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Research Article

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Human Infection with *Trypanosoma cruzi* Induces Parasite Antigen-Specific Cytotoxic T Lymphocyte Responses

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Abstract

Experimental models of Chagas' disease, an infection caused by the intracellular protozoan *Trypanosoma cruzi*, have demonstrated the crucial immunoprotective role played by $CD8^+$ T lymphocytes. These cells dominate inflammatory foci in parasitized tissues and their elimination from mice leads to uncontrolled parasite replication and subsequent death of the infected host. A trypomastigote surface antigen, TSA-1, and two amastigote surface molecules, ASP-1 and ASP-2, were recently identified as targets of $CD8^+$ cytotoxic T lymphocytes (CTL) in *T. cruzi*–infected mice. Until now, however, there was no evidence for the development of parasite-specific CTL in *T. cruzi*–infected humans. In this study, human CTL specific for TSA-1–, ASP-1–, and ASP-2–derived peptides were detected in the peripheral blood mononuclear cells from 21 of 24 HLA-$A2^+$ *T. cruzi*–infected patients. CTL recognition was antigen specific, A2-restricted, and $CD8^+$ T cell–dependent. Demonstration of human CTL against *T. cruzi* and against target molecules identified using the murine model provides important information for the optimal design and evaluation of vaccines to prevent or ameliorate Chagas' disease. (*J. Clin. Invest.* 1998. 102:1062–1071.) Key words: CTL • *Trypanosoma cruzi* • Chagas' disease • *trans*-sialidase • HLA

Introduction

Trypanosoma cruzi, the etiologic agent of Chagas' disease, infects an estimated 18 million people in Latin America, and 90 million individuals are at risk of infection (1, 2). After a commonly mild acute illness, a chronic phase ensues and 10–20 yr post infection nearly 30% of infected individuals develop cardiac and/or gastrointestinal lesions, which generally lead to death (3). Globally, Chagas' disease causes each year > 50,000 deaths, one million new cases and the loss of 2.74 million disability-adjusted life years (2). Although some reduction in the transmission of Chagas' disease has been achieved through vector control and blood bank screening measures, major obstacles that have prevented South and Central American countries from eradicating *T. cruzi* include behavioral differences among vector species, operational costs to maintain active con-

trol programmes, existence of multiple animal reservoirs, parasite persistence in chronically infected patients, and the lack of chemotherapies for the treatment during the chronic phase of the infection (2). For these reasons, vaccines constitute an attractive approach that could significantly contribute to control the transmission of Chagas' disease. To date, however, there are no vaccines in development against *T. cruzi*.

Experimental murine models of *T. cruzi* infection have been shown to faithfully mimic the various aspects of Chagas' disease including the immune mechanisms thought to operate in controlling the parasite in humans. While a strong antibody response contributes to the control of acute parasitemia, a potent type 1 cytokine production and a $CD8^+$ T cell–mediated response are largely responsible for the immune control of tissue parasites (4). That $CD8^+$ T cells are of critical importance in resistance to *T. cruzi* is supported by their predominance in inflammatory foci of parasitized tissues (5–8), by the correlation between failure to survive the acute stage of infection, increased tissue parasite loads and an absent inflammatory response in mice made deficient of this T cell subset (9–11), and by the ability of $CD8^+$ T cells from infected mice to act as cytotoxic T lymphocytes (CTL)[1] against *T. cruzi*–infected cells (12). Recent efforts to elucidate the molecules contributing to such MHC class I–restricted responses have led to the identification of amastigote surface protein (ASP)-1 and ASP-2, two surface proteins on the intracellular amastigote stage (13, 14), and trypomastigote surface protein (TSA)-1, a surface antigen on the cell-invasive trypomastigote form (15), as the first bona fide targets of CTL in *T. cruzi*–infected mice (16, 17). Interestingly, these three molecules are all members of the same subfamily of *T. cruzi trans*-sialidase proteins (18). CTL against ASP-1, ASP-2, and TSA-1 destroy parasite-infected cells in vitro (16, 17) presumably by the recognition of parasite-derived peptides on the surface of infected cells. Besides their cytolytic activity, the adoptive transfer of IFN-γ– and TNF-α–producing CTL lines specific for a single TSA-1 epitope protect naive mice against lethal *T. cruzi* infection (16). Finally, it has been recently shown that potent CTL responses participate in the protective anti–*T. cruzi* immune response induced in mice genetically immunized with plasmid DNA encoding the genes for each of these antigens (B. Wizel and N. Garg, unpublished). Thus, on the basis of these findings, we have initiated efforts to develop vaccines aimed at enhancing, among other effective anti–*T. cruzi* immune responses, the induction of protective $CD8^+$ T cells.

For vaccines against Chagas' disease to be developed, however, the relevant immune responses in *T. cruzi*–infected humans should first be elucidated. With the solid evidence dem-

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1. *Abbreviations used in this paper:* ASP, amastigote surface protein; CTL, cytotoxic T lymphocytes; CR, complete RPMI; GPI, glycosylphosphatidylinositol; TCM, T cell medium; TSA, trypomastigote surface protein.

onstrating the immunoprotective role played by CTL in the murine model of human Chagas' disease, and with the identification in such a model of ASP-1, ASP-2, and TSA-1 as CTL target molecules, we sought to investigate whether *T. cruzi*–specific CTL responses are induced in humans infected with this parasite and whether such CTL include cells, which recognize the same set of parasite molecules identified in *T. cruzi*–infected mice. We restricted our analysis to the HLA-A2 allele due to its high frequency of expression in individuals of various ethnic backgrounds (19, 20) including those living in Chagas' disease endemic areas of Latin America (21) and due to the availability of an HLA-A2.1 transgenic mouse model (22) where potential *T. cruzi*–derived CTL epitopes could be identified before seeking an equivalent response in humans. The present study reports that 21 of 24 *T. cruzi*–infected HLA-A2$^+$ individuals tested produce CTL against this parasite and that such CTL identified 11 HLA-A2.1–restricted epitopes from the ASP-1, ASP-2, and TSA-1 *trans*-sialidase molecules.

Methods

Cell lines and culture reagents. The following cell lines were used in peptide binding assays and as target cells in the murine and human CTL assays: HLA-A2.1-HMy2.C1R (C1R-A2.1; 23) and HLA-B8-HMy.C1R (C1R-B8; 24) are HMyC1R cells (HLA-A$^-$, -B*3503^{low}, -Cw4$^+$; 25, 26) that express transfected HLA-A2.1 and HLA-B8 genes, respectively (supplied by Dr. W.E. Biddison, National Institutes of Health, Bethesda, MD); JY is an EBV-transformed B cell line expressing alleles HLA-A2.1, -B7 and -Cw7; 721.221-A2.1/K^b and Jurkat A2.1/K^b (obtained from Dr. L. Sherman, Scripps Clinic and Research Foundation, La Jolla, CA) are, respectively, stable transfectants of the human class I null mutant B cell line 721.221 (27) and Jurkat human T cell leukemia line (HLA-A3$^+$, -B7$^+$) expressing the product of the HLA-A2.1/K^b chimeric gene (α1 α2 domains of HLA-A2.1 and α3, transmembrane and cytoplasmic domains of H-2K^b; 28); RMA-S is a low H-2^b expressor and TAP.2 peptide transporter deficient T cell lymphoma line (29; provided by Dr. H.-G. Ljunggren, Karolinska Institute, Stockholm, Sweden). All cell lines were grown in complete RPMI (CR) consisting of RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 20 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 U penicillin/50 μg/ml streptomycin (all from Life Technologies, Grand Island, NY). Transfected lines were cultured in CR containing 0.2 mg/ml of G418 sulfate (Life Technologies). T cell medium (TCM) used in the HLA-A2.1/K^b transgenic mouse studies was prepared by supplementing CR with 50 μM 2-ME. For the human studies, TCM was prepared by substituting heat-inactivated AB$^+$ human serum (Pel-Freeze, Brown Deer, WI) for FCS.

Synthetic peptides. Sequences of the *T. cruzi* antigens ASP-1, ASP-2, and TSA-1 (13–15) were inspected for the presence of 9– or 10–amino acid sequences conforming to the expanded HLA-A2.1 motif (30). This search yielded 570 motif-bearing segments. Following a computer algorithm analysis to take into account the role of secondary anchors in peptide binding to HLA-A2.1 molecules (30, 31; J. Sidney, unpublished data), 145 sequences were selected for peptide synthesis. Other peptides produced were: HLA-A2.1–binding hepatitis B virus core HBc$_{18–27}$ F$_6$→Y peptide (FLPSDYFPSV; 32); H-2 I-A^b–restricted Th epitope HBc$_{128–140}$ (TPPAYRPPNAPIL; 33); and the H-2 K^b–restricted CTL epitopes *T. cruzi* TSA-1$_{515–522}$ (VDYNF-TIV; 16) and OVA$_{257–264}$ (SIINFEKL; 34). All peptides were synthesized at Cytel Corporation (San Diego, CA) or at University of Georgia by the Molecular Genetics Instrumentation Facility (Athens, GA) using Fmoc chemistry and purified to > 95% homogeneity by reverse-phase HPLC. Peptide purity was assayed by HPLC and their composition verified by mass spectrometric analysis. Lyophilized peptides were dissolved at 12 to 20 mg/ml in DMSO, aliquoted and stored at −70°C.

