Further Characterization of Protective *Trypanosoma cruzi*-Specific CD4⁺ T-Cell Clones: T Helper Type 1-Like Phenotype and Reactivity with Shed Trypomastigote Antigens

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We previously reported the isolation from immune mice of a panel of murine clonal T-cell lines which specifically recognize antigens expressed by the trypomastigote stage of the protozoan parasite *Trypanosoma cruzi*, the causative agent of human Chagas' disease. Our analysis indicated that distinct clones which recognize common as well as strain-specific antigenic determinants were represented. The immunoprotective potential of several of these T-cell clones was demonstrated by adoptive transfer of protection to naive syngeneic recipients. Here we report that these T-cell clones are all of the T_{H1} phenotype, as determined from their lymphokine secretion patterns. Significant levels of stimulatory activity for each clone were detected in trypomastigote supernatants, and the release of this activity was time and temperature dependent. Seven of 10 T-cell clones tested responded to nitrocellulose-immunoblotted trypomastigote proteins in the range of 90 to 47 kDa; no fewer than six distinct epitopes residing on at least five distinct polypeptide species were recognized by this panel of clones. Two clones (2G8 and 4B10) previously shown to protect in vivo responded to immunoblotted proteins in the range of 65 to 53 and 90 to 80 kD, respectively. Stimulatory activity for the latter clone was shown to be expressed on the surface of trypomastigotes and to bind specifically to wheat germ agglutinin, indicating that its target antigen is an 85-kDa trypomastigote surface glycoprotein.

T lymphocytes are known to play a critical role in host resistance to the protozoan parasite *Trypanosoma cruzi*, the causative agent of human Chagas' disease (25, 44, 49, 59). However, their precise functional role(s) in immunity has yet to be determined. To facilitate study of the T-cell response to this parasite, we previously established a continuous *T. cruzi* antigen-specific murine T-cell line from the splenocytes of a *T. cruzi*-immune C57BL/6 mouse by cyclic propagation in vitro with irradiated trypomastigotes (31). Preliminary characterization of this line and a panel of clones derived from it indicated that these T cells bore the CD4⁺ surface marker and that their recognition of parasite antigen was class II major histocompatibility complex (MHC) (Ia^b) restricted. Specificity analysis indicated that the antigens recognized by these clones were species, trypomastigote stage, and predominantly *T. cruzi* strain specific. Furthermore, because the "parent" line and several clones were found to be capable of transferring protection from otherwise lethal challenge infection to naive syngeneic recipients, we decided to characterize these clones further.

Recent data suggest that two subpopulations of murine CD4⁺ T cells can be functionally distinguished by their lymphokine secretion profiles (54). Thus, so-called T helper type 1 (T_{H1}) cells secrete interleukin-2 (IL-2), gamma interferon (IFN-γ), and lymphotoxin and appear to be involved mainly in cell-mediated immune responses, whereas T helper type 2 (T_{H2}) cells secrete IL-4, IL-5, and IL-10 and are involved principally in humoral immune responses. Evidence from studies of *Leishmania major* infections in mice indicate that the type of T helper response generated

during infection is a critical determinant of outcome; a predominant T_{H1} response was found to be associated with resistance to this intracellular pathogen, while a predominant T_{H2} response was found to be associated with disease progression (19, 51).

We report here that these *T. cruzi*-specific cloned T-cell lines secrete IL-2 and IFN-γ upon activation and are therefore of the T_{H1} phenotype. Furthermore, the parasite antigens recognized by these clones are shed by living trypomastigotes in a time- and temperature-dependent manner. Activity for one of these clones, 4B10, which was previously shown to protect mice in vivo, migrates at 90 to 80 kDa, as determined by T-cell immunoblotting, is expressed on the trypomastigote surface, and binds specifically to wheat germ agglutinin (WGA) lectin, indicating that the target antigen of this protective T-cell clone is an 85-kDa glycoprotein species.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice were obtained from the Animal Facility, Research Institute of Scripps Clinic, or from the National Cancer Institute, Bethesda, Md.

Parasites. The Y strain of *T. cruzi* was used in all of these experiments and was originally obtained from Ruth Nussensweig (36). Growth and harvesting of cell culture-derived trypomastigotes were done as described previously (31).

