Intranasal Vaccinations with the trans-Sialidase Antigen plus CpG Adjuvant Induce Mucosal Immunity Protective against Conjunctival Trypanosoma cruzi Challenges

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Trypanosoma cruzi is an intracellular protozoan parasite capable of infecting through mucosal surfaces. Our laboratory has previously elucidated the anatomical routes of infection after both conjunctival and gastric challenge in mice. We have shown that chronically infected mice develop strong immune responses capable of protecting against subsequent rechallenge with virulent parasites through gastric, conjunctival, and systemic routes of infection. We have also shown that intranasal immunizations with the unique T. cruzi trans-sialidase (TS) antigen protect against gastric and systemic T. cruzi challenge. In the current work we have investigated the ability of purified TS adjuvanted with CpG-containing oligonucleotides to induce immunity against conjunctival T. cruzi challenge. We confirm that intranasal vaccinations with TS plus CpG induce TS-specific T-cell and secretory IgA responses. TS-specific secretory IgA was detectable in the tears of vaccinated mice, the initial body fluid that contacts the parasite during infectious conjunctival exposures. We further show that intranasal vaccinations with TS plus CpG protect against conjunctival T. cruzi challenge, limiting local parasite replication at the site of mucosal invasion and systemic parasite dissemination. We also provide the first direct evidence that mucosal antibodies induced by intranasal TS vaccination can inhibit parasite invasion.

Trypanosoma cruzi is an intracellular parasite and the causative agent of Chagas’ disease. An estimated 16 to 18 million people in Latin America are infected, and up to 40% of those will develop the manifestations of chronic Chagas’ disease, including cardiac arrhythmias, cardiomyopathy, megaeosophagus, and/or megacolon. To date there is no efficacious treatment for chronic infection, nor is a vaccine to prevent infection currently available. The parasite is not capable of infecting the mammalian host through intact skin but can infect through breaks in the skin or mucosal surfaces, such as the gastric mucosa after oral challenge or nasal-associated mucosa after conjunctival contamination (8, 12). The conjunctival route of T. cruzi infection is a common mode of parasite transmission. In fact, unilateral palpebral edema known as Román˜a’s sign, which occurs after conjunctival parasite contamination, has been recognized as a marker for acute Chagas’ disease since the 1930s (15).

We are currently investigating whether a vaccination protocol using the T. cruzi trans-sialidase (TS) antigen can induce mucosal immunity protective against conjunctival parasite challenge in susceptible BALB/c mice. The TS gene is a member of the largest gene family of T. cruzi and an important virulence factor for parasite infection. T. cruzi is not capable of synthesizing its own sialic acid; however, this molecule is required for host cell invasion by the parasite (5). TS both cleaves sialic acid residues from the surface of host cells and transfers them to the parasite surface.

TS is an immunodominant protein inducing strong antibody and cell-mediated responses during human infection (13). The catalytic domain contains an H-2kd-restricted CD8+ T-cell epitope (IYNVGQVSI) and at least one unidentified CD4+ T-cell epitope (6). DNA vaccines encoding the catalytic domain of TS have been shown to induce immunity protective against systemic T. cruzi challenge (3, 4, 6, 16). It has also been shown that both CD4+ and CD8+ T-cell epitopes are required for this TS-specific protective immunity (6). We have recently shown that intranasal vaccination with TS protein adjuvanted with CpG can induce immune responses protective against systemic and oral T. cruzi challenge (11).

In the current work we demonstrate that intranasal vaccination with the catalytic domain of TS combined with an oligonucleotide containing Toll-like receptor 9 (TLR-9)-triggering CpG motifs induces strong type 1 cellular immune responses as well as production of mucosal secretory IgA (sIgA) present in fecal extracts (FE) and in tears. We also demonstrate that these immune responses are protective against both systemic and conjunctival T. cruzi challenge and that opsonization with vaccine-induced TS-specific mucosal antibodies can inhibit parasite infection after conjunctival challenge. This is the first direct evidence that a vaccine-induced mucosal antibody response can inhibit T. cruzi infection after conjunctival challenge. These data provide further evidence that the development of vaccines protective against T. cruzi infection is a reasonable research goal and that these efforts should be directed toward generating type 1 immune responses and production of secretory IgA.

