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Immunolocalization and Challenge Studies Using a Recombinant *Vibrio cholerae* Ghost Expressing *Trypanosoma brucei* Ca²⁺ ATPase (TBCA2) Antigen

Kiantra Ramey,

Morehouse School of Medicine, Department of Microbiology, Biochemistry, and Immunology, BMSB Room 349D, 720 Westview Drive SW, Atlanta, GA 30310, Tel: 404-752-1765, Fax: 404-752-1179, kramey@msm.edu.

Francis O. Eko,

Morehouse School of Medicine, Department of Microbiology, Biochemistry, and Immunology, BMSB Room 333, 720 Westview Drive SW, Atlanta, GA 30310, Tel: 404-752-1584, Fax: 404-752-1179, feko@msm.edu.

Winston E. Thompson,

Morehouse School of Medicine, Department of Obstetrics and Gynecology and Cooperative Reproductive Science Research Center, 720 Westview Drive SW, Atlanta, GA 30310, Tel: 404-752-1715, wthompson@msm.edu.

Henry Armah,

University of Pittsburgh Medical Center, Department of Pathology, A711 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA 15261, Tel: 412-647-5550, Fax: 412-802-6079, armahh2@upmc.edu.

Joseph U. Igietseme, and

Centers for Disease Control and Prevention, National Centers for Infectious Diseases/ Scientific Resources Program, Mail Stop C17, 1600 Clifton Road, Atlanta, GA 30333, Tel: 404-639-3352, jbi8@cdc.gov.

Jonathan K. Stiles*

Morehouse School of Medicine, Department of Microbiology, Biochemistry, and Immunology, BMSB Room 349D, 720 Westview Drive SW, Atlanta, GA 30310, Tel: 404-752-1585, Fax: 404-752-1179, jstiles@msm.edu.

Abstract

Human African trypanosomiasis is a neglected disease caused by *Trypanosoma brucei* spp. A parasite cation pump (Ca²⁺ ATPase; TBCA2) essential for survival and cation homeostasis was identified and characterized. It was hypothesized that targeting this pump using a *Vibrio cholerae* ghost (VCG)-based vaccine could protect against murine *T. brucei* infection. mRNA and protein expression of TBCA2 was differentially expressed in blood and insect stages of parasites and immunolocalized in the pericellular membrane and the flagellar pocket of bloodstream forms. Antigen-specific antibodies and Th1 cytokines, interleukin-2, interferon-gamma, and tumor necrosis factor-alpha were induced in rVCG-TBCA2-immunized mice and *in vitro* on antigen stimulation of splenic immune T cells, but the corresponding Th2-type response was

unremarkable. Despite an increased median survival of 6 days in vaccinated mice, the mice were not protected against infection. Thus, immunization of mice produced robust parasite-specific antibodies but failed to protect mice against parasite challenge.

INTRODUCTION

Human African trypanosomiasis (HAT; sleeping sickness) is a neglected fatal vector-borne disease caused by *Trypanosoma brucei* spp. Sixty million people are at risk of infection with HAT, and 30,000 new cases and an equal number of deaths are reported annually in sub-Saharan Africa.¹ Currently, available anti-trypanosomals are extremely toxic and may cause post-treatment encephalopathy in treated patients.² The lack of suitable drugs for treating trypanosomiasis is caused by the limited understanding of molecular and physiologic mechanisms used by parasites to maintain homeostasis and proliferate. The ideal anti-trypanosomal agent will target vital physiologic processes and/or non-variant parasite-derived surface molecules without adversely affecting the human host or inducing resistance.