T-cell lines and hybrids. T-cell lines and clones were derived from the spleen of a *T. cruzi*-hyperimmune C57BL/6 mouse and have been maintained and propagated in vitro as described previously (31). Four clones (2D6, 2C5, 1A7, and 2C7) derived from the same T-cell population in addition to those originally reported have been included in the present analysis. T-cell activation was measured by a standard 72-h

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proliferation assay as described previously (31). An immortalized T-cell hybridoma derived from clone 4B10 and designated H4B10 was used in some of the experiments. This hybrid, which exhibited specificity identical to that of the original clone (data not shown), was produced by fusing 3- to 4-day antigen-activated clone 4B10 T cells with the AKR-derived mouse thymoma BW5147 and selection on HAT (hypoxanthine-aminopterin-thymidine) medium (50). Antigen-specific activation of this T-cell hybridoma was quantified by assessing the ability of supernatants from stimulated cultures to support the growth factor-dependent proliferation of the murine T-cell line CTLL-2 (12). T-cell hybrids (5×10^4) and 5×10^5 irradiated syngeneic spleen cells per microwell were cultured together in the presence of antigen. After 30 h of incubation, 100 μ l of supernatant per well was harvested from replicate wells and transferred to a new microwell plate. CTLL-2 cells (5×10^3 in 100 μ l) were then added, and, after 16 to 20 h of incubation, the wells were pulsed with [3 H]thymidine, and incorporation was determined 4 to 8 h later.

Lymphokine assays. The identity of the growth factor lymphokine secreted by the various T-cell clones was determined by a monospecific bioassay (12). Briefly, supernatants from activated T-cell clones were incubated with either an anti-IL-4 monoclonal antibody (MAb) (11B11; American Type Culture Collection [ATCC]) or a mixture of two IL-2 receptor (IL-2R) MAbs (7D4 and PC61; ATCC), and the effect of these treatments on their ability to support the growth of CTLL-2 cells was assessed. MAbs were prepared from mouse ascitic fluid by ammonium sulfate precipitation. Thirty-hour supernatants were assayed for IFN- γ production with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Genzyme).

Subcellular antigen fractionation. Trypomastigotes of the Y strain of *T. cruzi* were obtained from the supernatants of mouse 3T3 fibroblast cell cultures 5 to 7 days after trypomastigote infection as described previously (31). Trypomastigote suspensions were subjected to several slow spins ($50 \times g$, 5 min) to remove amastigotes and host cell debris, washed twice ($1,200 \times g$, 12 min) in phosphate-buffered saline (PBS)-1% glucose, resuspended to 5×10^8 organisms per ml in lysis buffer (20 mM Tris [pH 7.5], 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 μ g of aprotinin per ml), and freeze-thawed three times by alternate exposure for 10 min to the vapor phase of liquid nitrogen and a 37°C waterbath. Following centrifugation at $100,000 \times g$ for 75 min, the supernatants were removed, and proteins were precipitated by the addition of 10 volumes of cold acetone and overnight incubation at -20°C. Precipitated proteins, designated the soluble (S) fraction, were pelleted by centrifugation ($10,000 \times g$, 20 min), dried in a Speed-Vac concentrator, and stored at -20°C.

To obtain water-insoluble trypomastigote proteins (I), the freeze-thawed $100,000 \times g$ trypomastigote pellet was resuspended to 5×10^8 organism equivalents per ml in lysis buffer containing 0.5% Nonidet P-40 (NP-40). The suspensions were rocked overnight at 4°C. Insoluble material was pelleted at $100,000 \times g$ for 75 min, and the solubilized proteins in the supernatant were precipitated by the addition of cold acetone and prepared as described above. Prior to use in T-cell assays, the dried antigen was sterilized by irradiation (100 krad, cobalt source) and reconstituted with sterile PBS. The protein content of antigen preparations was determined by a modified Lowry assay (38).

Preparation of shed parasite antigen. Washed, cell culture-derived trypomastigotes (5×10^7 /ml) were incubated in

T-cell medium at 4, 26, or 37°C. At intervals, aliquots were removed, and after viability determinations were made, parasites were eliminated by two successive rounds of centrifugation ($13,000 \times g$, 10 min). Supernatants were then filtered through a 0.2- μ m filter and aliquoted into microplates, which were frozen at -20°C until use.

