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Antigenic Variation in Clones of *Trypanosoma brucei* Grown in Immune-Deficient Mice

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We have produced monoclonal antibodies against six variant surface glycoproteins from early variant antigen types (VATs) of the IsTaR 1 serodeme of *Trypanosoma brucei brucei*. We have used these in fixed cell immunofluoresence assays to follow the VAT composition of populations of each early VAT when passaged through irradiated mice. The IsTat 1.A and 1.7a populations were stable for more than 30 days (~150 generations), but 1.1a, 1.3a, 1.5a, and 1.11a all changed to 1.A within this time. The time and rate of this antigenic switch were characteristic for each VAT. Growth rates of the VATs were determined when they were both grown separately and grown with 1.A. It appeared that the order of growth rates was 1.7a > 1.A = 1.1a > 1.11a > 1.5a > 1.3a. We have generated theoretical curves for the replacement of one VAT by another based on differences in their growth rates and the rate at which one VAT switches to another (switch frequency). These curves closely match those derived experimentally. We postulate that the differences in growth rates between VATs and the different switch frequencies for VATs may be sufficient to generate the loosely defined sequence of VATs seen in chronic infections.

African trypanosomes have long been known to undergo antigenic variation whereby they are able to change the variant surface glycoprotein (VSG) in the surface coat and thus evade the immune system of their host (3). Although intensive research effort is being applied to the elucidation of the molecular mechanisms underlying the switch from expression of one VSG gene to another (1, 12), confusion still remains as to the nature of antigenic variation at the level of the infecting trypanosome population. It has been shown that in chronic infections with *Trypanosoma brucei*, different variant antigen types (VATs) appeared in a loosely defined sequence, with some VATs always appearing early and others late (2, 9, 20). Kosinski (6) has concluded that random generation of different VATs and their subsequent selection by differing growth rates is not sufficient to explain the orderliness observed in chronic infections, although Seed (13) and Miller and Turner (9) have suggested that competition between coexisting VAT populations may cause their growth rates to differ from those measured in VATs growing alone.

In this study, we have confirmed the observations of Doyle et al. (5) that antigenic variation can occur in the absence of the host immune system by showing antigenic variation when IsTaR 1 early VATs are passaged for extended periods of time through irradiated (immune-deficient) mice. We have used these results to propose a model to explain the sequence of VATs seen in chronic infections.

MATERIALS AND METHODS

Trypanosomes. The derivation of the IsTaR 1 serodeme of *T. brucei brucei* from the cryo-preserved stock EATRO 164 has been described in detail elsewhere (17). The VATs used in this study were cloned from populations present in rat (IsTat 1.A) and early in a chronic infection of a deermouse (*Peromyscus leucopus*) (IsTats 1.1a, 1.3a, 1.5a, 1.7a, and 1.11a). Characterization of the VSGs purified from each of the VATs by the method of Strickler et al. (16) has been presented by Milhausen et al. (8).

Animals. Random bred mice of the BALB/c and C57/Bl strains were used. At 18 to 24 h before injection with trypanosomes, they received 900 rad of total body irradiation from a ^{60}Co source.

Growth of trypanosomes. Stocks of cloned trypanosomes from each VAT were stored in liquid nitrogen. Before each experiment, a vial of each VAT to be used was thawed and the cells were inoculated intraperitoneally (i.p.) into an irradiated mouse. After 2 to 3 days, when the parasitemia had reached 5×10^8 to 5×10^9 per ml, trypanosomes were harvested, suspended in 0.06 M sodium phosphate (pH 8.0)–0.0255% NaCl–0.5% glucose (PSG). At this stage, cell counts were made and smears were made for checking of the homogeneity of the population by immunofluorescence assay (IFA). Fresh irradiated mice were then inoculated i.p. with 10^7 cells of each VAT (for VAT stability experiments and growth rate determination experiments) or 5×10^6 trypanosomes of each of two different VATs (for competition experiments) to commence the experiments. The parasitemia in each animal was then monitored from tail blood, and smears were made for IFA when necessary. When the parasitemia reached 5×10^8 to 1×10^9 trypanosomes per ml, 10^7 trypanosomes (from tail blood) were transferred to a new irradiated mouse. These passages were continued at 2- to 3-day intervals for the length of the experiment.