MATERIALS AND METHODS

Parasites, mice, and challenge protocol. The Tulahue`n strain of T. cruzi was maintained by passage through BALB/c mice (Harlan, Indianapolis, IN) and the reduviid vector Dipetalogaster maximus. To generate culture-derived metacyclic...
trypomastigotes (CMT), T. cruzi epimastigotes were cultured in modified Gracce’s medium (Sigma, St. Louis, MO) for 7 to 14 days. Parasite concentrations were determined by hemacytometer count, and percentages of CMT were determined by DiffQuik staining (Dade International Inc., Miami, FL). Blood form trypomastigotes (BFT) were maintained by passage through BALB/c mice. Immune BALB/c mice were challenged with these CMT preparations by atraumatic routes, as described previously [10]. All preparations of recombinant TS and phase 10 protein contained less than 15 endotoxin units/mg of protein. One month after the second immunization, mice were either challenged subcutaneously with 5,000 BFT and monitored for survival or challenged with 3 × 10^5 CMT by contaminative placement on the conjunctiva for protection and immune studies.

**Real-time PCR detection of T. cruzi in conjunctiva-associated tissues.** Parooid and submandibular lymph nodes, nasal cavities with associated nasolacrimal ducts, and spleens were harvested from immunized mice challenged conjunctivally with CMT. DNA samples were purified using a DNeasy kit (Qiagen, San Diego, CA), and total DNA concentrations were adjusted to 20 to 40 ng/ml. Primers (5’AACCCACGAGCAACAACAA3’ and 5’TGGCATCTGCACAACTGAT3’) were used to specifically amplify a 65-bp fragment of cruzipain, with a protocol similar to one we have previously described [10]. However, instead of using a real-time PCR detection system, our previous method of generation was detected using a 6-carboxyfluorescein (FAM)/AAX/N,N,N,N-tetramethyl-6-carboxyhydroxamidine (TAM) probe (5’TGGCCAGAGGCTGGGAC3’) (Synthegen, Houston, TX). TaqMan PCR master mix (Applied Biosystems, Foster City, CA), 900 nM each primer, and 100 to 200 ng of sample DNA. A standard curve was generated using positive control DNA harvested from a known concentration of T. cruzi epimastigotes grown in pure culture. These reactions were run in an ABI Prism 7700 sequence detector (Applied Biosystems) using the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Analysis was performed using Sequence Detection Systems version 1.9a software (Applied Biosystems). Results are reported as T. cruzi molecular equivalents (ME).

**Assays to assess T-cell proliferation.** Spleen and lymph node cells were harvested from memory-immune and naive mice 3 days after parasite challenge. Total cells were plated at a concentration of 2 × 10^6 cells/ml in 96-well round-bottomed microtiter plates. Cells were incubated with medium alone or 2 μg/ml recombinant trans-sialidase protein. Plates were incubated at 37°C with 5% CO_2 for 72 h. Supernatants were then harvested and cells plated with 0.5 μCi/well [3H]thymidine (Amersham Biosciences Corp, Piscataway, NJ). Plates were incubated for 6 hours more, and cells were harvested with a Tomtec Mach-IIIM cell harvester (Tomtec, Hamden, CT) onto glass filter mats. Radiolabeling of DNA synthesis was counted using a Wallac Trilux 1450 Microbeta liquid scintillation counter (Perkin-Elmer, Boston, MA). Results are reported as disintegrations per minute (dpm).