HLA-A2.1 peptide binding assay. The binding affinity of synthetic peptides to purified soluble A2.1 molecules was quantitated by measuring the inhibition of binding of a radiolabeled standard probe peptide as previously described (30, 35). In brief, ^{125}I-labeled HBc$_{18–27}$ F$_6$→Y probe peptide (1–10 nM), A2.1 molecules (5–500 nM) affinity purified from JY cell detergent lysates, and human β2-microglobulin (1 μM; Scripps Laboratories, San Diego, CA) were incubated for 48 h at room temperature in the presence of a cocktail of protease inhibitors. The percentage of A2.1-bound labeled peptide was determined by size exclusion gel filtration chromatography on a TSK 2000 column (TosoHaas, Montgomeryville, PA). In competitive inhibition assays, test peptides were included at various doses (1.2 ng/ml-120 μg/ml) and the concentration necessary to inhibit 50% (IC$_{50}$) of the binding of the radiolabeled peptide was calculated. The concentration of A2.1 yielding approximately 15% binding of radiolabeled probe (∼ 10 nM) was used in all inhibition assays. Each experiment included HBc$_{18–27}$ as a positive control (mean IC$_{50}$ of 5 nM). Peptide binding affinity was scored according to their IC$_{50}$ as follows: high, < 50 nM; intermediate, 50–500 nM; low, 500–10,000 nM; negative, > 10,000 nM. The measured IC$_{50}$s are reasonable approximations of the true Kd values inasmuch as under the conditions of this assay [label] < [A2.1] and IC$_{50}$ ≥ [A2.1].

Induction and assay of CTL from T. cruzi–infected and peptide immunized A2.1/K^b transgenic mice. HLA-A2.1/K^b (A2.1/K^b) transgenic mice (22; bred from pairs obtained from Dr. L. Sherman through Harlan Sprague-Dawley, Inc., Indianapolis, IN) were used to induce specific CTL responses by *T. cruzi* infection (16) and by peptide immunization (36). In brief, 6–12-wk-old mice were infected with 10^3 *T. cruzi* blood form trypomastigotes (Brazil strain), and after 3 mo, animals were challenged subcutaneously in the base of the tail with 2 × 10^5 tissue culture–derived trypomastigotes. For peptide-induced CTL, mice were immunized subcutaneously at the base of the tail with an IFA emulsion containing equimolar amounts of PBS/5% DMSO-dissolved putative CTL peptide (50 μg/mouse) and HBc$_{128–140}$ (140 μg/mouse). 4–6 mo postinfection or 11 d after peptide immunization, mice were sacrificed and immune splenocytes (3 × 10^7) were stimulated in vitro with peptide-coated syngeneic irradiated LPS blasts (1 × 10^7) in 10 ml TCM using upright T25 flasks (Corning Inc., Corning, NY). To prepare LPS blasts, syngeneic splenocytes (4.5 × 10^7) were washed and suspended in 30 ml TCM containing LPS (25 μg/ml; Sigma, St. Louis, MO) and dextran sulfate (7 μg/ml; Sigma) and incubated for 72 h at 37°C in upright T75 flasks (Corning). After irradiation (3,000 rad), LPS blasts were washed, incubated with CTL peptide (100 μg/ml) for 1 h at 37°C and washed once more before being added to splenocytes from *T. cruzi* chronically infected mice and from animals primed with the respective peptide. After 2 d at 37°C, splenocyte cultures were made to 5% with Con A supernatant (rat T-STIM without Con A, Collaborative Biomedical Products, Bedford, MA). At day 6 of culture, resultant effector cells were tested for their cytolytic activity in a ^{51}Cr release assay (16). Effector cells were washed, suspended in TCM, and plated in round bottom 96-well plates (100 μl/well) in the presence of stimulating peptide (10 μg/ml) and thrice washed Jurkat A2.1/K^b or 721.221-A2.1/K^b target cells (5 × 10^3/100 μl/well) that had been incubated overnight at 37°C with ^{51}Cr (100 μCi of a sterile Na$_2$^{51}CrO$_4$ solution; Amersham Life Science Corp., Arlington Heights, IL) and stimulating or irrelevant control HBc$_{18–27}$ peptide (10 μg/ml). After centrifugation (500 *g* for 2 min), plates were incubated for 5 h at 37°C. Supernatants were collected (Skatron, Inc., Sterling, VA) and released ^{51}Cr expressed as the mean percentage specific lysis of triplicate wells, calculated as follows: 100 × ([experimental release – spontaneous release]/[maximum release – spontaneous release]). Maximum and spontaneous release were determined in wells containing no effectors in the presence or absence of 2% Triton X-100, respectively. Spontaneous release was < 22% of maximum release in all experiments. Standard errors were invariably < 3% of the mean. A CTL response was considered positive when, at

the highest effector to target cell ratio (between 40:1 and 60:1), a difference of $\geq 10\%$ lysis was obtained by subtracting the percentage lysis for control peptide from percentage lysis for the test peptide.

Selection of study subjects. This study was approved by the Institutional Review Boards for the use of Human Subjects as Research Participants of the Military Health Services in Guatemala, Universidad del Valle de Guatemala and University of Georgia. Subjects were adult military personnel stationed at the bases of Chiquimula, Zacapa, Jutiapa and Jalapa, 4 contiguous Chagas' disease endemic regions in southeastern Guatemala, where they had been lifelong residents. After explaining the requirements for participation and obtaining signed informed consent, 744 volunteers (Chiquimula, code A; Zacapa, code B; Jutiapa, code F; Jalapa, code G) were serologically tested for *T. cruzi* infection using standardized ELISA (Center for Disease Control and Prevention), indirect hemagglutination and indirect immunofluorescent tests. Positive diagnosis was confirmed by an additional ELISA (Gull Labs, Salt Lake City, UT) and an agglutination assay using latex beads adsorbed with antigens from an autochthonous *T. cruzi* strain (37). Of 93 (12.5%) seropositive patients, 31 were not longer accessible for HLA-A2 serological typing as 26 left their posts upon completion of their military service, and five voluntarily withdrew from the study. 24 of 62 (38.7%) subjects were typed as HLA-A2$^+$ and selected for subsequent CTL studies (23 males and 1 female, mean age 22.8 yr, range 18–43 yr). Selected patients were in the indeterminate asymptomatic chronic phase of Chagas' disease. The uninfected HLA-A2$^+$ control group consisted of five Guatemalans from the same endemic area (all males, mean age 26.2 yr, range 21–33 yr) and six Caucasian natives from the United States (five males and one female, mean age 30.5 yr, range 27–37 yr). An additional control group from the *T. cruzi*–infected population consisted of 6 HLA-A2$^-$ subjects (five males and one female, mean age 21 yr, range 18–29 yr). All participants were serologically negative for HIV infection.

HLA typing. Participants were typed as HLA-A2$^+$ by staining 100 μl of heparinized whole blood with HLA-A2.1–specific mAb BB7.2 (38; ATCC; 10 μg/ml) followed by FITC-labeled F(ab′)$_2$ goat anti–mouse IgG (1/50 dilution; Southern Biotechnology, Birmingham, AL). Each staining step was for 30 min at 4°C and followed by two washings with PBS/1% BSA/0.05%NaN$_3$. Stained cell samples were suspended in 2 ml of FACS Lysing/Fixing Solution (1/10 dilution; Becton Dickinson, San Jose, CA), vortexed gently, and incubated for 10 min at room temperature in the dark. The resulting erythrocyte-free samples were washed twice and remaining nucleated cells analyzed by immunofluorescence microscopy. HLA-A2 haplotype was verified by flow cytometric analysis (EPICS Elite; Coulter Electronics, Hialeah, FL) of isolated PBMC (10^6) that had been stained for immunofluorescence as described above.

Isolation of PBMC and stimulation with synthetic peptides. Blood specimens from patients (45–55 ml) and control subjects (45 ml) were drawn by venipuncture into heparinized syringes and, in the case of Guatemalan samples, transported by land from the Chagas' disease endemic sites to Guatemala city for processing. Within 7 h of blood collection, PBMC were isolated by density gradient centrifugation on Lymphocyte Separation Medium (Organon Teknika, Durham, NC) and CTL cultures were initiated following a previously described method (39). In brief, PBMC were washed three times with RPMI 1640, suspended in TCM, and cultured at 37°C in 24-well plates (3 × 10^6/2 ml/well) in the presence of individual synthetic peptides (10 μg/ml) for 6 d at 37°C. At day 2 of culture, human rIL-2 (Cetus Corp., Emeryville, CA) was added to each well (50 U/ml). After incubation for 6 d, effector cells from PBMC cultures were tested for CTL activity in a standard 5-h ^{51}Cr release assay (40).

Preparation of target cells and assay of human CTL generated from PBMC cultures. Target cells to assess cytolytic activity of human CTL cultures consisted of HLA-A2.1–matched C1R-A2.1 cells incubated overnight at 37°C in the presence of ^{51}Cr (100 μCi) and stimulating or control HBc$_{18–27}$ peptide (10 μg/ml). In some experiments, similarly treated A2.1$^+$ JY cells were also used as target cells.

^{51}Cr-labeled C1R-B8 (A2.1$^-$B8$^+$) cells pulsed with the stimulating peptide were used as HLA-mismatched target cells. After extensive washing, target cells were suspended in TCM and added (5 × 10^3/100 μl/well) along with the pulsing peptide (10 μg/ml) to triplicate wells of round bottom 96-well plates containing the appropriately stimulated PBMC (100 μl/well). Target cells were also added to sets of triplicate wells plated with effector cells that had been depleted of CD8$^+$ and CD4$^+$ T cells. Immunomagnetic depletion was performed using magnetic beads coated with anti-CD8 or anti-CD4 antibodies (Dynal Inc., Great Neck, NY). CTL activity of depleted effector cell populations was tested only at the highest E/T ratio (between 40:1 and 60:1). Similarly, the highest E/T ratio was the only tested against mismatched control target cells. The ^{51}Cr release assay (39, 40) was then conducted as described for the transgenic mouse system. In all experiments, spontaneous release ranged between 13 and 26% of maximum release, and the standard errors were within 0.6 and 4% of the mean. As for the murine studies, a CTL response was considered positive when the percent net specific lysis calculated as the difference between percent specific lysis of test and control pulsed targets was $\geq 10\%$.