Enzyme immunoassay (EIA). Microtiter plate wells were coated with the purified VSG by incubation with 0.4 μg of purified VSG in 40 μl of 0.02M phosphate-buffered saline (PBS) per well overnight at 37°C. Alternatively, microtiter plate wells were coated with water-lysed bloodstream or procyclic stage (from in vitro culture) trypanosomes, using the equivalent of 4×10^6 cells per well, or were coated with 0.4 μg of concanavalin A in a similar fashion. The coating solution was then discarded, and the nonspecific protein binding sites were blocked by the addition to each well of 60 μl of 5% bovine serum albumin (BSA) in PBS and incubation

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TABLE 1. Reactivity of VSG-specific McAbs

VSG[a]	No.	Class	Reaction with the following VAT[b]: 1.A	1.D	1.1	1.3	1.5	1.5	1.11	No. reacting as measured by: Live-cell IFA	Western blots[c]	Immunoprecipitation of in vitro-translated VSG
A	4	G	**+++** +	– –	– –	++ –	– –	– –	– –	0/4	4/4	3/3
1.1	8	G	– –	**+++** **++**	**+++** **++**	– –	– –	– –	– –	0/7	7/7	2/2
1.3	1	G	**+++** +	– –	– –	++ –	– –	– –	– –	0/1	1/1	
1.3	14	G	– –	– –	– –	**+++** **+/–**	– –	– –	– –	10/14	1/14	2/6
1.5	16	G	– –	– –	– –	– –	**+++** +	– –	– –	1/16	16/16	7/7
1.7	8	G	– –	– –	– –	– –	– –	**+++** **+/–**	– –	1/8	1/8	
1.11	4	G	– –	– –	– –	– –	– –	– –	**+++** +	4/4	0/4	1/2
1.11	1	G	– –	**+** –	**++** –	– –	– –	– –	**+++** **++**	1/1	0/1	

[a] VSGs were used to immunize mice before fusion.

[b] Boxes around symbols indicate reaction in IFA with acetone-fixed cells. In each pair, the upper symbol indicates reaction in EIA with purified VSG; the lower symbol indicates reaction in EIA with whole-cell lysates. Symbols: +++, titer $>10^5$; ++, titer = 10^3 to 10^5; +, titer = 1 to 100; +/–, titer = 1; –, no reaction at any dilution.

[c] Reactivity in immunoblot analysis with sodium dodecyl sulfate–2-mercaptoethanol-denatured VSG.

for 90 min at 37°C. The BSA was discarded, 40 μl of culture supernatant or ascites fluid diluted in PBS–1% BSA was added, and incubation continued for a further 45 to 60 min. The plates were washed twice with 50 to 100 μl of PBS–1% BSA; then 40 μl of a 1/500 dilution of horseradish peroxidase-conjugated goat antimouse immunoglobulin G (IgG)+IgM +IgA antiserum (HRP-GAM) (Cappel Laboratories) was added and incubated for 60 min as before. The plates were washed three times before 40 μl of the colorimetric reagent (0.1 mg of *o*-phenylenediamine per ml–0.003% H_2O_2 in PBS) was added and the plates were incubated for 15 to 20 min in the dark. The reaction was stopped by the addition of 25 μl of 8 N H_2SO_4 per well, and the plates were photographed.

Production and characterization of anti-VSG monoclonal antibodies. BALB/c mice were injected i.p. with 60 μg of purified VSG in Freund complete adjuvant. After 4 weeks, and again 3 days before fusion, the mice were injected i.p. with 15 μg of VSG in 2 mM Tris (pH 7.5)–0.5 mM EDTA. Spleen cells from the immunized mice were fused with NS-1 myeloma cells by the polyethylene glycol centrifugation method described by Nowinski et al. (11). Fused cells were plated in 96-well plates with selective medium and a thymocyte feeder layer. The culture supernatants of hybridomas were screened for anti-VSG antibody after 9 to 10 days by means of the EIA described above. The antigen fixed to the plate was 0.4 μg of purified VSG. Cells secreting antibody that reacted with the immunizing VSG by EIA were cloned by limiting dilution, expanded, and injected i.p. into pristane-primed BALB/c mice for production of ascites fluid. The pooled ascites fluids from each clone were then analyzed by EIA, using purified VSGs and lysates of whole trypanosomes as antigen.

The ascites fluids were also characterized by means of IFA with both live and fixed trypanosomes (described below), immunoblot analyses ("Western" blots) as described by Towbin et al. (19) and Smit et al. (15), and immunoprecipitation of in vitro-synthesized VSG (8).