Preparation of biotinylated trypomastigote surface antigens. Trypomastigotes were surface biotinylated with *N*-hydroxy-succinimidoiminobiotin hydrobromide (NHS)-iminobiotin (Pierce) in dimethylformamide as described by Beard et al. (9). Viability was maintained throughout the labeling procedure, as determined by microscopic examination. Following exposure to NHS-iminobiotin, parasites were washed three times ($1,200 \times g$, 12 min) in PBS-1% glucose, and pellets were frozen at -20°C. Then, 5×10^9 thawed trypomastigotes were solubilized in 0.5% NP-40 containing 1 mM PMSF and 5 mM iodoacetamide. Cell debris was removed by centrifugation at $13,000 \times g$ for 10 min. The lysate was then made to 50 mM ammonium carbonate (pH 11), 1 M NaCl, 1 mM PMSF, and 1 mM iodoacetamide and applied to a column containing 2 ml of immobilized avidin (Pierce) equilibrated in the same buffer containing 0.05% NP-40. Flowthrough material was collected, the column was washed with 25 column volumes of equilibration buffer, and bound material was eluted by the addition of low-pH elution buffer (50 mM sodium acetate [pH 4.0], 1 M NaCl, 1 mM PMSF, 5 mM iodoacetamide). Eluted material was immediately neutralized by collection into microcentrifuge tubes containing 0.3 ml of 2 M Tris (pH 6.8). The flowthrough and eluted fractions were concentrated and subjected to acetone precipitation to remove detergent. Dried proteins were reconstituted in PBS, assayed for protein content, and sterilized by irradiation prior to use in T-cell assays.

T-cell immunoblotting. Trypomastigote proteins were solubilized in lysis buffer (20 mM Tris [pH 7.5], 1 mM PMSF, 0.5 μ g of aprotinin per ml) containing 0.5% NP-40, and 40 μ g per lane was separated on 8% polyacrylamide-sodium dodecyl sulfate (SDS) gels under reducing conditions (26). Proteins were electrophoretically transferred to a nitrocellulose (NC) membrane (Schleicher and Schuell; 0.2 μ m) by standard procedures for Western immunoblotting (10). After being washed for 1 h in distilled water, the NC membrane was dried, and lanes containing trypomastigote proteins were cut into strips. Molecular weight ranges for each strip were determined by comparison with India ink-stained lanes containing molecular weight standards. Replicate NC strips corresponding to each size range were further processed by the procedure described by Abou-Zeid et al. (1). Following several washes (6,000 rpm for 3 min in a microcentrifuge) in T-cell medium (31), NC particles were resuspended in 1 ml of T-cell medium and sterilized by irradiation (100 krad). Before the NC particles were used as an antigen, the tubes were vortexed, and large particles and aggregates were allowed to settle out for 3 min, leaving a fine suspension of NC particles. Dilutions of these were aliquoted directly into 96-well assay plates, which were frozen at -20°C until use. Two preparations of NC-bound *T. cruzi* antigen with slightly different size ranges were used in these experiments.

WGA affinity chromatography. Released antigen from a total of 3×10^9 to 5×10^9 trypomastigotes incubated for 24 to 48 h at 4°C in PBS-1% glucose supplemented with 1 mM PMSF was pooled and passed over a 1-ml WGA-Sepharose (Sigma) column preequilibrated with loading buffer (30 mM Tris [pH 8.0], 0.15 M NaCl, 1 mM PMSF). Flowthrough material was collected, and the column was washed with loading buffer until the optical density at 280 nm was less

TABLE 1. Production of IL-2 but not IL-4 by antigen-activated *T. cruzi*-specific T-cell clones

Supernatant	Proliferation (mean cpm of [³ H]thymidine) in the presence of:		
	No antibody	Anti-IL-4	Anti-IL-2R
Clones			
1A7	35,968	36,966	947
2A5	17,182	14,401	526
2C1	7,162	5,868	493
2C7	42,735	42,876	9,260
2C10	13,013	13,405	763
2D6	22,893	24,464	595
2G8	2,351	2,238	292
8F9	12,067	20,031	215
H4B10	22,701	37,026	105
Controls ^a			
IL-2	80,301	81,323	5,813
IL-4	8,556	571	903

^a Control supernatants contained 50 U of IL-2 or IL-4 per ml.

than 0.01 U. Bound material was specifically eluted with 3 to 5 ml of 0.25 M *N*-acetylglucosamine (Sigma). Flowthrough and eluted material was spin dialyzed against PBS, concentrated on Centricon-10 spin columns, and frozen at -20°C until use. Protein concentrations were determined, and the antigen was irradiated prior to use in T-cell assays.

