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Organization of Two Invariant Surface Glycoproteins in the Surface Coat of *Trypanosoma brucei*

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The surface coat of *Trypanosoma brucei*, formed by about 10^7 molecules of the membrane-form variant surface glycoprotein (mfVSG) per cell, is generally considered to constitute a barrier against the access of antibodies directed to invariant surface proteins. The recent characterization of two invariant surface glycoproteins (ISGs) with apparent molecular masses of 65 and 75 kDa (ISG65 and ISG75; 70,000 and 50,000 molecules per cell, respectively), which are both predicted to be composed of large extracellular domains, single transmembrane α-helices, and small intracellular domains, enabled a critical test of this hypothesis. Although ISG65 is distributed over the entire surface of the parasites, it is not accessible to antibodies or to the proteinase trypsin in live cells provided the mfVSG is also proteinase resistant. ISG75 is similarly distributed; its accessibility to antibodies depends on the expressed mfVSG, and it is sensitive to trypsin in a variant clone in which the mfVSG is proteinase resistant. Vaccination experiments using recombinant proteins to a mixture of the native ISGs were unsuccessful. ISG65 but not ISG75 elicited an antibody response in chronically infected mice. The results strengthen the view of the protective properties of the variant surface glycoprotein coat by steric hindrance and suggest that additional factors such as low abundance or low immunogenicity of invariant surface proteins may prevent a control of the disease by the humoral immune response.

African trypanosomes are unicellular protozoan parasites responsible for widespread chronic disease in humans and domestic animals in Africa. They live extracellularly in the blood and tissues of their mammalian hosts and evade the humoral immune response by antigenic variation of the major surface antigen, the membrane form of the variant surface glycoprotein (mfVSG), which covers the cells as a dense coat (11). Since the repertoire of genes coding for antigenically different variant surface glycoproteins (VSGs) is very large (7, 28), vaccination against trypanosomiasis based on the variant antigen is believed to be impossible. As an alternative, it has been argued for some time that trypanosomes must contain additional proteins at their surface which may not undergo antigenic variation and that deliberate immunization against such antigens may provide protection against the disease (5, 27). Candidate proteins may be receptors involved in the uptake of high-molecular-weight ligands (8, 9, 25) or solute transporters such as the glucose carrier (6, 15, 26).

Recently, two invariant surface glycoproteins (ISGs) specific for the bloodstream stage of *Trypanosoma brucei* have been described (30, 31; see also reference 20 for the characterization of similar proteins). These have been designated ISG65 and ISG75 and have apparent molecular masses of 65 and 75 kDa, respectively. Initially identified by surface biotinylation experiments, these polypeptides belong to a group of minor surface proteins which are found in a series of parasite clones expressing different VSGs. Gene cloning and sequencing suggest that both proteins are composed of large extracellular domains, single transmembrane α-helices, and small intracellular domains. Furthermore, as judged by immunofluorescence experiments on fixed parasites, ISG65 and ISG75 are distributed over the entire cell surface. Their abundance has been estimated to be about 0.5% of that of the VSG (70,000 to 50,000 versus 10^7 molecules per cell, respectively). Within the limitations imposed by the biotinylation strategy, no surface proteins having a similar overall organization as that of the two ISGs and exceeding their abundance are likely to exist in bloodstream-form trypanosomes.

The characterization of ISGs has enabled us, first, to study how these proteins are arranged in the mfVSG coat and, second, to test the hypothesis of whether they can be of use as a vaccine against the infection.

MATERIALS AND METHODS

Trypanosomes. Bloodstream forms of *T. brucei* MITat (variant clones MITat 1.2 and MITat 1.4 [10]) and AnTat serodemes (variant clone AnTat 1.1 [29]) and strain 247 (14) were grown in mice or rats.

Preparation of antigens. Concanavalin A (ConA)-binding proteins, which contain ISG65 and ISG75 as major components, were isolated from MITat 1.4 bloodstream forms and reconstituted into liposomes as described previously (31). Parts of the extracellular domains of ISG65 and ISG75 were expressed as fusion proteins together with glutathione *S*-transferase from *Schistosoma japonicum*. The production of Fus65a and Fus75a-glutathione *S*-transferase has been described (30). For generating Fus65b, a 1,026-bp *BclI-PstI* fragment from pSW14 (30) was blunt ended and subcloned into the blunt-ended *Hin*dIII-*Eco*RI sites of the expression vector pGEX-KG (16). Fus75b was obtained by subcloning a blunt-ended 1,050-bp *SstI-Sma*I fragment of pSW331 (30) into the blunt-ended *Xho*I site of pGEX-KG. The recombinant constructs were designated pGEX-SW14b and pGEX-SW331a, respectively. pGEX-SW14b encodes amino acids 15 to 355 of mature ISG65, and pGEX-SW331a encodes amino acids 4 to 354 of mature ISG75. Screening of transformants, purification of

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fusion proteins, and thrombin cleavage have been described (30).