Results

Selection of T. cruzi trans-sialidase–derived HLA-A2 binding test peptides. The previously identified target antigens of anti–*T. cruzi* murine CTL, ASP-1, ASP-2, and TSA-1 (16, 17), were selected as the candidate molecules to study whether humans infected with *T. cruzi* generate CTL responses to this parasite. Sequences of these three *trans*-sialidases were scanned for the presence of 9– or 10–amino acid segments conforming to the HLA-A2.1 expanded motif (30). This search yielded 217 ASP-1, 186 ASP-2, and 167 TSA-1 motif-bearing segments of which 25, 20, and 100 sequences, respectively, were synthesized as peptides after being identified by a computer algorithm as having the highest probability of A2.1 binding (30, 31; J. Sidney, unpublished data). Following quantitative molecular binding assays, the 145 peptides were classified as high (IC$_{50}$ < 50 nM; 4 peptides), intermediate (IC$_{50}$ 50–500 nM; 13 peptides), low (IC$_{50}$ 500–10,000 nM; 45 peptides) and negative (IC$_{50}$ > 10,000 nM; 82 peptides) binders, according to their A2.1-binding affinity. It has been previously demonstrated that the vast majority of peptide epitopes capable of eliciting a CTL response display HLA class I affinities of 500 nM or less (36). Occasionally, this affinity threshold has been raised to 1,000 nM (41–44). Accordingly, the 17 high and intermediate binders, and the six binders with an IC$_{50}$ of > 500 nM but $\leq$ 1,000 nM, were selected for further study (Table I).

CTL responses in T. cruzi–infected and peptide-immunized A2.1/K^b transgenic mice. Before assessing the recall CTL response of PBMC from *T. cruzi*–infected donors to ASP-1–, ASP-2–, and TSA-1–derived A2.1-binding peptides, putative A2.1-restricted CTL responses were first investigated using *T. cruzi*–infected and peptide-immunized A2.1/K^b transgenic mice. For this purpose, 4–12 wks after challenge infection with *T. cruzi* or 11 d after peptide immunization, splenocytes were stimulated in vitro with individual peptides and the CTL activity of effector cells was tested against A2.1/K^b expressing target cells pulsed with the homologous peptide or with HBc$_{18–27}$ negative control peptide. Antigen-specific CTL responses could be demonstrated in splenocyte cultures from both *T. cruzi*–infected and peptide-immunized A2.1/K^b transgenic mice (Fig. 1). Using Jurkat A2.1/K^b and 721.221-A2.1/K^b target cells, CTL responses were reproducibly detected for a total

Table I. T. cruzi Trans-sialidase–Derived HLA-A2.1–Binding Peptides

| | | Sequence‡ | | | | | | | | | | Binding§ |
Antigen	Residues*	1	**2**	3	4	5	6	7	8	**9**	**10**	(IC$_{50}$, nM)
ASP-1	668–677	L	**L**	G	L	W	G	L	T	G	**L**	1.3
	666–674	L	**L**	L	L	G	L	W	G	**L**		22
	50–58	V	**L**	A	K	D	G	T	E	**V**		67
	589–597	S	**V**	Y	V	D	A	K	L	**V**		102
	71–79	I	**A**	G	G	V	M	A	V	**V**		196
	508–516	F	**V**	N	H	D	F	T	V	**V**		300
	486–494	F	**M**	G	A	G	S	K	A	**V**		631
	665–674	P	**L**	L	L	L	G	L	W	G	**L**	643
ASP-2	135–143	G	**I**	R	P	Y	E	I	L	**A**		33
	302–311	W	**V**	F	P	E	S	I	S	P	**V**	109
	678–686	F	**L**	Y	N	R	P	L	S	**V**		146
	551–559	F	**V**	N	H	R	F	T	L	**V**		300
	624–633	M	**L**	Q	D	G	N	K	G	S	**V**	840
TSA-1	818–826	V	**L**	L	P	S	L	F	L	**L**		36
	818–827	V	**L**	L	P	S	L	F	L	L	**L**	99
	819–827	L	**L**	P	S	L	F	L	L	**L**		125
	641–649	F	**L**	Y	N	R	P	L	S	**V**		146
	89–97	K	**L**	F	P	E	V	I	D	**L**		203
	824–833	F	**L**	L	L	G	L	W	G	F	**A**	207
	514–522	F	**V**	D	Y	N	F	T	I	**V**		240
	398–407	L	**L**	Y	S	D	D	A	L	H	**L**	317
	825–834	L	**L**	L	G	L	W	G	F	A	**A**	534
	423–431	R	**L**	T	E	E	L	N	T	**I**		871
	231–239	I	**V**	M	G	N	G	T	L	**V**		998

*Numbering system refers to the ASP-1 and ASP-2 (*T. cruzi* Brazil) (13, 14) and to the TSA-1 (*T. cruzi* Peru) (15) sequences. ‡Nonamer or decamer residue peptides bearing expanded HLA-A2.1 motif (30). Anchor residues at position 2 and at position 9 or 10 are in boldface. Sequence in single letter amino acid code. §Binding is expressed as the nanomolar concentration of peptide capable of inhibiting by 50% the binding of ^{125}I-labeled HBc$_{18–27}$ F$_6$→Y standard peptide to purified soluble A2.1 molecules (IC$_{50}$; 30, 35).

of 12 peptides: six ASP-1 (Fig. 1 *A*), two ASP-2 (Fig. 1 *B*), and four TSA-1 (Fig. 1 *C*) peptides. CTL priming by *T. cruzi*–infection and by peptide immunization was confirmed by the ability of positive control H-2K^b-restricted TSA-1$_{515–522}$ CTL peptide to sensitize RMA-S cells (H-2^b) for lysis by similarly stimulated effector cells from *T. cruzi*–infected or TSA-1$_{515–522}$–immunized A2.1/K^b mice (Fig. 1 *C*). Splenocytes from chronically infected animals recognized 9 of 22 A2.1-binding peptides with a peptide-specific net CTL activity that ranged between 11.3 and 22% (E/T ratio 50:1). In the case of peptide-immunized mice, 11 of 19 peptides generated effector cells with net CTL activity in the range of 11.5 and 38.9% (E/T ratio 50:1). Of the 12 CTL peptides, 2 ASP-1, and 1 ASP-2 peptides were immunogenic by peptide immunization but repeatedly failed to expand CTL in splenocyte cultures from mice with active *T. cruzi* infection. With the exception of a TSA-1 peptide that was not tested in immunization experiments but for which a recall CTL response was generated following infection with *T. cruzi*, the remaining four ASP-1, one ASP-2, and three TSA-1 peptides were immunogenic in both parasite-infected and peptide-immunized A2.1/K^b mice. All but 1 of the 12 peptides capable of eliciting CTL, bound to A2.1 molecules with an IC$_{50}$ of ≤ 300
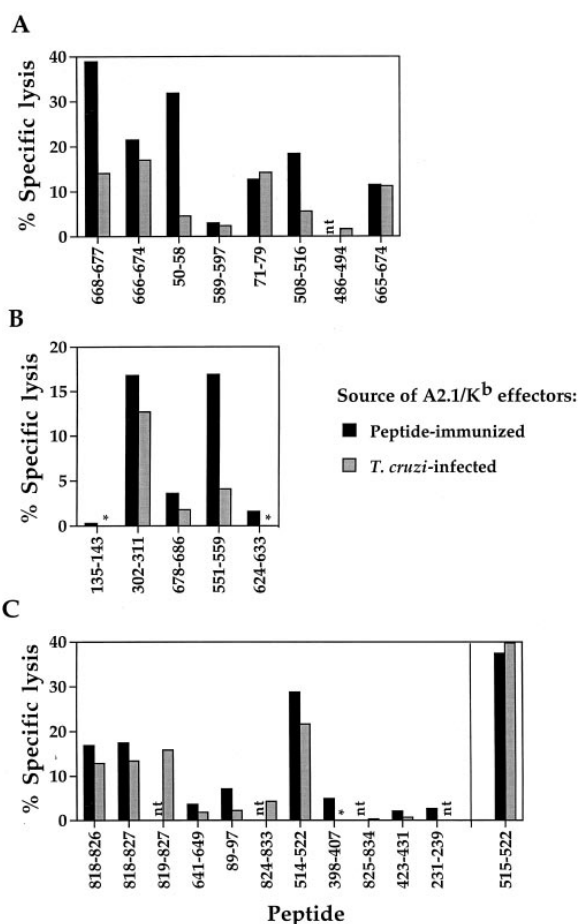

Figure 1. CTL immunogenicity and antigenicity of *T. cruzi trans*-sialidase–derived peptides in A2.1/K^b transgenic mice. The indicated ASP-1 (*A*), ASP-2 (*B*), and TSA-1 (*C*) peptides were used to immunize mice and for the in vitro stimulation of splenocytes from peptide-primed and *T. cruzi*–infected animals. After 6 d of culture, effector cells were assayed for specific CTL activity in a 5-h ^{51}Cr release assay against Jurkat A2.1/K^b or 721.221-A2.1/K^b target cells sensitized with the stimulating or negative control HBc$_{18–27}$ peptide (10 μg/ml) at various E/T ratios. As a positive control for infection- and peptide-induced CTL responses, TSA-1$_{515–522}$ (16; *C*) stimulated effector cells were tested against RMA-S (H-2^b) target cells sensitized with TSA-1$_{515–522}$ or negative control OVA$_{257–264}$ peptide (10 μg/ml). Data represent net percent peptide-specific lysis of one experiment at an E/T ratio of 50:1. Net peptide-specific lysis was calculated by subtracting the ^{51}Cr release by target cells pulsed with HBc$_{18–27}$ peptide from the ^{51}Cr release by target cells pulsed with the test peptide. ^{51}Cr release by HBc$_{18–27}$ peptide-pulsed targets ranged between values below background (*; considered as zero) to 5.1%. Not tested (*nt*). Results are representative of three independent experiments.

nM. These data indicate that the immunogenic A2.1/K^b-restricted epitopes identified as a result of peptide immunization and natural antigen processing during an active *T. cruzi* infection in transgenic mice represent potential A2.1-restricted epitopes in HLA-A2$^+$ *T. cruzi*–infected humans.