The original screening of culture supernatants from the eight successful fusions by EIA, using the immunizing VSG as antigen, identified 554 positive wells. Those 82 cell lines which gave the strongest reactions were cloned and expanded into mice to obtain ascites fluid. Of the ascites fluids examined, 55 were specific for single VSGs and 1 cross-reacted with other VSGs (Table 1). Each of these 56 monoclonal antibodies (McAbs) reacted strongly by IFA on acetone-fixed cells, and their specificity was consistent with that determined by EIA with purified VSG. Furthermore, VAT-specific reactions were observed in an EIA when trypanosome cell lysates (at least 99% pure for each VAT) were used as the antigen fixed to the plate (Table 1). When lysates of procyclic stages (which lack VSG) were used in the EIA, no reaction was observed with the VAT-specific McAbs, showing that the antigen was restricted to the bloodstream stage of the life cycle (data not shown).

Several VSG-specific McAbs were obtained against each of the six VATs used, and all were of the IgG class (Table 1). Most ascites fluids had titers in excess of 10^6 in EIAs involving purified VSG. Lower titers were obtained when cell lysates were used as antigens. This probably reflects a decrease in the amount of specific antigen (VSG) bound to the wells due to competition with numerous other antigens for the binding sites on the plate.

Of the 56 VAT-specific McAbs, 17 reacted with live cells. These detected epitopes exposed on the surface of live 1.3a, 1.5a, 1.7a, and 1.11a trypanosomes. In all cases, the fluorescence observed was weak, even at high concentrations of McAb. None of the McAbs against 1.A or 1.1a VSGs detected such epitopes.

From the 56 VAT-specific McAbs, single McAbs specific for each of the six VATs were chosen and used in the fixed-cell IFA to identify each VAT in the subsequent experiments of the study. These McAbs were 1H3-8(1.A), 3A5-3(1.1a), 6F4-8(1.3a), 2G10-1(1.5a), 5D2-13(1.7a), and 2C10-11(1.11a).

Fixed-cell IFA. Blood containing ca. 10^7 to 10^9 trypano-

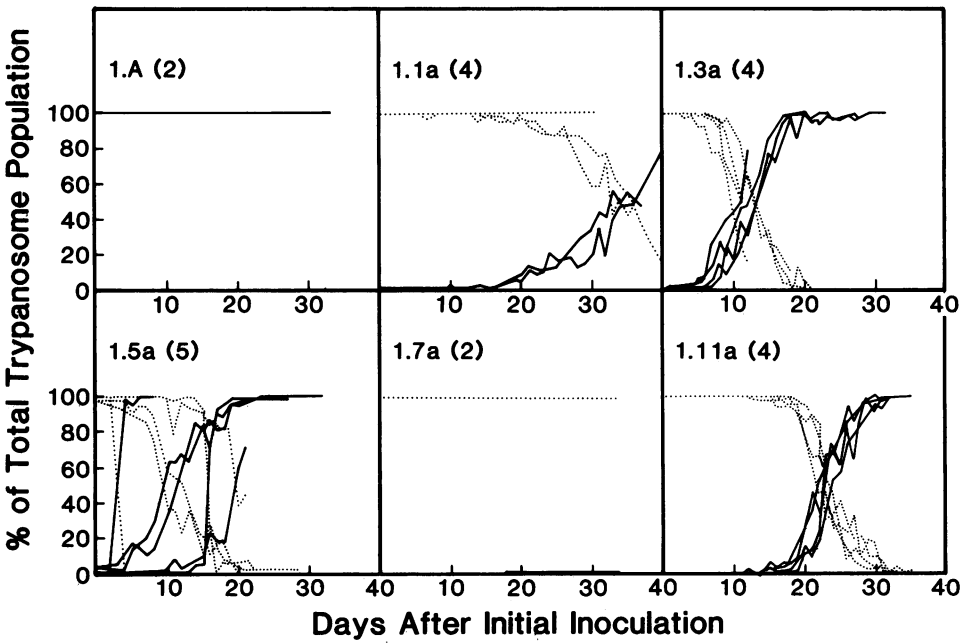

FIG. 1. Stability of the IsTaR 1 early VATs during prolonged passed through irradiated mice. Solid lines indicate the percentage of the population which is 1.A. Dotted lines indicate the percentage of the population still expressing the initial VAT. Numbers in parentheses indicate the series of mice used for each VAT.