Immunological techniques. Sera from infected mice were prepared from female C57BL/6 mice infected with 10^5 *T. brucei* AnTat 1.1 bloodstream forms (>90% long slender forms). At 6-day intervals, three mice were killed for the preparation of pooled serum. A Chinchilla Bastard rabbit was immunized with 0.35 mg of Fus65b in 50% complete Freund's adjuvant (CFA; Difco, Detroit, Mich.), boosted with 0.175 mg of Fus65b in 50% incomplete Freund's adjuvant 4 weeks later, and bled after 2 more weeks. The antibody titer expressed as the reciprocal dilution of serum at 50% maximum binding in an enzyme-linked immunosorbent assay (ELISA) was >12,800.

Vaccination experiments were performed in female C57BL/6 mice (about 10 weeks old; five animals per group). Each group was immunized intraperitoneally with the following recombinant proteins: Fus65b, Fus75a-glutathione *S*-transferase, Fus75b (about 10 μg per mouse), and a mixture of Fus65b and Fus75b (10 μg of each per mouse) in 50% CFA or Fus65b (10 μg per mouse) in 50% Hunter's Titermax (CytRx Corporation, Atlanta, Ga.). Each mouse of one group received about 10 μg of ConA-binding proteins reconstituted into liposomes. Control groups were immunized with CFA or with 10 μg of recombinant glutathione *S*-transferase in CFA per mouse. At day 46, mice were boosted with the same antigen in incomplete Freund's adjuvant. At day 54, serum was prepared from tail blood, and the antibody titer was analyzed by ELISA. At day 77, mice were boosted as described above with 5 μg of antigen per mouse. At day 88, mice were infected intraperitoneally with 10^4 trypanosomes of either strain 247 or variant clone AnTat 1.1. Parasitemia was estimated from day 92 onwards as described previously (19).

ELISAs were performed in polyvinyl chloride microtiter plates coated with 0.5 μg of recombinant protein per well by evaporation from 50 μl of water. After washing once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4 [pH 7.2]), the plates were incubated with blocking buffer (5% nonfat milk powder in PBS) for 60 min at 37°C. Serial dilutions of antisera (1:50 to 1:1,600) in blocking buffer were applied for 60 min at 37°C. After three washings with PBS, the plates were incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG)-IgM (1:5,000; Dianova, Hamburg, Germany) for 60 min at 37°C. After three washings with PBS and one with phosphatase buffer (50 mM $NaHCO_3$, 2 mM $MgCl_2$ [pH 9.6]), the bound phosphatase activity was determined spectrophotometrically at 405 nm by using *p*-nitrophenyl phosphate (1 mg/ml) as a substrate.

For flow cytometric analysis, trypanosomes were fixed in 2% formaldehyde in PBS for 30 min at room temperature. Cells were washed with PBS–5% bovine serum albumin (BSA), and unspecific binding sites were blocked by incubation for 10 min in PBS–5% BSA. Thereafter, cells were treated with primary and secondary antibodies for 60 min. Alternatively, cells were incubated for 60 min at 0 or 37°C with primary antibody diluted in medium (2) containing 15% inactivated fetal bovine serum. After three washings with PBS–5% BSA, cells were fixed as described above and treated with secondary antibody. The following antibodies were used at the indicated dilutions: rabbit anti-Fus65b IgG (0.3 mg/ml), rabbit anti-Fus65b F(ab) (0.5 mg/ml), mouse anti-Fus75b serum (1:50), mouse serum against ConA-binding proteins (1:50), fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (1:250, Dianova), biotinylated goat anti-mouse IgG (1:100; Sigma, Deisenhofen, Germany), FITC-labeled streptavidin (1:100; Amersham, Braunschweig, Germany), and mouse anti-rabbit IgG (1:2,000; Dianova). Flow cytometry was performed with a fluorescence-activated cell sorter (FACS IV; Becton Dickinson & Co., Sunnyvale, Calif.). F(ab) fragments were prepared as described previously (18).