Many studies have indicated that cell membrane cation pumps (cation ATPases) are involved in cation translocation, signal transduction, proliferation, cation homeostasis, and apoptosis in pathogenic eukaryotes and are potential targets for cation pump inhibitor therapy.^{3–13} For example, cytosolic calcium ion concentration $[Ca^{2+}]_i$ in blood stages of *T. brucei* is significantly below that encountered in extracellular milieu. Therefore, parasites require effective Ca^{2+} pumps to maintain $[Ca^{2+}]_i$ homeostasis, survival, and proliferation. The lack of interest in these proteins as vaccine targets is partly because of the prevailing concept that vaccines will not be effective against trypanosomes because of the very densely packed variable surface glycoprotein (VSG) coat that protects underlying proteins embedded in the plasma membrane from antibodies. The results of studies on adenyl cyclase and the low-density lipoprotein (LDL) receptor of *T. brucei* indicated a clustering of such molecules in a specialized membrane region called the “flagellar pocket” located at the anterior end of the parasite where its flagellum originates.^{14–16} Small antibody fragments, but not larger lectins or conventional antibody fragments, are able to penetrate the less dense flagellar pocket VSGs and the dense VSG coat to target their epitope.¹⁷ Membrane fractions from the flagellar pocket have been shown to protect mice against infection by *T. b. rhodesiense*, and antibodies directed against the membrane LDL receptor inhibit the growth of *T. brucei* cultivated *in vitro*.^{18,19} Studies also showed that African trypanosomes express T-lymphocyte triggering factor (TLTF), which is localized to small vesicles near the flagellar pocket (secretory apparatus), which triggers CD8⁺ T lymphocytes to proliferate and secrete interferon (IFN)- γ , a growth factor for *T. brucei*.²⁰ There are about five protein homologs of membrane structures found in the flagellar pocket of *T. brucei* used by trypanosomes for Ca^{2+} homeostasis during proliferation in human blood. These include Ca^{2+} pumps (ATPases), Na^+/Ca^{2+} exchangers, Ca^{2+} release channels, Ca^{2+} uniporters, and electroneutral Na^+/Ca^{2+} exchangers. These membrane proteins have not been adequately explored as potential anti-trypanosome targets.

We previously identified, cloned, sequenced, and generated antibodies of two plasma membrane-like cation ATPases (TBCA1 and TBCA2) that are significantly upregulated in blood stages of *T. brucei* but not in culture procyclics (insect stage). TBCA1 was confirmed to be a plasma membrane Na^+/K^+ ATPase,²¹ whereas TBCA2 is a Ca^{2+} ATPase (Stiles and others, unpublished data). Treatment of procyclic *T. brucei* with commercially available cation pump inhibitors such as ortho-vanadate and thapsigargin inhibited parasite proliferation *in vitro*.^{12,22–24} These findings indicate that the cation pumps are essential for parasite proliferation. Therefore, our hypothesis is that the pumps and their orthologs in

members of the tritryps (*T. brucei*, *T. cruzi*, and *Leishmania* spp.) are important for transition from their insect vector (*Glossina* spp.) to their mammalian host blood. Furthermore, this also suggests that inhibition of the pumps by immunoreacting specific antibodies with their target antigens on parasite surfaces may prevent *T. brucei* development and proliferation.

Recombinant *Vibrio cholerae* ghosts (VCGs) are gram-negative bacteria without the cytoplasmic content through the expression of lysis gene *E*. The bacterial ghosts possess adjuvant properties, maintain the structural and functional integrity of expressed antigens, and are great vehicles for delivery of multiple antigens to primary antigen presenting cells (APCs).²⁵ Previously, rVCG technology has been used to deliver chlamydial antigens resulting in the induction of specific immunity and protection against *Chlamydia trachomatis* infection in mice.²⁵ These studies test whether a *T. brucei* pump immunogen can be generated that could induce protective immune responses against *T. brucei* infection in a murine model using the novel rVCG delivery platform. An rVCG carrier-based subunit system expressing TBCA2 was constructed and tested for induced immunoprotective responses in rVCG-TBCA2-immunized mice. The results indicated that antigen-specific antibodies and Th1 cytokines, interleukin-2 (IL-2), IFN γ , and tumor necrosis factor- α (TNF α) are significantly induced in rVCG-TBCA2-immunized mice and *in vitro* on antigen stimulation of splenic immune T cells, respectively, albeit with an unremarkable Th2-type response.

MATERIALS AND METHODS

Trypanosomes

Blood stage *T. brucei* (GuTaT 10.0) trypomastigotes obtained from Dr. John Donelson, University of Iowa, was used for immunolocalization, challenge studies, nucleic acid, and protein isolation, as well as a source of stock parasites. Parasites were administered subcutaneously to naive Balb/c mice until a peak parasitemia of 10^6 parasites/mL was attained. For nucleic acid and protein isolation as well as storage, parasites were harvested by heart puncture into heparinized Vacutainer tubes and rapidly separated from blood cells and platelets by the histopaque isolation methods and processed accordingly.²⁶

Nucleic acid purification and polymerase chain reaction amplification

Trypanosoma brucei genomic DNA was isolated using a Qiagen DNA isolation kit (Qiagen, Valencia, CA). Total cellular RNA was extracted from parasites using Tri Reagent (Molecular Research Center, Cincinnati, OH), and mRNA was isolated from total RNA with Oligotex resin (Qiagen). RNA and DNA were quantitatively analyzed by UV spectrophotometry at 260 nm and verified by ethidium bromide staining of aliquots after electrophoresis through agarose gels.

