T-Cell Responses to the Trypanosome Variant Surface Glycoprotein Are Not Limited to Hypervariable Subregions

Taylor R. Dagenais, Karen P. Demick, James D. Bangs, Katrina T. Forest, Donna M. Paulnock, and John M. Mansfield

Departments of Bacteriology and Medical Microbiology and Immunology; Microbial Sciences Building, 1550 Linden Drive, University of Wisconsin—Madison, Madison, Wisconsin 53706

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Variable subregions within the variant surface glycoprotein (VSG) coat displayed by African trypanosomes are predicted sites for T- and B-cell recognition. Hypervariable subregion 1 (HV-1) is localized to an internal amphipathic alpha helix in VSG monomers and may have evolved due to selective pressure by host T-cell responses to epitopes within this subregion. The prediction of T-cell receptor-reactive sites and major histocompatibility complex class II binding motifs within the HV-1 subregion, coupled with the conservation of amino acid residues in other regions of the molecule sufficient to maintain secondary and tertiary VSG structure, prompted us to test the hypothesis that Th cells may preferentially recognize HV-1 subregion peptides. Thus, we examined the fine specificity of VSG-specific T-cell lines, T-cell hybridomas, and Th cells activated during infection. Our results demonstrate that T-cell epitopes are distributed throughout the N-terminal domain of VSG but are not clustered exclusively within HV-1 or other hypervariable subregions. In contrast, T-cell-reactive sites were not detected within the relatively conserved C-terminal domain of VSG. Overall, this study is the first to dissect the fine specificity of T-cell responses to the trypanosome VSG and suggests that evolution of a conserved HV-1 region may be unrelated to selective pressures exerted by host T-cell responses. This study also demonstrates that T cells do not recognize the relatively invariant C-terminal region of the VSG molecule during infection, suggesting that it could serve as a potential subunit vaccine to provide variant cross-specific immunity for African trypanosomiasis.

The plasma membrane of African trypanosomes is covered by a dense surface coat comprised of variant surface glycoprotein (VSG) homodimers (4, 8–10, 45). VSG molecules are immunodominant antigens that elicit B- and T-cell responses capable of providing temporal protection for the host during infection (15, 20, 26, 40). B-cell responses directed at surface-exposed determinants of VSG eliminate parasites from the bloodstream, whereas polarized VSG-specific Th1-cell responses contribute to the production of gamma interferon (IFN-γ), a critical component of relative host resistance that controls the parasite burden within extravascular tissues (17, 20, 31). However, trypanosomes repeatedly evade complete immune elimination by switching their VSG coats through a process of antigenic variation. Replacement of VSG coats with antigenically distinct surface coats permits trypanosomes to escape from existing B- and T-cell responses and requires the host to make new temporally protective responses throughout infection.

VSGs are separated into different families based on N-terminal and C-terminal proteolytic domains, sequence homologies, and the number and distribution of cysteine residues (5). Alignment of different Trypanosoma brucei VSGs within class and type subgroups has demonstrated that the primary amino acid sequences of VSG N-terminal domains are extremely diverse. However, VSGs share secondary and tertiary structural elements and fold in a similar manner (3, 5, 6, 38). This occurs because critical amino acid residues are the same or similar at key structural sites within VSG molecules and likely reflects the need to maintain integrity of the VSG coat structure during the process of antigenic switching.

Previous studies have revealed the presence of defined “hypervariable” (HV) subregions in VSG molecules (3, 19, 38), in addition to conserved structural features. Sequence comparisons among members of the evolutionarily related T. brucei 117 VSG gene family revealed three well-defined regions of amino acid hypervariability (19). As predicted by VSG structural modeling, two of these HV regions (HV-2 and HV-3) map to solvent-exposed domains of VSG and are consistent with selective pressure leading to variability in exposed B-cell epitopes in the coat structure (Fig. 1A). A different HV region (HV-1) (Fig. 1A) is present within internal amphipathic α-helices of the VSG homodimer; sequence variation in this subregion is predicted to be the result of selective pressure from T-cell responses to peptides generated by antigen processing and presentation (3, 19). However, these predictions have not been tested experimentally, and the presence of such HV subregions is somewhat controversial since they do not seem to be conserved among different strains and isolates of Trypanosoma brucei (21, 32, 33). Sequence comparisons across VSG classes and types essentially revealed the presence of many microvariable sites rather than well-defined HV subregions (21, 32). However, this variability is clearly not random (21), and studies to date have not replicated the VSG sequence lineage study that revealed HV subregions for the T. brucei brucei 117 gene family.
Restriction of T-cell responses to specific VSG sub-sequences such as the HV-1 region is entirely plausible because (i) host T cells have the potential ability to recognize relatively small peptide sequences anywhere within the entire VSG molecule, but antigenically variant parasites cannot alter all identical or cross-reactive VSG peptides due to highly conserved secondary and tertiary structural features of VSGs; (ii) the absence of functional cross-reactivity in studies of VSG-specific Th-cell responses to date suggests that not all VSG peptides, including invariant sequences, serve as T-cell-reactive sites; and (iii) alterations in antigen-presenting cell (APC) function may occur, including the inability to process and present VSG to T cells as infection progresses (11; B. Freeman et al., unpublished data), which may result in altered patterns of VSG-specific T-cell reactivity. Thus, from both an immunological and a parasitological perspective, the hypothesis that T cells may be limited in their recognition of VSG peptides to HV subregions is both plausible and testable.