RESULTS

Lymphokine secretion patterns of T-cell clones. Recent data suggest that two distinct CD4⁺ T helper subpopulations which differ in lymphokine secretion patterns and function exist in the mouse (54). In light of our ability to transfer protection with several of these clones (31), it was of interest to determine their T helper phenotypes. When supernatants from antigen-activated *T. cruzi*-specific T-cell clones or the T-cell hybrid clone H4B10 were incubated with CTLL-2 cells in the presence of MAbs against either mouse IL-4 or IL-2R, only the anti-IL-2R MAb significantly inhibited the proliferation of CTLL-2 cells, indicating that all of these clones secrete IL-2 rather than IL-4 (Table 1). Supernatants were also tested for the presence of IFN- γ with a commercial ELISA kit. As shown in Table 2, all of the T-cell clones tested were found to secrete measurable quantities of IFN- γ after specific stimulation. The one T-cell hybrid tested, H4B10, did not produce IFN- γ in response to antigen stimulation. While this result was puzzling, considering that this hybrid does produce IL-2 (Table 1), T-cell clones often

TABLE 2. IFN- γ production by antigen-activated *T. cruzi*-specific T-cell clones

Clone	IFN- γ production ^a (ng/ml)	
	Stimulated	Unstimulated
1A7	1.8	<0.1
2D11	62.2	0.1
2G8	93.8	1.5
2C10	34.3	<0.1
2C1	12.6	<0.1
2D6	61.3	0.6
2C7	49.4	<0.1
H4B10	0.9	0.4

^a Measured in 30-h supernatants with a commercial ELISA kit (Genzyme).

lose the capacity to produce lymphokines, including IFN- γ , when fused to form T-cell hybrids, presumably as a consequence of chromosome loss (46).

T-cell responses to fractionated trypomastigote antigens. Our previous analyses of the specificity of these clones were performed with whole organisms. To localize recognized antigens, we initially separated trypomastigote antigens into two fractions, containing predominantly water-soluble (S) proteins or water-insoluble (I) proteins. When T-cell responses to these fractions were tested, all of the clones proliferated to both fractions (Fig. 1). For several of the clones (2D6, 4B10, and 1A7), specific activity was significantly higher (2- to 10-fold) in the I fraction. Examination of I and S fractions derived from ³⁵S-labeled trypomastigotes by SDS-polyacrylamide gel electrophoresis (PAGE) revealed a number of common protein bands (data not shown).

T-cell responses to shed trypomastigote antigens. Because the previous experiments had shown that some of this stimulatory activity was soluble, we next determined whether the antigens recognized by these clonal T-cell lines were being shed by trypomastigotes. As presented in Fig. 2, significant levels of stimulatory activity for all of the clones were released into supernatants by cultured living trypomastigotes. While antigenic activity was released minimally at 4°C, more rapid release occurred at 26 or 37°C and could easily be detected in supernatants within 1 h of initiation of the culture. Activity for two of the eight clones tested, 4B10 and 2D6, was released even at 4°C. Release of this antigenic material was not due to parasite death, because viability in these cultures was maintained at high levels throughout the time course (data not shown). SDS-PAGE analysis of supernatants obtained from ³⁵S-labeled trypomastigotes at similar time points confirmed the time-dependent release of labeled material (data not shown).

To examine the physical form of this released T-cell stimulatory activity, living trypomastigotes were allowed to shed antigen at 26°C for 24 h, and then antigenic material was fractionated by differential centrifugation. As presented in Fig. 3 for several of these T-cell clones, most of the antigenic material was found in either the 1,000 $\times$ g pellet or the 100,000 $\times$ g supernatant.

T-cell immunoblotting. Further experiments to identify the target antigens recognized by these clones were performed by the technique of T-cell immunoblotting (1). As shown in Fig. 4, 7 of the 10 clones tested proliferated to specific *T. cruzi* antigen-containing NC fractions. In each case, proliferation was induced by one or sometimes two contiguous fractions. Clones reacting predominantly with single NC fractions included 2C5 (53 to 47 kDa) and 1A7 (65 to 59 kDa), and 4B10 (90 to 80 kDa), while clones recognizing two contiguous fractions included 2D6 (71 to 65 and 65 to 59 kDa) and 2G8, 2D11, and 2A5 (65 to 59 and 59 to 53 kDa).

