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Efficient Targeting of Conserved Cryptic Epitopes of Infectious Agents by Single Domain Antibodies

AFRICAN TRYPANOSOMES AS PARADIGM*

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Antigen variation is a successful defense system adopted by several infectious agents to evade the host immune response. The principle of this defense strategy in the African trypanosome paradigm involves a dense packing of variant surface glycoproteins (VSG) exposing only highly variable and immuno-dominant epitopes to the immune system, whereas conserved epitopes become inaccessible for large molecules. Reducing the size of binders that target the conserved, less-immunogenic, cryptic VSG epitopes forms an obvious solution to combat these parasites. This goal was achieved by introducing dromedary Heavy-chain antibodies. We found that only these unique antibodies recognize epitopes common to multiple VSG classes. After phage display of their antigen-binding repertoire, we isolated a single domain antibody fragment with high specificity for the conserved Asn-linked carbohydrate of VSG. In sharp contrast to labeled concanavalin-A that stains only the flagellar pocket where carbohydrates are accessible because of less dense VSG packing, the single domain binder stains the entire surface of viable parasites, irrespective of the VSG type expressed. This corroborates the idea that small antibody fragments, but not larger lectins or conventional antibody fragments, are able to penetrate the dense VSG coat to target their epitope. The diagnostic potential of this fluorescently labeled binder was proven by the direct, selective, and sensitive detection of parasites in blood smears. The employment of this binder as a molecular recognition unit in immuno-toxins designed for trypanosomosis therapy becomes feasible as well. This was illustrated by the specific trypanolysis induced by an antibody::β-lactamase fusion activating a prodrug.

Several infectious agents, *e.g.* human immunodeficiency virus, *Hepatitis*, *Plasmodium*, and *Trypanosomes*, have acquired a system of antigenic variation to circumvent the immune response of their host (1–4). The paradigm of this defense mechanism can be found in the African trypanosomes (4–6). The key player in their antigenic variation mechanism is the variant-specific surface glycoprotein (VSG)[1] dimer of which 10^7 identical copies form a dense layer covering the entire surface of the unicellular parasite (7, 8). At regular time intervals a new VSG type appears on the surface of the parasite, preventing recognition of the parasites by an upcoming anti-VSG response directed against the previous VSG type. Obviously, the trypanosomes possess a vast repertoire of VSG genes and multiple diversification mechanisms to express one VSG after the other so that only one type is expressed at the time (5, 9, 10).

The comparison of the primary structures of VSGs taking into account the position and number of conserved cysteine residues and *N*-glycosylation sites revealed the presence of three VSG subclasses represented by AnTat 1.1 (class I), MITat 1.1 (class II), and MITat 1.5 (class III) (11, 12). However, even VSGs belonging to the same subclass expose only unique and immunogenic regions to the solvent when present on the coat of the parasite (13–15). Therefore, trypanosomes have adopted a strategy of actively forcing the immune system to produce antibodies against regions that change continuously. Consequently, most monoclonal antibodies raised against VSG recognize a determinant with an accessible location on the parasite (13, 14). Isolation of a monoclonal antibody with a pan-reactivity toward multiple VSGs on viable parasites appears to be far more challenging, because the conserved epitopes become cryptic upon the assembly of VSGs on the membrane of the parasite. To be effective as an anti-trypanosome agent it seems that molecules are required that infiltrate the dense VSG coat to reach a conserved epitope common to the majority of the VSG molecules. The VSGs contain such conserved epitopes, *e.g.* an Asn-linked high mannose oligosaccharide ($GlnNAc_2$-Man_{5-9}) is conserved among all VSG of *Trypanosoma brucei brucei* subspecies (16). This particular carbohydrate epitope should be a preferred target to design specific drugs because it is absent from glycoproteins of the parasite host (17).

To circumvent the inaccessibility of the conserved epitopes we introduced dromedary antibodies. The serum of *Camelidae* contains a high proportion of functional antibodies devoid of light chains (18). The antigen-binding fragment of these so-called heavy-chain antibodies (HCAbs) is comprised in one single domain of only 15 kDa. This dedicated variable domain referred to as VHH is the smallest intact antigen-binding frag-

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[1] The abbreviations used are: VSG, variant-specific surface glycoprotein; CCM, 7-(4-carboxibutamido)-cephalosporin; FITC, fluorescein isothiocyanate; Fv, variable fragment; HCAb, heavy-chain antibody; PBS, phosphate-buffered saline; PDM, phenylenediamine mustard; TNF-α, tumor necrosis factor-α; VHH, variable fragment of heavy chain of HCAb; ELISA, enzyme-linked immunosorbent assay; DAPI, 4′,6-diamidino-2-phenylindole.

ment derived from *bona fide* antibodies (19). After immunization of dromedaries, or llamas, antigen-specific single domain VHHs can easily be isolated by the use of the phage-display technology (19–21). Because VHHs are about 10× smaller than conventional antibodies it is tempting to speculate that these recombinant antibody fragments exhibit a more efficient VSG coat-penetrating capacity and reach epitopes that are inaccessible for conventional antibodies. In addition, it has been reported that the antigenic regions for VHHs or HCAbs on a protein are different from those for the antigen-binding site of conventional antibodies (20, 21). This aspect, together with their smaller size, suggests that VHHs constitute a yet unexplored pool of candidates to target new epitopes such as those scattered on the surface of living trypanosomes that are non-antigenic or inaccessible for conventional antibodies. The benefits of VHHs in this respect should not only be limited to African trypanosomes but could be extended to include other infectious agents relying on an active antigenic variation mechanism (1–3).

