurring (Fig. 1). As many as 1 in 37 trypanosomes (2.7 percent) were in the process of switching on day 5 in mice infected with WRATat 1.10–derived metacyclic trypanosomes. However, the actual rate of switching from MVAT to BVAT may have been higher than that observed because the antibodies used identified only 3 of a total of 16 MVAT's and 3 of an unknown number of BVAT's. Alternatively, the rate of switching could have been lower than the apparent rate if VSG messenger RNA turnover was occurring slowly. The latter possibility seems unlikely because this turnover is thought to occur within hours (*13*).

A high switching frequency at a time of high parasitemia may provide an ideal stage in the trypanosome life cycle for studying genetic events that result in antigen switching. Analysis of the genomic context of MVAT and BVAT VSG genes during changes in their expression should provide insight into transcription regulatory mechanisms. This analysis will be possible through the use of complementary DNA probes such as those recently cloned for two MVAT VSG's (*14*). A complete understanding of the process whereby trypanosomes switch surface proteins may lead to new approaches for control of these parasites.

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Insect Resistance to the Biological Insecticide *Bacillus thuringiensis*

Abstract. *Resistance to the spore–crystal protein complex of* Bacillus thuringiensis, *the most widely used and intensively studied microbial insecticide, has been presumed to be unlikely to occur. In this study it was found that* Plodia interpunctella, *a major lepidopteran pest of stored grain products, can develop resistance to the insecticide within a few generations. Resistance increased nearly 30-fold in two generations in a strain reared on diet treated with* Bacillus thuringiensis *and after 15 generations reached a plateau 100 times higher than the control level. Resistance was stable when selection was discontinued. The resistance was inherited as a recessive trait.* Plodia interpunctella *strains collected from treated grain bins were more resistant than strains from untreated bins, indicating that the resistance can develop quickly in the field.*

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There have been few reports of insect resistance to microbial insecticides, leading to the presumption that insects are unlikely to become resistant to these agents (*1–3*). A few investigators have selected laboratory strains of Lepidoptera resistant to insect viruses (*1, 2*) and of Diptera resistant to the β-exotoxin of *Bacillus thuringiensis* (*1, 2, 4*). However, attempts to select insects resistant to commercial formulations containing the spores and δ-endotoxin complex of *B. thuringiensis* (BT), the most widely used and intensively studied microbial insecticide, have been unsuccessful (*1–3*). I now report that *Plodia interpunctella*, a lepidopteran pest of stored grain and grain products, can develop resistance to a commercial BT formulation within a few generations and that such resistance has been detected in bins of BT-treated grain.

As part of a large-scale study of the effectiveness of BT in controlling *P. interpunctella* in stored grain, insects were collected from native populations in treated and untreated grain storage facilities over a five-state area. Laboratory colonies were established from each population, and the susceptibilities of the colonies (strains) to a commercial formulation of BT were determined (*5*). The median lethal concentration (LC_{50}) of BT for 77 colonies ranged from 6.8 to 60.2 mg per kilogram of diet, with a mean of 22.5. The dose-mortality relations were essentially parallel to an average slope of 1.568 (range, 1.030 to 2.132). This range in LC_{50}'s is similar to that reported earlier for a smaller number of laboratory colonies (*6*). However, in my study the colonies established from populations in BT-treated bins were significantly less susceptible than colonies from populations in untreated bins [mean (± standard error) LC_{50}'s for treated and untreated bins, 25.1 ± 2.0 (n = 31) and 20.7 ± 1.7 (n = 46) mg/kg, respectively (P = 0.009, analysis of variance) (Fig. 1) (*7*). Slopes of the dose-mortality relations for the two groups of colonies did not differ significantly. Although the difference in mean LC_{50}'s is small, it does indicate a selective effect of BT on the insect populations. Large differences would not be expected because the insects were collected within a short time (1 to 5 months) after the grain was treated.

To determine whether a resistant colony could be selected in the laboratory, I selected a colony from a population that was not being effectively controlled by BT. Beginning with the 20th laboratory generation, the colony was subcultured on larval diet treated with BT at 62.5 mg/kg, a dose expected to produce 70 to 90 percent larval mortality (*8*). Survival in the first generation was 19 percent. In the second generation survival increased to 44 percent, in the third to 63 percent, and in the fourth to 82 percent. Thereafter, survival fluctuated between 68 and 89 percent, comparable to the unselected colony's survival rate of 71 to 89 percent on untreated diet. There was a corresponding rapid increase in the LC_{50} for the colony (Fig. 2A). After two generations of selection the LC_{50} increased to 27 times the average level for the unselected colony and continued to increase more gradually to 97 times after 15 generations. There was no consistent shift in the slope of the dose-mortality relations between the resistant colony and the unselected colony.

After nine generations of selection at a BT dose of 62.5 mg/kg, selection pressure was increased by subculturing the resistant colony on a diet treated with BT at 500 mg/kg. In the first generation survival was 32 percent, in the second

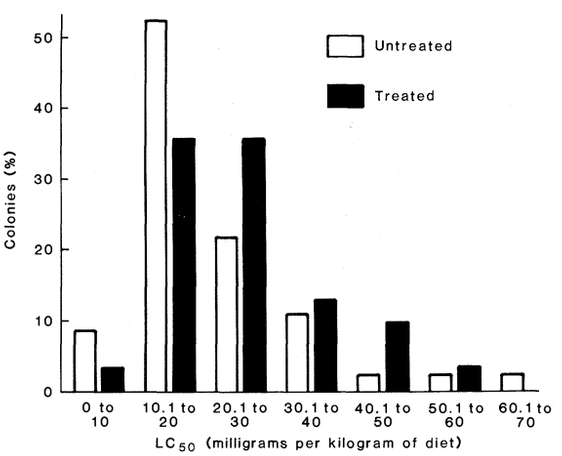

Fig. 1. Frequency distribution of LC_{50} values for *P. interpunctella* colonies collected from untreated and BT-treated bins of grain.