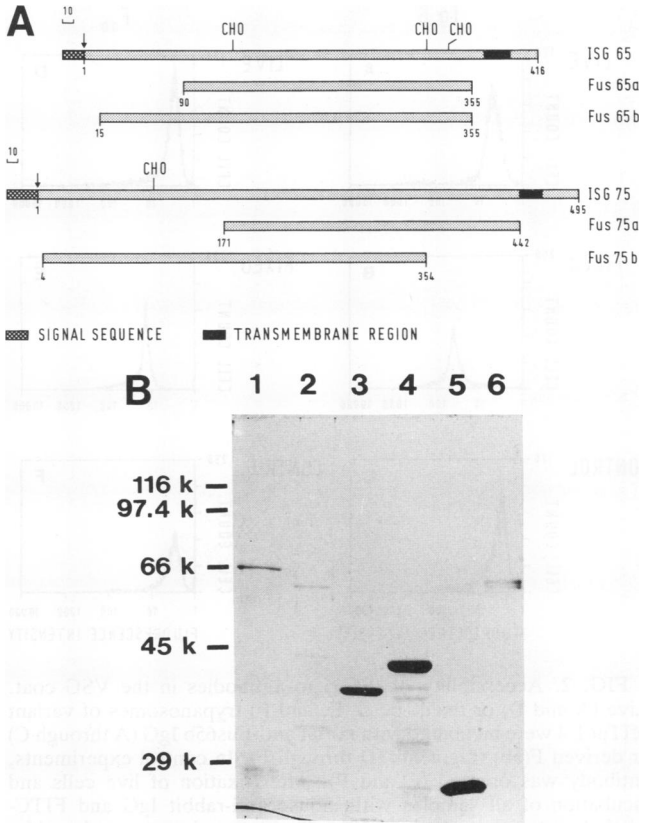

FIG. 1. Expression of ISG65 and ISG75 in *E. coli*. (A) Schematic description of the primary sequences of ISG65 and ISG75. CHO, potential glycosylation sites. Below each protein, the fragments used for the generation of fusion proteins in *E. coli* are shown. (B) SDS-PAGE of purified recombinant proteins. Lanes: 1, Fus75a-glutathione *S*-transferase; 2, Fus75b; 3, Fus65a; 4, Fus65b; 5, recombinant glutathione *S*-transferase; 6, sVSG of variant clone AnTat 1.1.

Other techniques. Trypsin digestion experiments using MITat 1.4 trypanosomes, sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE), and immunoblotting were performed as described previously (31).

Nucleotide sequence accession numbers. The nucleotide sequences of ISG65 and ISG75 are available from the GenBank or EMBL data bank under accession numbers M86709 (ISG65), M86710, and M86711 (ISG75).

RESULTS

Expression of ISG65 and ISG75 in *Escherichia coli*. Since ISG65 and ISG75 could be purified from *T. brucei* bloodstream forms only in small amounts (31), segments of ISG65 and ISG75 were expressed as fusion proteins with glutathione *S*-transferase in *E. coli*. Figure 1A depicts schematically the primary structures of ISG65 and ISG75 as well as the regions chosen for expression as recombinant proteins. To

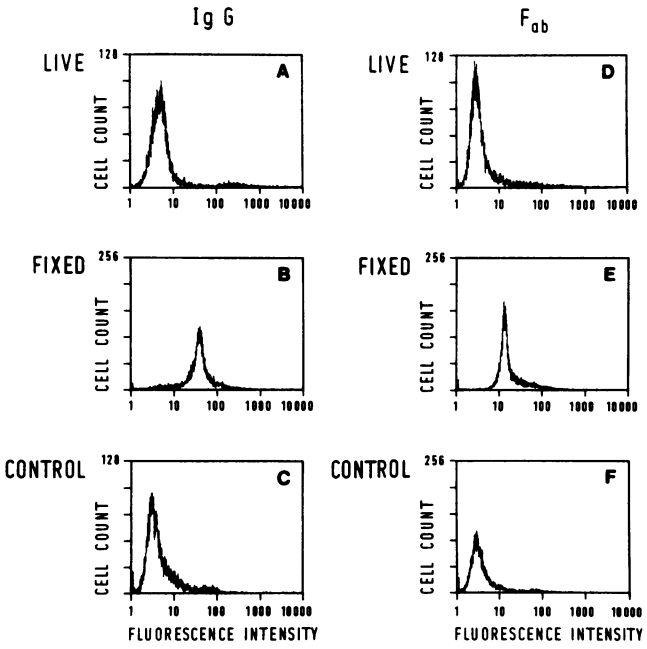

FIG. 2. Accessibility of ISG65 to antibodies in the VSG coat. Live (A and D) or fixed (B, C, E, and F) trypanosomes of variant MITat 1.4 were incubated with rabbit anti-Fus65b IgG (A through C) or derived F(ab) fragments (D through F). In control experiments, antibody was omitted (C and F). After fixation of live cells and incubation of all samples with mouse anti-rabbit IgG and FITC-labeled goat anti-mouse IgG, 10^4 cells per sample were analyzed by flow cytometry.

obtain soluble fusion proteins, the hydrophobic signal sequences at the NH_2 termini and the transmembrane α-helices near the COOH termini were excluded from the constructs. The fusion proteins were isolated by affinity chromatography on glutathione-agarose beads from the soluble fraction of bacterial lysates and cleaved by thrombin (not successful in the case of Fus75a); the Fus65a recombinant protein was further purified by ion-exchange chromatography. As shown by SDS-PAGE (Fig. 1B), Fus65a (lane 3, 37.5 kDa; yield, 2 mg/liter of bacterial culture), Fus65b (lane 4, 41 kDa; yield, 1.5 mg/liter), Fus75a-glutathione *S*-transferase (lane 1, 67 kDa; yield, 1.6 mg/liter), and recombinant glutathione *S*-transferase (lane 5, 25.5 kDa) were nearly homogeneous, whereas Fus75b (lane 2, 43 kDa; yield, ca. 0.1 mg/liter) could not be obtained in pure form. In an ELISA, all four recombinant proteins were recognized by mouse antiserum against ConA-binding proteins isolated from *T. brucei*; this mixture contained native ISG65 and ISG75 (31). The titer of this antiserum was considerably higher against the Fus65 constructs than the Fus75 constructs (not shown).