Analysis of the human CTL response to potential HLA-A2.1–restricted trans-sialidase–derived epitopes in T. cruzi–infected patients. Assessment of *T. cruzi*–specific CTL responses in HLA-A2$^+$ infected individuals required a study site with a high prevalence of human exposure to this parasite so that sufficient numbers of HLA-A2$^+$ volunteers could be iden-

tified from the infected population. The accessibility of all or the majority of the selected volunteers at a centralized field site was an additional essential criterion to select the study subjects. As such, 24 *T. cruzi*–infected HLA-A2+ Guatemalans who had lived their entire lives in the Chagas' disease endemic areas of Chiquimula, Zacapa, Jutiapa, or Jalapa were selected from 744 recruits stationed at the military bases of the same regions and tested for anti–*T. cruzi* CTL reactivity. To demonstrate anti–*T. cruzi* human CTL, 20 of the 23 *trans*-sialidase–derived A2.1-binding peptides were selected and tested for their ability to target A2.1+ cells for lysis by PBMC from *T. cruzi*–infected donors that had been stimulated in vitro for 6 d with the respective peptide. While high concordance of peptide immunogenicity exists between A2.1/K^b mice and A2.1+ humans, some peptides not immunogenic for CTL from the former have been reported to be frequently recognized by CTL from the latter (44). Accordingly, we selected for future studies 20 peptides, including 10 peptides immunogenic in the A2.1/K^b murine system, and 10 additional peptides, which had failed to induce in vitro recall CTL responses. These peptides included seven ASP-1, four ASP-2, eight TSA-1, and the nonamer shared by ASP-2 and TSA-1 (Table II). Due to the limited blood sample volume obtained from each volunteer and because PBMC from the same single blood sample were required for CTL testing, depletions of CD8+ and CD4+ T cells, MHC restriction analysis, and the generation of autologous EBV-immortalized B cell lines for use in future studies, it was not possible to test the response of all donors to each of the 20 peptides. Thus, PBMC were stimulated with 4–10 peptides, and each peptide was tested with 4–21 donors.
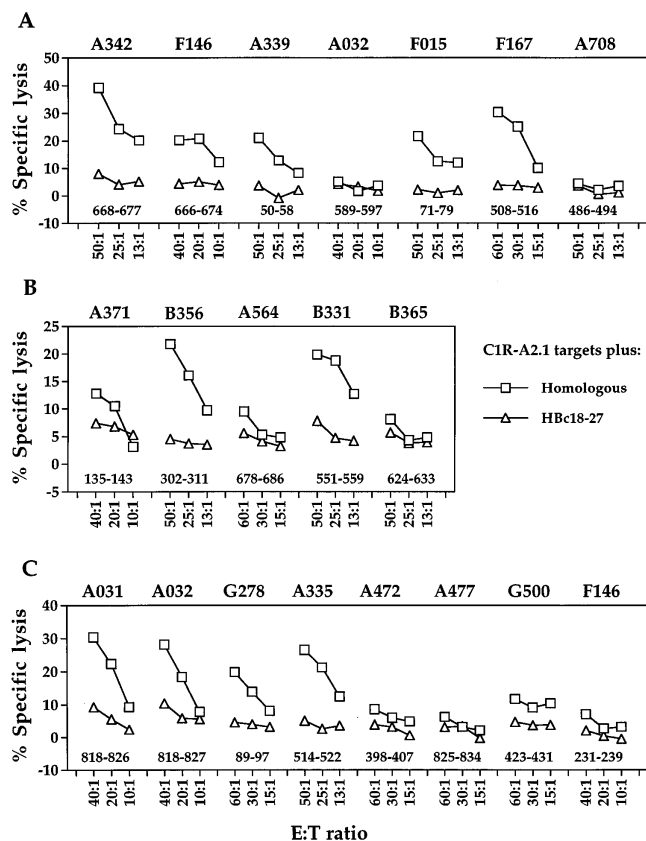
T. cruzi trans–sialidase peptide-specific CTL activity (percent net specific lysis ≥ 10%) was detected in association with 11 of the 20 peptides. All 11 peptides bound to A2.1 molecules with an IC_{50} of ≤ 300 nM (Table II and Fig. 2). As illustrated for a subset of HLA-A2+ patients, effector cells obtained after PBMC stimulation with each of the five ASP-1 (Fig. 2 *A*), two ASP-2 (Fig. 2 *B*), and four TSA-1 (Fig. 2 *C*) peptides lysed C1R-A2.1 cells sensitized with the stimulating peptides but displayed minimal or no lytic activity against the same target cells pulsed with HBc_{18-27} negative control peptide. The five ASP-1 (Fig. 3 *A*), two ASP-2 (Fig. 3 *B*) and four TSA-1 (Fig. 3 *C*) peptides were only able to target C1R-A2.1 but not HLA-disparate C1R-B8 cells for lysis by appropriately stimulated effector cells. Stimulated CTL also failed to recognize JY target cells (HLA-A2.1, -B7, -Cw7) unless sensitized with the homologous peptide (data not shown). Moreover, in all cases tested, the CTL activity displayed by undepleted effector cells was minimally affected by the depletion of CD4+ T cells but it was eliminated or significantly reduced by the depletion of CD8+ T cells (Fig. 3). Altogether, these results indicate that *T. cruzi*–infected subjects generated an antigen multispecific, HLA-A2.1–restricted, and CD8+ T cell–dependent CTL response against the *trans*-sialidase ASP-1, ASP-2, and TSA-1 molecules.

Human CTL activity was demonstrated in 21 of the 24 parasite-infected HLA-A2+ patients. For each responder, the frequency of peptides that were capable of eliciting recall CTL responses varied between 30% (3 of 10 tested) and 100% (4 of 4 tested). While it is evident that most patients had developed a polyclonal CTL response to ASP-1, ASP-2, and TSA-1, three

Table II. Summary of Frequency and Magnitude of CTL Responses in T. cruzi–infected Patients

Peptide	Sequence	Binding (IC_{50}, nM)	Frequency of response*		% Net specific lysis[‡]	
			Positive/total	(%)	Range	(Mean)
ASP-1$_{668}$	LLGLWGLTGL	1.3	14/18	(78)	18.1–34.2	(25.3)
ASP-1$_{666}$	LLLLGLWGL	22	3/4	(75)	12.6–17.0	(15.5)
ASP-1$_{50}$	VLAKDGTEV	67	13/18	(72)	13.2–28.7	(16.7)
ASP-1$_{589}$	SVYVDAKLV	102	0/4	(0)		
ASP-1$_{71}$	IAGGVMAVV	196	4/4	(100)	12.9–19.4	(16.2)
ASP-1$_{508}$	FVNHDFTVV	300	6/7	(86)	14.0–35.8	(24.2)
ASP-1$_{486}$	FMGAGSKAV	631	0/4	(0)		
ASP-2$_{135}$	GIRPYEILA	33	0/7	(0)		
ASP-2$_{302}$	WVFPESISPV	109	4/4	(100)	12.3–19.1	(16.5)
ASP-2$_{678}$	FLYNRPLSV	146	0/4	(0)		
ASP-2$_{551}$	FVNHRFTLV	300	1/4	(25)	12.1	(12.1)
ASP-2$_{624}$	MLQDGNKGSV	840	0/4	(0)		
TSA-1$_{818-826}$	VLLPSLFLL	36	3/4	(75)	16.7–29.3	(20.0)
TSA-1$_{818-827}$	VLLPSLFLLL	99	18/21	(86)	15.8–34.7	(20.6)
TSA-1$_{641}$	FLYNRPLSV	146	0/4	(0)		
TSA-1$_{89}$	KLFPEVIDL	203	9/13	(69)	10.8–29.3	(21.3)
TSA-1$_{514}$	FVDYNFTIV	240	17/21	(81)	11.9–46.8	(24.0)
TSA-1$_{398}$	LLYSDDALHL	317	0/4	(0)		
TSA-1$_{825}$	LLLGLWGFAA	534	0/6	(0)		
TSA-1$_{423}$	RLTEELNTI	871	0/6	(0)		
TSA-1$_{231}$	IVMGNGTLV	998	0/4	(0)		

*Frequency of response represents the number of positive patients from the total number of patients tested. Respective percentages are presented in parentheses. [‡]Percent net specific lysis was measured by subtracting the ^{51}Cr release by target cells pulsed with negative control HBc_{18-27} peptide from the ^{51}Cr release by target cells pulsed with the test peptide. ^{51}Cr release by HBc_{18-27} peptide-pulsed targets ranged between values −3.3% (considered as zero) to 11.7%. Presented range and mean net peptide-specific lysis were calculated from positive CTL responses only.