Here we report on the isolation of a VHH, specific for the conserved carbohydrate epitope of VSGs. Unlike other, larger molecules, it is shown that this VHH penetrates the dense VSG coat of the living parasite and stains trypanosomes independent of the expressed VSG class. The efficacy of this antibody fragment as a small molecular recognition unit in a more complex construct opens promising routes to develop new diagnostic and therapeutic tools to combat trypanosomosis. An illustrative example is included of how this might be accomplished.

EXPERIMENTAL PROCEDURES

Purification of VSG AnTat 1.1, MITat 1.1, MITat 1.5, and Deglycosilated VSG—Frozen stabilates of *Trypanosoma brucei brucei* bloodstream parasites expressing the respective VSG were expanded by infection of rats (Charles River). Rats with systemic parasitaemia (typically 4–5 days post infection) were exsanguinated, and parasites were purified from heparinized blood by DEAE-cellulose (DE52, Whatman) chromatography. VSG was isolated via ion-exchange chromatography and gel filtration (7, 22). Deglycosilated AnTat1.1 was prepared by incubating 100 μg VSG for 16 h at 30 °C with 10 units of *N*-glycosidase-F (Roche Applied Science). Deglycosilated protein was recovered from the flow-through of the sample passed over concanavalin-A column (Amersham Biosciences) equilibrated with PBS and was further purified by gelfiltration.

Dromedary Immunization—A dromedary (*Camelus dromedarius*) received six injections of 1 mg VSG AnTat 1.1 at weekly intervals (20). 45 days after the first injection serum was collected and separated into its different IgG subclasses by differential adsorption on HiTrap-Protein-A and HiTrap-Protein-G columns (Amersham Biosciences) as described previously (21). At the same time, anti-coagulated blood was collected to isolate lymphocytes with Uni-Sep (Sopagen). Peripheral blood lymphocytes were counted, and aliquots of 5×10^6 cells were pelleted and stored at −80 °C until further use.

Dot-blot Analysis—Each VSG (2 μg) (AnTat 1.1, MITat 1.1 or MITat 1.5) was spotted onto a nitrocellulose membrane and dried, and residual protein-binding sites were blocked for 2 h with PBS with 1% (w/v) bovine serum albumin. The membrane was incubated sequentially with one of the fractionated dromedary IgG isotype (5 μg/ml in PBS), a rabbit anti-dromedary IgG serum, and a goat anti-rabbit-IgG antibody conjugated to horseradish peroxidase (Sigma). In between the successive 2 h incubations was a washing step with PBS-0.1% Tween 20. Thirty minutes after adding the substrate (methanol/4-chloro-1-nafthol in PBS/H_2O_2) the reaction was stopped by washing the membrane with water.

Solid-phase Binding ELISA—Different VSG (AnTat 1.1, deglycosilated AnTat 1.1, MITat 1.1, or MITat 1.5) at 1 μg/ml 0.1 M $NaHCO_3$, pH 8.2, were coated overnight (4 °C) to 96-well plates. Residual protein-binding sites were blocked (2 h, room temperature) with 1% casein in PBS. After incubation with serial dilutions of dromedary IgG subclasses, bound dromedary IgG was detected with a rabbit anti-dromedary IgG serum and a goat anti-rabbit IgG antibody conjugated to alkaline-phosphatase (Sigma). Ten minutes after addition of the substrate *p*-nitrophenyl phosphate (Sigma), the optical density was measured at 410 nm.

The carbohydrate specificity of our VHHs was tested by ELISA. Wells of peptide immobilizer plates (SanverTech) were coated overnight (4 °C) with oligomannose-9 (Oxford Glycoscience) (0.1 μg/ml 0.1 M $NaHCO_3$, pH 8.2). Overcoating was omitted and the VHH (in triplicate) was loaded in 1:2 serial dilutions starting at 10 μg/ml. Bound VHH was detected using a mouse anti-His antibody and a goat anti-mouse IgG antibody conjugated to horseradish peroxidase. Thirty minutes after adding peroxidase substrate, the reaction was stopped with 0.1 N H_2SO_4 and the optical density was measured at 450 nm (690 nm was used as reference filter).

For the concanavalin-A competition experiment, we coated VSG AnTat 1.1 (1 μg/ml 0.1 M $NaHCO_3$, pH 8.2) overnight at 4 °C on 96-well plates. Residual protein-binding sites were blocked for 2 h at room temperature with 1% casein in PBS. The VHH (starting from 10 μg/ml, in triplicate) was serially diluted in PBS or 50 μg concanavalin-A/ml PBS and applied to the wells. Detection of bound VHH was as for the carbohydrate ELISA.

VHH Library Construction and Selection of Binders—The preparation of mRNA from lymphocytes, cDNA generation, VHH amplification (with oligo-dT primers), and cloning into a phage-display vectors was performed as described (21). Retrieval of VSG specific VHHs was done by three consecutive rounds of panning on antigen immobilized at 10 μg/well in microtiter plates (21). After the last panning individual colonies were picked and expression of the VHH gene was induced with 1 mM isopropyl β-D-thiogalactopyranoside. The recombinant VHH, extracted from the periplasm, was tested for antigen recognition by ELISA.

