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Characterization of the T-Cell Proliferative Response to a Purified Glycopeptide Antigen (GP-25) Present on the *Trypanosoma cruzi* Cell Surface

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A glycoconjugate (GP-25) was previously purified from *Trypanosoma cruzi* and shown to be a major immunogenic constituent of the parasite cell surface, capable of inducing specific humoral responses in the vast majority of patients with Chagas' disease. In the present study, the T-cell proliferative response to GP-25 was studied in mice immunized with *T. cruzi* fractions or whole parasites. Recognition of GP-25 by proliferating T cells requires the participation of syngeneic, accessory spleen cells and is specifically blocked by anti-Ia antibodies. Furthermore, recognition of GP-25 is influenced by the MHC haplotype of accessory antigen-presenting cells. Short-term, GP-25-specific T-cell lines were used to demonstrate the specificity of anti-GP-25 T cells and to show that this glycoconjugate is not involved in T-cell cross-reactivity with heart antigens. T cells primed with nonpathogenic trypanosomatids are able to recognize the purified *T. cruzi* GP-25 molecule, indicating that T cells recognize a GP-25 epitope which is shared among trypanosomatids.

Infection of humans with the intracellular protozoan *Trypanosoma cruzi* causes Chagas' disease, a widespread parasitic disease endemic to and highly prevalent in South America (1). Recently, a purified glycopeptide molecule (GP-25) was obtained by aqueous extraction from *T. cruzi* epimastigotes, and its carbohydrate and amino acid composition was determined (5). GP-25 is a heat-resistant, soluble antigenic material with a molecular weight of 25,000. It bears a carbohydrate portion containing galactose, mannose, glucose, and xylose at a molar ratio of 35:13:1:1. It has been demonstrated (6) that (i) the vast majority of patients with Chagas' disease have serum antibodies specifically reacting with GP-25, while normal individuals and patients with unrelated parasitic infections lack immune reactivity to GP-25; (ii) GP-25 is located at the parasite surface; and (iii) GP-25 is expressed in all stages of the parasite life cycle, including the infective, insect-derived metacyclic forms. GP-25 was detected in all in vitro-grown *T. cruzi* strains tested so far (6). Collectively, these data lend support to the use of anti-GP-25 cellular responses as a model system to probe not only the basic aspects of T-cell responses to a defined antigen from *T. cruzi* but also the changes in regulation of specific T-cell responses during natural and experimental Chagas' disease.

In this report we describe some functional aspects of T-lymphocyte proliferative responses to the purified GP-25 molecule in animals immunized with either *T. cruzi* aqueous extracts or intact epimastigote forms.

Mice of selected strains were immunized with 25 μg of a partially purified aqueous extract from *T. cruzi* epimastigotes, prepared as described elsewhere (5), emulsified in complete Freund adjuvant (CFA; $H_{37}Ra$; Difco Laboratories, Detroit, Mich.). In addition, mice were immunized with heat-killed culture forms of one of the following trypanosomatids: *T. cruzi*, *Crithidia deanei*, *Herpetomonas samuelpessoai*, *Leptomonas samueli*, and the plant trypanosomatid *Phytomonas davidi*. The parasites were emulsified in CFA and injected (10^7 per animal) into the hind footpads. One week to 1 month after immunization, the draining lymph nodes were removed and lymph node cells (LNC) were fractionated over nylon wool columns, as described elsewhere (4). Accessory cells were syngeneic, mitomycin C-treated splenocytes taken from nonimmune animals. Short-term T-cell lines were prepared by culturing GP-25-immune T lymphocytes (5×10^6) with accessory cells (1×10^6) that had previously been pulsed with GP-25 and purified as described elsewhere (5) at 25 μg/ml for 1 h at 37°C. To assess T-cell proliferation, LNC (4×10^5) or T-cells (1×10^5 to 2×10^5) plus an equal number of syngeneic accessory splenocytes were cultured with purified *T. cruzi* glycoconjugates, purified protein derivative (PPD; Connaught Laboratories, Willowdale, Ontario, Canada), or a suitable dilution of a heart extract prepared by sonication of a pool of hearts from albino mice. Cultures were done in 0.2 ml of RPMI medium supplemented with 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), antibiotics, and 5% fetal calf serum in 96-well round-bottom microtiter plates (Linbro, Hamden, Conn.) for 3 days in a humid environment with 5% CO_2 at 37°C. Eighteen hours before the cultures were harvested, 1 μCi of tritiated thymidine ([^{3}H]TdR; 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to each well. Cultures were harvested with a semiautomated harvesting device onto fiber glass filters, and the amount of [^{3}H]TdR incorporated onto DNA was measured by liquid scintillation spectroscopy. The standard error of the mean rarely exceeded 10% of the mean and is omitted for simplicity in some cases reported herein.