Accessibility of ISG65 and ISG75 to antibodies in the VSG coat. Bloodstream forms of variant clone MITat 1.4 were incubated with anti-ISG65-specific antibodies and FITC-labeled secondary antibodies and then analyzed by flow cytometry (Fig. 2). Incubation of formaldehyde-fixed cells with anti-Fus65b IgG resulted in a fivefold increase in fluorescence intensity compared with that of a control (Fig. 2B and C). Specific fluorescence in MITat 1.4 bloodstream forms was observed only in fixed, not live, cells (compare Fig. 2A through C). The same behavior was also observed for F(ab) fragments prepared from anti-Fus65b IgG (Fig. 2D through F).

Trypanosomes of the variant clones MITat 1.2 and MITat 1.4 were incubated with preimmune or anti-Fus75b serum or with serum against ConA-binding proteins and then treated with biotinylated secondary antibodies and FITC-labeled streptavidin. For live MITat 1.2 bloodstream forms incubated with anti-Fus75b antiserum, a small but highly reproducible increase in specific fluorescence could be observed (compare Fig. 3D through F). This increase amounted to 1.7 ± 0.26-fold in four independent experiments and was independent of the incubation temperature (4 or 37°C). In contrast, no increase could be detected in three independent experiments when MITat 1.4 (Fig. 3A through C) or MITat 1.6 trypanosomes (not shown) were used. If cells were fixed before the incubation with antibody (Fig. 3G through M), the fluorescence intensity increased about 3-fold for anti-Fus75b or 20-fold for serum against the mixture of ConA-binding proteins. Furthermore, the reaction of fixed cells was independent of the variant clone used.

Protease digestion experiments. Previous experiments (31) have shown that ISG65 and ISG75 could be completely degraded by incubation of trypanosomes of variant clone MITat 1.6 with the protease trypsin; in this clone, the VSG is also trypsin sensitive. In contrast, the mfVSG of MITat 1.4 cells is known to be trypsin resistant (Fig. 4B), and so is ISG65 (not shown). However, ISG75 could be completely degraded in this variant in a few minutes (Fig. 4A).

Formation of antibodies in infected mice. C57BL/6 mice were infected with 10^5 trypanosomes of variant clone AnTat 1.1. As shown in Fig. 5A, mice controlled the initial rise in parasitemia efficiently, while, thereafter, the trypanosome load remained high until the animals succumbed after about 40 days. At 6-day intervals, three mice were sacrificed, and pooled sera were tested by ELISA for the presence of antibodies against Fus65a or Fus65b, recombinant glutathione *S*-transferase, or purified AnTat 1.1 soluble VSG (sVSG; Fig. 1B, lane 6). Antibodies against Fus65a or Fus65b could be detected after 12 or 6 days, respectively (Fig. 5B). In both cases, the titer increased until day 24 and declined thereafter. The antibodies against Fus65b present from day 12 onward belonged mainly to the IgG subclass. Between days 24 and 30, the antibody titer against Fus65a and Fus65b in infected mice was about eight times lower than in sera from mice immunized repetitively with ConA-binding proteins (data not shown). Remarkably, in three independent experiments, we were unable to detect antibodies against the recombinant proteins corresponding to ISG75 in these mice. Finally, the titer against AnTat 1.1 sVSG was maximal at day 6 and decreased slowly thereafter. Whereas the antibodies present at day 6 belonged to the IgM subclass, specific IgG could be detected at later times.

Vaccination experiments. Groups of five C57BL/6 mice were immunized with the recombinant proteins or the mixture of ConA-binding proteins reconstituted into liposomes, as detailed in Materials and Methods. Control groups received recombinant glutathione *S*-transferase, adjuvant only, or no treatment. As judged by an ELISA with antisera obtained after the first booster injection, all immunized mice except one produced antibodies against the respective antigens. The reciprocal serum dilutions at 50% maximum binding were about 3,000 for antisera against Fus65b, Fus 75a-glutathione *S*-transferase, glutathione *S*-transferase, and the mixture of ConA-binding proteins and about 2,000 for the anti-Fus75b serum. After a further boost, mice were infected intraperitoneally with 10^4 trypanosomes of either variant