TBCA2 ATPase sequence was obtained by polymerase chain reaction (PCR) amplification from intron-less parasite genomic DNA using the oligonucleotide primers GANAAN-ACNGGNACNCTNAC (forward) and TCNTTNACNCCN-TCNCCNGT (reverse). The primers incorporated the codon bias observed in trypanosomatid genes²⁷ and were designed to recognize two highly conserved amino acid motifs of cation ATPase genes, the ATP phosphorylation site, DKTGTLLT, and the ATP binding site, TGDGVND.^{28,29} These sites bound the large central cytoplasmic loop, which lies between the four amino terminal and six carboxyl terminal membrane spanning domains of P-type ATPases. Negative controls, which omitted template DNA, were included in each amplification experiment. PCR products (in plasmid vector, pT7Blue; Invitrogen, Carlsbad, CA) were sequenced by the dideoxy chain termination method using a modified T7 polymerase (Sequenase; United

States Biochemical, Cleveland, OH). Internal oligonucleotide primers were designed to complete the sequence on both strands. DNA sequence data were deposited in the GenBank database under the accession numbers AF_145723 and AF_145722. These sequences have since been verified to be localized on chromosomes 8 and 9 (Tb_09.244.2570 and Tb_927.8.1160, respectively) on the recently completed genome sequence of *T. brucei* (www.sanger.ac.uk/Projects/).

The *tba2* gene, coding for the immunogenic 1,022-bp fragment of the Ca²⁺ ATPase pump, *TBCA2*, was obtained by PCR amplification from the pT7blue plasmid vector. The sequence was amplified with the Expand High Fidelity PCR System (a unique mix of Taq and Pwo DNA polymerases; Roche, Mannheim, Germany) using the oligonucleotide primers EF24F (5'-GCATGctcgagTGACAAGACTGGTA-3') and EF25R (5'-AGAGctgc-agTATCATTTCAGCCGT-3'), which contain *Xho*I and *Pst*I restriction sites (lowercase), respectively. The amplification reaction was carried out in a GeneAmp PCR System (Perkin-Elmer, Waltham, MA), and an amplified PCR product of the correct size (~1,022 bp) was isolated from a 0.8% agarose gel and purified with the QIAquick PCR purification kit (Qiagen).

TBCA2 mRNA expression analysis

DNase treated *T. brucei* total RNA (2 µg) from different blood and insect stages was reverse transcribed and amplified by PCR using the QIAGEN OneStep RT-PCR kit (Qiagen) per the manufacturer's instructions. The TBCA2 oligonucleotide primer pair (sense, ACAGCGAAGATGCCGA, anti-sense, CTTGGTTAACTCCTGC) was used to amplify a 308-nucleotide product. A primer pair (sense: GCGAACGAAATCCGTAATGT, anti-sense CGTCTCCAAGTCAGCTTTC), which amplifies a 300-nucleotide fragment of the paraflagellar rod gene (PFR-A; GenBank accession no. X14819), was included to permit normalization of mRNA production by reverse transcription. PFR-A is equally expressed in *T. brucei* blood and insect stages.³⁰ The number of cycles needed to attain products in the linear range of PCR was determined before performing semi-quantitative RT-PCR. Negative controls, which omitted *T. brucei* template RNA, were included in each amplification experiment.

Production of monoclonal antibody against TBCA2

A proprietary web-based gene analysis software program (GREASE; <http://fasta.bioch.virginia.edu/fasta/grease.htm>) was used to identify a potential antigenic region of TBCA2, CNRYLSSEGREEEPLT, for antibody production. Bioinformatic analysis and BLAST searches of this sequence in NCBI databases showed it to be unique. This 15-mer peptide was conjugated to keyhole limpet hemocyanin (KLH) through activated EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] or the carbodiimide method. A monoclonal antibody (mAb) against this peptide was synthesized at the Morehouse School of Medicine Core Monoclonal Antibody Laboratory by previously described methods.³¹ The antibody was purified, analyzed, and titered by enzyme-linked immunosorbent assay (ELISA); minimum reactive titer was ~1:400. TBCA2 mAb was used to immunolocalize the TBCA2 pump in whole parasites (blood stages) and in Western blots to estimate protein size and expression during development.