When LNC from animals immunized with *T. cruzi* aqueous extracts were stimulated in vitro with purified GP-25, a proliferative response was observed (Table 1, experiment 1). This response was observed in mice from A/J, B10, BALB/c, B10BR, B10.A, and C3H strains and was selectively abolished by previous treatment of the responding cells with a rat monoclonal anti-Thy-1.2 antibody and complement (data not shown). The addition of a monoclonal anti-Ia antibody to the culture markedly suppressed T-cell proliferation to GP-25

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TABLE 1. LNC proliferative response to GP-25 and its blockade by anti-Ia antibodies

Expt and LNC stimulus[a]	Addition	[^{3}H]TdR incorporation	% Suppression
1[b]			
Medium		1,323	
GP-25		6,426	
PPD		13,222	
2[c]			
GP-25		2,097	
GP-25	Anti-Ia[d]	894	57.4
PPD		10,584	
PPD	Anti-Ia	10,074	4.8
LPS		28,321	
LPS	Anti-Ia	26,695	5.7

[a] Concentrations for LNC stimulation were 25 μg/ml for GP-25, 20 μg/ml for PPD, and 20 μg/ml for lipopolysaccharide (LPS).

[b] B10 LNC immunized with GP-25-containing extract in CFA. Results are given as mean counts per minute of triplicate cultures.

[c] B10.A LNC immunized with GP-25-containing extract in CFA. Results are given as the difference between antigen-stimulated and control cultures.

[d] Anti-Ia is a monoclonal anti-IE$^{k/d}$ antibody (Litton Bionetics, Kensington, Md.) with a final concentration of 2% culture fluid.

(Table 1, experiment 2) without interfering with both anti-PPD T-cell responses and lipopolysaccharide-induced B-cell mitogenesis. This result suggests that T-cell recognition of GP-25 depends on a concomitant recognition of self Ia molecules expressed by accessory cells, as demonstrated for conventional, soluble protein antigens (7). The failure to inhibit anti-PPD responses is not surprising since PPD is a complex antigen that could be restricted by Ia epitopes not recognized by this particular monoclonal reagent. Mice immunized with CFA alone gave no proliferative response to GP-25 (data not shown).

To demonstrate that T-cell recognition of GP-25 requires syngeneic accessory cells, we prepared a short-term T-cell line specific for GP-25 and restimulated the recovered T cells with GP-25 in the absence or the presence of freshly prepared splenic accessory cells (Table 2, experiment 1). As demonstrated, immune T cells could recognize GP-25 only when syngeneic accessory cells were added to the culture. To show that T-cell recognition of GP-25 is restricted by the major histocompatibility complex phenotype of the accessory cell, we prepared a short-term line composed of (B10 × B10.A)F_1 GP-25-immune T cells that was stimulated for 1 week with GP-25-pulsed B10 accessory cells. When this cell line was restimulated without GP-25 but with each of the semisyngeneic parental accessory cells, a somewhat high background proliferation was noted (Table 2, experiment 2). However, in the presence of purified GP-25, only B10, but not B10.A, accessory cells could induce a significant antigen-specific proliferative response in F_1 T cells. Since B10 and B10.A mice differ only at the *H-2* locus, this result indicates that T-cell recognition of GP-25 is restricted by major histocompatibility complex gene products expressed by accessory cells.

To determine the relationship between T cells recognizing GP-25 and T cells recognizing other *T. cruzi* antigens, short-term T-cell lines were generated from LNC immune to a mixture of aqueous and phenolic *T. cruzi* extracts (Fig. 1). Responding T cells were restimulated with PPD, with a heart extract, and with purified and semipurified fractions from *T. cruzi*. Galactomannan III is a glycoconjugate isolated from *T. cruzi* phenolic extracts (J. O. Previato, P. A. Gorin, and L. Mendonça-Previato, Abstr. Annu. Meet. Chagas' Dis., Caxambú, Brazil, 1982). The "R"-protein fraction is a heat-denatured protein fraction from aqueous *T. cruzi* extract. While whole LNC showed proliferative responses to each of the *T. cruzi* fractions, besides responding to PPD and the heart extract, short-term T-cell lines specific for PPD and GP-25 showed a rather selective reactivity profile with specificity for the selected antigen (Fig. 1). The results with the GP-25 cell line show that GP-25-reactive T cells do not cross-react with other *T. cruzi* glycoconjugates or with heart antigens, at least in this system.