Construction of the cAb-An33::β-lactamase Conjugates—The cAb-An33::β-lactamase conjugate was constructed by PCR amplification, and insertion of the cAb-An33 sequence, the llama γ2c hinge sequence, and the β-lactamase gene followed by a His_6 tag between the NcoI and EcoRI sites of pHEN4 vector (20, 21). Firstly, the particular his-tagged β-lactamase was amplified from *Enterobacter cloacae* P99 strain (23) by PCR using primers "βL forward" (5′-CATGCCATGGGC-ACGCCAGTGTCAGAAA AA-3′) and "βL reverse" (5′-CGCGAATTCT-TAATGATGATGATGATGCTGTAGCGCCTCGAGG-3′) (restriction sites underlined) and ligated as a NcoI-EcoRI fragment into pHEN4. Secondly, the cAb-An33 gene linked to the llama γ2c hinge gene (19) was engineered as a NcoI-NcoI fragment using primers "A4Short forward" (5′-CATGCCATGACTCGCGGCCCAGCCGGCCATGGC-3′) and "CEAMH reverse" (5′-CATGCCATGGGAGCTTTGGGAGCTTTGG-AGCTGGGGTCTTCGCTGTGGTGCGCTGAGGAGACGGTGACCTGG-GT-3′) (restriction sites underlined) encoding the 15-amino acids-long llama γ2c hinge (AHHSEDPSSKAPKAP). The plasmid containing the cAb-An33 gene, as obtained after the pannings, was used as template. The PCR fragment was digested by NcoI and ligated in the expression vector containing the β-lactamase gene, linearized by NcoI.

Expression and Purification of Single Domain Antibodies and Conjugates—VHH genes of the clones that scored positive in ELISA were recloned into an expression vector pHEN6 as NcoI-BstEII fragments (21).

Production and purification of recombinant antibody fragments and of the cAb-An33::β-lactamase conjugate was performed as described (21, 24). Recombinant protein was > 95% pure as evaluated by Coomassie-stained SDS-PAGE. The absorption at 280 nm of the fractions and the extinction coefficient, as calculated from the amino acid sequence, were used to determine the protein concentration.

Immunofluorescent Microscopy and Flow Cytometry Analysis—The VHH, polyclonal rabbit antibodies and cAb-An33::β-lactamase conjugate were FITC-labeled (25), and the amount of labeling was determined spectrophotometrically. The bloodstream-form of *T. brucei brucei* (AnTat 1.1, MITat 1.1, and MITat 1.5) was used in immunofluorescent microscopy and flow cytometric analysis. Aliquots of 10^7 parasites were incubated for 30 min in an ice-bath with 10% normal rabbit serum/PBS prior to adding different FITC-labeled VHHs (5 μg), FITC-labeled polyclonal rabbit anti-VSG, or FITC-labeled concanavalin-A (Sigma) (5 μg/ml). After 30 min the parasites were pelleted and extensively washed with PBS/10% normal rabbit serum to remove unbound antibodies and analyzed in FACSvantage Fluorescence-Activated Cell Sorter (BD Biosciences) or by immunofluorescence microscopy (Nikon ECLIPSE E600 with phase contrast, 40–100× magnification). Two μl DAPI staining (Roche) was added to 10 μl sample prior to microscopic imaging with the corresponding filter. The same field was imaged with the FITC filter and both images were superimposed.

Trypanolysis Assay—Purified parasites (10^6 parasites/ml phosphate-saline-glucose buffer) incubated with TNF-α (10^4 units/ml) were monitored for 6 h. The experiment was repeated in absence or presence of cAb-An33 or cAb-An04 (1 μg/ml) added to the parasite culture 1 h prior to TNF-α addition. The data shown are typical results from 3 independ-

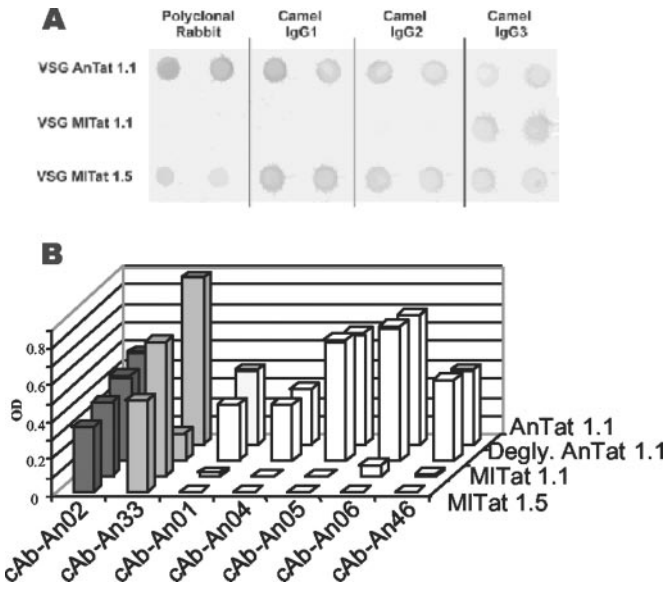

FIG. 1. **Antigen specificity of rabbit and dromedary IgGs and dromedary VHHs.** *A*, dot-blot analysis of polyclonal rabbit IgG and camel conventional antibodies (*IgG1*) and heavy-chain antibodies (*IgG2* and *IgG3*) on VSGs of different classes, *i.e.* AnTaT 1.1, MITat 1.1, and MITat 1.5. *B*, ELISA signals on AnTat 1.1, deglycosilated AnTat 1.1, MITat 1.1, and MITat 1.5 obtained with different recombinant VHHs retrieved from pannings of the "immune VHH library."

ent experiments performed in triplicate (average standard deviation 1–2%).