The discovery of VSG-specific T-cell responses serves as the experimental foundation for the present study (28, 29, 40).
Th-cell responses to VSG determinants were originally characterized in *Trypanosoma brucei rhodesiense* LouTat 1-infected animals (20, 40). In this model system, VSG-specific T-cell responses were mediated by CD4+ T-cell receptor (TCR) αβ+ cells, were major histocompatibility complex class II (MHC-II) restricted, and required processing of the VSG molecule by APCs in order to activate Th cells (11, 40); the production of IFN-γ by polarized VSG-specific Th1 cells was subsequently shown to be linked definitively to host resistance during infection (17, 20, 41). Thus, the discovery of protective T-cell responses to VSG gave credence to the idea that T cells may have provided selective pressure for the development and retention of specific HV subregions in VSGs. However, there is an inherent conundrum in these predictions and observations because VSG sequences contain numerous potential T-cell epitope sites as well as amino acid sequences predicted to bind to MHC-II molecules; these types of sites are present throughout the molecule and clearly are not restricted to HV-1 or other HV subregions. Thus, either APCs selectively process and present a subset of VSG peptides to T cells, as discussed above, or T cells exhibit a limited repertoire of receptors specific for VSG epitopes.

To determine the extent of VSG peptide recognition by T cells, we created VSG-specific T-cell lines and a panel of VSG-specific T-cell hybridomas derived from Th cells activated by exposure to *T. b. rhodesiense* LouTat 1 VSG during infection. Preliminary studies revealed that infected mouse dendritic cells (DCs) and, to a lesser extent, macrophages (MPs) presented VSG peptide–MHC-II complexes ex vivo to VSG-specific T cells from infected mice (11) and to T-cell lines and hybridomas; the magnitudes of different T-cell hybridoma responses to APCs from infected animals suggested that a variety of VSG peptides may be processed and presented during infection. In the present study, therefore, we determined the fine specificity of VSG-specific Th cells by utilizing T-cell hybridomas, Th cells from infected mice, and a peptide library based on the known sequence of the LouTat 1 VSG molecule. The fine specificity of VSG-specific T-cell hybridomas mapped to multiple peptides outside the HV-1 region, which were distributed throughout the N-terminal domain of the molecule. An examination of infected mouse T cells ex vivo revealed a wider spectrum of T-cell-reactive sites, some of which colocalized with the T-cell hybridoma-reactive sites, and others that revealed new T-cell-reactive sites that were similarly distributed throughout the VSG molecule. None of the T cells examined in this study recognized peptides associated with the conserved C-terminal domain, however.

Overall, these results represent the first direct evidence for specific Th-cell epitopes within the VSG molecule and show that T-cell-reactive sites within VSG homodimers map to multiple microvariable subregions rather than to a defined HV subregion. Furthermore, the possibility of targeting T cells to the relatively invariant C-terminal domain of VSGs raises the prospect of inducing cross-VSG protective immunity against African trypanosomiasis.

MATERIALS AND METHODS

**Mice.** H-2Kb-compatible female and male B10.BR/SgSnJ mice between 8 and 12 weeks of age were purchased from The Jackson Laboratories (Bar Harbor, ME) and used for all experimental infections. Trypanosomabibstables were expanded in outbred Swiss mice (Harlan Sprague-Dawley, Madison, WI) in order to provide parasites for the experimental infections. All animals were handled according to National Institutes of Health and University of Wisconsin—Madison guidelines and were housed in facilities accredited by the American Association for Accreditation of Laboratory Animal Care.

**Trypanosomes.** *Trypanosoma brucei rhodesiense* clone LouTat 1 was used for all infections and was grown from frozen stables as described previously (42). Briefly, Swiss mice were immunosuppressed with cyclophosphamide treatment (300 mg/kg body weight; Sigma, St. Louis, MO) (42) concurrent with intraperitoneal injection of LouTat 1 trypanosomes. After 5 days, infected blood was collected and trypanosomes were isolated as described below. Experimental mouse groups were infected by intraperitoneal injection of 10⁷ trypanosomes diluted in 10 mM phosphate-buffered saline (PBS), pH 8, containing 1% (wt/vol) glucose (PBSG).