Immunocytochemistry

The procedure used to immunolocalize TBCA1 has been described in detail previously.³² Axophor red-labeled mouse monoclonal TBCA2 was used at a dilution of 1:200. *T. brucei* has a single mitochondria spanning the whole body, thus mitochondrial staining^{33,34} enabled us to distinguish sub-pellicular expression of TBCA2 from mitochondrial expression after counter staining with 4,6-diamidino-2-phenylindole (DAPI; Molecular

Probes, Eugene, OR) and mounting in Vectashield (Vector Laboratories, Burlingame, CA). Mounted slides were examined using an Olympus laser scanning confocal microscope (Olympus America, Melville, NY) imaging system equipped with a 15-mW krypton/argon laser. Control slides carried out with the omission of the primary antibody exhibited negative immunostaining.

Construction of the expression vector, pTBCA2

The *tbca2* PCR product was digested with *Xho*I and *Pst*I enzymes, purified, and subcloned into the *Xho*I/*Pst*I-cleaved membrane-targeting vector, pKSEL5-2,³⁵ under the transcriptional control of the *LacPo* promoter. The resulting recombinant expression plasmid (designated pTBCA2) contained the *T. brucei tbca2* gene inserted between the *LacZ'* and *L'* genes (i.e., - *L'* targeting). The plasmid was restriction digested and sequenced to certify the size and orientation of the cloned insert.

Purification of recombinant TBCA2 protein

Plasmid pTBCA2 was introduced into the *V. cholerae* 01 strain HI by electroporation, and clones containing the plasmid and designated HTBCA2 were isolated. HTBCA2 was grown to mid-stationary phase (OD₆₀₀ ~1.86) at 37°C and 180 rpm in 250 mL of brain heart infusion (BHI) broth, and the bacteria were collected by centrifugation at 5,000g for 10 minutes. The rTBCA2 protein was purified using the CellLytic B II purification method according to the manufacturer's instructions (Sigma, St. Louis, MO). The bacterial cell pellet was mixed with CellLytic B II (5 mL/g cell paste) containing deoxyribonuclease I (final concentration, 5 µg/mL). After extraction, the mixture was centrifuged at 25,000g for 15 minutes to pellet insoluble material. The supernatant containing the soluble proteins was carefully removed. The amount of the recombinant TBCA2 protein was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue staining (data not shown) and Western blotting analysis.

Western blot analysis of recombinant TBCA2 expression

To confirm TBCA2 protein expression in *T. brucei*, 50 µg of parasite protein extracts was subjected to one-dimensional PAGE, as described previously.³³ *T. brucei* parasites (10⁶) were harvested by centrifugation, washed twice in phosphate-buffered saline (PBS), and resuspended in 500 µL SDS-PAGE sample buffer (50 mmol/L TRIS, pH 6.8, 100 mmol/L dithiothreitol, 2% SDS, 1% bromophenol blue, 10% glycerol) containing a protease inhibitor cocktail of leupeptin, aprotinin, EDTA, and Pefabloc (Boehringer Mannheim, Indianapolis, IN). Cell lysates were prepared by heating at 90°C for 5 minutes, and 20 µL lysates separated by 12% SDS-PAGE was transferred onto 0.2 µmol/L nitrocellulose membranes using a Royal Genie electrophoretic blotter (Idea Scientific, Minneapolis, MN) at 350 mA for 5 hours. Blots were incubated for 1 hour in Tris-buffered saline (TBS) containing 0.05% Tween-20 and 5% nonfat dried milk and subsequently left overnight at 4°C with mouse mAb to TBCA2 (1:1,000). Membranes were incubated with the appropriate secondary antibody for 2 hours at room temperature, and antibody binding was detected by chemiluminescence (Amersham, Arlington Heights, IL). Protein content was normalized using α -tubulin.

Expression of recombinant TBCA2 (rTBCA2) in *V. cholerae* 01

The expression of TBCA2 by HTBCA2 cells was evaluated by immunoblotting analysis. Cultures of HTBCA2 and control HI (pKSEL5-2) were grown to early-log phase under appropriate conditions, and rTBCA2 expression was induced by the addition of 2 mmol/L IPTG (isopropyl- β -thiogalactopyranoside; Roche Diagnostics). Samples were removed at different time intervals, solubilized in sample buffer, and separated by SDS-PAGE as

previously described.^{36,37} The CelLytic B II–purified rTBCA2 subjected to the same conditions was also included. After protein transfer, rTBCA2 was detected using TBCA2 mAb (see above).