somes per ml was smeared on glass slides, air dried, and fixed in cold acetone for 5 min. Aliquots (10 μl) of 1/100 dilutions of the McAb ascites fluids in PBS–1% BSA were added to 1-cm^2 sections of the slides and incubated in a humid chamber at 37°C for 30 to 60 min. The slides were washed gently with PBS, and a 10-μl aliquot of a 1/40 dilution of fluorescein isothiocyanate (FITC)-conjugated goat antimouse serum (FITC-GAM) (Cappel Laboratories) was added to each section. The incubation and washing was repeated, and the slides were mounted in 50% glycerol (pH 7.6) and scored by fluorescence microscopy. Generally, between 50 and 300 cells were counted for each McAb.

Live-cell IFA. Live-cell IFAs were performed by mixing, in a 96-well microtiter tray, 3×10^6 live trypanosomes (in 15 μl of PSG) with 20 μl of the ascites fluids diluted 10 or 100 times in PSG. The cells were incubated on ice for 20 min and then washed twice with 150 μl of cold PSG. An aliquot (20 μl) of FITC-GAM(IgG) (1/40 dilution in PSG) was added to each well, and the cells were incubated and washed as before. After the final wash, the cells were suspended in ~10 μl of PSG containing 50% glycerol and 1% formaldehyde and then examined for fluorescence.

Immunoblot analysis: Western blots. VSG preparations were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli and Favre (7). Samples containing 1 to 10 μg of purified VSG were loaded onto 7.5 to 15% polyacrylamide gradient gels. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose sheets by the method of Towbin et al. (19). The sheets were then incubated in 5% BSA for 1 h at 37°C to block remaining protein binding sites. After being washed twice in PBS, the sheets were cut into 0.5- to 1.0-cm strips which were then incubated for 2 h at 37°C in a 1/100 dilution of ascites fluid. The strips were then washed twice in PBS and incubated for 1 to 2 h at 37°C in a 1/1,000 dilution of HRP-GAM (specific for either IgG or IgM). After another two washes in PBS, the regions of antibody binding on each strip were visualized by incubation in 0.5 mg of 4-chloro-1-napthol per ml–0.25% H_2O_2 in 16.7% methanol until a purple color developed (5 to 10 min) (15). The reaction was then terminated by several washes in PBS.

Immunoprecipitation of in vitro-synthesized products. RNA from homogeneous VAT populations were isolated and translated in vitro as described elsewhere (8). The products, radiolabeled with [^{35}S]methionine, were incubated with 5 μl of rabbit antisera or 0.1 μl of ascites fluid. The antigen-antibody complexes were collected directly with Formalin-fixed *Staphylococcus aureus* or with *S. aureus* precoated with affinity-purified goat antimouse immunoglobulin (gift of J. Ledbetter, Genetic Systems Corp.). After thorough washing as previously described (8), samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

Analysis of results and generation of theoretical curves. Exponential curve fitting analysis of the results from the growth rate determination experiments was performed with a Hewlett-Packard HP-25 calculator. Only parasitemias between 5×10^6 and 5×10^8 trypanosomes per ml were included in the analyses. The same calculator was used to generate theoretical curves for VAT switching and competitive growth. The formula used was:

$$\%An = 100([X_{n-1} \cdot f] + [A_{n-1} \cdot 2^{\frac{gX}{gA}}])/([2X_{n-1}] + [X_{n-1} \cdot f] + [A_{n-1} \cdot 2^{\frac{gX}{gA}}])$$

where $\%An$ is the percentage of population which is variant A after n generations; A_{n-1} or X_{n-1} is the total number of cells of variant A or X after $n - 1$ generations; gA or gX is the generation (doubling) time of variant A or X; and f is the switch frequency, i.e., the proportion of X cells that switch to A cells per generation.

Thus, the percentage of cells was calculated after each generation of X cells. The program was initiated with 10^7 cells of variant X alone for VAT switching theoretical

TABLE 2. Comparison of generation times of IsTaR 1 VATs

VAT	Mean generation time[a] (h) (SD)	Statistical difference with[b]:					
		1.A	1.1a	1.3a	1.5a	1.7a	1.11a
1.A	5.2 (0.4)	—					
1.1a	4.9 (0.5)	—	—				
1.3a	5.5 (0.5)	—	—	—			
1.5a	5.0 (0.2)	—	—	—	—		
1.7a	4.7 (0.3)	—	—	$P < 0.1$	—	—	
1.11a	5.4 (0.4)	—	—	—	—	$P < 0.1$	—

[a] Generation times were obtained from regression analyses of parasitemias in three mice.