Additional properties of the antigen recognized by "protective" clone 4B10. To determine whether clone 4B10's target antigen was expressed at the parasite surface, we surface-biotinylated trypomastigote proteins and purified them by affinity chromatography on immobilized avidin. Following acetone precipitation to remove detergent, the flowthrough and avidin-binding eluted fractions were compared for their ability to stimulate T-cell hybrid clone H4B10. As the data indicate (Fig. 5), the avidin-binding fraction contained significantly higher activity than the non-avidin-binding fraction, suggesting that the target antigen for this clone is expressed on the parasite surface.

Because two major trypomastigote surface glycoproteins of 85 (6) and 90 (6, 8, 65) kDa occur in the size range

FIG. 1. Proliferative responses of *T. cruzi*-specific T-cell line ST1 and various ST1-derived T-cell clones stimulated with water-soluble (○) or water-insoluble (●) trypanostigote antigens. Antigen was prepared as described in Materials and Methods; 10^4 rested T cells and 4×10^5 irradiated (3,000 rads) syngeneic spleen cells along with dilutions of antigen were incubated in triplicate for 3 days at 37°C in 5% CO_2 . At 12 to 16 h prior to harvest, $1 \mu\text{Ci}$ of [^3H]thymidine was added to each well. Well contents were harvested onto glass fiber filters, and radioactivity was counted by liquid scintillation.

recognized by clone 4B10 (90 to 80 kDa), we next tried to determine whether one of these proteins was the target of this particular clone. Because these proteins show differential affinity for WGA (6, 9, 24), we examined whether activity for this clone bound specifically to WGA. As presented in Table 3, while almost all (>90%) of the stimulatory activity for the T-cell clones 2G8 and 2D6 was found in the non-WGA-binding fraction, activity for clone H4B10 was significantly enriched in the WGA-binding fraction, suggesting that this clone does recognize an 85-kDa glycoprotein species.

DISCUSSION

We previously reported the isolation and preliminary characterization of a panel of CD4^+ , MHC class II-restricted *T. cruzi*-specific T-cell clones (31). Our demonstration of immunoprotective function in these T cells prompted us to characterize them further and to attempt to determine their antigen specificity.

One previously unresolved issue was the T helper pheno-

type of these T cells. Our finding that these *T. cruzi*-specific T-cell clones are of the T_{H1} helper type, as evidenced by their secretion of IL-2 and IFN- γ (Tables 1 and 2), is consistent with the ability of several of these clones to transfer protection, since IFN- γ has been shown to play a critical role in resistance to *T. cruzi* infections (39, 41, 52, 63). Presumably, one important protective role of IFN- γ in *T. cruzi* infections is the activation of intracellular killing mechanisms in macrophages (39, 63) and perhaps other cell types (39). We previously demonstrated that several of these T-cell clones were capable of inducing intracellular killing of *T. cruzi* when cultured with infected peritoneal macrophages (31). Such macrophage-activating activity has been reported to correlate well with resistance (32). In addition to their role in cell-mediated immune responses, T_{H1} T cells are also known to exhibit antibody helper function, especially in immunoglobulin G2 (IgG2) antibody responses (54). Interestingly, previous analyses of antibodies capable of passively transferring resistance against *T. cruzi* infection indicated that these are primarily of the IgG2 isotype (55), suggesting the possibility that parasite-specific T_{H1} T cells

FIG. 2. Time course of release of T-cell target antigens by cultured trypomastigotes. (Left panels) Proliferative responses of various T-cell clones to supernatants derived from living trypomastigotes cultured at 4°C (○), 26°C (△), or 37°C (□) for 1, 21, or 42 h. T cells were stimulated with 10⁶ trypomastigote equivalents of supernatant antigen, and assays were performed as described in the legend to Fig. 1. (Right panels) Proliferative responses of T-cell clones to whole irradiated trypomastigotes.

may also participate in the generation of protective antibody responses. Furthermore, considering that CD8⁺ T cells have been shown to be involved in resistance to experimental *T. cruzi* infections (58), an additional possible role for CD4⁺ T_{H1} T cells and their soluble factors may be in promoting the growth and differentiation of protective parasite-antigen-specific CD8⁺ cytotoxic T-lymphocytes (53).