Production of recombinant *V. cholerae* ghosts expressing TBCA2 (rVCG-TBCA2)

Vibrio cholerae 01 strain H1-TBCA2 (containing the cloned *tbca2* gene) was co-transformed with the lysis plasmid pDKLO1³⁸ to yield clone H1-TBCA2E. Transcription of gene *E* from pDKLO1 is directed by the activity of the *Pm/xylS* promoter-repressor system of the *Pseudomonas TOL* plasmid.³⁸ Bacteria were grown in BHI broth containing ampicillin (100 µg/mL) and kanamycin (25 µg/mL) at 37°C to mid-log phase, and IPTG was added to the growing culture to induce for *tbca2* gene expression. After induction, cell lysis was achieved by adding 3-methyl benzoate (to 5 mmol/L) to induce gene *E* expression. At the end of lysis, cultures were harvested by centrifugation, resuspended in a low ionic buffer or distilled water, and washed with PBS. Harvested ghosts were resuspended in PBS and lyophilized. The efficiency of E-mediated killing of vibrios was estimated by plating samples on BHI agar as previously described.³⁹ Results indicated a 100% killing efficiency; no colony-forming units were found on plates at any dilution. Lyophilized VCGs were weighed, and the number of cfus per milligram of VCG was calculated.

Immunization protocol

Five- to 8-week-old female BALB/c mice (Jackson laboratory, Bar Harbor, ME) were housed in laminar flow racks under pathogen-free conditions at a constant temperature of 24°C with a cycle of 12 hours of light and 12 hours of darkness and were fed mouse chowder and water *ad libitum*. Balb/c mice were used because of their known susceptibility to trypanosomes.⁴⁰ Immunizations were carried out using a 1-mL syringe fitted with a 27-gauge needle. Two groups of mice (10/group) were vaccinated intraperitoneally with either 3 mg of lyophilized rVCG-TBCA2 or rVCG alone. Each milligram of lyophilized rVCG-TBCA2 or rVCG corresponded to $\sim 3.3 \times 10^9$ CFU. All immunizations were given in 50 µL of PBS while under phenobarbital anesthesia, and animals were boosted twice at two week intervals.

Humoral responses to TBCA2

Blood was collected by periorbital bleeding, and the serum was pooled for each group of animals. A solid-phase ELISA procedure was used to determine TBCA2-specific antibody titers in the sera of immunized and control mice. Briefly, 96-well microtiter plates (Nunc, Rochester, NY) were coated with 10 µg/well of purified TBCA2 in PBS and 100 µL of mouse IgA or mouse IgG2a at 4°C overnight. Plates were blocked with 1% bovine serum albumin diluted in PBS at room temperature for 1 hour and washed once with wash buffer (PBS, pH 7.4, 0.05% Tween 20). Sera were added to the wells and incubated at room temperature for 1 hour and washed three times with wash buffer. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or IgG2a (Accurate Chemical, Westbury, NY) diluted 1:3,000 in blocking solution containing 1% bovine serum albumin was added and incubated for 1 hour at room temperature. The plates were washed three times with wash buffer, and the HRP-substrate (Sigma) was added and incubated for 35 minutes. Absorbance was read on a Microplate Autoreader spectrophotometer (Bio-tex Instruments, Winooski, VT) at 492 nm.

Antigen-specific Th1/Th2 cytokine detection and quantification

Six weeks after immunization, enriched immune T cells were prepared from the spleen by the nylon wool enrichment procedure as previously described.⁴¹ Purified T cells contained at least 95% CD3⁺ cells, as determined by fluorescence-activated cell sorting (FACS)

analysis. Cells were cultured at 37°C in 5% CO₂. The level of antigen-specific Th1 or Th2 response was assayed by measuring the antigen-specific Th1/ Th2 cytokine (IFN γ , TNF α , IL-2, IL-4, and IL-10) profile of each cell population. Briefly, purified T cells were plated in a serial doubling dilution in duplicate 96-well tissue culture plates at 2×10^5 cells/well and cultured with wild-type APCs and *T. brucei* lysates as antigen (10 μ g/mL) for 5 days. Background cultures contained wells without APCs or antigen. Supernatants were harvested and assayed for cytokines using a Bio-Plex multiplex cytokine assay kit in combination with the Bio-Plex Manager software (Bio-Rad, Hercules, CA). The mean and SD of all replicate cultures were calculated. The concentration of each cytokine was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values ($\pm$ SD) of triplicate cultures for each experiment. Results were derived from three independent experiments.

Challenge studies

Two groups of mice (10/group) were immunized intraperitoneally three times 2 weeks apart with either 3 mg of rVCG-TBCA2 or rVCG. Six weeks after immunization, mice were challenged intraperitoneally with 1×10^6 washed *T. brucei* parasites^{42,43} in 30 μ L of PBS while under phenobarbital anesthesia. Infections were monitored by assessing the level of parasitemia in individual animals every 3 days after challenge. Survival was assessed by counting the number of mice remaining alive at day of count and expressing it as a percentage of starting number of mice (Kaplan-Meier curve). Results were derived from three independent experiments.