[b] Statistical comparisons were made by using Student's *t*-test.

curves, and 5×10^6 cells of each variant for competitive growth theoretical curves.

RESULTS

Stability of each VAT in irradiated mice. To determine whether each of the IsTaR 1 early VATs would undergo antigenic change without immune selection, we passaged each VAT through irradiated (immune-deficient) mice for 30 to 40 days (ca. 15 passages). Parasite counts and smears of tail blood from the mice were made every day, and the trypanosome populations were examined by IFA with McAbs for determination of their VAT compositions (Fig. 1). The behavior of the different VATs can be roughly divided into three categories. IsTat 1.A remained unchanged for the 33 days of the experiment, with 1.A cells accounting for 100% of the trypanosomes in every smear examined. IsTat 1.7a behaved similarly, except that for the final 10 to 15 days a very small percentage of the cells (<1.0%) in the population were 1.A. In the second category, IsTat 1.1a initially remained unchanged for 30 days, with only 1 to 2% of the total population becoming 1.A in the final 10 to 15 days. However, when the experiment was repeated in another two series of mice and extended for a longer period of time, it was found that the population gradually changed from 1.1a to 1.A, with more than 90% of the cells being 1.A by 50 days in one case. Finally, the three remaining VATs, 1.3a, 1.5a, and 1.11a, also underwent antigenic change to 1.A during the course of the experiment. In these cases, however, the replacement of the initial population by 1.A was much more rapid.

In all cases, the only VAT ever seen, apart from that used to initiate the population, was 1.A. In a number of other experiments in immune-competent rats and deer mice (*Peromyscus*), 1.A was invariably the first VAT to arise in a relapse from any other IsTat VAT, and it comprised 100% of the cells in each relapse population (10; unpublished data). Consequently, 1.A is the most predominant VAT of the IsTaR 1 serodeme. It is, therefore, not surprising that other IsTaR 1 VATs can switch to 1.A and that 1.A cells can replace the initial population even in the absence of immune selection.

Growth rate determinations. In light of the results shown in Fig. 1, we decided to determine whether any possible differences in growth rates between the different VATs could explain the replacement of the 1.1a, 1.3a, 1.5a, and 1.11a populations by 1.A. Trypanosomes from each of the six VATs were grown in irradiated mice, and the numbers of parasites in the blood were periodically counted. Regression analyses were performed on the resulting data for determination of the generation or doubling times for each VAT. Parasitemias of greater than 5×10^8 trypanosomes per ml were not included in the analyses since the growth rate of the parasite declined at such high parasitemias. Table 2 shows the generation times determined for each VAT and the results of pairwise comparisons between them with Student's *t* test. Only 1.7a and 1.3a ($P < 0.1$) and 1.7a and 1.11a ($P < 0.1$) showed significant differences in generation times.

Growth competition between different VATs. Our inability to show any significant difference between the generation times of 1.A and other VATs was due largely to the high standard deviations obtained when the generation times for each VAT were estimated. We therefore decided to undertake a series of competitive growth experiments in which irradiated mice were inoculated with similar numbers of trypanosomes of two different VATs. The much greater sensitivity of this procedure would enable us to distinguish between VATs with similar growth rates. Mice were injected with mixtures of 5×10^6 1.A trypanosomes and 5×10^6 trypanosomes of another VAT. The trypanosome population was passaged through irradiated mice while the VAT composition of the population was followed by fixed-cell IFA (Fig. 2). As in the stability experiment (Fig. 1), three categories of results were obtained: in the 1.A-1.7a mixture, the 1.7a population had outgrown the 1.A population to some extent after 9 days; in the 1.A-1.1a mixture, both 1.A and 1.1a existed at approximately equal levels for the duration of the experiment. Finally, when 1.A was mixed with 1.3a, 1.5a, or 1.11a, the 1.A population outgrew the other partner within 7 to 8 days. The results of these experiments would predict, therefore, that 1.7a grew faster than 1.A, which grew at a rate similar to that of 1.1a but faster than 1.3a, 1.5a, or 1.11a, at least when each of these VATs was mixed with 1.A. It should be noted that these results largely agree (with the exception of 1.5a and 1.1a) with the order of generation times shown in Table 2, despite the lack of statistically significant difference between these generation times. The results also fit well with those shown in Fig. 1, in which, in irradiated mice, VATs 1.3a, 1.5a, and 1.11a give way to 1.A, whereas 1.1a does so only very slowly and 1.7a does not at all for the length of the experiment.