Next, we attempted to demonstrate that intact parasites could induce proliferating, anti-GP-25 T cells. Mice were immunized with heat-killed epimastigote forms of *T. cruzi* in CFA, and immune LNC were tested for secondary proliferative responses to purified GP-25 (Table 3). The results (Table 3) indicated that *T. cruzi* epimastigotes are potent inducers of anti-GP-25 T cells. Since anti-GP-25 antibodies were found to react only with *T. cruzi* in a species-specific fashion (6), we tested the specificity of anti-GP-25 T cells against a number of nonpathogenic trypanosomatids. Mice were immunized with each trypanosomatid species in CFA, and the immune LNC were tested for a proliferative response against the purified *T. cruzi* GP-25 molecule (Table 3). Surprisingly, the results showed that T cells immune to any trypanosomatid tested become primed to respond to *T. cruzi* GP-25. These results indicate that one or more epitopes on the *T. cruzi* GP-25 molecule are shared with molecules from nonpathogenic insect and plant trypanosomatids. The fact that anti-GP-25 antibodies react with *T. cruzi* in a species-specific manner (6) suggests that T cells and antibodies recognize different epitopes on the glycoconjugate molecule and that T cells recognize epitopes which are more conserved amongst trypanosomatids than the epitopes recognized by anti-*T. cruzi* GP-25 antibodies. One indication that this is indeed the case came from studies with periodate-modified GP-25 molecules. LNC from BALB/c mice immune to *T. cruzi* GP-25 proliferated in response to purified GP-25 (1,806 cpm with medium alone and 4,829 cpm in the presence of 25 μg of GP-25 per ml). If the sugar residues on the GP-25 molecule were previously oxidized by periodate treatment, immune LNC still responded to the oxidized molecule (3,831

TABLE 2. Accessory cell dependence and major histocompatibility complex restriction of anti-GP-25 T-cell proliferative response

Expt and T-cell stimulus[a]	Accessory cells	[^{3}H]TdR incorporation (cpm) ± SEM
1[b]		
Medium	None	1,308 ± 215
GP-25	None	1,083 ± 227
Medium	BALB/c	2,637 ± 708
GP-25	BALB/c	7,933 ± 722
2[c]		
Medium	B10	5,007 ± 427
GP-25	B10	10,945 ± 903
Medium	B10.A	5,223 ± 184
GP-25	B10.A	5,685 ± 145

[a] GP-25 was used in the second culture at 25 μg/ml.

[b] Responder T cells derived from a 3-day BALB/c T-cell line maintained with GP-25 (25 μg/ml). T-cell blasts were isolated on a Ficoll gradient to deplete dead accessory cells and dead T cells.

[c] Responder T cells derived from a 7-day (B10 × B10.A)F_1 T-cell line maintained with GP-25-pulsed B10 accessory splenic cells.

