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Do Self-Heart-Reactive T Cells Expand in *Trypanosoma cruzi*-Immune Hosts?

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Anti-heart T-cell activity was evaluated by a lymph node cell proliferative assay in isogenic strains of mice immunized with several *Trypanosoma cruzi* epimastigote and trypomastigote antigenic preparations. In addition, chronically infected animals were boosted with trypomastigote antigens and their lymph node cells were tested by in vitro proliferative responses. Our results indicated that (i) use of allogeneic sources of heart antigens may induce alloreactive responses in *T. cruzi*-immune T cells, (ii) specific autoimmune T-cell reactivity against self-heart constituents could not be demonstrated after immunization of the host with *T. cruzi*, and (iii) a proportion of chronically infected mice showed a small but detectable level of auto-anti-heart T-cell reactivity. These results argue against the notion that *T. cruzi* epitopes cross-reactive with self-heart tissue play a role in initiating T-cell-mediated autoimmunity. Anti-heart autoreactive T cells, generated in a proportion of the animals, may result from heart lesions associated with the infection process.

Infection of humans by the protozoan parasite *Trypanosoma cruzi* leads to Chagas' disease, a debilitating endemic disease widespread in South and Central America (3). In a proportion of affected individuals, the chronic phase of the disease is characterized by inflammatory damage to the cardiac tissue, which progresses until congestive heart failure ensues (1). The nature of the events linking the host immune response to *T. cruzi* infection and the subsequent inflammatory damage to heart tissue is unknown, but one hypothesis proposes that heart-reactive autoimmune lymphocytes could arise in the course of an immune response to cross-reactive *T. cruzi* antigens, as occurs in organ-specific autoimmune diseases (6, 16). A putative cross-reaction occurring between a given *T. cruzi* epitope and its normal counterpart expressed by the host heart tissue has been suggested (14) as the basis for anti-heart autoaggression during Chagas' disease. Although some evidence for the induction of autoimmune lymphocytes exists (6), there is no indication that these responses are primarily involved in the pathogenesis of cardiac lesions or whether they reflect stimulation of the host immune system after damage to the heart by some other primary mechanism. The evidence against such an autoimmune basis for the chronic heart disease has already been reviewed (9). Because of their initiator and effector roles in induction of autoimmune diseases, T lymphocytes must be critically involved at the level of immune recognition of any putative antigenic epitope shared by *T. cruzi* parasites and heart tissue.

In this report, we describe our efforts to demonstrate self-heart-reactive proliferating T lymphocytes in mice immunized with distinct antigenic preparations from *T. cruzi*.

Mice of the BALB/c (*H-2*^{d}) and B10.A (*H-2*^{a}) isogenic strains were immunized with one of the following preparations emulsified in complete Freund adjuvant (CFA; $H_{37}RA$; Difco Laboratories, Detroit, Mich.): (i) saline (control groups); (ii) a mixture containing 25 μg of a partially purified aqueous extract (*T. cruzi* glycopeptide antigen Gp-25), 25 μg of *T. cruzi* epimastigote phenolic extract (the glycoconjugate galactomannan 3), and 25 μg of a heat-denaturated protein fraction (R protein) from an aqueous extract prepared as previously described (11); (iii) heat-killed, cultured *T. cruzi* epimastigotes (10^7 per animal injected into the hind footpads); (iv) heat-killed bloodstream *T. cruzi* Y trypomastigotes (10^7 per animal); (v) heat-killed, cultured *T. cruzi* CL metacyclic forms (10^7 per animal). In addition, BALB/c mice were infected with 10^2 living *T. cruzi* Y blood forms and boosted twice with heat-killed *T. cruzi* trypomastigotes (10^7 per animal) in CFA. Proliferative assays were performed at least 6 months after infection and approximately 2 weeks to 1 month after the last booster. At 2 weeks to 1 month after immunization, the draining lymph nodes were removed, and lymph node cells (LNC) were either used as a source of T cells or passed through nylon wool columns (8) to obtain nonadherent lymphocytes (NWNAL). Whenever NWNAL were used, they were supplemented with irradiated (2,200 rads) syngeneic splenocytes as a source of accessory cells (AC). Short-term T-cell lines were prepared as previously described (5). To assess T-cell proliferation, LNC (4×10^5) or NWNAL (2×10^5; supplemented with 10^5 AC) were cultured with purified *T. cruzi* glycoconjugates Gp-25 and galactomannan 3, R protein, heat-killed parasites, or purified protein derivative (PPD; Connaught Laboratories, Willowdale, Ontario, Canada). In addition, sonic extracts were prepared from a pool of hearts from Swiss albino mice or from C57BL10 (B10) congenic strains B10.A (*H-2*^{a}) and B10.BR (*H-2*^{k}). Extracts were prepared in serum-free culture medium, filtered through 0.45-μm (pore size) filters, aliquoted, and stored frozen until use. Protein content was determined as described by Lowry et al. (10). In addition, a fraction enriched in heart cell membranes was obtained from BALB/c whole extracts by ultracentrifugation (7). Cultures were done in 0.2 ml of RPMI 1640 medium supplemented with 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (20 mM), antibiotics, and 5% AB human serum (or fetal bovine serum for cultures lasting more than 5 days) in 96-well flat-bottom microtiter plates (Linbro, Hamden, Conn.) for 3 or 5 days in a humid environment with 7% CO_2