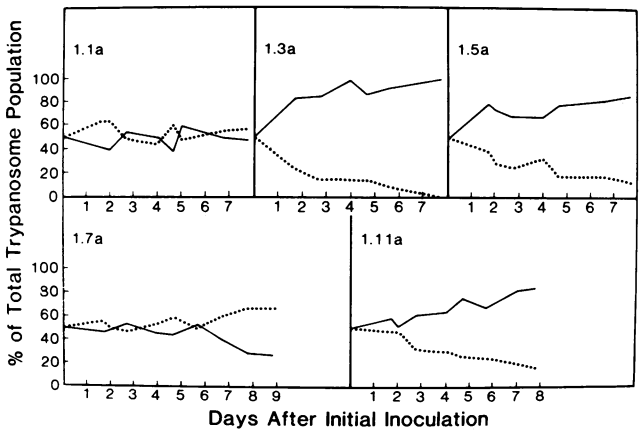

FIG. 2. Proportions of each VAT after inoculation of irradiated mice with a mixture of two different VATs. In all cases, 50% of the initial inoculum was 1.A; the other VAT is indicated above each graph. Solid lines indicate the percentage of the population expressing 1.A. Dotted lines indicate the percentage of the population expressing the other starting VAT. Each graph represents the average for two series of mice started with the same initial inoculum.

DISCUSSION

IsTat 1.A replaces other IsTaR 1 early VATs when clones of these VATs are grown in irradiated mice (Fig. 1), demonstrating that antigenic variation can occur in the absence of immune selection. This confirms the conclusion of Doyle et al. (5), who found antigenic variation could occur in in vitro cultures of *T. brucei* bloodstream forms. However, although Doyle (4) was able to demonstrate the presence of heterologous VATs in cloned VAT populations passaged in irradiated mice, he was not able to demonstrate significant replacement of the initial VAT population by these heterologous VATs for at least 38 days. This may be simply a reflection of the growth rates of VATs concerned.

As shown here and elsewhere (10), VAT 1.A is the predominant variant of the IsTaR 1 serodeme, since it invariably arises in the first relapse population from any other IsTaR 1 VAT. However, it does not have the fastest growth rate (Table 2 and Fig. 2); 1.7a is faster and 1.1a has a similar growth rate. Thus, its predominance cannot be explained solely on the basis of a faster growth rate. Miller and Turner (9) also found that the order of appearance of VATs did not correlate with growth rate, and using computer analysis of published data, Kosinski (6) concluded that random generation of different VATs and selection by growth rates alone could not produce the degree of variant orderliness reported in the literature. Thus, a factor (or factors) other than differences in growth rate must play a role in determining the order of variant appearance in chronic infections. Since it has been shown (2, 9, 20) that the order of appearance of VATs is in an imprecise but somewhat predictable manner, it would appear that the order of appearance is neither totally random nor rigidly programmed. Since some VATs appear to be expressed with high probability after others (9, 10), it would appear that for each VAT there exists a different probability that it will switch to another particular VAT. Conversely, each VAT has a certain, distinct probability of arising from another VAT. We have defined the probability of one particular VAT arising from another as its "switch frequency;" this represents the proportion of cells in a particular VAT population which will switch to another particular VAT per generation.