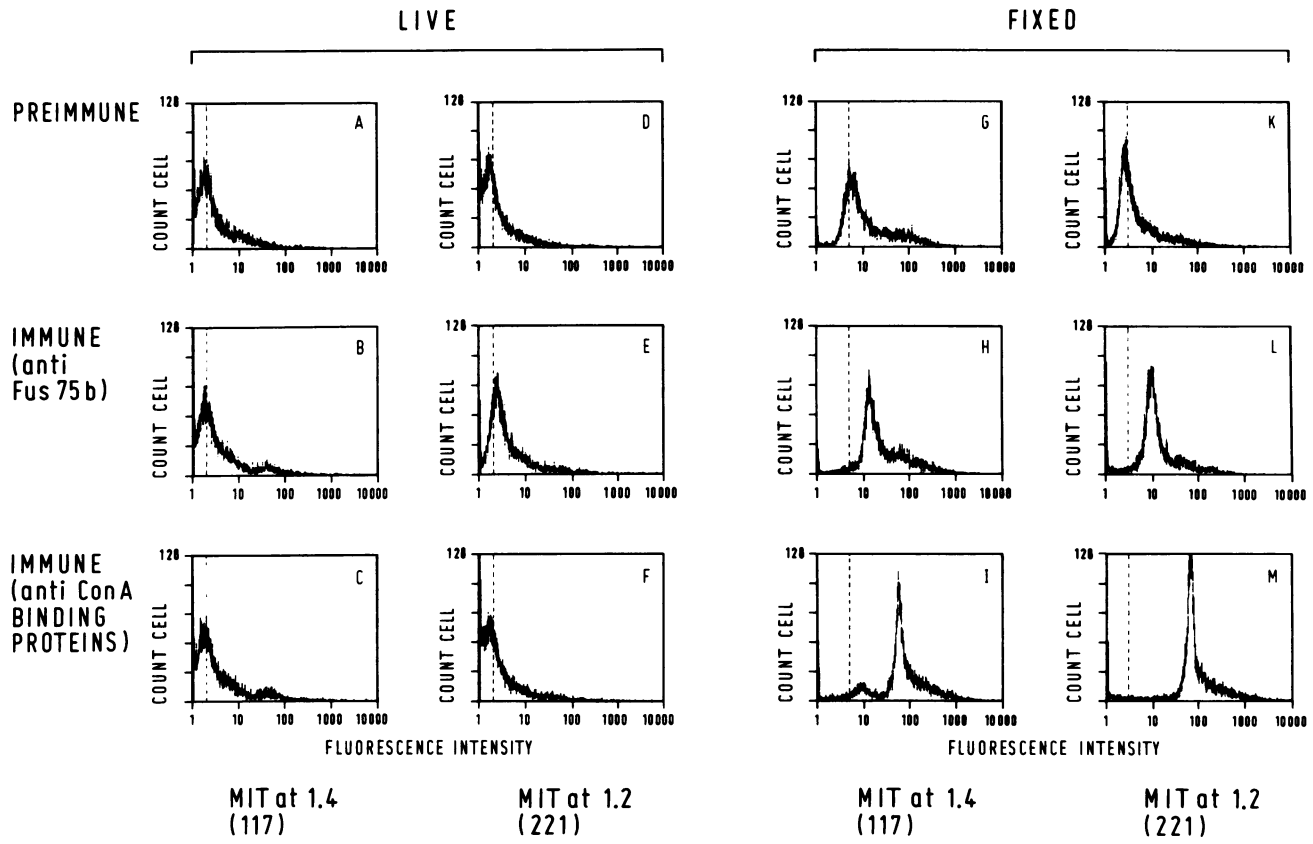

FIG. 3. Accessibility of ISG75 to antibodies in the VSG coat. Trypanosomes of variant clone MITat 1.2 or MITat 1.4 were incubated before (live) or after fixation (fixed) with preimmune serum, mouse anti-Fus75b serum, and mouse anti-ConA-binding proteins (dilution 1:50). After incubation with biotinylated secondary antibodies and FITC-labeled streptavidin, aliquots were analyzed by flow cytometry.

AnTat 1.1 or strain 247, and the parasitemia was monitored for 7 weeks. The course of parasitemia (see example in Fig. 5A) was the same in vaccinated and control mice, and all mice but one died of the infection within 10 to 12 weeks. It should be added that all three strains (MITat, AnTat, and 247) expressed similar amounts of ISG65 and ISG75 as judged by immunoblot and fluorescence-activated cell sorter analysis. Furthermore, our vaccination protocol did not select for trypanosomes that express less ISG65 or ISG75. About 5 weeks after infection, tail blood of mice from groups immunized with Fus65b, Fus75b, the mixture of ConA-binding proteins, or adjuvant only was transferred to NMRI mice. As judged by immunoblot, trypanosomes isolated from these mice contained the same amounts of ISG65 and ISG75 (data not shown).

DISCUSSION

For both the localization and the vaccination experiments, the quality of the antibodies against the antigens used in this study are of prime importance. First, the mixture of ConA-binding proteins containing only traces of VSG were purified under nondenaturing conditions (31). Therefore, the immune response against this mixture, which contains ISG65 and ISG75 as major components, is expected to include antibodies against conformational epitopes exposed at the surface of these polypeptides. Second, the nucleic acid sequences for both ISG65 and ISG75 predict highly hydrophilic extracellular domains connected to hydrophobic transmembrane helices (30). The fusion proteins and the recombinant polypeptides obtained by thrombin cleavage were found in the soluble fraction of *E. coli* lysates rather than insoluble inclusion bodies, suggesting that they may assume a three-dimensional structure with certain features shared by their native trypanosomal counterparts. This view is supported by the observation that antisera against Fus65a and Fus75a-glutathione *S*-transferase can be used to immunoprecipitate ISG65 and ISG75, respectively, from *T. brucei* detergent extracts (30). Therefore, the recombinant proteins elicit an antibody response that appears to include the recognition of epitopes of the native proteins. However, the titers of the antisera remained low even after multiple immunization of mice.