Cytotoxicity Assays—The LD_{50} of the phenylenediamine mustard parental drug (PDM) and the 7-(4-carboxibutamido)-cephalosporin mustard prodrug (CCM) were determined by incubating 10^6 *T. brucei brucei* AnTat 1.1 parasites (2.5 h, 30 °C) in phosphate-saline-glucose buffer with PDM or CCM (0–150 μM) (26).

To test the toxic effect of the VHH conjugated to β-lactamase and prodrug we incubated 10^6 *T. brucei brucei* AnTat 1.1 or MITat 1.5 parasites with 5 μg cAb-An33::β-lactamase (1 h, 4 °C). After washing off unbound material, various concentrations of CCM were added and the surviving parasites were counted. This experiment was repeated 7 times in triplicate, and congruent results were obtained (standard deviation of 1%, according to *t* test analysis).

RESULTS

Identification of VSG-specific VHHs—A dromedary and a rabbit were immunized with VSG purified from *T. brucei brucei* AnTat 1.1. The conventional antibody subclass (*i.e.* the IgG1), and the HCAb subclasses (*i.e.* IgG2 and IgG3) were fractionated from the dromedary serum (18, 21). These fractionated dromedary IgGs and the rabbit anti-AnTat 1.1 serum were used in a dot-blot assay to evaluate their capacity to recognize epitopes shared by the three different VSG classes (I-III), represented by AnTat 1.1, MITat 1.1, and MITat 1.5, respectively (11, 12) (Fig. 1*A*). The conventional antibodies from rabbit and dromedary, as well as the HCAb of the IgG2 class, all recognize epitopes on the VSG AnTat 1.1 and MITat 1.5. Remarkably, the HCAbs of the IgG3 isotype bind all three VSG classes to a similar extent. Hence other parts of the immunogen (AnTat 1.1) seem to be antigenic for the IgG3. Moreover, from the signal intensity it is inferred that the dromedary IgG3 isotype contains a detectable titer of antibodies that associate with epitopes common to VSGs of different classes.

Because the antigen-binding fragment of a dromedary HCAb is comprised within one single domain (*i.e.* the VHH) it was decided to isolate these small molecular recognition units via recombinant phage-display technology. The VHH repertoire present in an aliquot of 5×10^7 lymphocytes was cloned in a phage-display vector (20, 21, 27). Pannings of this VHH library with immobilized AnTat 1.1 yielded seven VSG-specific VHHs (GenBank™ accession numbers AY263485-AY263491). All these VHH genes encoded the framework-2 hallmark amino acids that distinguish the variable domain of a heavy chain of a HCAb from that of a conventional antibody (19). These seven VHHs were produced in *Escherichia coli*, purified, and their specificity and possible cross-reactivity with other VSGs was analyzed on AnTat 1.1, MITat 1.1, MITat 1.5, and deglycosilated AnTat 1.1 via solid-phase ELISA (Fig. 1*B*). Five VHHs recognize AnTat 1.1, independent of its glycosilation status, but fail to bind the MITat 1.1 and MITat 1.5. These VHHs are probably directed against unique epitopes present in the hypervariable region of AnTat 1.1. Interestingly, two VHHs, the cAb-An02 and cAb-An33, associate with all three VSG molecules. However, the antigen-binding capacity of the former is hardly influenced upon deglycosilation of the antigen, whereas the latter interacts poorly with the deglycosilated AnTat 1.1.

Carbohydrate Specificity of cAb-An33—The binding properties of cAb-An33 were further analyzed because it is better expressed than cAb-An02, gives higher signals in ELISA, and seems to recognize the carbohydrate moiety of VSG, a part of the molecule that is expected to be solvent accessible in the trypanosomal coat. The carbohydrate specificity of cAb-An33 was confirmed by competition with lectins, by inhibition of TNF-α trypanolysis, and by direct binding to VSG peptide fragments or oligosaccharides.

First, the binding curves of a serial dilution of the cAb-An33 from 10 to 0.01 μg/ml in a solid-phase ELISA with coated AnTat 1.1 are indicative for an affinity in the nM range. The binding is, however, competed by the presence of concanavalin-A (Fig. 2*A*), a lectin known to bind the carbohydrates attached to VSG (28). Because a direct interaction between the cAb-An33 and concanavalin-A could not be demonstrated in an ELISA approach nor by biosensor, it is clear that the lectin competes specifically with the antibody fragment for its epitope. The association of any other VSG-specific VHH with AnTat 1.1 is not impeded by an excess concanavalin-A.