**VSG purification.** Trypanosomes were passed over a PBSG-equilibrated DEAE-cellulose column (DE52; Fisher Scientific, Hanover Park, IL) in order to remove red blood cells (RBCs) and leukocytes (25). Parasites were eluted, washed several times in PBSG by centrifugation, and enumerated by use of a hemacytometer. VSG purification was performed as previously described (10, 37, 40, 46). Trypanosomes were resuspended to 10⁷ organisms/ml in 0.3 M zinc acetate containing 0.1 mM tosyl lysine chloromethyl ketone. Following a 15-min incubation at 4°C, trypanosomes were centrifuged at 3,000 × g at 4°C for 10 min, and the supernatant fluid was reserved at 4°C. An equal volume of 10 mM phosphate buffer containing 0.1 mM tosyl lysine chloromethyl ketone was used to resuspend the pellet. This mixture was incubated for 20 min at 37°C before centrifugation at 10,000 × g at 4°C for 15 min. The supernatant and previously reserved fluids were combined and centrifuged at 300,000 × g at 4°C for 1 h, and the resulting supernatant fluid was concentrated by centrifugation through a Centricon-30 column (Amicon Corp., Danvers, MA). The concentrated supernatant was subsequently passed over a DEAE-cellulose column equilibrated with 10 mM phosphate buffer, pH 8; VSG was eluted from the column and subjected to electrophoretic analysis. VSG was detected as a single 62-kDa band on sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions (see example in Fig. 1B).

**VSG peptide isolation, sequencing, and synthesis.** Purified VSG was subjected to digestion with clostridin (proteolytic site specificity, R-X-X) followed by conventional reversed-phase high-performance liquid chromatography (HPLC) and size-exclusion HPLC to obtain distinct VSG proteolytic fractions (Fig. 1B). Peptide fractions were subsequently analyzed by SDSL-polyacrylamide gel electrophoresis and by inclusion in culture with APCs plus VSG-specific Th-cell lines. Peptides from HPLC fractions exhibiting T-cell reactivity were subjected to N-terminal amino acid sequence analysis by the University of Wisconsin—Madison Biotech Facility, and the locations of the peptide sequences within the VSG were determined by overlay with the known *T. b. rhodesiense* LouTat 1 VSG amino acid sequence (38). The LouTat 1 VSG primary sequence was derived by reverse transcription-PCR (RT-PCR) cloning and sequencing to determine the expressed VSG gene sequence and by protein sequence analyses to confirm the start site of the mature protein as well as the overall substituents of the protein sequence, as we described previously (22, 38, 39; our unpublished protein sequence determinations). For the present study, overlapping peptides were synthesized to represent the entire LouTat 1 VSG molecule. VSG peptides of 15 amino acids in length with an 11-amino-acid overlap were synthesized by Sigma Genosys (St. Louis, MO). Lyophilized peptides were resuspended in dimethyl sulfoxide and dialyzed or diluted in PBS to prepare 1 mM stocks, which were aliquoted and stored at −80°C. These peptides were added to APCs as noted below.

**Flow cytometry.** Fluorescein isothiocyanate- or allophycocyanin-conjugated anti-CD3 and phycoerythrin-conjugated CD4 were purchased from BD Biosciences Pharmingen (San Diego, CA). Isotype-matched antibodies (Abs) with the proper fluorochromes were used as controls. T-cell lines or hybridomas were incubated with FcBlock (BD Biosciences) to block nonspecific staining prior to staining for cell surface antigens on ice for 30 min. Cells were washed twice with flow buffer (PBS-0.5% bovine serum albumin [BSA]), fixed in 1% paraformaldehyde (Polysciences), and analyzed on a FACSCalibur flow cytometer, using Cell Quest (BD Biosciences) and FlowJo (Tree Star, San Carlos, CA) software.

**Isolation of APCs.** APCs used in this study were derived from several sources. In some experiments (with T-cell hybridomas [see below]), naïve irradiated spleen cells were used, while in others purified DCs were derived from spleens of uninfected or infected mice for use. Adherent spleen cells, largely MPs, were isolated from uninfected mice as follows. Spleens were disrupted in complete RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 16 mM HEPEs, and 50 µg/ml gentamicin) and passed through 40-µm nylon filters to obtain a single-cell suspension. Cells were treated...
with ACK lysis buffer to remove erythrocytes, washed twice with complete RPMI medium, plated, and incubated for 2 h at 37°C in 5% CO₂. Plates were subsequently washed thoroughly with PBS to remove nonadherent cells, and MPs were harvested after incubation at 4°C for 15 min in ice-cold PBS by being scraped with a rubber policeman or being removed from plates by use of Accutase (Innovative Cell Technologies, Inc., San Diego, CA). MPs were washed, resuspended in complete RPMI medium, and characterized by flow cytometry prior to use.

Splenic DCs were isolated by enzymatic digestion of spleen tissue with DNase I (Invitrogen, Carlsbad, CA) and Liberase (Roche Applied Science, Indianapolis, IN) in complete RPMI medium for 30 min at 37°C and 5% CO₂. DNase I and Liberase concentrations were determined empirically for each day of infection to account for changes in spleen size and composition. After 5 min of incubation with EDTA (final concentration, 0.01 M), spleen fragments were passed through 40-μm nylon filters and washed with Ca²⁺- and Mg²⁺-free PBS-0.5% BSA-5 mM EDTA (MACS buffer); erythrocytes were lysed with ACK lysis buffer. The remaining cells were overlaid onto 14.1% Nycoplasma (Sigma) and spun at 1,900 rpm for 20 min at 4°C. For flow cytometry, low-density enriched cells were resuspended in PBS-0.5% BSA-2 mM EDTA-0.05% azide (flow buffer) and treated as described below. For DC isolation, low-density cells collected from the interface were washed twice with MACS buffer, labeled with anti-CD11c magnetic beads (Miltenyi Biotec Inc., Auburn, CA), and passed over two consecutive MACS columns (Miltenyi Biotec Inc.) according to the standard protocol provided by the manufacturer. CD11c⁺ DCs were washed and resuspended in complete RPMI medium prior to use.

