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Technical Note

Using *T. brucei* as a biological epitope-display platform to elicit specific antibody responses

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ABSTRACT

The African trypanosome (*Trypanosoma brucei*) is transmitted by the bite of the tsetse vector to the mammalian bloodstream where it exists as a completely extracellular parasite. As a result of this exposure, the parasite elicits a robust immune response that is almost exclusively antibody mediated, and is extremely specific to the trypanosome coat displayed on the surface. This coat is comprised of ~11 million copies of a single gpi-linked molecule (the variable surface glycoprotein or VSG) and can therefore be used as a powerful platform for the immunogenic display of antigenic determinants. Here we describe a method to display repetitive, ordered arrays of linear epitopes on the surface of *T. brucei* and to then use the engineered organisms to generate specific anti-epitope antibody responses, upon injection into mice. This method offers an alternative approach to generating anti-peptide antibodies, and could be a useful option in cases where more traditional methods have failed.

1. Introduction

T. brucei cells are densely covered with 5.5×10^6 homodimers of a single molecule, the variable surface glycoprotein (VSG). This VSG coat shields other surface proteins from host antibodies. At the same time, it presents a repetitive structure which greatly facilitates epitope presentation to the antibody producing B-cell lymphocytes and therefore elicits robust antibody responses (Overath et al., 1994). As a result, during infection of its mammalian host, trypanosomes are removed from the bloodstream through antibody binding (Borst, 2002).

Taking advantage of the ability to display more than one VSG on the surface as well as the natural ability of the trypanosome to elicit highly specific antibody responses to the predominant coat, we have engineered *T. brucei* to carry a chimeric coat composed of the predominant VSG, VSG 427-2 (also known as VSG221 or MITat1.2) of the Lister 427 strain and an internally expressed identical VSG but into which we

inserted in-frame the FLAG peptide epitope. We report here that exogenous peptides inserted at a number of different VSG surface loops leads to their display on the coat of the parasite. We have also immunized mice with engineered organisms (either live or formalin-fixed), and found that the immunization rapidly elicited specific anti-peptide antisera. Overall, these proof-of-principle experiments suggest the utility of the African trypanosome as a tool with which to generate epitope-specific antibody responses.

2. Materials and methods

2.1. Plasmid constructs

A DNA fragment comprising of full-length VSG427-2 (also known as VSG221 or MITat1.2) (residues 1–476) was amplified by PCR from cDNA derived from *Trypanosoma brucei* (Lister 427). The full-length VSG427-2 was used as a PCR template to generate VSG427-2 variants.

For overexpression of internally tagged VSG427-2 variants in *T. brucei*, (termed FLAG-VSG), tagged VSGs were cloned into the HindIII and BamHI sites of the pUB39 plasmid (generously provided by Dr. G. Cross, Rockefeller University).

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2.2. *T. brucei* strains, growth and transfection

The bloodstream-form trypanosomes derived from the Lister 427 (VSG427-2 expressing) strain were cultured in HMI-9 and transfected as described previously (Wirtz et al., 1999).

2.3. FACS analysis

T. brucei were prepared and stained for FACS according to standard procedures. The reagents used for staining cells for FACS analysis were an anti-VSG221-FITC conjugated rabbit polyclonal antibody (a gift of Dr George Cross), the monoclonal ANTI-FLAG® M2-FITC conjugated antibody (Sigma-Aldrich Cat. number F4049), and the PE labeled monoclonal anti-HA (Miltenyi Biotec Cat. number 120-002-687).

2.4. Parasitic infection and clearance of mice

The transgenic bloodstream-form trypanosomes derived from the Lister 427-2 (MITat1.2; VSG 221) used in this study were introduced into C57BL/6 mice by intraperitoneal (i.p.) injection. Specifically, we injected live $2\text{--}3 \times 10^3$ parasites, or 10^7 formalin fixed cells, in HMI-9, in a volume of 200 μL , using a 25G^{5/8} needle. Six days after infection, the mice were injected with 20 μg of G418, which has been shown to lyse the parasites and result in clearance (Murphy et al., 1993). Boosts were conducted 21 days after initial injection. The Rockefeller University Institutional Animal Care and Use Committee has reviewed and approved the details of this study (IACUC protocol number 10001).

2.5. ELISA

96 well microtiter plates were coated with 100 μL of a PBS solution containing a recombinant Flag tagged protein (10 ng/ μL) overnight. The wells were washed three times with PBS-0.5% Tween 20 and then blocked for 2 h with PBS-0.5% Tween 20-3% milk-5% sucrose. The plates were incubated with 100 μL containing various dilutions of serum in PBS-0.5% Tween 20-3% milk for 2 h followed by three washes with PBS-0.5% Tween 20. The plates were then incubated with a 100 μL of PBS-0.5% Tween20 containing a 1/5000 dilution of anti-mouse IgGs peroxidase conjugated antibody (Southern Biotech) for 1 h followed by three washes with PBS-0.5% Tween20. The plates were developed by adding 100 μL of ABTS chromogen substrate solution from Invitrogen and the OD was determined at 405 nm with an ELISA reader.