Figure 2. Specificity of CTL responses to *trans*-sialidase–derived peptides in *T. cruzi*–infected patients. PBMC from representative HLA-A2[+] *T. cruzi*–infected responding donors were stimulated in vitro with the indicated ASP-1– (*A*), ASP-2– (*B*), and TSA-1– (*C*) derived peptides (10 μg/ml). Effector cells obtained after a 6-d culture were tested for recognition of C1R-A2.1 target cells pulsed overnight with the homologous peptides or with negative control HBc$_{18-27}$ peptide (10 μg/ml) in a 5-h ^{51}Cr release assay at the indicated E/T ratios.

subjects failed to display peptide-specific net CTL activity ≥ 10% to any of the tested *trans*-sialidase–derived peptides.

Responding subjects recognized the 11 CTL peptides with a net specific lytic activity that ranged from 10.8% to 46.8% (E/T ratios ranging between 40:1 to 60:1; Table II). For each group of the *T. cruzi trans*-sialidase–derived antigenic peptides, the magnitude of human CTL responses varied, with average net peptide-specific lysis values ranging from 15.5 to 25.3% for the ASP-1 group (E/T ratios ranging between 40:1 to 60:1), from 12.1 to 16.5% for the ASP-2 pair set (E/T ratio 50:1), and from 20 to 24% for the TSA-1 peptide series (E/T ratios ranging between 40:1 to 60:1; Table II). Similarly, the frequencies of responding patients for each series of CTL peptides ranged between 72 and 100% for the ASP-1–, between 25 and 100% for the ASP-2–, and between 69 to 81% for the TSA-1–derived peptides (Table II).

To ascertain whether the observed in vitro expansion of CTL from *T. cruzi*–infected patients reflected previous in vivo exposure to this parasite, the 11 peptides associated with CTL activity were used to stimulate PBMC isolated from blood samples of five HLA-A2[+] *T. cruzi*–seronegative Guatemalans (Fig. 4). Generated effector cell populations failed to lyse C1R-A2.1 target cells sensitized with the stimulating peptides.
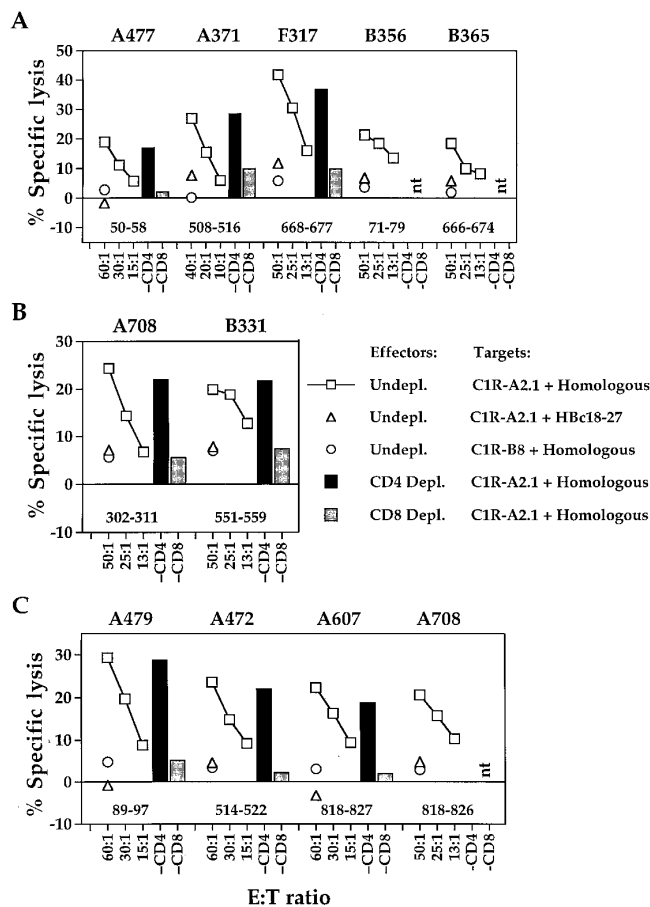
Figure 3. Demonstration of HLA-A2.1–restricted and CD8^{+} T cell–dependent CTL activity to *T. cruzi trans*-sialidase–derived peptides. PBMC from the indicated volunteers were cultured for 6 d with each of the ASP-1 (*A*), ASP-2 (*B*), and TSA-1 (*C*) CTL peptides (10 μg/ml). CTL activity of undepleted effector cells was tested at the indicated E/T ratios against C1R-A2.1–matched (A2.1^{+}) target cells pulsed with the homologous CTL peptide or with negative control HBc$_{18-27}$ peptide (10 μg/ml). Peptide-pulsed (10 μg/ml) C1R-B8 cells were used as mismatched (A2.1^{−}) target cells. After depletion of CD4^{+} or CD8^{+} T cells, cytotoxicity of depleted effector cell populations was measured at the highest E/T ratio against C1R-A2.1 cells sensitized with the homologous peptide; *nt*, not tested.

Similar results were obtained using PBMC from six uninfected HLA-A2[+] Caucasian Americans (data not shown). These findings clearly demonstrated that human CTL responses to *T. cruzi* are indeed parasite primed in vivo.

Discussion

Demonstration in *T. cruzi*–infected humans of the immune responses proven to be relevant in resistance to *T. cruzi* infection in mice (4) has important implications for the development of vaccines that could prevent the onset or minimize the severity of Chagas' disease. Herein, we provide the first evidence that *T. cruzi* induces parasite-specific class I MHC-restricted CD8^{+} T cell responses in infected humans. Furthermore, we document the identification of 11 minimal HLA-A2.1–restricted CD8^{+} T cell epitopes in ASP-1, ASP-2, and TSA-1.
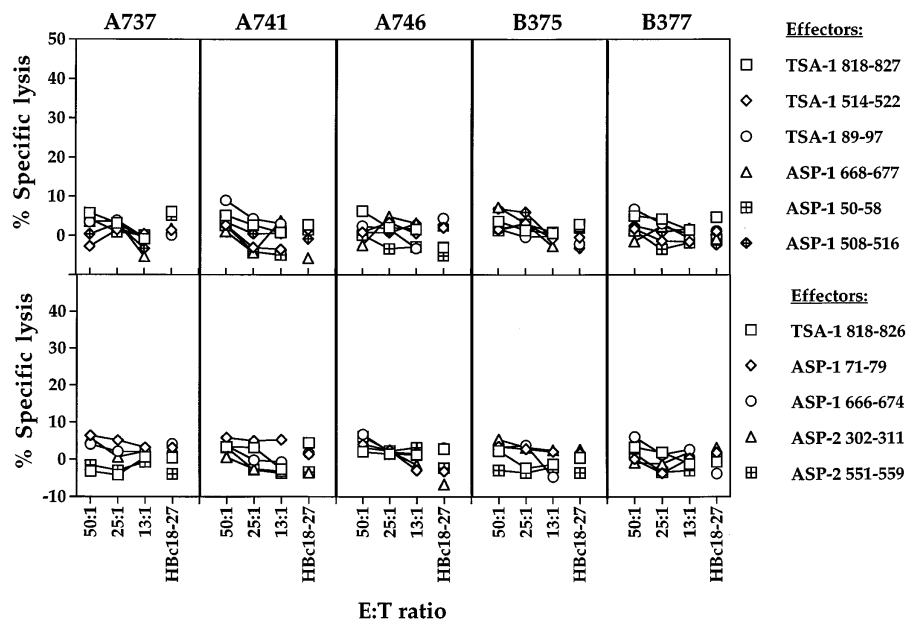
Figure 4. *T. cruzi*–specific CTL response in healthy uninfected volunteers. PBMC from 5 HLA-A2[+] *T. cruzi*–seronegative Guatemalans were stimulated individually with each of the 11 *T. cruzi trans*-sialidase CTL peptides (10 μg/ml). After 6 d, effector cells were tested against C1R-A2.1 target cells pulsed with the same peptides or with irrelevant HBc$_{18-27}$ peptide (10 μg/ml) in a 5-h ^{51}Cr release assay at the indicated E/T ratios. For clarity, the CTL activity against HBc$_{18-27}$ peptide-pulsed targets is only shown at an E/T ratio of 50:1.

Before the initiation of the present investigation, a major impediment to the study of human CTL responses to *T. cruzi* was that the parasite molecules gaining access into the class I processing and presentation pathway had not been identified. Nearly 6 yr after the initial evidence that an intact CD8^{+} T cell compartment is required for survival of *T. cruzi* infection in mice (45), we identified the previously reported trypomastigote antigen, TSA-1 (15), and two newly discovered amastigote proteins, ASP-1 and ASP-2 (13, 14), as the first three target molecules of murine CTL elicited during infection (16, 17). With these molecules in hand, we examined a human system and asked: (1.) are antiparasite CTL responses induced in *T. cruzi*–infected humans? and (2.) do human CTL recognize the same set of parasite molecules recognized by murine CTL? To answer these questions, which constitute a prerequisite towards the consideration of such molecules as vaccine candidates, we structured a strategy whose core was chosen to be the HLA-A2 system. The main reason for this selection was that in humans living in various Chagas' disease endemic countries of Latin America, HLA-A2 is the most prominent HLA allele, with phenotypic frequencies in the 21–57% range (21) in good agreement with the 25–64% range at the global level (19, 20).