Statistical analysis

The differences in the level of cytokine and antibody production as well as protection were determined by the Student *t* test. The level of significance was determined at $P < 0.05$.

RESULTS

Construction of pTBCA2 vector expressing TBCA2 protein

Construction of plasmid pTBCA2 was accomplished by placing the *tbcA2* coding region between and in frame with the *LacZ'* and $-L'$ anchors in pKSEL5-2 under the transcriptional control of the *lac* promoter (Figure 1). DNA sequencing confirmed that the cloned gene was in frame with the $-L'$ fusion anchor and that the integrity of the plasmid construct was maintained. Expression of the recombinant protein (rTBCA2) was confirmed by Western blot analysis using mouse mAbs. TBCA2 was present mainly in the soluble fraction (Figure 2) but was not detectable in the test strain containing the plasmid vector alone. Two forms of rTBCA2 with equivalent band intensities were detected. The 43-kd TBCA2 protein form co-migrated with the CellLytic B II-purified TBCA2 and corresponded with the molecular mass predicted for the TBCA2- L' fusion protein. The form with a higher molecular mass (~48 kd) most likely represents the *LacZ'*-TBCA2- L' fusion protein.

TBCA2 mRNA expression analysis

TBCA2 mRNA transcripts in blood stage trypomastigotes (animal or *in vitro* derived) were detected and upregulated compared with culture procyclics, indicating increased expression of TBCA2 during *Trypanosoma* development (Figure 3).

Localization of TBCA2 in *T. brucei*

Trypanosoma brucei TBCA2 protein was immunolocalized using Axophor red-labeled *T. brucei* TBCA2 mAb (Molecular Probes). TBCA2 was localized within the sub-pericellular

regions with intense staining in regions surrounding the nucleus and flagellar pocket as well as the flagellum in blood stages as previously observed for TBCA1²¹ (Figure 4).

Production of rVCG expressing TBCA2

Recombinant VCGs expressing TBCA2 were produced by protein E-mediated lysis of bacterial cells as previously reported.²⁵ Induction of the *Pm* promoter by the addition of methyl benzoate to the growing bacterial culture resulted in rapid de-repression and protein E-mediated lysis. The extent and rate of lysis were quantified from the decrease in turbidity per unit of time, measured by the absorbance of the lysing culture at 600 nm. Electron microscopic analysis of protein E-lysed *V. cholerae* 01 cells showed no gross alterations in cellular morphology in comparison with unlysed cells. Except for the lysis tunnel, the morphology of the cell, including all cell surface structures and appendages, was unaffected by the lysis event (data not shown).

Induction of TBCA2-specific antibodies

To assess the immunogenicity of our rVCG-TBCA2 construct, induction of IgA and IgG2a responses at 3 and 6 weeks after immunization were measured by titrating the serum of rVCG-TBCA2-immunized and control mice against the trypanosomal TBCA2 antigen. Significant levels of antigen-specific IgA and IgG2a antibodies ($P < 0.01$), which remained elevated up to 6 weeks after immunization, were detected in the serum of immunized mice (Figure 5A and B). There were no detectable TBCA2 antibody levels in the serum of rVCG-immunized control mice.

Induction of TBCA2-specific immune responses

Splenic immune T cells were isolated 6 weeks after immunization and stimulated with TBCA2 *in vitro*. The rVCG-TBCA2 construct significantly induced an elevated antigen-specific Th1 (IL-2, IFN γ , and TNF α) response (Figure 6A) on re-stimulation. The amounts of IL-2, IFN γ , and TNF α produced by immune T cells was significantly greater compared with levels produced by controls ($P < 0.01$). The amounts of IL-4 ($P = 0.25$) and IL-10 ($P = 0.07$) produced by splenic T cells from rVCG-TBCA2-immunized mice were not significantly different from control mice (Figure 6B).

Challenge studies

Despite an increased median survival of 6 days in vaccinated mice, none of the mice were protected against infection, and all vaccinated animals died within 3 weeks after challenge, in three independent experiments (data not shown).