We previously showed that two of these trypomastigote-specific T-cell clones, 2G8 and 4B10, were able to protect mice from a lethal *in vivo* challenge with *T. cruzi* (24). In the present study, these protective clones were found to recognize shed (Fig. 2) trypomastigote stage antigens of 63 to 57 and 90 to 80 kDa, respectively (Fig. 4). While the antigen reactive with clone 2G8 was not further identified, a strong candidate is the 60-kDa trypomastigote stage antigen recently shown to elicit T-cell responses in mice (62). Our finding that clone 4B10's target antigen is surface expressed (Fig. 5) and binds specifically to WGA lectin (Table 3) effectively implicated the 85-kDa surface glycoprotein, because this surface protein is nearly unique among try-

mastigote proteins in its ability to bind to WGA (2, 6, 9, 24, 33). Previous studies demonstrated that the 85-kDa surface protein is important in host cell attachment (27, 28) and invasion (3, 28, 64). Trypomastigote proteins of this approximate size have been found to exhibit fibronectin/collagen receptor activity (85 kDa) (33), C3 convertase decay accelerating factor activity (87 to 93 kDa) (21), and neuraminidase/sialidase activity (85 kDa) (22, 36, 56). Recent studies at the genetic level suggest that the 85-kDa trypomastigote surface protein is a member of a multigene family (14, 23, 37) whose products may be simultaneously coexpressed on the surface of trypomastigotes (23). Conserved sequence homology to bacterial proteins suggests that these 85-kDa surface proteins may function as sialidases (22, 36, 56). Unfortunately, we have so far been unsuccessful in our attempts to identify the actual parasite protein recognized by clone 4B10 with several of the available recombinant 85-kDa protein family members, including TSA-1 (14), pTt34 (56, 57), and SA85-1.1 (22, 23).

We have also shown that all of the antigens recognized by

FIG. 3. Proliferative responses of T-cell clones to shed trypanomastigote antigens fractionated by differential centrifugation. Trypomastigotes (10^8 /ml) cultured in T-cell medium were allowed to shed antigen at 26°C for 24 h, and total cultures were then subjected to serial centrifugation steps at increasing speeds. At each step, the pelleted material was retained and the supernatants were centrifuged at the next higher speed. Each pellet was resuspended to the original volume with T-cell medium, and these, along with the $100,000 \times g$ supernatant, were irradiated (100 krad) to sterilize them and diluted into assay wells. Doses tested were 5×10^6 (solid bars), 1×10^6 (dark hatched bars), 2×10^5 (stippled bars), and 4×10^4 (light hatched bars) parasite equivalents per well. Proliferation assays for T-cell clones were performed as described in the legend to Fig. 1. IL-2 secretion by activated T-cell hybrid clone H4B10 was measured by using the growth factor-dependent T-cell line CTLL-2 as described in Materials and Methods.

this panel of *T. cruzi*-specific T-cell clones are released rapidly and in substantial quantities by living trypanomastigotes (Fig. 2). That these clones recognize shed antigenic material is consistent with current notions of host antigen processing which hold that exogenous, mainly soluble, anti-

gens constitute the primary source of antigenic peptides for class II MHC binding and recognition by CD4⁺ T cells (15). The spontaneous release of antigens by cultured trypanomastigotes has been noted previously by a number of investigators (4, 21, 34, 43). Among these shed antigens are the 85-kDa/68-kDa fibronectin receptor complex (33, 61), the 87- to 93-kDa C3 convertase decay accelerating factor (21), the sialidase/SAPA/trans-sialidase protein (11, 18, 22, 35, 40, 47), and a 160-kDa trypanomastigote surface protein (60).