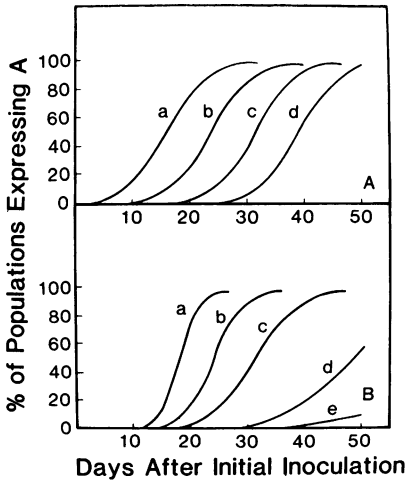

FIG. 3. Theoretical curves for VAT switching, showing the effect of different switch frequencies (A) and generation times (B). In A, the generation time of variant *A* (*gA*) was 5.0 h and the generation time of variant *X* (*gX*) was 5.5 h. The switch frequency (*X* to *A*) was 10^{-3} (a); 10^{-4} (b); 10^{-5} (c), or 10^{-6} (d). In B, *gA* was 5.0 h, and the switch frequency was 10^{-5} throughout. The generation time of *X* was 6.0 h (a); 5.7 h (b); 5.5 h (c); 5.3 h (d), or 5.2 h (e).

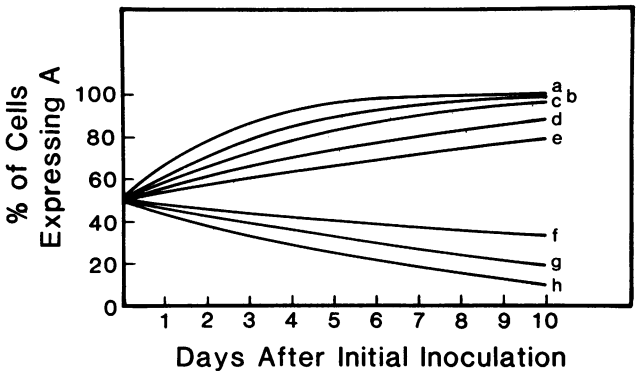

FIG. 4. Theoretical curve for competition between VATs, showing the effects of various generation times. The theoretical curves were started with an inoculation of 5×10^6 cells each of variant *A* and variant *X* at day 0. The switch frequency (*X* to *A*) was 10^{-5}, and *gA* was 5.0 h. Generation time of *X* (*gX*) was 6.0 h (a); 5.7 h (b); 5.5 h (c); 5.3 h (d); 5.2 h (e); 4.9 h (f); 4.8 h (g), or 4.7 h (h).

Using a simple equation which takes into account only differences in growth rate and switch frequencies (see above), we constructed a series of theoretical curves in which one VAT population (starting with 10^7 trypanosomes) is replaced by another (Fig. 3). In Fig. 3A, in which variant *A* (generation time, 5.0 h) replaces variant *X* (generation time, 5.5 h), we show the effect of differences in the frequency with which *X* switches to *A*. The slopes of the curves are the same with widely differing switch frequencies, but the time taken for 1.A to replace 1.X is delayed by ca. 7.5 days with each 10-fold decrease in switch frequency. The effect of differing growth rates on the replacement of *X* by *A* may be seen in Fig. 3B. As expected, the rate of overgrowth by variant *A* is faster when the difference between the generation times of *A* and *X* is larger. When the generation times are more similar (e.g., 5.0 h and 5.2 h; Fig. 3B, curve e), the replacement of variant *X* by variant *A* is very slow, even though variant *X* is continually switching to variant *A*. Thus, it can be seen that differences in growth rates between two VATs and changes in the switch frequency of one to the other exert different effects on the way in which one VAT replaces another in the absence of immune selection. The switch frequency mostly determines the time it takes for the faster-growing variant to reach detectable levels in the population. The rate of replacement of the slower-growing variant is largely determined by the difference in growth rate between the two variants. With the same equation used to generate the theoretical curves in Fig. 3, we generated theoretical curves for two VATs with differing growth rates mixed in equal proportions and allowed to grow together. In Fig. 4, we have assumed that variant *X* switches to variant *A* with a switch frequency of 10^{-5}, and while keeping the generation time of variant *A* constant at 5.0 h, we have varied the generation time of variant *X*. Even small differences in generation times (e.g., 0.1 h in curve f) can lead to appreciable differences in the proportion of each variant present on day 10. We also found that varying the switch frequency had very little effect on the curves. Thus, the type of experiment shown in Fig. 2 should be able to demonstrate small differences between the generation times of different VATs. When the results shown in Fig. 2 were compared with the theoretical curves generated