Our strategy started with the use of an extended HLA-A2.1 binding motif (30) and a computer algorithm for the stringent and rapid selection of 9– and 10–amino acid sequences within ASP-1, ASP-2, and TSA-1 bearing an increased predictability of binding to A2.1 molecules. This refined search dictated the selection of 145 out of 570 candidate peptides for synthesis. Once synthesized, a standardized quantitative molecular binding assay (35) was used to further select those peptides that were capable of binding to A2.1 molecules with an affinity of < 1,000 nM. Although 17 of the 23 chosen peptides were selected because they bound with an IC$_{50}$ of ≤ 500 nM, the affinity threshold frequently associated with immunogenicity (36), the selection of the six remaining peptides was based on reports about CTL epitopes having binding affinities of ∼ 1,000 nM (41–44).

We next tested the ability of selected peptides to elicit recall CTL responses from splenocytes of *T. cruzi*–infected and peptide-immunized A2.1/K^b transgenic mice (22). Because these mice are endowed with an antigen processing machinery and a CTL repertoire representative of HLA-A2.1[+] individuals, they are a valuable tool to predict human CTL epitopes (22, 36, 44, 46–48). It was found that 12 of the 23 A2.1-binding peptides expanded A2.1/K^b murine CTL in vitro. Detected CTL responses were antigen-specific and restricted by the A2.1 chimeric molecule inasmuch as Jurkat A2.1/K^b and 721.221-A2.1/K^b target cells only share the A2.1/K^b class I molecule with generated effector cells and none of such CTL lysed HBc$_{18-27}$-sensitized target cells. It should be noted that the magnitude of CTL responses detected in *T. cruzi*–infected mice was, on average, 41% lower than the magnitude of responses detected for peptide-primed animals. This difference in magnitude could have been attributed, at least in part, to the immunodominant and overwhelming CTL response to parasite-derived epitopes restricted by the predominant H-2^b class I molecules, rather than by the transgene-encoded A2.1/K^b molecules (16, 17, 22). Regardless of the mechanisms underlying this discrepancy, these findings indicated that infection of A2.1/K^b mice with *T. cruzi* does lead to A2.1/K^b-restricted ASP-1–, ASP-2–, and TSA-1–specific CTL responses.

Based on the aforementioned results, we next asked whether antiparasite CTL responses are induced in humans infected with *T. cruzi*. 20 of the 23 A2.1-binding peptides were selected for their ability to elicit recall CTL responses from PBMC of 24 HLA-A2[+] *T. cruzi*–infected volunteers. Because it has been shown that CTL responses for some peptides can be heterogeneous among various patients and that reactivities can vary from experiment to experiment for a single patient (40, 49–51), each of the 20 selected peptides was assayed with cells from at least four patients and defined as antigenic when CTL activity was detected in at least one of the assays. PBMC from 21 of the 24 HLA-A2[+] patients identified 11 of the 20 (55%) peptides as antigenic. Specifically, and in keeping with the notion that a threshold of 500 nM determines the capacity of peptide epitopes to be immunogenic (36), five of seven (71%) ASP-1, two of five (40%) ASP-2, and four of nine (44%) TSA-1 peptides bound to A2.1 molecules with affinities

≤ 300 nM. All 11 of these peptides were able to sensitize HLA-A2.1$^+$ target cells for specific lysis by the appropriate peptide-stimulated effector cells, and in all cases tested, CTL responses were found to be mediated by CD8$^+$ T cells. These results and the fact that none of the 11 uninfected HLA-A2$^+$ or the six *T. cruzi*–infected HLA-A2$^-$ controls (data not shown) had detectable CTL responses to the identified epitopes indicate that CD8$^+$ T cells are primed in vivo by *T. cruzi* infection and that a population of these cells, after selective in vitro expansion, recognizes ASP-1–, ASP-2–, and TSA-1–derived peptides in the context of HLA-A2.1 molecules.

Although peptide-specific CTL responses were detected for the vast majority of *T. cruzi*–infected HLA-A2$^+$ patients (21 of 24; 87.5%), such responses were not detected for three volunteers. Repeated serology at the Centers for Disease and Control Prevention (Atlanta, GA) using two additional tests confirmed the seropositivity of these patients to *T. cruzi*. It is conceivable that these "nonresponders" may have been of an A2 subtype within the A2 supertype allele family (31, 52) in which none of the identified CTL epitopes was immunogenic or promiscuously recognized on target cells positive for the most frequent HLA-A2.1 subtype (20, 53). Alternatively, the observed CTL nonresponsiveness might also be explained by holes in the T cell repertoire, a stage in the infection where CTL responses are immunosuppressed, and infection by *T. cruzi* strains with sequence variations in the identified epitopes. Future studies might attempt to determine if the CTL nonresponsiveness of infected individuals is associated with disease severity or if it is also observed against yet to be defined non–*trans*-sialidase CTL target molecules.

The fact that most *T. cruzi*–infected Guatemalans had detectable CTL responses to ASP-1–, ASP-2–, and TSA-1–derived peptides is notable, inasmuch as the sequences from the two amastigote proteins (13, 14) and that from the trypomastigote antigen (15) were respectively obtained from the Brazil and Peru strains. This finding suggests that the sequences of such CTL peptides are conserved among the Guatemalan strains of *T. cruzi* infecting the responding patients. However, it is also possible that even if these *trans*-sialidase molecules were polymorphic at the level of the identified CTL epitopes, their conserved sequences might still be encoded in other, as yet unidentified, related genes. ASP-1, ASP-2, and TSA-1 are only three members of the subfamily II of *trans*-sialidases, which in turn belongs to a superfamily of molecules encoded by hundreds if not thousands of highly homologous and largely unsequenced genes (18).

Two groups of CTL epitopes identified in this study are of special interest and deserve to be discussed. In the first group, ASP-1$_{508}$, ASP-2$_{551}$, and TSA-1$_{514}$ are peptides with a significant degree of sequence homology, map to similar regions within their respective proteins and display strikingly similar intermediate A2.1-binding affinities (300 nM, 300 nM, and 240 nM, respectively). Interestingly, the COOH-terminal octameric sequences of these three nonamers represent murine CTL epitopes restricted by H-2K^b molecules (16, 17) of which TSA-1$_{515–522}$ has been characterized as a protective determinant (16). Because analysis of the homologous regions in the sequenced members of this large subfamily of *trans*-sialidases reveal conservation of A2.1 anchor motif residues, it is tempting to speculate that such segments may behave as antagonists or agonists of potentially protective CTL epitopes. This altered peptide ligand hypothesis (54) is currently under investi-gation. In the second group, ASP-1$_{668}$, ASP-1$_{666}$, TSA-1$_{818–826}$, and TSA-1$_{818–827}$ represent peptides that map at the COOH-terminal hydrophobic domain of ASP-1 and TSA-1. These sequences, as in the majority of *T. cruzi* glycosylphosphatidyli-nositol (GPI)-anchored surface proteins, represent putative GPI signal peptides. It is unclear how these good A2.l-binding CTL epitopes are generated. However, it is possible that the GPI signal sequences might be cleaved in the parasite's endo-plasmic reticulum (ER)/post-ER compartment and then se-creted into the cytosol of the host cell where they undergo fur-ther processing. If this is the case, this would be the first demonstration of hydrophobic sequences downstream the GPI cleavage-attachment site of GPI-anchored proteins contribut-ing to the generation of CTL epitopes. This scenario is analo-gous to the NH$_2$-terminal ER translocation signal sequences from several proteins of viral and parasitic origin, which have been shown to contain immunodominant CTL determinants (55, 56). Alternatively, precursor proteins containing these hy-drophobic domains may enter the cytosol of the host cell via the default secretion pathway that has been reported for *T. cruzi* proteins, which fail to receive GPI anchors (57).

The murine model of Chagas' disease has provided com-pelling evidence linking CD8$^+$ T cells and resistance to *T. cruzi* infection (4, 16). In this study, however, due to the cross-sec-tional design and the analysis of only indeterminate asymp-tomatic chronically infected patients, it was not possible to es-tablish a correlation between CTL responses and protective immunity. Despite this caveat, we suggest a possible role for the detected CD8$^+$ T cells in controlling the multiplication and spread of parasites in infected humans, a role that is supported by studies documenting that CD8$^+$ T cells constitute the main cell type infiltrating chagasic hearts (7, 58) and that the para-site load in heart tissue is directly correlated with the severity of cardiomiopathy (59, 60) and inversely correlated with the number of IFN-γ–producing CD8$^+$ T cells (61). Interestingly, we have recently shown in mice that IFN-γ–producing TSA-1$_{515–522}$–specific CD8$^+$ CTL lines transfer protection adoptively (16). Considerable work will be necessary to examine the con-tribution of CTL found in PBMC to the control of parasite burden in infected hearts and to determine whether a correla-tion with protective immunity can be established through epitope-specific CTL precursor frequency analysis of PBMC from patients with different clinical forms of Chagas' disease and living in different geographic areas of Latin America.