**Enzyme-linked immunospot (ELISPOT) assay to assess IFN-γ production.** Millifilter HA 96-well microtiter plates with nitrocellulose bases (Millipore, Manassas, VA). Plates were blocked with phosphate-buffered saline (PBS) containing 10% fetal bovine serum (FBS) to prevent nonspecific binding. To assess antigen-specific IFN-γ secretion by both CD4 + and CD8 + TS-specific T cells, total lymph node or spleen cells were cultured with A20J cells permanently transfected with a plasmid expressing T. cruzi trans-sialidase (TS). Wells containing nontransfected A20J cells were used as negative controls. To evaluate relative levels of CD8 + T-cell responses, a previously described H-2Kb-restricted epitope (IVYGQVSVI) derived from the TS antigen was used to stimulate lymphocyte cultures (6). A total of 2 × 10^5 mononuclear cells were plated per well and pulsed with 2 μM TS peptide. The numbers of IFN-γ-producing cells were detected by the addition of biotinylated anti-IFN-γ (Pharmingen, San Diego, CA), followed by addition of streptavidin conjugated to horseradish peroxidase (HRP) (Jackson Immunoresearch Laboratories, West Grove, PA) and 3-amino-9-ethylcarbazole substrate precipitation. Results are reported as the number of spot-forming cells (SFC) per million cells.

**Harvest of mucosal secretions to assess antigen-specific secretory IgA responses.** T. cruzi-specific secretory IgA levels were measured in tears and fecal extracts obtained from memory-immune and naive BALB/c mice. Mice were anesthetized with a standard dose of 60 mg/kg ketamine and 5 mg/kg xylazine. The conjunctival and ocular surfaces of five mice from each group were washed with 5 μl PBS containing 10% fetal calf serum (FCS). These washes were pooled and the final volume adjusted to 50 μl. Fecal pellets were collected from five naive mice and pooled for each group. The pellets were dissolved in PBS containing 10% FBS by vortexing for 15 min at room temperature. Extracts were clarified by centrifugation. TS-specific sIgA responses were also measured in tears and fecal extracts from six mice intranasally vaccinated with TS CpG and six mice intranasally vaccinated with NC CpG. Samples were prepared as described above; however, in this case the samples were not pooled. The supernatants and tear preparations were serially diluted and added to Immulon II HB enzyme-linked immunosorbent assay (ELISA) plates coated with either 20 μg/ml T. cruzi lysate or 5 μg/ml recombinant TS protein to measure sIgA levels in memory-immune and TS CpG vaccinated mice, respectively. Plates were incubated overnight at 4°C and washed with PBS-0.05% Tween 20. Biotinylated goat-anti-mouse IgA (Southern Biotechnology Associates, Burlingame, AL) was added to detect bound IgA antibodies. Plates were incubated for 2 h at room temperature, washed, and developed with streptavidin conjugated to horseradish peroxidase, followed by the addition of 3,3’,5,5’-tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO). Plates were analyzed at 450 nm referenced to 650 nm.

**Assessment of protection provided by opsonization of CMT with fecal extracts (FE) from memory-immune and TS CpG-vaccinated mice.** For opsonization studies, parasites (10^8 CMT/ml) were mixed 1:1 with sterile filtered (0.2 μm) FE from TS CpG-vaccinated mice and incubated at room temperature for 30 min. For in vivo studies, mice were inoculated with 5 μl of each opsonized parasite suspension (containing 2 × 10^5 CMT) per eye as described above. Mice were sacrificed after 10 days and DNA isolated from the draining lymph nodes. Parasite replication was measured by real-time PCR as described above.