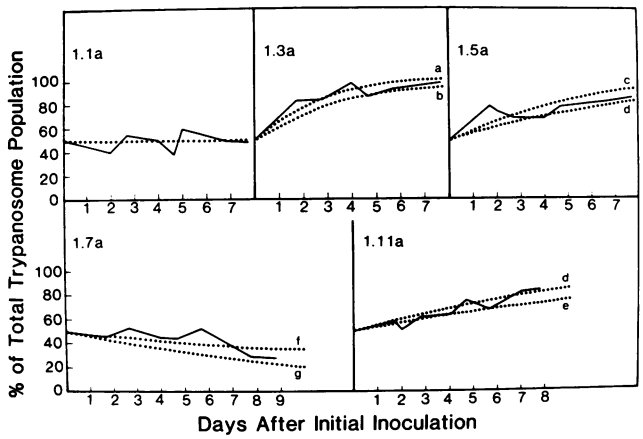

FIG. 5. Comparison of theoretical curves for growth competition between two variants with experimental results obtained. Solid lines indicate the percentage of the total population expressing VAT 1.A (from Fig. 2). Dotted lines represent theoretical curves for the results from Fig. 4. All are based on a generation time of 5.0 h for variant *A* and switch frequencies of 10^{-5}, and on generation times for variant *X* as indicated in Fig. 4.

in Fig. 4 (Fig. 5), we were able to predict the generation times of the IsTaR 1 early VATs, assuming a generation time of 5.0 h for 1.A. These generation times (1.1a, ~5.0 h; 1.3a, ~5.7 to 6.0 h; 1.5a, ~5.3 to 5.5 h; 1.7a, ~4.8 to 4.9 h; 1.11a, ~5.2 to 5.3 h) fall within the ranges predicted from the growth rate determination experiments and have a similar rank order (Table 2). Seed (13) suggested that competition between VATs grown in mixed population can result in their having different apparent growth rates when grown together than they have when grown separately. This does not appear to have been the case in the experiments shown in Fig. 2.

The results presented in Fig. 1 show that each VAT, with the exception of 1.5a, was very consistent in that the rate at which it was replaced by 1.A and the time at which this replacement occurred were constant when different mice were used and the starting cells were thawed from different frozen stocks. The results for 1.5a, however, were variable, with complete replacement with 1.A occurring in only 4 days on one occasion. A similar phenomenon may occur with 1.1a since the results from Fig. 1 would suggest a generation time of ca. 5.3 h (compared with 5.0 h for 1.A), whereas the results from Fig. 2 (and Table 2) suggest a generation time of ca. 5.0 h. This may reflect (genomic) heterogeneity within the 1.5a population which may affect growth rates and switch frequencies to 1.A. Another possibility is that the 1.A populations which replace different VATs at different times may have different growth characteristics. Seed (13) found that clones which appeared serologically identical behaved differently in growth competition experiments. IsTat 1.A-expressing clones relapsed from different early VATs have been shown to differ with respect to the genomic organization of their VSG genes (10).

The model we have presented here is limited in a number of ways. Most importantly, it does not take into account the immune response of the host. In an immune-competent host, as each VAT reaches a sufficiently high number to elicit an immune response, it will be removed from the population and prevented from reappearing in significant numbers. This allows slower-growing VATs to replace those faster-growing ones which have been destroyed by the immune system of the host. The model also ignores the possible effects of sequestration of antigenically different populations in the brain (14) and lymph nodes (18). Differential sequestration of certain VATs in these tissues may well alter their proportion of the population in the peripheral circulation. Another problem is that all six VAT clones examined were isolated relatively early (within the first 44 days) in a chronic infection. This may explain their similar growth rates. Examination of clones isolated late in an infection may reveal more widely differing growth rates.

We believe that the results presented here are consistent with two trypanosomal factors modulating the sequence of expression of VATs. By varying the frequency with which each VAT can be expressed, as well as having different growth rates for different VATs, trypanosomes are able to maintain a loosely ordered sequence of VATs in a chronic infection. Thus, some VATs with a high switch frequency and a relatively high growth rate will always appear early in infections (e.g., 1.A and other predominant VATs), whereas those with low switch frequencies and low growth rates will appear only late in infection. They will not be present early in an infection in sufficient numbers to elicit an immune response from the host. Thus, the parasite is able to ensure that it will survive for long periods in a single host. The mechanisms by which trypanosomes cause different VATs to have different growth rates and different switch frequencies is not yet clear. As we have suggested elsewhere (10), it is possible that those VSG genes which are present in telomeric locations are more easily expressed than those with intrachromosomal locations, and in this way the trypanosome may control the switch frequency for different VATs.

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