Antibodies to ConA-binding proteins bind to lightly fixed but not live trypanosomes (Fig. 3). In the case of ISG65, specific antibodies or derived Fab fragments have no access to the antigen in live cells (Fig. 2). Trypsin can degrade ISG65 in variants in which the VSG is sensitive to the protease in live cells (31) but not in a variant (MITat 1.4) where the VSG is trypsin resistant. In contrast, ISG75 is sensitive to trypsin cleavage in MITat 1.4 (Fig. 4), and a small amount of antibody binding to live cells is observed in one (MITat 1.2) but not in another (MITat 1.4) variant clone (Fig. 3). These data indicate that steric hindrance by the VSG coat to access of antibodies or trypsin is less effective for ISG75 than ISG65. When MITat 1.2 trypanosomes were incubated with anti-Fus75b antibodies and then injected into a mouse, the parasites were not eliminated (unpublished

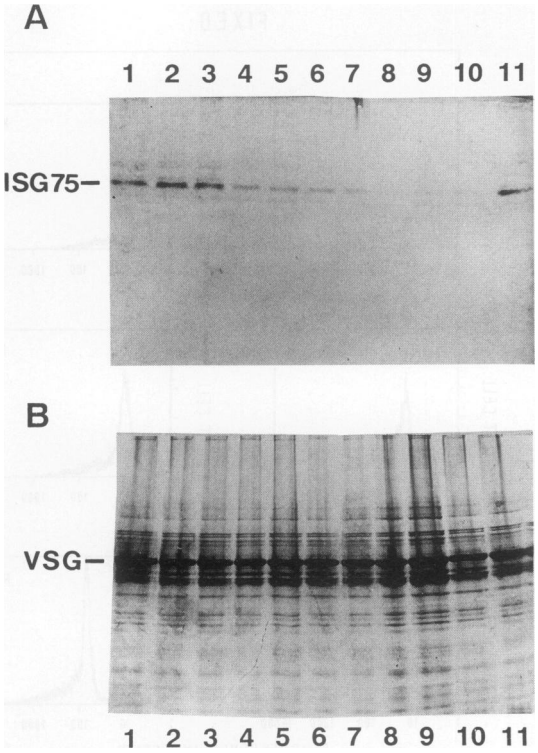

FIG. 4. Digestion of ISG75 by trypsin. Bloodstream forms of variant MITat 1.4 were treated with trypsin for 0, 1, 2, 3, 4, 5, 6, 8, 10, or 12 min (lanes 1 to 10, respectively) or left untreated (lane 11). (A) Lysates from 5×10^6 cells in SDS were separated by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane and probed with anti-Fus75b serum. (B) Lysates from 2×10^6 cells were separated by SDS-PAGE and stained with Coomassie blue. The dominant band at 61 kDa is VSG as confirmed by immunoblotting experiments.

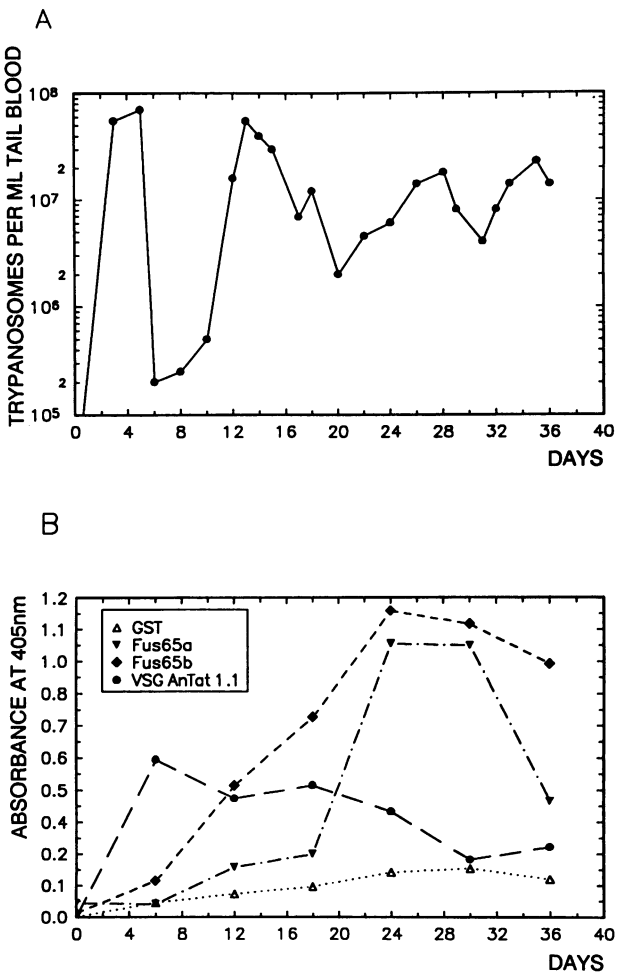

FIG. 5. Detection of antibodies against ISG65 in sera of *T. brucei*-infected mice. C57BL/6 mice were infected with 10^5 trypanosomes of variant clone AnTat 1.1. (A) The course of parasitemia is shown. (B) At 6-day intervals, sera were tested at a 1:50 dilution against the recombinant proteins (Fus65a and -b), the sVSG of variant clone AnTat 1.1, and recombinant glutathione *S*-transferase (GST) by an ELISA with goat anti-mouse IgG-IgM coupled to alkaline phosphatase. On the ordinate, the absorbance of *p*-nitrophenol released from *p*-nitrophenyl phosphate is plotted. The figure shows one of three independent experiments.