3. Results

Structural analysis of the N-terminal domain of Lister VSG 427-2 (VSG221 or MITat1.2), revealed a compact homodimeric structure (Blum et al., 1993). The fact that the loops are well defined in the structure as well as their resistance to proteolytic cleavage indicates that they contribute to the overall structural integrity and function of this molecule. To assess whether insertions of a short peptide sequence such as the FLAG epitope (DYKDDDDK) within these loops would be tolerated, we designed a series of internally tagged VSGs using VSG427-2 as a backbone (termed FLAG-VSGs; Fig. 1)

(Blum et al., 1993). In conjunction, we also created a series of internally tagged VSG427-2 constructs where we deleted portions of the VSG loop coding regions and replaced them with the short peptide sequences.

T. brucei parasites require surface expression of the VSG in order to survive. We therefore expressed the tagged VSGs from an internal (ectopic) location, while leaving the endogenous VSG intact. In so doing, issues of potential lethality (e.g. should the tagged protein hinder proper VSG assembly) were circumvented and an analysis of live trypanosomes which expressed FLAG-VSG, the endogenous VSG alone or a mixture of both could be carried out. We generated 2–5 clones per construct and tested their expression in whole trypanosome extract by dot blot (not shown) and FACS analysis to ascertain surface expression (Fig. 1b). Most of the *T. brucei* clones transfected with constructs where the FLAG sequence was inserted within VSG loops were well expressed, and some of those were also displayed on the surface (Fig. 1b, clones 1,3,5,6,7). Surprisingly, none of the clones transfected with constructs where the FLAG sequence replaced a portion of a VSG loop, were expressed (not shown), suggesting that these sequences are structurally important to the organism, and are not simply providing targets to the humoral immune system.

Overall, our chimeric clones fell into three categories, with regard to surface expression: (a) clones that displayed only VSG427-2 but not FLAG-VSG on the surface (e.g. Fig. 1b, clones 2, 4, 8, 9), (b) clones that displayed VSG427-2 as well as low levels of FLAG-VSG on the surface (which were further binned into clones expressing low levels of the chimeric protein vs. those containing a population with no surface expression and a subpopulation with low surface expression, (Fig. 1b, clones 1 and 6) and finally (c) clones that prominently displayed the FLAG-VSG chimera on the surface (Fig. 1b, clones 3, 5, 7). Since the FLAG-VSG coat was expressed ectopically, all our *T. brucei* clones also co-expressed VSG427-2 from the endogenous locus (a representative experiment for clone 7 is shown in Fig. 1c). We conclude that certain locations within the exposed VSG loops on the surface of *T. brucei* are not only tolerant to peptide insertions, but can also display exogenous peptides with high efficiency.

To assess whether the humoral immune system could respond to the FLAG-VSG coat by producing anti-FLAG antibodies, we injected a FLAG-VSG clone into C57BL/6 mice (clone 3, displaying high levels of FLAG on the surface – Fig. 1b). We confirmed infection by assessing the level of parasitemia through a tail nick five days after injection, and then drug cleared the trypanosomes from the bloodstream the next day (Murphy et al., 1993). We then collected small amounts of sera at several timepoints after clearance and assessed the antibody affinity maturation response by ELISA. As a comparison, we also injected untransfected *T. brucei* (ZM control), as well as FLAG peptide conjugated to KLH (not shown).

As expected, infected mice mounted a robust and specific immune response against the FLAG peptide that was prominently displayed on the surface of the chimeric trypanosomes (Fig. 2a). Additionally, this antibody response could be amplified after a single boost, with day 42 representing 21 days post boost (Fig. 2a). We conclude that