DISCUSSION

Currently, 60 million individuals are at risk of infection of HAT, and commercially available anti-trypanosomals have toxic side effects and cause post-treatment encephalopathy in patients.^{1,2} Previous attempts to develop vaccines against trypanosomiasis have been unsuccessful because of the parasite's highly immunogenic variant surface glycoproteins comprising the surface coat and the phenomenon of antigenic variation.^{44,45} Previous reports indicated that immunizing with flagellar pocket proteins from *T. brucei rhodesiense* provided partial protection in *T. brucei rhodesiense*-infected mice and naturally occurring cases of *T. congolense* and *T. vivax* in cattle.^{18,46} Immunizations with purified paraflagellar rod proteins also reduced parasitemia and provided 100% protection against *T. cruzi* infection.⁴⁷ Recent studies showed that intramuscular immunization with rVCG expressing cloned subunit antigens is a suitable delivery system for testing vaccine candidates in mice challenged with live virulent organisms.²⁵ This rVCG technology was used to deliver

chlamydial antigens resulting in the induction of local and systemic Th1 responses in addition to partial protection against *Chlamydia trachomatis* infection in naive mice after the transfer of immune T cells from immunized mice.²⁵ In this study, we evaluated the utility of a subunit candidate Ca²⁺ ATPase construct based on the novel rVCG delivery system.

Protein expression of TBCA2 using rVCG technology was confirmed by Western blot analysis using mouse anti-TBCA2 mAbs. *T. brucei* Ca²⁺ ATPase mRNA was expressed differentially, and its protein localized in the flagellar pocket and sub-pericellular and surrounding nuclei regions of bloodstream parasites using mouse anti-TBCA2 mAbs.

Intraperitoneal immunization of naïve mice with rVCG-expressing TBCA2 resulted in an unbalanced Th1/Th2-type profile. The production of type I cytokines and increased synthesis of IgG2a antibodies are necessary for host resistance to African trypanosomiasis.^{48–51} IgA and IgG2a responses at 3 and 6 weeks after immunization were significantly higher than controls. The difference between the two antigen-specific antibody isotypes were noted at 6 weeks after immunization, with IgA levels decreasing as IgG2a levels increased, whereas control levels remained static. On trypanosomiasis infection, IgA is produced by B cells, which is indicative of a Th2 response. A T-independent B-cell response is necessary for early control of trypanosomiasis.⁵² At the same time, Th1 cells promote isotype switching to IgG2a. A previous study showed 60% of mice survived a trypanosome infection with concomitant production of specific IgG2a antibodies after immunization with *T. brucei* flagellar pocket fractions.⁵³ Anti-parasitic IgG2a antibodies mediate phagocytosis of *T. congolense* by macrophages while inducing the production of NO, which is known to be cytotoxic to *T. brucei* and *T. congolense*.⁵⁴ The increasing production of IgG2a in immunized mice indicates rVCG-TBCA2 directs the immune response toward a robust Th1-type profile, which is expected to be protective.

Analysis of splenic immune T lymphocytes from rVCG-TBCA2-immunized mice showed increased production of proinflammatory markers (INF γ , TNF α , and IL-2), but IL-4 and IL-10 production was not significantly different from controls on re-stimulation with TBCA2 *in vitro*. Th1 cytokine responses are beneficial and crucial in providing resistance to African trypanosomiasis by limiting parasite growth during an early infection.⁵⁵ Despite the beneficial effects of Th1 cytokine production, deleterious effects may also occur if overexpressed in the host, particularly INF γ , as a result of decreased IL-10 production. Robust IL-10 production maintains protective immune responses during a *T. brucei* infection, whereas sustained INF γ production in *T. brucei*-infected IL-10 knockout mice resulted in increased mortality of mice compared with infected wild-type mice.⁵⁵ In this study, rVCG-TBCA2 induced IL-4 and IL-10 production that was not statistically different from controls, but the reasons are unclear and are currently being studied.

The lack of robust IL-10 production probably intensified the deleterious inflammatory effects of INF γ . However, it seems that increased INF γ levels were likely the reason for the observed increase in parasitemia resulting in fatal disease in mice. *T. brucei* have been shown to use INF γ as a growth factor to proliferate profusely, which could overwhelm the mice.^{56,57} Thus, our findings corroborate those of previous studies indicating that an increase in circulating INF γ levels results in decreased survival of immunized mice. Immunization with the TBCA2 subunit vaccine failed to reduce parasitemia and protect *T. brucei*-infected mice despite a median survival of 6 days longer than controls.