VSG-specific Th-cell lines and T-cell hybridomas. For the purpose of deriving Th-cell lines, spleens were taken from mice 2 weeks after infection with T. b. rhodesiense LouTat 1 infection, as we described previously (40). The organs were disrupted in tissue culture medium, and the cell suspension was filtered through a 70-μm-pore-size nylon strainer to remove debris. Following hypotonic lysis of RBCs, cells were washed and plated at 1.25 × 10⁶/ml with 6.25 × 10⁵/ml APCs (adherent splenocytes harvested from naive B10.BR mice, as described above, were irradiated [3,000 R] as a source of APCs for these T-cell cultures) and 50 μg/ml VSG in medium supplemented with 20% FBS and 2 mM NAD⁺-methyl-L-arginine (Sigma). After 1 week of incubation at 37°C and 5% CO₂, proliferating cells were fed with DMEM-T (Dulbecco’s modified Eagle’s medium [DMEM] containing 10% FBS, 5% R-SN [Collaborative Biomedical Products], and 5% P388D1 cell supernatant containing interleukin-1 [IL-1]) and incubated for 2 weeks at 37°C and 5% CO₂. Live cells were collected using Histopaque-1077 (Sigma) and were cultured in DMEM-T at 10⁵ cells/ml with 10⁵ APCs/ml and 50 μg VSG/ml for 2 weeks; this step was repeated once more prior to use of TH-cell line. Cells were maintained in culture by repeated removal of dead cells and reculturing in DMEM-10% FBS at 10⁶/ml with 10⁵ APCs/ml and 50 μg VSG/ml.

For derivation of T-cell hybridomas, T lymphocytes were isolated from spleens of B10.BR mice after 1 week of infection with T. b. rhodesiense LouTat 1 by centrifugation over nylon wool columns or by cell sorting. T cells were comprised at a 1:1 ratio with BW5147 TCR αβ⁺ thymoma cells and with 50% polyethylene glycol 1500 (Sigma) to induce cell fusion. Cells were washed, resuspended in DMEM-20 containing syngeneic thymocytes (feeder cells), and incubated at 37°C and 5% CO₂. Nonhybridized cells were depleted by hypoxanthine-aminopterin-thymidine-supplemented DMEM-20, and proliferating cell populations were expanded. Candidate populations exhibiting reactivity to VSG were cloned by several rounds of limiting dilution and then reselected for VSG specificity and CD3/TCR expression.

TCR Vβ analysis. VSG-specific T-cell hybridomas were assessed for Vβ gene usage by RT-PCR. RNAs were extracted using Ultraspec RNA according to the manufacturer’s directions and were treated with DNase to remove contaminating DNA. RT-PCR was performed on all RNA samples by use of an Access RT-PCR kit (Promega, Madison, WI). Vβ gene segments were amplified using Vβ gene-specific oligonucleotide primers and a common Cβ primer previously published by Pannetier et al. (36). cDNA synthesis and subsequent cycles of PCR were carried out in a thermocycler under the following conditions. CDNA was synthesized during a 45-min incubation at 45°C, followed by a 2-min incubation at 94°C to VSG-specific T-cell lines (10⁷ cells) or T-cell hybridomas (10⁷ cells) in 50 μl VSG or 10 μM VSG peptides at 37°C and 5% CO₂. Supernatant fluids were collected after 24 h and stored at −20°C prior to analysis. For analysis of IL-2, 5 × 10⁶ IL-2-dependent CTLL-2 cells were cultured with 100 μl supernatant for 24 h, followed by the addition of 50 μl [H]thymidine (1 μCi/ml in complete RPMI medium) for another 24 h. IL-2 was measured by [H]thymidine incorporation by CTLL-2 cells (TIB-214; ATCC). In some instances, IL-2 and IFN-γ levels were determined by cytokine-specific enzyme-linked immunosorbent assay (ELISA; BD Biosciences). For all ELISA results, dedicated ELISA reader software was used to provide a linear curve that interprets all data along a standard protein curve to infinity. Experimental optical densities below the lower end of the standard range were interpolated as negative values.