Second, the interaction of TNF-α and the carbohydrate moiety of the VSG, probably that exposed in the flagellar pocket, induces rapid lysis of parasites (29, 30). This *in vitro* TNF-α-mediated *T. brucei* AnTat 1.1 lysis is effectively inhibited by the presence of cAb-An33 (Fig. 2*B*). It is a specific inhibition because the co-incubation of TNF-α with any of the other VSG-specific VHH, *e.g.* cAb-An04, failed to protect the trypanosomes from lysing. There is no indication for a TNF-α and cAb-An33 interaction, so the antibody fragment and the area harboring the lectin-like activity of TNF-α compete with each other for the same target.

Third, from all peptides fractionated after digesting AnTat 1.1 with various proteases (30), only the fractions containing peptides carrying carbohydrates (revealed by orcinol staining) give a signal in a direct ELISA with cAb-An33.

Finally, in a direct ELISA, only the cAb-An33 binds significantly to oligomannose-9, a complex carbohydrate structure resembling the Asn-linked oligomannose of trypanosomes (16). In contrast, all other VSG-specific VHHs failed to interact with this oligomannose-9 as exemplified by cAb-An46 in Fig. 2*C*.

Interaction of VSG Carbohydrate-specific VHHs with Living Trypanosomes—The capacity of cAb-An33 to recognize its epitope on living trypanosomes was analyzed via flow cytometry and microscopy. Flow cytometry shows that FITC-labeled cAb-An33 stains parasites of the three VSG classes, although not to the same intensity (Fig. 3*A*). However, the labeling intensity is always much larger than that obtained with FITC-labeled concanavalin-A. In contrast, the variant specific VHH (*e.g.* cAb-An46) labeled with FITC stains only parasites expressing AnTat 1.1 (Fig. 3*B*). The variants expressing VSGs of

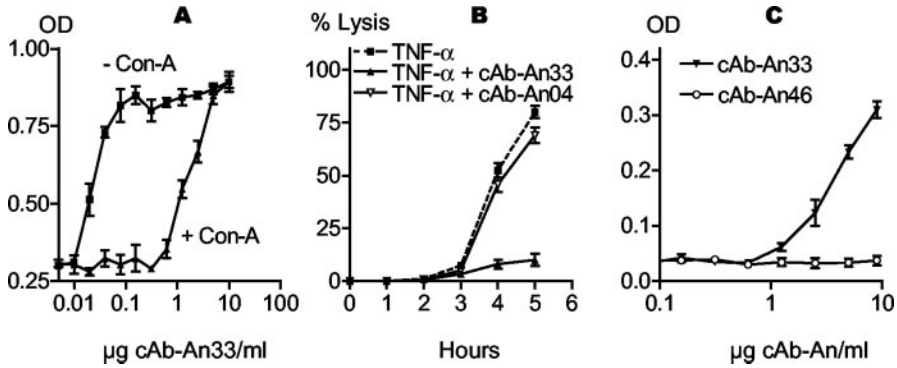

FIG. 2. **Carbohydrate specificity of cAb-An33.** *A*, ELISA of cAb-An33, serially diluted in PBS (−*ConA* curve) or in excess of concanavalin-A (+*ConA* curve) before applying the mixture to AnTat 1.1-coated wells. *B*, trypanolysis assay in which the fate of purified trypanosomes was followed for 6 h when incubated with TNF-α (*dotted line*, ■), or with TNF-α and cAb-An33/ml (▲), or with TNF-α and cAb-An04 (▽). *C*, ELISA of cAb-An33 (▼) and another AnTat 1.1-specific VHH (cAb-An46, ○) on oligomannose-9.