**RESULTS AND DISCUSSION**

**Intranasal TS CpG vaccination induces antigen-specific type 1 immune responses.** To characterize the immune responses induced by intranasal vaccination, we measured antigen-specific proliferative responses and IFN-γ production. Briefly, two doses of recombinant TS or negative control phase 10 (NC) protein (50 μg) adjuvanted with CpG 1826 (10 μg) were administered intranasally to BALB/c mice 1 week apart, as previously described [10]. One month after the final vaccination, mice were challenged via contamination of the ocular surface with 3 × 10^3 CMT as described previously [8]. Three days after challenge, mice were sacrificed, spleens harvested, and single-cell suspensions prepared. Spleen cells plated at 2 × 10^6 cells per well in 96-well microtiter plates were cultured with either medium alone or 2 μg/ml of recombinant TS protein. Proliferation was measured by [3H]thymidine incorporation as previously described [10]. Significantly higher levels of TS-specific proliferation were seen in wells containing spleen cells from TS CpG-vaccinated mice than in wells containing spleen cells from NC CpG-vaccinated controls (Fig. 1a) (n = 3/group; P < 0.01 by Mann-Whitney U test), with means of 212,340 versus 33,437 dpm, respectively.

To assay for antigen-specific T-cell IFN-γ responses, spleen cells were cocultured with A20J cells stably transfected with a plasmid encoding the signal peptide and catalytic domain of TS or with control A20J cells. The numbers of TS-specific IFN-γ secreting cells were determined by ELISPOT assay as described previously [10]. Significantly higher numbers of IFN-γ producing cells were seen in lymphocyte preparations harvested from TS CpG-vaccinated mice than in controls, with means of 75 and 2.5 spot-forming cells (SFC) per million spleen cells, respectively (Fig. 1b) (n = 3/group; P < 0.01 by Mann-Whitney U test). To assess antigen-specific CD8 + T-cell responses, we used a synthetic peptide encoding the H-2k4-
Intranasal vaccination with TS CpG stimulates slgA production. We have previously shown that conjunctival infection induces protective mucosal antibody responses (8). However, chronic infection is not a viable vaccination strategy. Therefore, we investigated whether our TS CpG vaccination protocol also could induce protective mucosal antibody responses. Fecal extracts (fecal pellets dissolved in PBS containing 10% FBS and clarified by centrifugation) and tears were prepared from either TS CpG-vaccinated mice or mice vaccinated with a negative control protein plus CpG and studied for levels of TS-specific slgA by ELISA. Briefly, Immulon II HB ELISA plates were coated with 5 μg/ml recombinant TS protein. Tears and fecal extracts (FE) were serially diluted and added to the coated plates. End point titers of slgA were determined by addition of a biotinylated anti-IgA antibody followed by streptavidin-HRP (Jackson Immunoresearch) and visualized with TMB substrate (Sigma, St. Louis, MO). Significantly higher titers of antigen-specific slgA were seen in both the FE and tears of the TS CpG-vaccinated mice (Fig. 2a and b). Differences between the TS-vaccinated and negative control groups were statistically significant to end point dilutions of 1:64 and 1:320 in FE and tears, respectively (n = 6/group; P < 0.05 by Student’s t test).

Inductions of both mucosal antibody responses and type 1 immunity are important goals for any vaccine strategy targeting intracellular pathogens that are capable of invading through a mucosal route. It is reasonable to assume that because humoral immunity characterized by generation of pathogen-specific antibodies is characteristic of type 2 immune responses and the cytokines involved in the generation of type 1 versus type 2 responses antagonize the generation of the other immune response, an immunization strategy resulting in a type 1 systemic response could inhibit the production of mucosal IgA. This has previously been shown not to be the case, and our current data clearly show that mucosal antibody responses develop in the context of a mucosally administered vaccine which stimulates a systemic type 1 response (9, 10, 12).

Mice immunized intranasally with trans-sialidase and CpG are protected against T. cruzi systemic challenge. Six- to 8-week-old BALB/c mice were immunized twice intranasally with TS or control protein and CpG 1826 as described above. One month after the second immunization, six mice from each group were injected subcutaneously with 5,000 BFT, a lethal dose for naïve BALB/c mice. Five out of six of the TS CpG-vaccinated mice survived the challenge, while none of the NC CpG-vaccinated mice survived (Fig. 3). These results are significantly different by the Fisher exact two-tailed test (P < 0.02). All deaths occurred between 15 and 21 days after challenge. Five of the six TS CpG-vaccinated mice survived for at least 2 months postinfection. These results demonstrate that a significant protective systemic immune response was generated by intranasal TS CpG vaccination.