In conclusion, these results indicate that immunizing mice with rVCG-expressing TBCA2 results in the production of parasite-specific antibodies and proinflammatory cytokines (IL-2, INF γ , and TNF α) but an unremarkable Th2-type response. This biased stimulation of proinflammatory cytokines with a recombinant antigen is novel and requires further study to

ascertain the mechanism involved. TBCA2 was localized on the pericellular membrane and nuclei of parasites, indicating that these Ca^{2+} pumps may serve a dual purpose of pumping out calcium at the nuclear membrane as well as pericellular membrane. The extensive distribution of the pumps on the pericellular membrane indicates that they may be suitably targeted in future anti-pump drug design. Despite an increased median survival of 6 days in vaccinated mice, none of the mice were protected against infection. Perhaps, a stronger Th2 response than was observed in this study may be vital for clearing parasites from circulation. This may require a modification of the vaccine design to include a Th2-enhancing feature. We are currently studying the possibility of targeting relevant Ca^{2+} pump antigens to induce a balanced Th1/Th2-type response in the host that could potentially control parasitemia and increase host survival. Thus, the results showed that the rVCG vaccine technology could potentially be used to alter host cytokine homeostasis but may require further modification to ensure protection against the trypanosomes.

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FIGURE 1.

Construction of pTBCA2. The *T. brucei* gene, *tbca2*, was amplified from the pT7blue plasmid vector with primers, which flanked either end of the *tbca2* open reading frame. The primers included the restriction sites *Xho*I and *Pst*I (underlined) used for cloning into the corresponding sites of the membrane targeting vector, pKSEL5-2, resulting in pTBCA2. *E'* and *L'* are target sequences for membrane anchorage. Expression of rTBCA2 from pTBCA2 is under the transcriptional regulation of the *lac* promoter (*lacPO*). *bla*, β -lactamase gene; *ColEI*, replication origin; *LacZ'*, 5'-sequences of β galactosidase gene (*lacZ*); *E'*, 5'-sequences of gene *E* from bacteriophage (λ X174; *L'*, 3'-sequences of gene *L* from phage MS2; mcs1, mcs2, mcs3, different multiple cloning sites; *tbca2*, gene coding for *T. brucei* calcium ATPase pump protein.

FIGURE 2.

Western immunoblot of TBCA2 detected with the mouse anti-TBCA2 monoclonal antibody. Mid-log phase cultures were induced with IPTG for 45 minutes and cell lysates assayed for rTBCA2 expression by Western blot. Lane 1, CellLytic B II-purified TBCA2 from *V. cholerae* HTBCA2 cells; Lane 2, *V. cholerae* HI cells containing the plasmid vector, pKSEL5-2; Lane 3, *V. cholerae* HI cells containing the recombinant plasmid, pTBCA2, expressing rTBCA2. Numbers to the left are approximate molecular masses, in kilodaltons.

FIGURE 3.

RT-PCR analysis of TBCA2 mRNA from *in vitro* (Lane 1) and bloodstage (Lane 2) *T. brucei* (GuTat 10.1). RT-PCR was run at linear range for both primer pairs using 2.0 μ g DNAase-treated total RNA. The TBCA2 oligonucleotide primer pair (sense, ACAGCGAAGATGCCGA, anti-sense, CTTGGTTAACTCCTGC) was used to amplify a 308-nucleotide product. mRNA was normalized by RT-PCR using a primer pair (sense: GCGAACGAA-ATCCGTAATGT, anti-sense CGTCCTCCAAG-TCAGCTTTC) to give a 300-bp PCR fragment of the paraflagellar rod gene (PFR-A; GenBank accession no. X14819).

FIGURE 4.

TBCA2 was localized using Axophor red in trypanosomes34 (A) and the nuclei and kinetoplasts of the parasites stained blue with DAPI (B). A and B were merged to show co-localization (C). Cells were embedded in Vectashield and viewed with a confocal-microscope imaging system (Olympus). This figure appears in color at www.ajtmh.org.

FIGURE 5.

Serum was collected from both control and rVCG-TBCA2-vaccinated mice at 3 and 6 weeks after immunization. IgA (**A**) was significantly greater in vaccinated mice at all times but began to decrease by Week 6. The same was observed for IgG2a (**B**). At Weeks 3 and 6, IgG2a significantly increased in vaccinated mice compared with controls. Bars represent SD of three experiments (* $P < 0.05$, ** $P < 0.01$).

FIGURE 6.

Splenic immune T cells were isolated 6 weeks after immunization and stimulated with TBCA2 *in vitro* to analyze Th1 (A) and Th2 (B) cytokine production. The vaccine construct significantly induced elevated antigen-specific Th1 cytokines: IL-2, IFN- γ , and TNF- α . However, the amounts of IL-4 and IL-10 production by splenic T cells from vaccinated mice were not significantly different ($P = 0.25$ and $P = 0.07$, respectively) from control mice. Bars represent SD of three experiments (** $P < 0.01$).