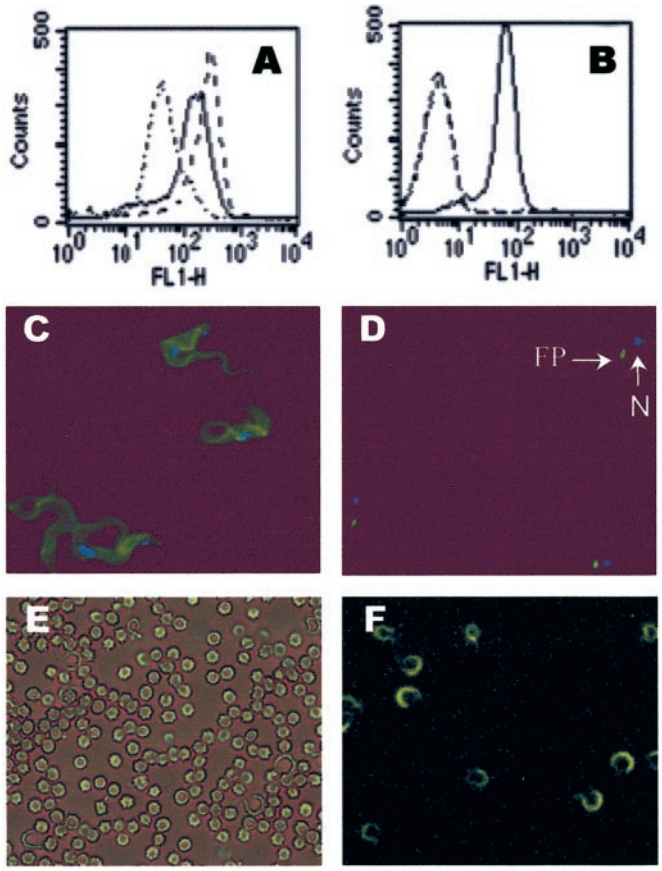

FIG. 3. **Staining of living trypanosomes.** Fluorescence-activated cell sorter results with FITC-labeled cAb-An33 (*A*) or cAb-An46 (*B*) on purified trypanosomes expressing AnTat 1.1 (*solid line*), MITat 1.1 (*dotted line*), or MITat 1.5 (*dashed line*). The profiles of the *dotted* and *dashed lines* overlap each other in *B*. *C*, immuno-microscopy of FITC-labeled cAb-An33 on purified trypanosomes expressing AnTat 1.1. The trypanosomes were stained with DAPI staining to visualize the nucleus. *D*, same as *C* but the cAb-An33 was replaced by FITC-labeled concanavalin-A. The nucleus (*N*) and the flagellar pocket (*FP*) of one trypanosome are indicated (*arrows*). *E* and *F*, blood of a trypanosome infected mouse incubated with FITC-labeled cAb-An33 illuminated with visible light (*E*) or UV (*F*).

other classes are not stained, and the profile is indistinguishable from an unstained sample of parasites, or trypanosomes incubated with an antibody fragment of non-VSG specificity. Thus, it seems that cAbAn-33, like concanavalin-A, detects various *T. brucei* parasites. Both proteins bind to the same sugars of the VSG (as inferred from Fig. 2*A*), however, they do so to a different extent as seen by immuno-fluoresence microscopy. The FITC-labeled concanavalin-A stains only the flagellar pocket of parasites (*green spots*, Fig. 3D*)*, adjacent to the nucleus (*blue DAPI-stained spot*, Fig. 3*D*). Apparently, the Asn-linked carbohydrates of VSG are only accessible for the 90 kDa large lectin when located in the flagellar pocket (28). In contrast, the FITC-labeled cAb-An33 stains the entire surface of the parasite (Fig. 3*C*). Hence, the cAbAn-33 succeeds in targeting its carbohydrate epitope even when present in the dense VSG coat of living parasites where access for larger molecules is seriously limited.

Due to the high fluorescence intensity and the high selectivity of cAb-An33 for the trypanosomal carbohydrates, it becomes possible to visualize the trypanosomes directly in blood smears (Fig. 3, *E–F*). Such a selective staining can certainly never be achieved with concanavalin-A because its broad recognition of sugars would stain the cells of the host of the parasite as well, and the weak labeling signal of the trypanosomal flagellar pocket would remain unnoticed.

Efficacy of cAb-An33 as Molecular Recognition Unit in a Bifunctional Construct—The single domain nature of the cAb-An33 makes it particularly suitable as modular building block into larger, more complex constructs (24), *e.g.* to generate an immuno-toxin. To address this possibility, we genetically linked the genes for this small, molecular recognition unit that specifically targets living trypanosomes with that of β-lactamase. The k_{cat}/K_m values of the β-lactamase for nitrocefin were determined at 1.1×10^7 M^{-1} s^{-1} for both, the free and the conjugated form, reflecting that the linkage to the antibody fragment does not hinder the catalytic activity of the enzyme. The antigen-binding capacity of the cAb-An33 moiety in the conjugate was confirmed in a direct ELISA on coated AnTat1.1, and with nitrocefin as chromogenic substrate (31). Thus, both the binding of the VHH and the enzymatic activity of the β-lactamase are well preserved in the fusion protein. The β-lactamase can also convert the mustard (CCM) into the highly toxic PDM (26). A 2.5 h incubation of 0 to 150 μM CCM to bloodstream form *T. brucei* AnTat 1.1 parasites in PSG buffer revealed an LD_{50} value of 20 μM for this prodrug, which is 20× higher than the parental drug PDM (Fig. 4). The sub-lethal doses of CCM (0.5–10 μM) become highly toxic for the trypanosomes when added to parasites washed extensively after a prior incubation with the cAb-An33::β-lactamase fusion. This indicates that sufficient enzyme remains attached by virtue of the trypanosome-specific antibody to generate the more toxic PDM from CCM. A similar result was obtained with trypanosomes that express the MITat 1.5, confirming that the broad trypanosome specificity of the cAb-An33 moiety is maintained in the conjugate. This prodrug activation is immunologically specific because exposing the parasites to a non-binding control conjugate cAb-Lys3::β-lactamase prior to CCM treatment is well tolerated by trypanosomes (Fig. 4) (cAb-Lys3 is a well studied VHH with specificity for chicken lysozyme (27, 32)).