We have shown that a purified protein vaccine adjuvanted with CpG and given intranasally is capable of inducing immunity protective against systemic T. cruzi challenge. T. cruzi can infect its mammalian host either through mucosal surfaces or...
through breaks in the skin. After initial invasion, the parasite replicates in local tissues, and it then disseminates hematogenously to distant sites (8, 12). The parasite is capable of infecting any nucleated cell. It is therefore critical that any vaccine directed toward controlling parasitemia protect against systemic parasite challenge. While our current vaccine strategy does not provide sterilizing immunity (parasites have been recovered from TS-immunized mice surviving challenge by transferring blood samples into SCID mice [unpublished observations]), we are currently working on using alternate vectors and vaccine strategies to achieve this goal.

Intranasal vaccination with TS CpG provides protection against a conjunctival T. cruzi challenge. Six- to 8-week-old BALB/c mice were vaccinated intranasally and challenged conjunctivally 4 weeks later with $2 \times 10^7$ CMT. Twelve days after challenge, mice from each group (TS CpG and NC CpG) were sacrificed and nasal cavities (including nasolacrimal ducts), draining lymph nodes (parotid and submandibular), and spleens harvested. DNA was purified using a DNeasy kit, and T. cruzi-specific real-time PCR was used as described previously (8) to determine the amounts of parasite replication in each tissue. Significantly less recoverable parasite DNA was found in the draining lymph nodes harvested from the TS CpG-vaccinated group than in those from the negative control group, with mean values of 462 and 195 T. cruzi ME/μg DNA, respectively (Fig. 4a) ($n = 6$ and 9/group, $P < 0.05$ by Mann-Whitney U test). Statistically significant differences in amounts of recoverable parasite DNA were also seen in the spleens of TS CpG-vaccinated mice compared with NC CpG-vaccinated controls (data not shown). These data demonstrate that mucosal immunity can be generated by intranasal vaccination with TS protein adjuvanted with CpG.

In addition to generating systemic immunity, we devised our vaccine strategy to specifically target mucosal immunity as well. By generating immune responses at the immediate site of infection, we hoped to decrease the number of parasites capable of infecting and replicating in local tissues and thereby decrease the overall parasite burden. It is not known what factors influence the development of Chagasic pathology in humans, as only 30 to 40% of those infected with T. cruzi will go on to manifest symptoms of Chagas’ disease. One reasonable possibility is that the lower the parasite burden, the less likely the individual is to develop Chagasic pathology. This possibility is supported by recent studies showing that treating patients chronically infected with T. cruzi with antiparasitic agents can delay and in some cases reverse pathological electrocardiogram (EKG) changes seen in cardiomyopathy due to Chagas’ disease (2, 7, 17, 18). It is possible that a vaccine to prevent the development of Chagas’ disease does not need to provide sterilizing immunity. Decreasing the parasite burden may be

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**FIG. 2.** Intranasal TS CpG vaccination induces secretory IgA production. Fecal extracts (FE) and tears (a and b, respectively) were harvested from TS CpG- and NC CpG-vaccinated mice. End point titers were determined by TS-specific ELISA. Differences between TS CpG- and control-vaccinated mice were statistically significant to end point dilutions of 1:64 and 1:320 in FE and tears, respectively ($n = 5$/group, $P < 0.04$ and 0.02, respectively, by Student’s $t$ tests). These results are representative of three experiments. Error bars indicate standard deviations.