The numbers of IFN-γ-secreting VSG-specific T cells present in primary lymphocyte populations were determined by enzyme-linked immunospot (ELISPOT) assay as we described previously (41), using ELISPOT kits purchased from R&D Systems (Minneapolis, MN). B10.BR mice were infected with 1 × 10⁶ trypanosomes; on day 5 of infection, cell suspensions were prepared from the spleens of infected as well as control uninfected mice. After lysis of RBCs, T cells were collected by passing cell suspensions over nylon wool columns as described above. Approximately 1 × 10⁶ irradiated APCs and 2 × 10⁵ T cells were aliquoted into wells of ELISPOT plates (R&D Systems) and stimulated with 10 μM of VSG peptide or 50 μg/ml purified VSG. The plates were incubated for 24 h in a humidified CO₂ incubator at 37°C. The ELISPOT assay was developed according to the manufacturer’s instructions, as follows. Plates were washed four times with PBS, and 100 μl of diluted IFN-γ detection antibody was added to each well. After an overnight incubation at 4°C, the plates were washed four times with PBS; diluted streptavidin-AP (100 μl/well) was added, and the plates were incubated for 2 h at room temperature. After the plates were washed, 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) chromogen (100 μl/well) was added, and the plates were incubated at room temperature for 1 h in the dark. The chromogen solution was washed from plates using double-distilled water, and the plates were air dried. Spot-forming cells were enumerated using a dissection microscope.

VSG structure. The primary structure of LouTat 1 VSG (GenBank accession no. X56643) was analyzed for predicted motifs by use of DNASTAR v6 software (Madison, WI). A model for the three-dimensional structure of the N-terminal domain of the LouTat 1 VSG sequence was calculated based on the X-ray crystal structure and conserved residues of the homologous MtTat 1.2 VSG molecule (PDB code 1VSG) (3). Default parameters were chosen for homology-based modeling in the “automated mode” of Swiss-Model (1). Structure figures were generated using PyMOL v1.1 (14); the V1-HV-2, HV-3 subregions and defined T-cell-reactive sites were mapped onto the three-dimensional (3D) structures.

RESULTS

VSG-specific Th-cell lines recognize multiple sites in the VSG N-terminal domain. Determination of T-cell specificity for VSG during African trypanosome infection was initiated with VSG-specific Th-cell lines derived from infection with LouTat 1 trypanosomes; T cells were established in culture and selected for responsiveness to VSG presented by APCs, as well as tested for CD3/TCR and CD4 expression, as we have previously described (40). The general peptide specificity of these cell lines was tested against different HPLC-separated peptide fractions of clostripain-digested VSG, using irradiated H-2Kb-compatible APCs from naive mice for presentation to T cells. As shown by one representative example in Fig. 1B, the uncloned Th-cell line 1-11 responded to intact soluble VSG as well as to several potential peptide fragments in fraction 3 of clostripain-digested VSG.

To further characterize the specificity of this representative Th-cell line, several distinct peptides from reactive HPLC fraction 3 were separated electrophoretically, extracted from gels, and partially sequenced. Mapping of these peptides onto the LouTat 1 VSG protein sequence revealed that one peptide region partially overlapped with the predicted HV-1 subregion—other peptides were distributed outside the HV-1 subregion of the molecule (Fig. 1B). This observation was the first indirect clue that Th-cell specificity may be directed to subse-
sequences other than, or in addition to, the HV-1 subregion of VSG. However, use of such uncloned T-cell lines did not enable us to identify the minimal reactive sites within the proteolytic fragments to which VSG-specific Th cells responded. An analysis of the LouTat 1 VSG sequence for structural motifs revealed that neither TCR nor MHC-II binding motifs were predicted to cluster specifically within the HV-1 (or other) subregion, but rather they were distributed throughout the LouTat 1 VSG molecule (see below). The difficulties in establishing and maintaining clonal Th-cell lines, coupled with the possibilities that Th cells with the strongest responses may predominate in culture and that proteolytic digestion of VSG may destroy a subset of T-cell epitopes, prompted us to create a panel of VSG-specific T-cell hybridomas with which TCR specificities could be identified by using overlapping peptides corresponding to the primary amino acid sequence of LouTat 1 VSG. Thus, mapping the fine specificity of T cells for VSG by this alternative means was clearly necessary.

**T-cell hybridomas recognize N-terminal but not C-terminal VSG epitopes.** VSG-specific T-cell hybridomas were derived from Th cells activated by infection of relatively resistant B10.BR mice (known VSG responders) (20) with *T. b. rhodesiense* LouTat 1. Thirteen hybridomas specific for LouTat 1 VSG were selected and characterized; all were VSG variant type specific and MHC-II restricted and expressed the CD3/TCR complex (data not shown). As an initial indicator of TCR diversity among the hybridomas, we performed RT-PCR with T-cell hybridoma RNAs to assess TCR Vβ usage (Table 1). A comparison of biological responses among several T-cell hybridomas that expressed the same Vβ chain suggested that these subsets actually were unique clones (Fig. 2). As shown, using APCs taken ex vivo from LouTat 1-infected mice, T-cell hybridomas 28A and 130A, which expressed the same Vβ chain, recognized their cognate antigens with different temporal patterns. Using APCs derived from the same infected mouse, 28A cells exhibited evidence for strong peptide recognition on days 4 and 7 postinfection, whereas 130A cells exhibited strong recognition on day 4 but a markedly diminished response on day 7. We were unable to characterize Vβ expression in two of the other hybridomas and identified two Vβ amplification products for another two hybridomas (Table 1).

The remainder of the hybridomas clearly expressed distinct Vβ genes, suggesting that a diverse TCR repertoire is generated in response to VSG determinants during infection.