DISCUSSION

A dromedary, single domain antibody-fragment with specificity for the Asn-linked carbohydrate of the trypanosomal VSG

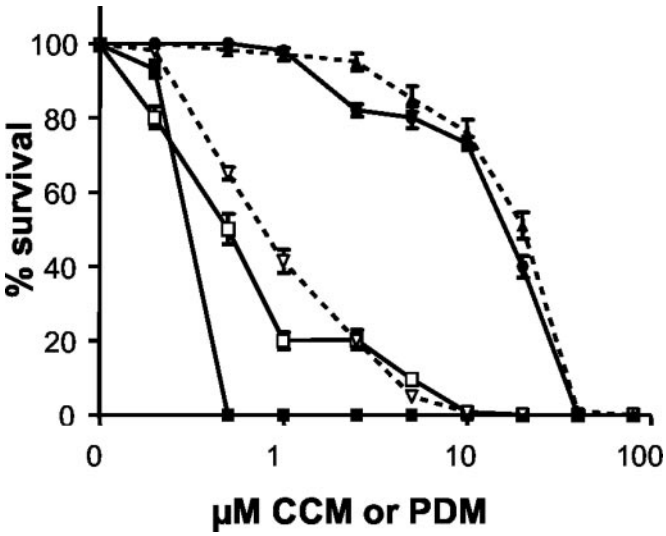

FIG. 4. **Survival of bloodstream-form *T. brucei* AnTat 1.1 and MITat 1.5.** The percentage of surviving parasites in presence of various amounts of PDM toxin (*dotted line*, ▽) or CCM prodrug (*dotted line*, ▲) were recorded to determine the LD_{50}. For prodrug therapy, parasites were incubated with VHH::lactamase fusion (cAb-Lys3::β-lactamase (●) or cAb-An33::β-lactamase (■)), washed to remove unbound conjugate, and incubated with various amounts of CCM. The number of surviving parasites was scored after 3 h. The experimental setup was repeated for MITat 1.5 parasites with cAb-An33 fusion protein (□).

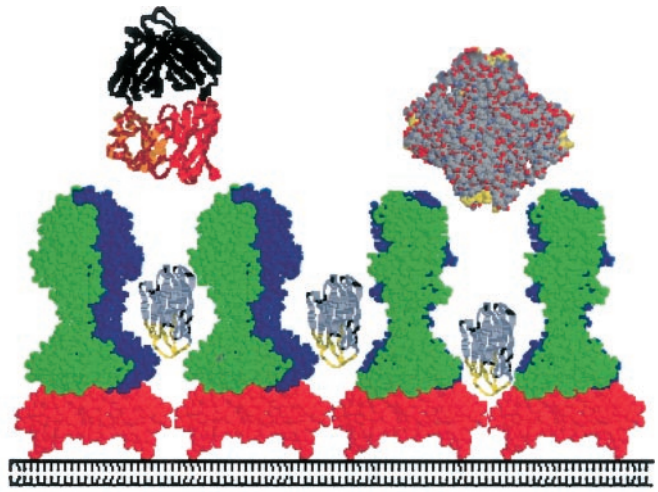

FIG. 5. **Model of coat-penetrating potential of single domain antibody fragments.** The structure of the VSG dimer (*green* and *blue*) shown from its widest (two molecules on the *left*) or narrowest sides (two molecules on the *right*), the *red part* represents the 130 amino acids for which no structure is available yet. The glycosylation site is present within this part. The structure of concanavalin-A (*top-right*, space filling with sugar-binding pockets in *yellow*), a Fab fragment (*top-left* ribbon representation; VH, *red*; VL, *orange*; CH1 and CL, *black*), and the structure of a VHH in *gray* with the antigen-binding loops in *yellow* are given. All structures are drawn to scale and the density of the VSG (shown unilateral) is as calculated in Ref. 35.

has been identified. This small, recombinant protein has the potential to detect trypanosome infections directly on blood smears and to become the molecular recognition unit in an immuno-toxin design for trypanosomosis therapy.

The reasons to prefer camel single domain antibody for this type of application is dictated by: (i) the small size of the antibody-fragment (19), and (ii) the density of the VSG molecules on the surface of the African trypanosomes exposing their hypervariable immunogenic areas and hiding the conserved epitopes of VSG dimers (13–15) (Fig. 5). From calculations of the number and size of VSG molecules and the total surface of the parasite it has been estimated that the VSG molecules should nearly contact each other (8, 33–35). This level of density is modeled in Fig. 5 where the crystallographic structure of the N-terminal part of the VSG dimer (Protein Data Bank code 2VSG) is viewed along its widest or narrowest side. (To compensate for the remaining 130 amino acids of which structural details are lacking, we duplicated the structure of the last 130 amino acids of the VSG and rotated it over 180° before adding it to the rest of the structural VSG model to obtain a more faithful representation of the actual VSG volume and VSG density on the coat of the parasite.) The hypervariable region of the VSG molecule is located at the top of these VSG models, whereas the Asn-linked carbohydrate is attached to the lower part of the molecule (within the *red part* in Fig. 5), deeply buried between the VSG molecules. The concanavalin-A lectin binds to the VSG sugars; however, this tetrameric molecule can only interact specifically with the carbohydrates exposed in the flagellar pocket (28). The same carbohydrates of the VSGs located on the remaining parasite surface are inaccessible for molecules of this size, as illustrated by Fig. 5. In the same vein, a monoclonal antibody against the carbohydrate moiety of the VSG, or even a Fab derived from such a monoclonal, is expected to be occluded from the carbohydrate in the trypanosomal coat. In contrast, a dromedary single domain antibody should gain an easier access to epitopes close to the membrane, cryptic for larger molecules, due to its smaller overall size (problate particle of 2.8 nm diameter and height of 4.4 nm).