experiments). In conclusion, the relatively small trypsin molecule is able to penetrate the coat, cleaving VSGs and ISGs, provided that suitable cleavage sites are approachable. Access of the larger antibody molecules to ISGs buried in the coat has to date been observed only for ISG75 in one variant clone. We assume that, in this case, the amount and the arrangement (i.e., mono- or divalent binding) of antibodies were not adequate for direct or complement-mediated uptake of the motile cells by macrophages in mice. An attempt to demonstrate the localization of the ISGs by immunoelectron microscopy failed presumably because of the low abundance of these proteins (27a).

Our results strengthen the concept for the protective properties of the highly extended VSG molecules (4, 13) by steric hindrance (for a review, see reference 1). It is well known that only those monoclonal antibodies against VSG that recognize exposed epitopes on the surface of live cells are protective (17, 21, 24). Antibodies binding to epitopes less accessible on living trypanosomes, as judged by a weak reaction in immunofluorescence experiments, fail to neutralize a challenge infection (21). Also, resistance to the alternative pathway of complement is guaranteed only as long as the coat remains intact (12, 22).

The conclusions on the surface organization of the ISGs are supported by the negative outcome of the vaccination experiments. Immunization with either the ConA-binding proteins or the recombinant ISGs did not influence the course of the infection. ISG65 appears to be generally more immunogenic than ISG75 because the titers in the serum against the ConA-binding proteins containing both proteins were higher against Fus65a or -b than against Fus75a or -b as judged by ELISA or immunoblots. More importantly, while anti-Fus65a or -b antibodies could be demonstrated readily in sera of infected mice (Fig. 5), no reaction against Fus75a or -b was discernible. Therefore, one important consideration with regard to a possible protective effect of antibodies against invariant proteins is their immunogenicity. Also, during infection, additional immune regulatory mechanisms which suppress antibody formation against ISG75 may be operative. The lack of a protective effect of the ISGs is in marked contrast to the highly immunogenic VSG where mice immunized with a small amount of protein even in the absence of adjuvant can survive a variant-specific challenge infection (3).

In summary, trypanosomes are able to express small

amounts of invariant antigens at their surfaces without compromising the protective function of their mfVSG coats, and therefore the prospects of a vaccine based on invariant surface proteins, which are evenly distributed over the trypanosome surface, are bleak. There remains the vague possibility that immunization against receptors involved in high-molecular-weight nutrient uptake may be more effective (see discussion in references 5, 23, and 27). However, if such proteins are accessible only to their ligands or antibodies in the concealed extracellular compartment of the flagellar pocket, potential trypanocidal mechanisms involving complement or macrophages may not be triggered effectively.