To determine the fine specificity of VSG-specific T-cell hybridomas, each hybridoma was cultured with APCs from uninfected mice and each of 124 overlapping 15-amino-acid peptides spanning the LouTat 1 VSG sequence. Of the 13 hybridomas tested, all of which recognized peptides derived from purified VSG that had been processed and presented by APCs, 6 hybridomas displayed detectable IL-2 responses to defined VSG peptides. Table 2 lists the peptides that were recognized by T-cell hybridomas and their amino acid positions within the VSG protein sequence. Figure 3 shows representative profiles of T-cell hybridomas responding to each of three different peptide sequences identified as immunodominant T-cell epitopes by virtue of the fact that other T-cell hybridomas also recognized the same or an overlapping peptide sequence at that site; responses to specific peptides were also confirmed by IL-2 ELISA (data not shown). The distribution of T-cell-reactive peptides throughout the VSG molecule and outside the HV-1 region contradicts the hypothesis that T-cell responses are restricted to the HV-1 subregion of VSG, since it is clear that none of the peptides are clustered exclusively within a specific subregion of VSG. Several of the T-cell-
reactive peptides contained predicted T-cell epitope and MHC-II binding motifs (see below). Interestingly, all of the peptides identified as Th-cell epitopes were expressed at high levels by APCs taken directly from infected mice (Fig. 2 and 4) and may represent immunodominant epitopes (also see Fig. 5A and 6A). We were unable to determine the fine specificity of the remaining VSG-specific T-cell hybridomas, although these cells as well as the other T-cell hybridomas recognized VSG determinants that are processed by APCs in vitro, as well as VSG peptides that are naturally presented by DCs and MPs during infection ex vivo (see examples in Fig. 2 and 4). Thus, relevant VSG epitopes are generated during infection for presentation to T cells. However, one finding of note was that none of the T-cell hybridomas recognized epitopes within the relatively conserved invariant sequences of the VSG C-terminal domain (see Fig. 6B), providing the first direct evidence that T cells may not recognize peptides derived from this domain of VSGs.

VSG epitope specificity and diversity confirmed using infected mouse Th cells ex vivo. It was important to determine whether Th-cell populations activated in vivo during infection recognized the same spectrum of peptides as VSG-specific T-cell hybridomas. Although the hybridomas were derived from Th cells activated in situ, there may have been skewing of the “representative repertoire” during the fusion and selection processes. Therefore, T cells isolated directly from infected mice were cocultured with irradiated APCs from naïve mice plus purified VSG or one each of the VSG peptides used above for epitope mapping in IFN-γ ELISPOT assays. The results clearly show that Th1 cells from infected mice recognize the same spectrum of VSG peptides as that identified with the T-cell hybridomas and that immunodominant epitopes identified with T-cell hybridomas are representative of the natural Th-cell response (Fig. 5A and B and 6A and B). Furthermore, additional T-cell-reactive sites were identified that, although recognized by fewer IFN-γ-secreting T cells than the immunodominant sites, illustrate a broader repertoire of T-cell epitopes within VSG. Thus, it is clear that Th1 cells generated by exposure to VSG peptides that are processed and presented during infection are specific for an array of sites that are widely distributed throughout the N-terminal domain of the VSG molecule; these sites are not clustered exclusively within any subregion of VSG, including the buried alpha helix or the solvent-exposed subregions that are predicted to contain HV subregions (Fig. 1A). Modeling of all T-cell-reactive sites onto the 3D structure of LouTat 1 VSG revealed the spatial distribution and orientation of such sites within the N-terminal domain of the VSG structure (Fig. 6C). However, as with the T-cell hybridoma approach, T-cell-reactive sites were not detected within the conserved invariant sequences of the VSG C-terminal domain (Fig. 6B; note that a 3D model of this domain could not be derived for LouTat 1 VSG).

DISCUSSION

Analyses of VSG sequence and structure have influenced current thinking and experimental approaches to understanding B- and T-cell responses to this protective antigen during infection with African trypanosomes. Comparative crystallographic analyses and molecular modeling of Trypanosoma brucei VSG molecules revealed that antigenically distinct VSGs may fold into very similar structures (3, 34, 38). This structural conservation may reflect a need to maintain dense packing of VSGs within the trypanosome surface coat during switching,
when new VSG homodimers intercalate into the existing coat structure. Furthermore, maintenance of a dense and rigid molecular surface coat may be important both for limiting B-cell responses to surface solvent-exposed epitopes and for providing a T-cell-independent signal to B cells during early trypanosome population outgrowth (18). Another level of “conservation,” namely, the conservation of HV regions at specific sites within the general structure of related molecules, has been noted among different VSGs. Identification of two major HV regions (HV-2 and -3) that are solvent exposed suggests that selection for amino acid hypervariability within such regions in different VSGs may have occurred in order to evade antibody-mediated destruction (19). Another HV region, HV-1, located within an amphipathic α-helix of VSG monomers that is not exposed on the VSG surface coat (Fig. 1A), is also conserved among structurally related VSGs and is predicted to have been selected by Th-cell pressure (3, 5, 19, 30, 38). The conservation of both VSG structure and sites of amino acid hypervariability within a structural motif that displays predicted and functional peptide binding to MHC-II molecules led to the hypothesis that different VSGs may be processed similarly so as to preferentially present peptides from the HV-1 region to Th cells. Thus, our studies of Th-cell specificity were designed to determine