A major hindrance that has discouraged researchers from attempts at development of vaccines against *T. cruzi* has been the widely debated autoimmune etiology in the pathogenesis of Chagas' disease (62), which in turn has seeded the fear that enhancing the antiparasite immune response will exacerbate rather than prevent disease. However, evidence continues to accumulate in humans and in rodents that it is the persistence of *T. cruzi* in the diseased tissue and not the parasite-induced immune responses to self molecules, which correlates best with the induction and severity of disease (63–66). Hence, it is our view that by inducing efficacious anti–*T. cruzi* immune re-sponses, vaccines may not only protect humans at risk of infec-tion, but they may also reduce or perhaps eliminate parasites from infected patients. Vaccines against *T. cruzi*, designed as a synthetic construct, plasmid DNA or as a recombinant infec-tious agent, would most likely need to incorporate several try-pomastigote- and amastigote-based components capable of in-ducing, among other effective antiparasite immune responses,

protective CD8+ T cells. The current studies represent a significant step toward the development of such vaccines as it has now been established that parasite-specific HLA restricted CD8+ CTL are generated in humans infected with *T. cruzi*, and three CTL target molecules have been identified. Having demonstrated the validity of the mouse model to identify CTL target antigens, work is now in progress to carry out the same studies for other *T. cruzi* molecules and to develop vaccines that may ultimately provide an effective control measure against Chagas' disease.

Acknowledgments

References

1. Moncayo, A. 1992. Chagas' disease: epidemiology and prospects for interruption of transmission in the Americas. *World Health Stat. Q.* 45:276–279.

2. UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Tropical disease research: progress 1975–1994, highlights 1993–1994. *In* Twelfth Programme Report of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). World Health Organization, Geneva. 125–134.

3. Santos-Buch, C.A., and A.M. Acosta. 1985. Pathology of Chagas' disease. *In* Immunology and Pathology of Trypanosomiasis. I. Tizard, editor. CRC Press, Boca Raton, FL. 145–183.

4. Tarleton, R. 1996. Immunity to *Trypanosoma cruzi. In* Host Response to Intracellular Pathogens. S. Kauffmann, editor. R.G. Landes Company, Austin, TX. 227–247.

5. Sato, M.N., E.H. Yamashiro-Kanashiro, M.M. Tanji, R. Kaneno, M.L. Higuchi, and A.J.S. Duarte. 1992. CD8+ cells and natural cytotoxic activity among spleen, blood, and heart lymphocytes during the acute phase of *Trypanosoma cruzi* infection. *Infect. Immun.* 60:1024–1030.

6. Sun, J., and R. Tarleton. 1993. Predominance of CD8+ T lymphocytes in the inflammatory lesions of mice with acute *Trypanosoma cruzi* infection. *Am. J. Trop. Med. Hyg.* 48:161–169.

7. Higuchi, M.L., P. Gutierrez, V.D. Aiello, S. Palomino, E. Bocchi, J. Kalil, G. Bellotti, and F. Pileggi. 1993. Immunohistochemical characterization of infiltrating cells in human chronic chagasic myocarditis: comparison with myocardial rejection process. *Virchows Arch. A. Pathol. Anat. Histopathol.* 423: 157–160.

8. Reis, D.D., E.M. Jones, S.J. Tostes, E.R. Lopes, G. Gazzinelli, D.G. Colley, and T.L. McCurley. 1993. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor-alpha+ cells and dominance of granzyme A+, CD8+ lymphocytes. *Am. J. Trop. Med. Hyg.* 48:637–644.

9. Tarleton, R.L., B.H. Koller, A. Latour, and M. Postan. 1992. Susceptibility of β2-microglobulin-deficient mice to *Trypanosoma cruzi* infection. *Nature.* 356:338–340.

10. Tarleton, R.L., J. Sun, L. Zhang, and M. Postan. 1994. Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitism in experimental Chagas' disease. *Infect. Immun.* 62:1820–1829.

11. Tarleton, R.L., M.J. Grusby, M. Postan, and L. Glimcher. 1996. *Trypanosoma cruzi* infection in MHC-deficient mice: further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. *Int. Immunol.* 8:13–22.

12. Nickell, S.L., G.A. Stryker, and C. Arevalo. 1993. Isolation from *Trypanosoma cruzi*-infected mice of CD8+, MHC-restricted cytotoxic T cells that lyse parasite-infected target cells. *J. Immunol.* 150:1446–1457.

13. Santos, M.A.M., N. Garg, and R.L. Tarleton. 1997. The identification and molecular characterization of *Trypanosoma cruzi* amastigote surface protein-1, a member of the *trans*-sialidase gene super-family. *Mol. Biochem. Parasitol.* 86:1–11.

14. Low, H.P., and R.L. Tarleton. 1997. Molecular cloning of the gene encoding the 83 kDa amastigote surface protein and its identification as a member of the *Trypanosoma cruzi* sialidase superfamily. *Mol. Biochem. Parasitol.* 88: 137–149.

15. Fouts, D.L., B.J. Ruef, P.T. Ridley, R.A. Wrightsman, D.S. Peterson, and J.E. Manning. 1991. Nucleotide sequence and transcription of a trypomastigote surface antigen gene of *Trypanosoma cruzi. Mol. Biochem. Parasitol.* 46: 189–200.

16. Wizel, B., M. Nunes, and R.L. Tarleton. 1997. Identification of *Trypanosoma cruzi trans*-sialidase family members as targets of protective CD8+ TC1 responses. *J. Immunol.* 159:6120–6130.

17. Low, H.P., M.A.M. Santos, B. Wizel, and R.L. Tarleton. 1998. Amastigote surface proteins of *Trypanosoma cruzi* are targets for CD8+ cytotoxic T lymphocytes. *J. Immunol.* In press.

18. Schenkman, S., D. Eichinger, M.E.A. Pereira, and V. Nussenzweig. 1994. Structural and functional properties of *Trypanosoma trans*-sialidase. *Annu. Rev. Microbiol.* 48:499–523.

19. Imanishi, T., T. Akaza, A. Kimura, K. Tokunaga, and T. Gojobori. 1992. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. *In* HLA 1991, Proceedings of the Eleventh International Histocompatibility; Workshop and Conference, Vol. 1, Ch. W15.1. K. Tsuji, M. Aizawa, and T. Sasazuki, editors. Oxford University Press, Tokyo. 1065–1077.

20. Krausa, P., M. Brywka, III, D. Savage, K.M. Hui, M. Bunce, J.L.F. Ngai, D.L.T. Teo, Y.W. Ong, D. Barouch, C.E.M. Allsop, et al. 1995. Genetic polymorphism within HLA-A*02: significant allelic variation revealed in different populations. *Tissue Antigens.* 45:223–231.

21. Santiago-Delpin, E.A. 1991. Histocompatibility profile of selected Latin American countries. *Transplant. Proc.* 23:1861–1864.

22. Vitiello, A., D. Marchesini, J. Furze, L.A. Sherman, and R.W. Chesnut. 1991. Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human-mouse class I major histocompatibility complex. *J. Exp. Med.* 173:1007–1015.

23. Hogan, K.T., N. Shimojo, S.F. Walk, V.H. Engelhard, W.L. Maloy, J.E. Coligan, and W.E. Biddison. 1988. Mutations in the alpha-2 helix of HLA-A2 affect presentation but do not inhibit binding of influenza virus matrix peptide. *J. Exp. Med.* 168:725–736.

24. DiBrino, M., K.C. Parker, J. Shiloach, R.V. Turner, T. Tsuchida, M. Garfield, W.E. Biddison, and J.E. Coligan. 1994. Endogenous peptides with distinct amino acid anchor residue motifs bind to HLA-A1 and HLA-B8. *J. Immunol.* 152:620–631.

25. Storkus, W.J., D.N. Howell, R.D. Salter, J.R. Dawson, and P. Cresswell. 1987. NK susceptibility varies inversely with target cell class I HLA antigen expression. *J. Immunol.* 138:1657–1659.

26. Zemmour, J., A.-M. Little, D.J. Schendel, and P. Parham. 1992. The HLA-A,B "negative" mutant cell line C1R expresses a novel HLA-B35 allele, which also has a point mutation in the translation initiation codon. *J. Immunol.* 148:1941–1948.

27. Shimizu, Y., and R. DeMars. 1989. Production of human cells expressing individual transferred HLA-A, -B, -C genes using an HLA-A, -B, -C null human cell line. *J. Immunol.* 142:3320–3328.

28. Irwin, M.J., W.R. Heath, and L.A. Sherman. 1989. Species-restricted interactions between CD8 and the α3 domain of class I influence the magnitude of the xenogeneic response. *J. Exp. Med.* 170:1091–1101.

29. Ljunggren, H., and K. Karre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants: analysis of the mechanism. *J. Exp. Med.* 162:1745–1759.

30. Ruppert, J., J. Sidney, E. Celis, R.T. Kubo, H.M. Grey, and A. Sette. 1993. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell.* 74:929–937.

31. Sidney, J., H.M. Grey, R.T. Kubo, and A. Sette. 1996. Practical, biochemical and evolutionary implications of the discovery of HLA class I supertypes. *Immunol. Today.* 17:261–266.

32. Bertoletti, A., C. Ferrari, F. Fiaccadori, A. Penna, R. Margolskee, H.J. Schlicht, P. Fowler, S. Guilhot, and F.V. Chisari. 1991. HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. *Proc. Natl. Acad. Sci. USA.* 88:10445–10449.

33. Milich, D.R., J.L. Hughes, A. McLachlan, G.B. Thornton, and A. Moriarty. 1988. Hepatitis B synthetic immunogen comprised of nucleocapsid T-cell sites and an envelope B-cell epitope. *Proc. Natl. Acad. Sci. USA.* 85:1610–1614.

34. Rötzschke, O., K. Falk, S. Stevanovic, G. Jung, P. Walden, and H.-G. Rammensee. 1991. Exact prediction of a natural T cell epitope. *Eur. J. Immunol.* 21:2891–2894.