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TABLE 1. Responses of T cells from *T. cruzi* epimastigote-immune mice to heart antigens[a]

LNC stimulus	[^{3}H]TdR incorporation (cpm) by:	
	T. cruzi-immune LNC[b]	Control LNC[c]
Medium	4,127	3,321
GP-25	17,407	5,060
Galactomannan 3	12,789	5,219
R protein	26,965	2,227
PPD	24,093	29,759
Swiss mouse heart antigen	9,880	4,282

[a] BALB/c LNC were stimulated with 25 μg of purified GP-25 or galactomannan 3 per ml, 20 μg of R protein or PPD per ml, or 13 μg of Swiss mouse-derived heart protein (crude sonic extract) per ml. [^{3}H]TdR uptake was assessed after 72 h in culture. Underlined values denote significant stimulation ($P < 0.05$).

[b] BALB/c mice were immunized with a mixture of *T. cruzi* epimastigote extracts as described in the text (group ii).

[c] Control BALB/c mice were immunized with saline alone emulsified in CFA.

at 37°C. At 6 h before harvesting, cultures were pulsed with 1 μCi of tritiated thymidine ([^{3}H]TdR; 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.). Cultures were done in triplicate and harvested with a semiautomated device onto fiber glass filters, and the amount of [^{3}H]TdR incorporated into DNA was measured by liquid scintillation spectrometry. The standard error was within 10% of the mean value and was omitted for simplicity. Statistical significance ($P < 0.05$) was determined by the Student *t* test.

When LNC from mice immunized with a mixture of *T. cruzi* epimastigote antigenic fractions (group ii) were stimulated with purified or semipurified *T. cruzi* antigens, a proliferative response was observed (Table 1). This response was abolished by treatment of the LNC with anti-Thy-1.2 and complement or by addition of monoclonal antibodies against major histocompatibility complex (MHC) class II molecules or to the L3T4 marker of helper T cells (data not shown), indicating that the response was dependent on MHC class II antigen-restricted, L3T4-positive T cells. When secondary cultures were carried out for 3 days, *T. cruzi*-immune T cells responded to a crude Swiss mouse sonic heart extract, while control T cells primed with adjuvant alone gave little, if any, response to either heart or *T. cruzi* antigens (Table 1). These results suggested that *T. cruzi* induced T cells reactive against normal heart tissue in a manner similar to that of previous studies using allogeneic and xenogeneic heart preparations to stimulate *T. cruzi*-immune T cells (13, 14). A major criticism of this approach is that extracts derived from outbred mice could contain disparate MHC antigens and thus the observed response could be alloreactive rather than a true auto-anti-heart response. We immunized B10.A (*H-2*^{a}) mice with *T. cruzi*-derived epimastigote antigens and tested immune LNC with heart antigens derived from different isogenic mouse strains. *T. cruzi*-immune T cells derived from B10.A (*H-2*^{a}) mice showed a proliferative response to heart antigens derived from both Swiss and B10.BR (*H-2*^{a}) mice (Table 2). More important, however, is the finding that these same responding T lymphocytes did not proliferate in response to a normal, syngeneic B10.A heart extract. Thus, most, if not all, of the T-cell proliferative response seen in *T. cruzi*-immune animals could be ascribed to T-cell alloreactivity against foreign MHC antigens contained in the heart preparation (probably presented by autologous accessory cells). Similar negative results were obtained with NWNAL derived from epimastigote-immune animals (data not shown).