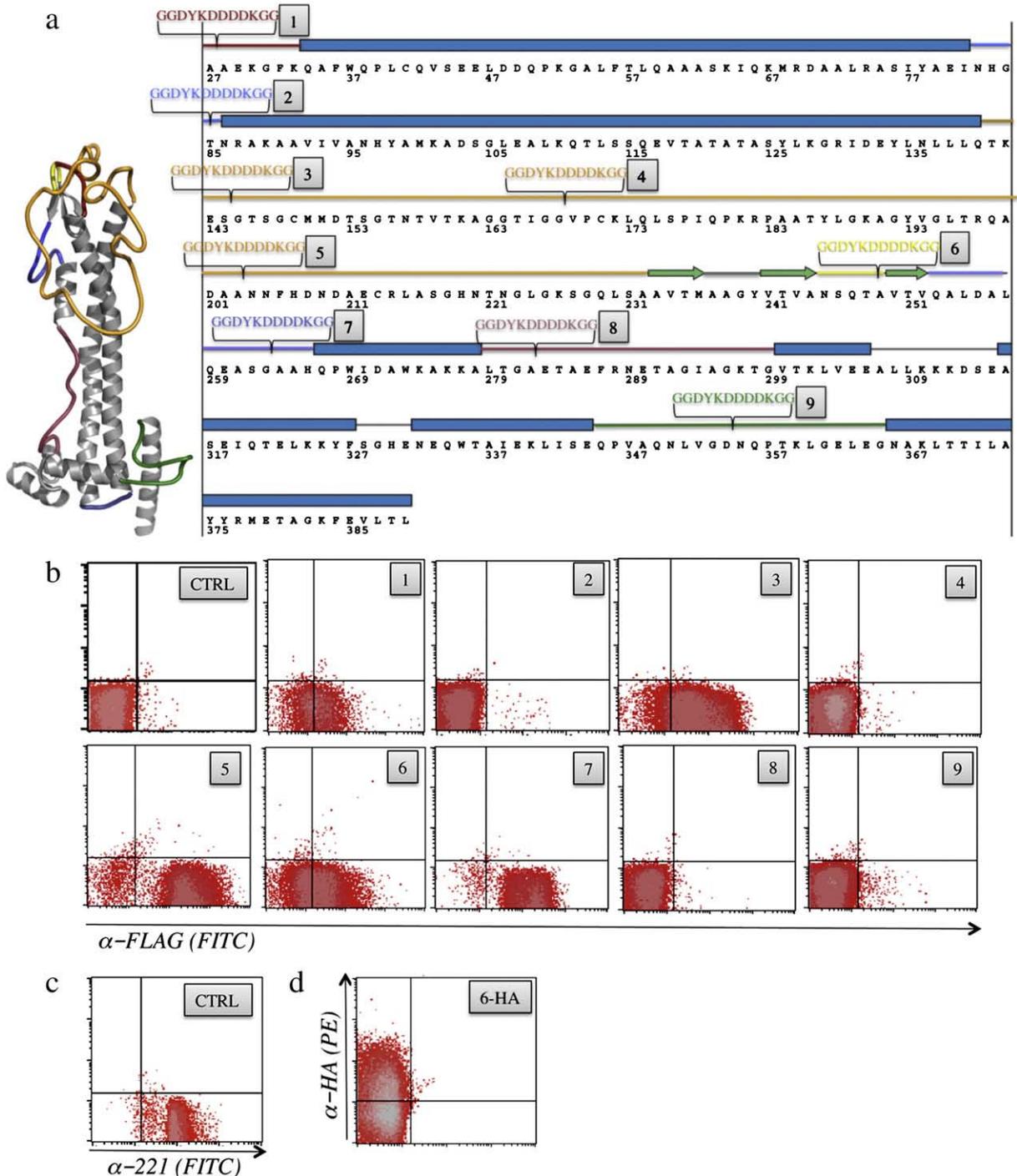


Fig. 1. Linear epitopes inserted at multiple positions within VSG loops can be displayed on the surface of *T. brucei*. (a) The structure of the N-terminal domain of VSG427-2 is shown on the left. Secondary structure elements are presented above the primary sequence of VSG427-2 where the blue rectangles represent alpha-helices and the green arrows represent beta-strands. The loops connecting the secondary structural elements are depicted in various colors that correlate the location of the loop on the structure with the secondary structure/primary sequence alignment. The location and sequence of the epitope insertions are presented within the single horizontal bracket above the sequence of VSG427-2 followed by a number which represents the transgenic trypanosome that expresses the chimeric VSG with the epitope in the indicated position. (b-d) Analysis of VSG427-2 chimeric surface expression by flow cytometry. Plots in (b) are stained with an anti-FLAG antibody conjugated to fluorescein (anti-FLAG-FITC); (c) represents the untransfected strain, expressing VSG427-2 (as assessed by surface staining with an anti-221-FITC antibody); (d) represents cells transfected with HA peptide; surface expression is assessed by staining with an anti-HA antibody conjugated to phycoerythrin (PE).