![Image of graph showing Th-cell responses to Trypanosome VSG and VSG peptides.](image)

**FIG. 5.** Infected mouse Th cells recognize LouTat 1 VSG and VSG peptides. (A) CD4^+^ T cells from mice infected with *T. b. rhodesiense* LouTat 1 were isolated and cultured with irradiated naïve mouse APCs and either purified VSG or one each of the VSG peptides spanning the LouTat 1 VSG sequence. T-cell responses were measured by ELISpot analysis, and the results are depicted as numbers of IFN-γ-expressing spot-forming cells (SFC). The VSG amino acid sequences to which T cells responded are noted above the peaks representing immunodominant sites. (B) T cells from infected mice were cultured with *T. b. brucei* 117 VSG, *T. b. rhodesiense* LouTat 1 VSG, or selected LouTat 1 VSG peptides. The responses were measured by IFN-γ ELISA and demonstrate the lack of variant cross-reactivity (40).
whether functional and protective MHC-II-restricted Th-cell responses to the VSG molecule are generated primarily against HV-1 region epitopes.

Th-cell lines and hybridomas were derived from VSG-specific Th cells activated during infection of relatively resistant B10.BR mice in order to map the specificity of these cells for discrete peptide sequences within the LouTat 1 VSG molecule. Our initial experiments using VSG-specific Th-cell lines suggested that Th cells may respond to peptide fragments that map outside the HV-1 subregion. Further analysis using VSG-
specific T-cell hybridomas and overlapping peptides spanning the LouTat 1 VSG sequence demonstrated specificity for multiple sites that were present throughout the N-terminal domain of the molecule. The number of different TCR Vβ chains expressed by VSG-specific T-cell hybridomas and the different patterns of peptide reactivity expressed by those T-cell hybridomas displaying identical Vβ chains (e.g., presumptive evidence for distinct Vα chains) clearly demonstrate that the VSG-specific T-cell response is not mono- or oligoclonal; such limited clonality might have been expected if T-cell responses were limited to a distinct HV subregion of VSG.

Confirmation of the T-cell hybridoma results was obtained by directly determining the VSG peptide-specific responses of infected mouse T cells, which showed that the same subsets of peptides were recognized by Th1 cells activated in situ and also that additional minor Th1-cell-reactive sites were detectable. Several T-cell responses mapped to peptides that overlapped with the HV-1 subregion, but the majority of T-cell-reactive sites were distributed throughout the N-terminal domain of the molecule, including the HV-2 and HV-3 subregions (Fig. 6).

It is unclear from these experiments whether all potential epitopes within the HV-1 region are processed or presented to T cells during infection. The specificity of several T-cell hybridomas, including two that recognize VSG peptides expressed at high levels in vivo (11), could not be identified using the overlapping VSG peptides prepared for this study. Additionally, some peptides may be expressed at levels too low to detect with the hybridomas in vitro or may be recognized by Th cells that did not withstand the hybridoma selection process. Therefore, the evolutionary advantage provided by maintenance of the HV-1 region has yet to be determined. As Field and Boothroyd (19) proposed, the amphipathic α-helix containing the HV-1 subregion may simply be able to accept extensive amino acid variation without detriment to the overall structure, or this region may also be involved in B-cell-specific responses, despite the fact that it is predicted to be buried in the surface coat structure. It is possible that early T-cell-independent B-cell responses to solvent-exposed HV-2 and HV-3 epitopes of the surface coat (18; 38) cause molecular “tufting” of the structure, which exposes regions of the VSG normally buried within the coat. One of these regions may be the HV-1 region.

Since some T-cell responses to VSG epitopes map outside the putative HV regions, one might predict a level of VSG cross-reactivity among antigenically distinct VSGs. Functionally, however, there is no evidence for such cross-reactivity in these experiments (see Fig. 5B, in which there is no demonstrable cross-reactivity of LouTat 1 VSG-specific T cells with the closely related T. b. brucei 117 VSG molecule) (18) or in our earlier VSG-specific Th-cell studies (40, 41). Thus, T-cell-reactive sites in VSGs probably represent variant sequence microheterogeneity among antigenically distinct VSGs (38). Aggregate VSG sequence database entries, as well as recent surveys of VSGs expressed by many different field isolates that included closely related trypanosome strains, suggest that such microheterogeneity is widespread in the N-terminal domains of VSGs (21, 32, 33). The data presented in our current study provide the functional evidence for such heterogeneity in terms of T-cell recognition.