**FIG. 3.** Intranasal TS CpG vaccination protects mice against a systemic T. cruzi challenge. BALB/c mice were vaccinated with either TS CpG or NC CpG and later challenged subcutaneously with 5,000 BFT. Five of the six TS CpG-vaccinated mice survived to an end point of 50 days, while none of the NC CpG-vaccinated mice survived ($n = 6$/group; $P < 0.02$ by Fisher’s exact two-tailed test). These results are representative of five experiments.
sufficient to prevent the clinical manifestations of chronic *T. cruzi* infection. Further studies are required to determine whether or not this is the case.

**TS-specific sIgA protects against conjunctival challenge.**

We also investigated whether the sIgA induced by TS CpG vaccination could inhibit *T. cruzi* infection. FE from TS CpG-vaccinated or control mice were mixed with CMT at a 1:1 ratio and incubated for 30 min at room temperature, and 6- to 8-week-old BALB/c mice were then challenged conjunctivally with approximately $3 \times 10^5$ CMT from each preparation. At 10 days after challenge, mice were sacrificed and draining lymph nodes harvested. DNA was purified and the amount of recoverable parasite DNA determined by real-time PCR. Figure 4b. shows that significantly lower levels of parasite replication were detected in the draining lymph nodes of mice challenged with parasites opsonized with FE from TS CpG-vaccinated mice ($n = 4$ group; $P < 0.03$ by Mann-Whitney U test). The mean amount of recoverable *T. cruzi* DNA from mice challenged with TS CpG FE-opsonized parasites was 158 *T. cruzi* ME/μg DNA, compared with a mean of 723 *T. cruzi* ME/μg DNA recoverable from mice challenged with naive FE-opsonized CMT, an approximately 5-fold decrease.

*T. cruzi* does not normally infect through the large intestine, where sIgA titers present in FE occur. Therefore, one could question the relevance of using sIgA obtained from FE in our opsonization studies (used instead of tears because of the increased total volumes obtained). After oral and conjunctival challenge, *T. cruzi* infects through gastric and nasal-associated mucosal tissues, respectively. The concentrations of secretory antibodies in gastric secretions are unknown and difficult to measure. However, we show in Fig. 2 that the concentrations of TS-specific sIgA are 5-fold higher in tears (which contact parasites inoculated conjunctivally) than in FE in TS-vaccinated mice. Therefore, the use of FE for our opsonization studies is likely to underestimate the relevance of sIgA for protection induced by TS vaccination.

These results indicate that a strong mucosal antibody response is generated by mucosal TS CpG vaccination, which is capable of inhibiting infection at the site of parasite entry. Although we have not purified the TS-specific sIgA and shown that sIgA and not contaminating IgG is responsible for these protective effects, we have quantified both total IgA and total IgG in fecal extracts and found that there was at least 5-fold more IgA than IgG and that specific depletion of IgA and not IgG has reduced the protective opsonizing effects of the immune fecal extracts (data not shown).

Our lab has previously shown that mucosal antibodies are associated with type 1 immune responses that are protective against subsequent mucosal *T. cruzi* challenges (8, 9, 10, 12). Studies from other groups have shown that opsonization with monoclonal antibodies can decrease the infectivity of parasites (14). Presented here are the first data, however, to show that mucosal antibodies induced *in vivo* by vaccination can decrease the infectivity of *T. cruzi*. Induction of mucosal immune responses is an important aspect of mucosal vaccine strategies, and IgA production has been used as a correlate of protective immunity. This is the first evidence in the *T. cruzi* model to directly indicate the importance of mucosal IgA.

We have shown that vaccination with TS protein adjuvanted with CpG provides protection against both systemic and conjunctival *T. cruzi* challenges. This protection is mediated by type 1 immune responses as demonstrated by an increased number of TS-specific IFN-γ-producing CD4$^+$ and CD8$^+$ T cells. We have also shown that TS CpG vaccination induces mucosal IgA production. This parasite-specific IgA prevents infection with opsonized parasites.
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