35. Sette, A., J. Sidney, M.-F. del Guercio, S. Southwood, J. Ruppert, C. Dahlberg, H.M. Grey, and R.T. Kubo. 1994. Peptide binding to the most frequent HLA-A class I alleles measured by quantitative molecular binding as-

says. *Mol. Immunol.* 31:813–822.

36. Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W.M. Kast, C.J.M. Melief, C. Oseroff, L. Yuan, J. Ruppert, et al. 1994. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J. Immunol.* 153:5586–5592.

37. Matta, V.L., M.P. De Leon, F. Nave, and M. Paz. 1993. Evaluacion y estandarizacion de los metodos serologicos para el diagnostico de la enfermedad de Chagas. *In* Enfermedades Tropicales en Guatemala Informe Anual No. 2. Japan International Cooperation Agency, Guatemala. 79–81.

38. Parham, P., and F.M. Brodsky. 1981. Partial purification and some properties of BB7.2 a cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant of HLA-A28. *Hum. Immunol.* 3:277–299.

39. Wizel, B., R.A. Houghten, K.C. Parker, J.E. Coligan, P. Church, D. Gordon, W.R. Ballou, and S.L. Hoffman. 1995. Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the *Plasmodium falciparum* sporozoite surface protein 2. *J. Exp. Med.* 182:1435–1445.

40. Wizel, B., R.A. Houghten, P. Church, J.A. Tine, D.E. Lanar, D.M. Gordon, W.R. Ballou, A. Sette, and S.L. Hoffman. 1995. HLA-A2 restricted cytotoxic T lymphocyte responses to multiple *Plasmodium falciparum* sporozoite surface protein 2 epitopes in sporozoite-immunized volunteers. *J. Immunol.* 155:766–775.

41. Cox, A.L., J. Skipper, Y. Chen, R.A. Henderson, T.L. Darrow, J. Shabanowitz, V.H. Engelhard, D.F. Hunt, and C.L. Slingluff, Jr. 1994. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science.* 264:716–719.

42. Lai, Z.-W., J. Kremer, E. Gleichmann, and C. Esser. 1994. 3,3′, 4,4′-tetrachlorobiphenyl inhibits proliferation of immature thymocytes in fetal thymus organ culture. *Scand. J. Immunol.* 39:480–488.

43. Alexander, J., C. Oseroff, J. Sidney, P. Wentworth, E. Keogh, G. Hermanson, F.V. Chisari, R.T. Kubo, H.M. Grey, and A. Sette. 1997. Derivation of HLA-A11/K^b transgenic mice: functional CTL repertoire and recognition of human A11-restricted CTL epitopes. *J. Immunol.* 159:4753–4761.

44. Wentworth, P.A., A. Vitiello, J. Sidney, E. Keogh, R.W. Chesnut, H.M. Grey, and A. Sette. 1996. Differences and similarities in the A2.1-restricted cytotoxic T cell repertoire in humans and human leukocyte antigen-transgenic mice. *Eur. J. Immunol.* 26:97–101.

45. Tarleton, R.L. 1990. Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with *Trypanosoma cruzi. J. Immunol.* 144:717–724.

46. Theobald, M., J. Biggs, D. Dittmer, A.J. Levine, and L.A. Sherman. 1995. Targeting p53 as a general tumor antigen. *Proc. Natl. Acad. Sci. USA.* 92:11193–11997.

47. Shirai, M., T. Arichi, M. Nishioka, T. Nomura, K. Ikeda, K. Kawanishi, V. Engelhard, S.M. Feinstone, and J.A. Berzofsky. 1995. CTL responses of HLA-A2.1-transgenic mice specific for hepatitis C viral peptides predict epitopes for CTL of humans carrying HLA-A2.1. *J. Immunol.* 154:2733–2742.

48. Vitiello, A., A. Sette, L. Yuan, P. Farness, S. Southwood, J. Sidney, R.W. Chesnut, H.M. Grey, and B. Livingston. 1997. Comparison of cytotoxic T lymphocyte responses induced by peptide or DNA immunization: implications on immunogenicity and immunodominance. *Eur. J. Immunol.* 27:671-678.

49. Sedegah, M., B.K. Lee Sim, C. Mason, T. Nutman, A. Malik, C. Roberts, A. Johnson, J. Ochola, D. Koech, B. Were, et al. 1992. Naturally acquired CD8+ cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. *J. Immunol.* 149:966–971.

50. Dupuis, M., S.K. Kundu, and T.C. Merigan. 1995. Characterization of HLA-A*0201-restricted cytotoxic T cell epitopes in conserved regions of the HIV type 1 gp160 protein. *J. Immunol.* 155:2232–2239.

51. Cerny, A., J.G. Hutchison, C. Pasquinelli, M.E. Brown, M.A. Brothers, B. Grabscheid, P. Fowler, M. Houghton, and F.V. Chisari. 1995. Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA-A2.1 binding motif. *J. Clin. Invest.* 95:521–530.

52. del Guercio, M.-F., J. Sidney, G. Hermanson, C. Perez, H.M. Grey, R.T. Kubo, and A. Sette. 1995. Binding of a peptide antigen to multiple HLA alleles allows definition of an A2-like supertype. *J. Immunol.* 154:685–693.

53. Fernandez-Vina, M.A., M. Falco, Y. Sun, and P. Stastny. 1992. DNA typing for HLA class I alleles. I. Subsets of HLA-A2 and of -A28. *Hum. Immunol.* 33:163–173.

54. Sloan-Lancaster, J., and P.M. Allen. 1996. Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu. Rev. Immunol.* 14:1–27.

55. Hundrisier, D., M.B.A. Oldstone, and J.E. Gairin. 1997. The signal sequence of lymphocytic choriomeningitis virus contains an immunodominant cytotoxic T cell epitope that is restricted by both H-2D^b and H-2K^b molecules. *Virology.* 234:62–73.

56. Doolan, D.L., S.L. Hoffman, S. Southwood, P.A. Wentworth, J. Sidney, R.W. Chesnut, E. Keogh, E. Appella, T.B. Nutman, A.A. Lal, et al. 1997. Degenerate cytotoxic T cell epitopes from *P. falciparum* restricted by multiple HLA-A and HLA-B alleles. *Immunity.* 7:97–112.

57. Garg, N., R.L. Tarleton, and K. Mensa-Wilmot. 1997. Proteins with glycosylphosphatidylinositol (GPI) signal sequences have divergent fates during a GPI deficiency: GPIs are essential for nuclear division in *Trypanosoma cruzi. J. Biol. Chem.* 272:12482–12491.

58. Higuchi, M.D., M.M. Reis, V.D. Aiello, L.A. Benvenutti, P.S. Gutierrez, G. Bellotti, and F. Pileggi. 1997. Association of an increase in CD8+ T cells with the presence of *Trypanosoma cruzi* antigens in chronic, human, chagasic myocarditis. *Am. J. Trop. Med. Hyg.* 56:485–489.

59. Higuchi, M.L., T. De Brito, M.M. Reis, A. Barbosa, G. Bellotti, A.C. Pereira-Barreto, and F. Pileggi. 1993. Correlation between *Trypanosoma cruzi* parasitism and myocardial inflammatory infiltrate in human chronic chagasic myocarditis: light microscopy and immunohistochemical findings. *Cardiovasc. Pathol.* 2:101–106.

60. Bellotti, G., E.A. Bocchi, A. Villela de Moraes, M. de L. Higuchi, M. Barbero-Marcial, E. Sosa, A. Esteves-Filho, R. Kalil, R. Weiss, A. Jatene, et al. 1996. *In vivo* detection of *Trypanosoma cruzi* antigens in hearts of patients with chronic Chagas' heart disease. *Am. Heart J.* 131:301–307.

61. Reis, M.M., M.D. Higuchi, L.A. Benvenutti, V.D. Aiello, P.S. Gutierrez, G. Bellotti, and F. Pileggi. 1997. An *in situ* quantitative immunohistochemical study of cytokines and IL-2R+ in chronic human chagasic myocarditis: correlation with the presence of myocardial *Trypanosoma cruzi* antigens. *Clin. Immunol. Immunopathol.* 83:165–172.

62. Kalil, J., and E. Cunha-Neto. 1996. Autoimmunity in Chagas disease cardiomiopathy: fulfilling the criteria at last? *Parasitol. Today.* 12:396–399.

63. Jones, E.M., D.G. Colley, S. Tostes, E.R. Lopes, J.C. Vnencak, and T.L. McCurley. 1993. Amplification of a *Trypanosoma cruzi* DNA sequence from inflammatory lesions in human chagasic cardiomyopathy. *Am. J. Trop. Med. Hyg.* 48:348–357.

64. Brandariz, S., A. Schijman, C. Vigliano, P. Arteman, R. Viotti, C. Beldjord, and M.J. Levin. 1995. Detection of parasite DNA in Chagas' heart disease. *Lancet.* 346:1370–1371.

65. Lane, J.E., D. Olivares-Villagomez, C.L. Vnencak-Jones, T.L. McCurley, and C.E. Carter. 1997. Detection of *Trypanosoma cruzi* with the polymerase chain reaction and in situ hybridization in infected murine cardiac tissue. *Am. J. Trop. Med. Hyg.* 56:588–595.

66. Tarleton, R.L., L. Zhang, and M.O. Downs. 1997. "Autoimmune rejection" of neonatal heart transplants in experimental Chagas disease is a parasite-specific response to infected host tissue. *Proc. Natl. Acac. Sci. USA.* 94:3932–3937.