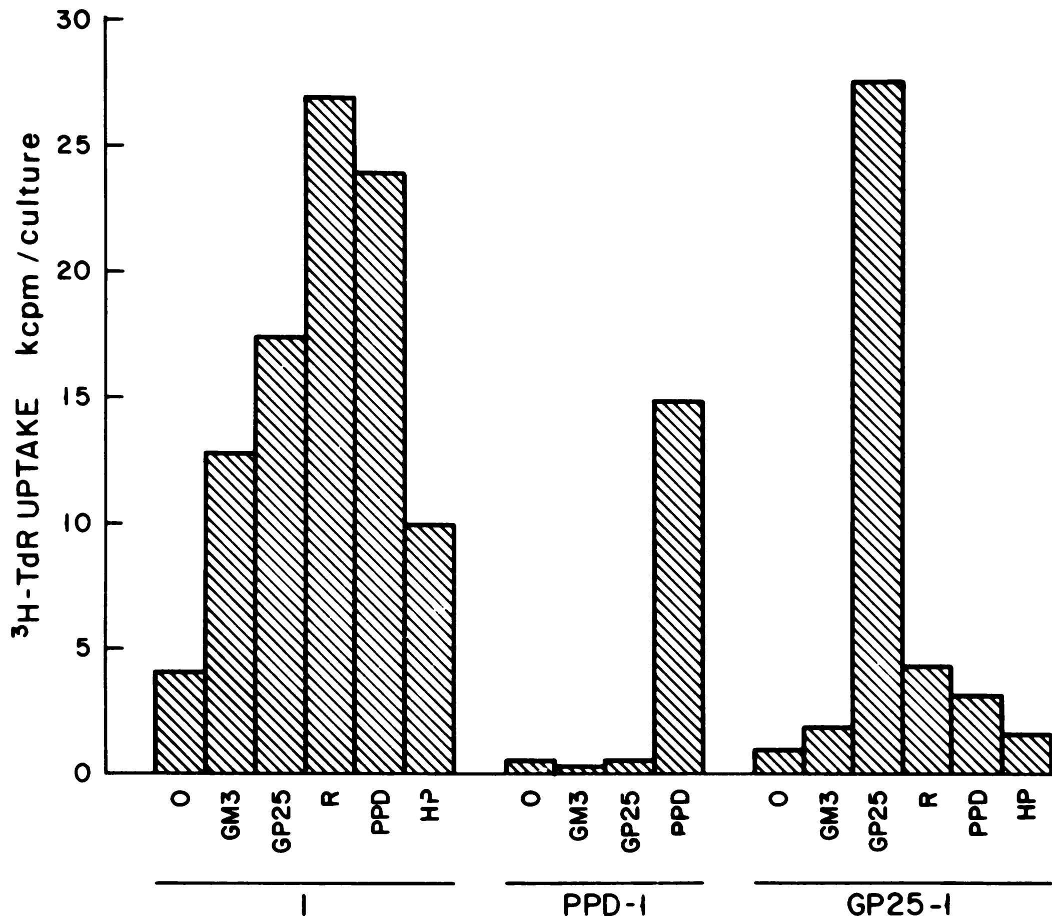

FIG. 1. Short-term T-cell line enriched for responsiveness to GP-25. BALB/c immune LNC (I) either were directly tested for proliferative responses to isolated *T. cruzi* antigens, PPD, and a heart extract (HP) or were passed through nylon wool columns and stimulated with both PPD-pulsed syngeneic accessory cells (PPD-1) and GP-25-pulsed syngeneic accessory cells (GP-25-1) for 1 week. Recovered cells were stimulated with the indicated antigens in the presence of fresh syngeneic accessory cells. Antigen concentrations for tertiary stimulation were 25 μg/ml for GP-25 and GM3 (galactomannan 3), 20 μg/ml for R fraction and PPD, and 13 μg of protein per ml for HP. [^{3}H]TdR uptake was measured after 72 h in culture.

cpm). However, this same oxidized molecule gave no specific reaction with rabbit anti-GP-25 antibodies in a radioimmunoassay (data not shown). This result suggests that the carbohydrate moiety of the GP-25 molecule is critical for recognition by antibody molecules, but not so critical for immune proliferating T cells, which might recognize a protein epitope as a major immunodominant antigen determinant. These findings are not surprising, since there is evidence that T and B cells usually recognize distinct epitopes on the same antigen molecule (2). The finding that anti-GP-25 T cells cross-react with nonpathogenic trypanosomatids is interesting, since there is evidence that previous immunization with nonpathogenic trypanosomatids leads to a T-cell-specific proliferative response to *T. cruzi* antigen (3) as well as to a protective state against experimental infection with *T. cruzi* (8). It remains to be seen whether anti-GP-25 T cells could be at least partially responsible for this protective role of insect trypanosomatids.

TABLE 3. T-cell proliferative response to *T. cruzi* GP-25 in mice immunized with whole trypanosomatids

T cells immune to[a]:	Expt	[^{3}H]TdR incorporation (cpm) ± SEM with:	
		Medium alone	GP-25[b]
T. cruzi	1	1,737 ± 49	8,574 ± 717
	2	1,988 ± 133	11,423 ± 578
T. conorhini	3	4,586 ± 334	26,678 ± 528
	4	1,096 ± 168	12,663 ± 1,082
H. samuelpessoai	5	4,668 ± 290	10,498 ± 1,058
	6	4,270 ± 230	8,909 ± 846
L. samueli	7	12,964 ± 423	26,028 ± 577
C. deanei	8	892 ± 83	2,374 ± 68
P. davidi	9	3,723 ± 243	8,017 ± 391

[a] B10.A mice immunized with 10^7 heat-killed culture forms of each trypanosomatid in CFA. Each experiment represents a pool of three animals.
[b] GP-25 was used at 25 μg/ml, final concentration.

Previous studies (6) demonstrated that anti-GP-25 antibodies are present in the vast majority of patients with Chagas' disease. Direct evidence for the biological relevance of anti-GP-25 T-cell responses has recently been obtained in our laboratory. The majority of patients with Chagas' disease show T-cell proliferative responses to purified GP-25, which is particularly evident after treatment of the responding cells with indomethacin or after passage over nylon wool columns (work in progress). This result indicates that, during natural infection by *T. cruzi*, T cells reactive to GP-25 are modulated by adherent cells, probably via prostaglandin

secretion. Thus, the purified GP-25 molecule, which has a known sugar and amino acid composition and appears to be an important antigen for cellular responses during naturally occurring *T. cruzi* infections, can be used to generate specific T-cell lines that could be useful in studies designed to dissect the cellular immune response in experimental models of Chagas' disease.

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