It could be argued that the size-restricted access of some conserved VSG epitopes on parasites can be overcome as well with a Fv, the smallest antigen-binding fragment of a conventional antibody. However, these molecules are twice as large compared with the VHH molecules. In addition, the Fv fragments are unstable entities that need a synthetic tether between the VH and VL domains to arrive at a single chain Fv or disulfide-stabilized Fv. Quite often, the linker provokes single chain Fv dimerization, and the disulfide-stabilized Fv fragments are more difficult to express in large quantities (36). For these reasons, the Fv constructs seemed less favorable for the envisaged application.

The further reason to choose dromedary antibodies lies in their recognition of epitopes that are less immunogenic for conventional antibodies. This feature was originally demonstrated by the large number of enzyme inhibiting HCAbs or VHHs present in serum of an immunized dromedary or in the pool of enzyme-specific VHHs (20). A structural explanation for this observation was provided by crystallographic analyses of VHHs complexed to their cognate antigens (32, 37, 38). A protruding loop of the lysozyme-specific VHH inserts in the cavity of the active site of the enzyme (27, 32). Protruding antigen-binding loops are rare in conventional antibodies, whereas VHHs have usually an extended complementary determining region 3 (CDR3) sequence, suggesting that protruding loops might occur frequently in VHHs (19). In another case, a binder with shorter CDR sequences was also able to interact with the substrate-binding pocket of amylase because the entire domain could insert in this groove on the enzyme surface (37). Such convex epitopes are definitely less antigenic for conventional antibodies. Hence, it seems that VHHs prefer a convex epitope, or cavities on the antigenic surface, whereas conventional antibodies associate preferably on flat surfaces (19, 38). The differential targeting between HCAbs and conventional antibodies was confirmed with the serum immunoglobulins of the dromedary immunized with AnTat 1.1. Only the IgG3 HCAbs recognized AnTat 1.1, MITat 1.1, and MITat 1.5, reflecting the presence of cross-reacting antibodies. Most likely, these cross-reacting antibodies interact with an epitope that is common to AnTat 1.1 and at least one other VSG subclass. The

cAb-An33 could be the VHH of such a pan-reactive HCAb as it recognizes the conserved carbohydrate of the VSG.

The sugar specificity of cAb-An33 was amply demonstrated by concanavalin-A competition, by inhibition of TNF-α trypanolysis, by recognition of various proteolytic peptides containing the Asn-linked high mannose oligosaccharide ($GlnNAc_2$-Man_{5-9}), by direct association with a synthetic oligomannose-9, and by a greatly reduced interaction with deglycosilated AnTat 1.1. In accordance with our model predictions, this binder could attach to its epitope present within the living parasites. Moreover, the high specificity for the trypanosomal sugar moiety, and the conservation of this epitope on various serotypes warrants the efficacy of cAb-An33 to detect trypanosomes directly in blood smears. Remarkably, the chemical coupling of small FITC groups on the VHH didn't lead to detrimental obstruction of the epitope targeting. Although parasites expressing other VSG classes might exhibit a reduced accessibility of the epitope, possibly resulting from an altered VSG packing density or from the presence of additional or extended VSG loops.

To obtain a cAb-An33-derived therapeutic agent for treatment of trypanosomosis it will be necessary to link an effector molecule to this small molecular recognition unit. As a proof of concept we chose the β-lactamase as fusion partner because of its multiple attractive features. First, it is a well characterized, small monomeric molecule of 30 kDa that is expressed successfully in prokaryotic (39) and eukaryotic cells. Second, it can be fused efficiently via its N-terminal end to the C-terminal end of a VHH, because the C-end and the antigen-binding loops of the antibody fragment are on opposite sides of the domain, and the N-terminal of the enzyme is distant from the catalytic site. Third, the enzyme has excellent catalytic properties for chromogenic substrates (*e.g.* nitrocefin) as well as cell-permeable fluorescent substrates (coumarin cephalosporin fluorescein) allowing a sensitive screening (40). Moreover, the enzyme can convert the CCM prodrug into a potent toxic PDM (26), so that the fusion can be used in an antibody directed antibody prodrug therapy. Fourth, the lactamase activity is absent in eukaryotic host cells, and finally, sensitive complementation tests are under development (41). The experiments with cAb-An33::β-lactamase on trypanosomes are very promising. Apparently, the antibody moiety of the conjugate is still able to detect the parasites, while preserving the nitrocefin hydrolyzing activity of the attached enzyme. For diagnostic purposes, this fusion product can be used in combination with nitrocefin to detect as low as 5 parasites/ml, a sensitivity that is comparable to the most sensitive, modern detection techniques for trypanosomosis (42, 43). In addition, the potential to eliminate the parasites with the same conjugate in a prodrug therapy with CCM was positively evaluated.

We surmise that the successful employment of single domain antibody derivatives to combat trypanosomosis could also be repeated to tackle various other infectious agents exhibiting any form of antigenic variation (1–4) because the basis for their success relies on their general properties: the small size and the capacity to recognize non-conventional epitopes of the variable component that become cryptic for larger antibodies when incorporated into the micro-organism.

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Efficient Targeting of Conserved Cryptic Epitopes of Infectious Agents by Single Domain Antibodies: AFRICAN TRYPANOSOMES AS PARADIGM

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