How and in what form these antigens are released is not clear. While some of these antigens may be secreted or excreted parasite products, our finding that significant levels of activity were present in water-insoluble parasite fractions (Fig. 1) suggested that some of these antigens might initially associate with the parasite surface membrane, as was shown to be the case for the target antigen recognized by clone 4B10 (Fig. 5). In addition, the finding that substantial levels of activity for two of the clones (4B10 and 2D6) were released even at 4°C suggests that antigen release can occur independently of normal secretory processes. Although recent studies reported that *T. cruzi* trypanomastigote surface proteins are shed as membrane vesicles (16), in the present study, we detected little or no activity in parasite fractions presumed to contain such membrane vesicles (i.e., $10,000 \times g$ and $100,000 \times g$ pellets; Fig. 3). Another possibility is that some of these shed antigens may be surface proteins released by phospholipase-mediated cleavage of glycosyl phosphatidylinositol linkages (32). Several *T. cruzi* surface proteins, including the 85-kDa gene family member neuraminidase/sialidase/shed acute-phase antigen (SAPA) proteins (17, 40, 45), appear to be glycosyl phosphatidylinositol linked. Because clone 4B10 was reactive with an 85-kDa surface glycoprotein, we investigated whether this activity was linked to the parasite surface via glycosyl phosphatidylinositol. Although we failed to see accelerated release of activity following treatment of trypanomastigotes with exogenous bacterial phospholipase C (30) (perhaps due to high endogenous activity), we did find that short-term incubation of *T. cruzi* trypanomastigotes in 10 mM ZnCl₂, which is known to inhibit endogenous trypanosome phospholipase C activity (20, 45), inhibited the release of activity by about 75% (data not shown). However, because noticeable changes in parasite movement and morphology were also observed, toxic effects could not be ruled out.

In conclusion, we have shown that this panel of *T. cruzi* antigen-specific T-cell clones, several of which were previously shown to protect mice from lethal challenge, display the T_{H1} phenotype. This finding is consistent with their ability to protect, since the T_{H1} T-cell-associated lymphokine IFN- γ has a protective function against *T. cruzi* infection both in vitro and in vivo (39, 41, 52, 63). In addition, our finding that all of the target antigens recognized by this panel of T-cell clones are spontaneously released by living trypanomastigotes suggests that shed parasite antigens constitute important targets for the host protective anti-*T. cruzi* T-cell response. Finally, our analyses implicated an 85-kDa trypanomastigote surface glycoprotein as the target antigen recognized by one of our protective T-cell clones. This finding, combined with previous studies demonstrating that 85-kDa trypanomastigote surface proteins are targeted by protective antibodies (3, 34), confirms the immunologic importance of the 85-kDa protein gene family and suggest that its members may be potential vaccine candidates.

FIG. 4. Proliferative responses of various *T. cruzi*-specific T-cell populations to NC-immunoblotted trypomastigote proteins. Trypomastigote proteins bound to an NC membrane were prepared as described in Materials and Methods, diluted 1:5 in tissue culture medium, and added to triplicate wells in 100- μ l volumes. Proliferation assays were performed as described in the legend to Fig. 1. Vertical scales show size in kilodaltons.

FIG. 5. Response of T-cell hybridoma clone H4B10 to iminobiotinylated trypomastigote surface proteins. Live trypomastigotes were surface biotinylated, and biotinylated proteins were purified from NP-40-treated parasite lysates by passage over an avidin-Sepharose column as described in Materials and Methods. Avidin-bound (●) and non-avidin-bound (○) materials were tested for their ability to activate lymphokine release by T-cell hybridoma H4B10. Tc Ag, *T. cruzi* antigen.

TABLE 3. Stimulatory activity for *T. cruzi*-specific T-cell hybridoma clone 4B10 binds specifically to WGA lectin

Clone	WGA present	Sp act ^a (U/ μ g of protein) in expt no.:				Mean sp act $\pm$ SEM	Mean % of total activity ^b
		1	2	3	4		
H4B10	—	1.0	2.4	0.7	0.3	1.1 $\pm$ 0.6	44.7 $\pm$ 5.2
	+	4.8	18.8	4.0	3.0	7.8 $\pm$ 3.7	55.3 $\pm$ 5.3
2G8	—			5.8	6.6	6.2 $\pm$ 0.4	96.6 $\pm$ 1.1
	+			0.7	0.6	0.7 $\pm$ 0.1	3.4 $\pm$ 1.1
2D6	—			7.1	6.1	6.6 $\pm$ 0.5	96.0 $\pm$ 0.3
	+			1.2	0.9	1.1 $\pm$ 0.2	4.0 $\pm$ 0.3

^a Specific activity was computed by plotting the log mean experimental [³H]thymidine uptake versus the log of protein concentration and finding the protein concentration required to generate 5,000 cpm (experimental minus control) of uptake.

^b Total activity was determined by multiplying the total protein recovered in each fraction by the specific activity.

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