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REFERENCES

1. **Balber, A. E.** 1990. The pellicle and the membrane of the flagellum, flagellar adhesion zone, and flagellar pocket: functionally discrete surface domains of the bloodstream form of African trypanosomes. Crit. Rev. Immunol. **10**:177–201.
2. **Baltz, T., D. Baltz, C. Giroud, and J. Crockett.** 1985. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rodesiense* and *T. gambiense*. EMBO J. **4**:1273–1277.
3. **Baltz, T., D. Baltz, R. Pautrizel, C. Richet, G. Lambin, and P. Degand.** 1977. Chemical and immunological characterization of specific glycoproteins from *Trypanosoma equiperduum* variants. FEBS Lett. **82**:93–96.
4. **Blum, M. L., J. A. Down, A. M. Gurnett, M. Carrington, M. J. Turner, and D. C. Wiley.** 1993. A structural motif in the variant surface proteins of *Trypanosoma brucei*. Nature (London) **362**: 603–609.
5. **Borst, P.** 1991. Transferrin receptor, antigenic variation and the prospect of a trypanosome vaccine. Trends Genet. **7**:307–309.
6. **Bringaud, F., and T. Baltz.** 1992. A potential hexose transporter gene expressed predominantly in the bloodstream form of *Trypanosoma brucei*. Mol. Biochem. Parasitol. **52**:111–122.
7. **Cabern, A., C. Biround, T. Baltz, and P. Maltern.** 1977. *Trypanosoma equiperdum*: etude des variations antigeniques au cours de la trypanosome experimentale du lapin. Exp. Parasitol. **42**:6–13.
8. **Coppens, I., P. Baudhuin, F. R. Opperdoes, and P. J. Courtoy.** 1988. Receptor for the low-density lipoproteins on the hemoflagellate *Trypanosoma brucei*—purification and involvement in growth of the parasite. Proc. Natl. Acad. Sci. USA **85**:6753–6757.
9. **Coppens, I., F. R. Opperdoes, P. J. Courtoy, and P. Baudhuin.** 1987. Receptor-mediated endocytosis in the blood-stream form of *Trypanosoma brucei*. J. Protozool. **43**:465–473.
10. **Cross, G. A. M.** 1975. Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. Parasitology **71**:393–417.
11. **Cross, G. A. M.** 1990. Cellular and genetic aspects of antigenic variation in trypanosomes. Annu. Rev. Immunol. **8**:83–110.
12. **Ferrante, A., and A. C. Allison.** 1983. Alternative pathway activation of complement by African trypansomes lacking a glycoprotein coat. Parasite Immunol. **5**:491–498.
13. **Freymann, D., J. Down, M. Carrington, I. Roditi, M. Turner, and D. Wiley.** 1990. 2.9 Å resolution structure of the N-terminal domain of a variant surface glycoprotein from *Trypanosoma brucei*. J. Mol. Biol. **216**:141–160.
14. **Geigy, R., and M. Kaufmann.** 1973. Sleeping sickness survey in the Serengeti area, Tanzania. Acta Trop. **30**:12–23.
15. **Gruenberg, J., P. R. Sharma, and J. Deshusses.** 1978. D-glucose transport in *Trypanosoma brucei*. Eur. J. Biochem. **89**:461–469.
16. **Guan, K., and J. E. Dixon.** 1991. Eucaryotic proteins expressed in *E. coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione-S-transferase. Anal. Biochem. **192**:262–267.
17. **Hall, T., and K. Esser.** 1984. Topological mapping of protective and nonprotective epitopes on the variant surface glycoprotein of the WRATat 1 clone of *Trypanosoma brucei rhodesiense*. J. Immunol. **132**:2059–2063.
18. **Harlow, E., and D. Lane.** 1988. Antibodies. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Herbert, W. J., and W. H. R. Lumsden.** 1976. *Trypanosoma brucei*: a rapid "matching" method for estimating the host's parasitemia. Exp. Parasitol. **40**:427–431.
20. **Jackson, D. G., H. J. Windle, and H. P. Voorheis.** 1993. The identification, purification and characterization of two invariant surface glycoproteins beneath the surface coat barrier of bloodstream forms of *Trypanosoma brucei*. J. Biol. Chem. **268**:8085–8095.
21. **Masterson, W. J., D. Taylor, and M. J. Turner.** 1988. Topological analysis of epitopes of a variant surface glycoprotein of *Trypanosoma brucei*. J. Immunol. **140**:3194–3199.
22. **Mosser, D. M., and J. F. Roberts.** 1982. *Trypanosoma brucei*: recognition *in vitro* of two developmental forms by murine macrophages. Exp. Parasitol. **54**:310–316.
23. **Overath, P., D. Schell, Y. D. Stierhof, H. Schwarz, and D. Preis.** 1992. A transferrin-binding protein in *Trypanosoma brucei*: does it function in iron uptake?, p. 333–347. *In* J. A. F. Op den Kamp (ed.), Dynamics of membrane assembly. NATO ASI Series, vol. H 63, Springer Verlag, Berlin.
24. **Pinder, M., A. van-Melick, and G. Vernet.** 1987. Analysis of protective epitopes on the variant surface glycoprotein of a *Trypanosoma brucei brucei* (DiTat 1.3) using monoclonal antibodies. Parasite Immunol. **9**:395–400.
25. **Schell, D., R. Evers, D. Preis, K. Ziegelbauer, H. Kiefer, F. Lottspeich, A. W. C. A. Cornelissen, and P. Overath.** 1991. A transferrin-binding protein of *Trypanosoma brucei* is encoded by one of the genes in the variant surface glycoprotein gene expression site. EMBO J. **10**:1061–1066.
26. **Seyfang, A., and M. Duszenko.** 1993. Functional reconstitution of the *Trypanosoma brucei* plasma-membrane D-glucose transporter. Eur. J. Biochem. **214**:593–597.
27. **Shapiro, S. Z., and T. W. Pearson.** 1986. African trypanosomiasis: antigens and host-parasite interactions, p. 215–274. *In* T. W. Pearson (ed.), Parasite antigens: towards new strategies for vaccines. Marcel Dekker, Inc., New York.

27a. **Stierhof, Y.-D., and K. Ziegelbauer.** Unpublished results.

28. **Van der Ploegh, L. T. H., A. Bernards, F. A. M. Rijsewijk, and P. Borst.** 1982. Characterization of the DNA duplication-transposition that controls the expression of two genes for variant surface glycoproteins in *Trypanosoma brucei*. Nucleic Acids Res. **10**:593–609.
29. **Van Meirvenne, N., P. G. Janssen, and E. Magnus.** 1975. Antigenic variation in syringe passaged populations of *Trypanosoma* (Trypanozoon) *brucei*. I. Rationalisation of the experimental approach. Ann. Soc. Belge Med. Trop. **55**:1–23.
30. **Ziegelbauer, K., G. Multhaup, and P. Overath.** 1992. Molecular characterization of two invariant surface glycoproteins specific for the bloodstream stage of *Trypanosoma brucei*. J. Biol. Chem. **267**:10797–10803.
31. **Ziegelbauer, K., and P. Overath.** 1992. Identification of invariant surface glycoproteins in the bloodstream stage of *Trypanosoma brucei*. J. Biol. Chem. **267**:10791–10796.