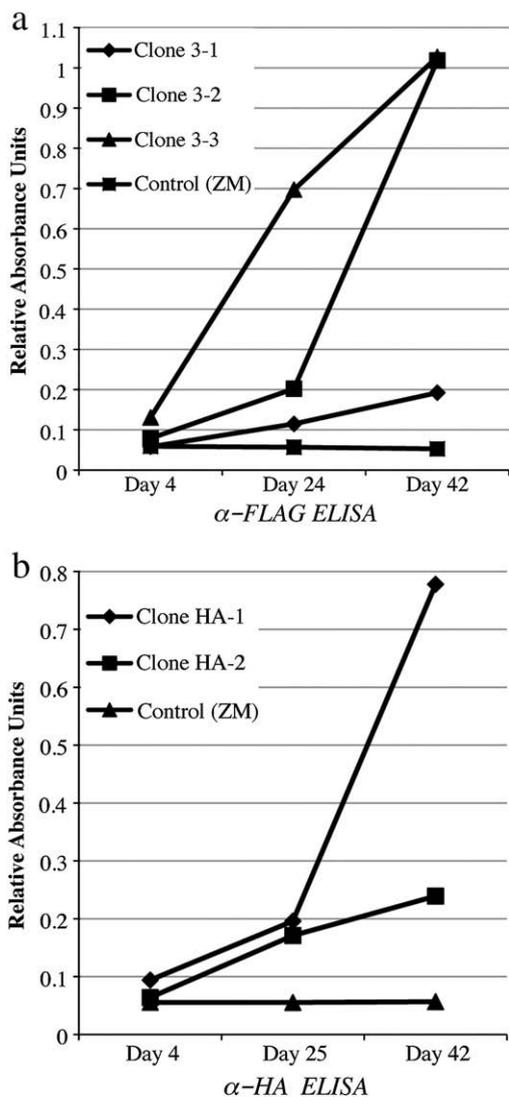


Fig. 2. Inoculation of mice with chimeric trypanosomes elicits specific anti-epitope antibodies. (a) Determination of the presence of anti-DYKDDDDK IgGs in the serum of immunized mice at various time points, by standard ELISA. Mice were boosted 21 days after initial immunization. (b) Similar as (a) with the exception that the presence of anti-YPYDVPDYA IgGs was determined.

chimeric trypanosomes can elicit a specific antibody response against an exogenous peptide displayed on their surface coat.

In the aforementioned experiments, live trypanosomes were injected into the host. To address whether live trypanosomes are required for stimulation of an immune response against an epitope in our tagged VSG, we generated freshly formalin fixed organisms. Trypanosomes can be fixed after a short treatment with formalin, however formalin fixation is likely to interfere with any epitope that contains lysines (such as FLAG). To assess whether fixed chimeric trypanosomes can elicit potent antibody responses such as those observed with the live organisms, we engineered trypanosomes to carry a chimeric VSG coat that displayed the HA peptide (YPYDVPDYA) on the surface (Fig. 1c, same

location as clone 3), a peptide which does not contain lysines. We were able to select a number of clones expressing HA-VSG on the surface (Fig. 1d and not shown). We then fixed one of the HA-VSG clones and the equivalent FLAG-VSG clone (Fig. 1b clone 3), and injected 10^7 fixed cells into C57BL/6 mice. These mice did not develop parasitemia, as expected (data not shown). However, they did mount a specific antibody response against HA (Fig. 2b), though not against FLAG (not shown). We conclude that fixed trypanosomes can also be used to elicit peptide-specific immune responses.

4. Discussion

T. brucei Lister 427 is a mammalian parasite with a completely extracellular life cycle, and a potent ability to stimulate the humoral immune response. Though it is one of the causative agents of N'agana in cattle, the Lister 427 strain is not infectious to humans, where at least two serum factors, the lipoprotein apoL1 as well as haptoglobin act as trypanosome lytic factors, offering natural immunity against this organism (Vanhamme et al., 2003; Vanhollebeke et al., 2008; Molina-Portela et al., 2008). We report here that host inoculation with *T. brucei* Lister 427 engineered to display peptide epitopes from exogenous sources, leads to the generation of antibody responses specific to the peptide. These antibodies can be elicited both after injection of live trypanosomes (followed by drug clearance), and importantly, also after injection of trypanosomes fixed with either formalin (Fig. 2b) or glutaraldehyde (not shown).

In vivo, inoculation with *T. brucei* leads to a neutralizing antibody response toward the prevailing coat, and to exquisite and long lasting memory, such that it is impossible to infect a mouse with a specific parasite after it has been exposed to it once (Dempsey and Mansfield, 1983). It is therefore possible that inoculation with engineered trypanosomes displaying peptides derived from pathogens could also function as a vaccine, in situations where high titers of specific, neutralizing antibody responses are desirable. However, whether inoculation with engineered parasites can protect the host against subsequent infection with the pathogen from which the peptide epitope was derived (e.g. influenza) remains to be seen.

While it is not clear what about trypanosomes elicits high affinity antibody responses in the host (a notion potentially related to the extremely high density, repetitive and ordered arrays of more than 10^7 epitopes displayed on the surface of the parasite), our experiments offer proof-of-principle of the concept that we can potentially manipulate such responses to our benefit. This should provide incentive for the study of otherwise neglected organisms, as understanding the host response they produce may be of biomedical utility in designing novel and effective therapies toward a range of human diseases.

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