TABLE 2. Alloreactivity of T cells that proliferate in response to heart antigens[a]

Stimulus[b]	[^{3}H]TdR incorporation (cpm)
Medium	4,100
GP-25	23,507[c]
Swiss mouse heart tissue	13,210[c]
B10.A (*H-2*^{a}) mouse heart tissue	4,614
B10.BR (*H-2*^{k}) mouse heart tissue	9,912[c]

[a] The responding T cells were LNC from a pool of B10.A (*H-2*^{a}) mice immunized with a mixture of *T. cruzi* epimastigote extracts in CFA.

[b] LNC were stimulated with 25 μg of purified Gp-25 per ml, 13 μg of Swiss mouse heart protein per ml, or 25 μg of either B10.A or B10.BR heart protein per ml. [^{3}H]TdR uptake was assessed after 72 h in culture.

[c] Significant ($P < 0.05$) stimulation.

Moreover, absence of response to a syngeneic heart extract excluded the possibility that this anti-heart response might be a consequence of priming against a heart tissue antigen(s) contained in the *T. cruzi* epimastigote culture medi n.

We were interested in the finding that the alloreactive T-cell response against Swiss heart extracts were detected in *T. cruzi*-immune animals but not in adjuvant-primed controls in a 3-day culture. If the culture period was extended to 5 days, a clear primary anti-heart T-cell response to allogeneic Swiss and B10.BR mouse heart extracts, but not to syngeneic B10.A mouse heart extracts, could be detected in control mice (Table 3). This result indicated that heart extracts prepared from allogeneic sources were indeed potent stimulators of primary T-cell alloreactivity in vitro. Since *T. cruzi*-immune T cells were already responsive to allogeneic heart tissue by day 3, it appeared that immunization with *T. cruzi* activated alloreactive T-cell precursors in vivo, possibly explaining accelerated alloreactive responses by day 3 in thee animals, as compared with control mice (Table 1). Previous observations that *T. cruzi* infection is able to activate a large fraction of host T lymphocytes (12) are compatible with this notion, since alloreactive precursors may be included among these in vivo activated T cells. These results should be compared with those of our previous studies using an auto-anti-heart T-cell activity in *Streptococcus*-primed animals (4). We have attempted to induce anti-heart T-cell autoimmunity by in vitro priming of normal initiator T lymphocytes with *T. cruzi* epimastigote-pulsed macrophages, followed by injection into the footpad and in vivo recruitment of lymph node lymphocytes. No response to self-heart extracts could be detected in recruited T cells

TABLE 3. Alloreactive response detected in control T cells by extension of the culture period[a]

Stimulus[b]	[^{3}H]TdR incorporation (cpm)
Medium	3,324
R	4,526
PPD	118,379[c]
Swiss mouse heart tissue	10,491[c]
B10.A (*H-2*^{a}) mouse heart tissue	3,894
B10.BR (*H-2*^{k}) mouse heart tissue	9,437[c]

[a] The responding T cells were LNC from a pool of control B10.A (*H-2*^{a}) mice immunized with saline in CFA.

[b] LNC were stimulated with 20 μg of R protein or PPD, 13 μg of Swiss mouse heart protein, or 25 μg of either B10.A or B10.BR mouse heart protein per ml. [^{3}H]TdR uptake assessed after 5 days in culture.

[c] Significant ($P < 0.05$) stimulation.

with this protocol (data not shown). Thus, even with the same assay which proved successful in revealing anti-self-heart T cells after priming with rheumatogenic streptococci (4), *T. cruzi* failed to induce such autoimmune T cells. These results are in agreement with a report by Todd et al. (15), showing that T cells from human Chagasic patients respond to heart extracts, but the response correlated with cellular responses to streptococcal antigens and not with responses to *T. cruzi* antigens.

We also immunized BALB/c mice with bloodstream (Y strain) and metacyclic (CL strain) trypomastigotes in adjuvant to obtain NWNAL and stimulated responding T cells supplemented with AC with the membrane-enriched fraction from syngeneic BALB/c mouse heart extracts. We performed experiments to demonstrate auto-anti-heart T cells in these animals. The results revealed a lack of T-cell reactivity to syngeneic heart membranes (Table 4). Auto-anti-heart T-cell response was unequivocally demonstrated in a single case (100,146 cpm), but in this experiment basal T-cell reactivity to AC alone was very high (53,783 cpm). Animals which were cage matched with the latter T-cell donors were negative in successive repeat experiments. We also tried to enrich self-heart-reactive NWNAL from pools of trypomastigote-immune animals by making short-term T-cell lines stimulated by heart membranes in the presence of AC, but the resulting T cells also failed to demonstrate any heart-specific response (data not shown). Finally, BALB/c mice were infected with living blood forms of *T. cruzi*. After 6 months of infection, the animals were boosted with killed trypomastigotes in CFA and assayed for anti-heart responses. About 33% of the mice tested showed low, although significant, anti-heart T-cell autoreactivity (Table 5).