Thus, it appears that natural selection for microheterogeneity throughout the VSG molecule resulted from B- and T-cell responses to exposed and buried residues. Interestingly, these findings bring into question the biological relevance of VSG gene modification resulting from segmental conversion among different silent or basic-copy VSG genes (2). New VSG genes resulting from such segmental conversion would contain large segments of distinct VSGs that, if expressed, would have to be unique in primary sequence in order to evade B- and T-cell recognition. The only way that such variant sequences could be expressed productively on viable trypanosomes during an ongoing infection would be if T cells were limited to recognizing specific (e.g., HV) subregions; otherwise, all potential target peptides in the new and old segments would have to differ for each such variant that arises. Based on our current results, therefore, we suggest that such segmental conversion among VSG genes would likely have a nonviable outcome for trypanosomes that express them during long-term infection in immunocompetent hosts.

The observation that VSG-specific T-cell responses are temporally limited during infection, being detectable within a few days of infection but largely undetectable after 10 days, has several bases. First, parasitemia representing the infecting LouTat 1 trypanosome population was eliminated by day 6 postinfection. Any parasites appearing after this time (e.g., on or after day 10) would be antigenic variants displaying a new VSG surface coat. Thus, VSG peptides derived from LouTat 1 would no longer be expressed significantly on APCs (11; Freeman et al., unpublished data). We also have shown that exposure of APCs to glycosylphosphatidylinositol residues of shed VSG renders the cells gradually unable to produce stable intracellular MHC-II–peptide complexes (Freeman et al., unpublished data). Thus, the absence of the prior VSG coupled with alterations in APC function contributes to the temporal nature of VSG-specific T-cell responses during infection.

Th-cell epitope selection may be influenced by a number of factors leading to TCR recognition of peptide–MHC-II complexes, including the antigen processing events that produce antigenic fragments and the binding of specific peptides within the MHC-II groove. Structural features such as disulfide bonds and noncovalent interactions influencing antigen stability have been associated with the efficacy of antigen presentation and epitope selection (7, 23, 27, 44). In addition, immunodominant T-cell epitopes have been associated with structurally stable sites in protein antigens; these sites are often flanked by unstable regions predicted to be preferentially cleaved during processing, thereby allowing adjacent stable regions to be exposed for binding to MHC-II (12, 13, 24). The stable sites could exist as a range of structures but tended to be hydrophobic due to the importance of hydrophobic residues in peptide binding to MHC-II (24). Although hydrophobic interactions may bias Th-cell epitopes to the protein interior, it has been suggested that peripheral epitopes may also bind MHC-II prior to proteolytic events that result in protein unfolding; the early binding to MHC-II could then prevent degradation of these epitopes during antigen processing in endosomal and lysosomal compartments (16, 35). Thus, theoretically, as shown by predicted epitope and MHC-II binding motifs, and practically, as shown by functional T-cell epitope mapping in this
study, a variety of peptides from different non-HV-1-specific subregions of VSG are presented to T cells (Fig. 6A, B, and C).

The absence of T-cell specificity for the relatively invariant C-terminal domain of VSGs is another key finding of this study. The fact that T cells did not recognize peptides from this domain in the peptide mapping approach is confirmed by the absence of any detectable cross-reactivity at the functional level when examining T-cell responses to different VSGs that share C-terminal domain homology (40) (Fig. 5B). In retrospect, it may be that an absence of T-cell reactivity to the C-terminal domain, coupled with the natural inaccessibility of antibodies to this buried region, resulted in a lack of selection for microheterogeneity within the C-terminal domain, thus largely maintaining sequence homogeneity within VSG type and class. The mechanism(s) that prevents potential T-cell recognition of this domain is unknown at present, but our recent studies on modulation of APC function during trypanosomiasis show a progressive inability to process and present new VSG peptides (11; Freeman et al., unpublished data). There is also the possibility that the C-terminal domain of VSGs may not be accessible to the enzymes that degrade the rest of the molecule during uptake and processing. Ultimately, these types of events may predispose APCs to display only a subset of N-terminal domain VSG peptides during infection. Thus, our work at once experimentally refutes the HV-1 subregion hypothesis but reveals, ironically, that the relatively invariant C-terminal subregion is not recognized by T cells. In other words, VSG-specific T-cell responses are limited to certain subregions, just not the one defined by the HV-1 hypothesis.

Overall, and in summary, this is the first study to characterize the specificity of Th cells activated during infection for distinct peptides of the VSG molecule. The demonstration that peptides from the HV-1 (or other HV) subregion are not preferentially presented to T cells during infection is a finding that prompts rethinking of the evolutionary advantage that this buried HV region may provide to the parasite, as well as the overall implications for conserved structure on processing and presentation of VSG peptides. Further analysis of Th-cell specificity and the identities of VSG-derived peptides presented during ongoing infection is necessary to understand how VSG is processed and presented by APCs, to identify the spectrum of immunodominant epitopes in different VSGs, and to understand the significance, if any, of the HV-1 region for host immune resistance. The discovery that T cells fail to recognize peptides associated with the relatively conserved C-terminal domain of VSGs opens the door to experimental approaches that may preferentially stimulate Th cells to provide cross-variant protection to the infected host. This is theoretically possible because although antibodies cannot access buried residues of the C-terminal domain, T cells could be generated specifically against peptides derived from this domain to provide variant cross-protection during African trypanosomiasis.

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References