One conclusion from our data is that several examples of anti-heart reactivity in *T. cruzi*-immune animals can actually derive from alloreactive or even xenoreactive T-cell responses to MHC determinants contained in heart antigenic preparations (although with accelerated kinetics when compared with control animals) and not from cross-reactions between autologous heart and *T. cruzi* epitopes. In trypomastigote-immune animals, anti-self-heart T-cell reactivity also remained elusive. Such a reaction was found in a single case, but there was a very high background T-cell response, suggesting that the anti-heart response was part of a more generalized polyclonal T-cell activation induced in the host. In addition, this response was not reproduced in repeat experiments with mice from the same group. Mice immunized with metacyclic forms from the CL strain also gave negative results (data not shown).

TABLE 4. Lack of T-cell reactivity to self-heart in trypomastigote-immune mice

Expt no.	*T. cruzi* strain which provided trypomastigotes for immunization[a]	[^{3}H]TdR incorporation (cpm)[b] with:	
		Medium	Self-heart membranes[c]
1	Y	5,777	5,210
2	CL	32,494	35,861
3	Y	34,260	28,544
4	Y	6,226	5,352

[a] BALB/c mice were immunized with 10^7 heat-killed trypomastigotes emulsified in CFA.

[b] Purified lymph node NWNAL were used as responders. The data shown were determined with irradiated syngeneic AC. Pools of two to four individual mice were used in each experiment.

[c] Cultures were stimulated with purified heart membranes. [^{3}H]TdR uptake was assessed after 72 h in culture.

TABLE 5. T-cell reactivity to self-heart in chronically infected and boosted BALB/c mice[a]

Mouse no.	[^{3}H]TdR incorporation (cpm)[b] in response to:				
	Medium	HA at 1/50	HA at 1/100	HA at 1/200	PPD
1	2,434	2,111	2,677		22,215
2	1,486	744	954		5,445
3	4,718	7,194	9,060		15,811
4	15,942	22,194	24,374		27,977
5	928	1,308	943	1,346	2,670
6	1,709	1,652	2,348	1,904	37,871
7	1,324	1,135	2,707	2,798	3,790
8	1,274	1,248	2,295	4,353	8,968
9	3,045	5,787	3,389	3,231	12,207
10	14,236	15,002	15,003		20,153
11	5,264	5,904	4,994	3,987	8,254
12	5,197	8,191	4,887	5,191	23,652

[a] BALB/c mice were infected with 50 bloodstream forms of *T. cruzi* Y, reinfected after 7 months, and immunized at 1 year after the first infection with killed trypomastigotes in CFA as described in the text.

[b] Lymph node proliferative assay (3 days) performed as described in the text. Mean values showing significant differences ($P < 0.05$) are underlined. HA; BALB/c crude heart sonic extract.

The data derived from chronically infected and boosted animals revealed that a proportion (around 33%) of the animals showed a small but consistent level of anti-self-heart T-cell responses. The cardiac lesions associated with the initial phases of *T. cruzi* infection could be involved in the priming of auto-anti-heart T cells in these mice. It is unclear why the responses were so small or why they could be demonstrated only in a proportion of the animals. Aging is a possible factor associated with the low levels of response, since the animals were boosted 6 months after infection. In addition, strong regulatory influences within the T-cell idiotypic network may operate to down regulate auto-anti-heart T-cell reactivity, and it is possible that the strength of this suppressive regulation is variable from animal to animal. A pathogenic role of these T cells in perpetuating the cardiac damage in these animals is speculative. Although we have no direct evidence for such a role, it is noteworthy that in infected isogenic mice cardiac lesions characteristic of the chronic phase were not found in all animals, at least when evaluated by electrocardiographic alterations, but only in a proportion of the mice (2). We are currently investigating whether there is a positive correlation between T-cell anti-heart reactivity and the presence of histologically detectable cardiac damage in chronically infected mice. Our results caution against protocols using nonsyngeneic heart antigens to demonstrate autoimmune T lymphocytes and oppose the notion of the cross-reaction model of heart damage in Chagas' disease. Our results also show that auto-anti-heart T lymphocytes can develop in chronically infected animals, possibly as a consequence of cardiac damage.

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