Brief Definitive Report

Immunoprotective *Leishmania major* Synthetic T Cell Epitopes

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Summary

Using the predictive algorithm of Rothbard and Taylor (1988. *EMBO J.* 7:93) and the primary structure of gp63 (Button, L., and M.R. McMaster. 1988. *J. Exp. Med.* 167:724; Miller, R.A., S.G. Reed, and M. Parsons. 1990. *Mol. Biochem. Parasitol.* 39:267) we have been able to delineate the structures of a number of gp63 T-cell epitopes which stimulate the proliferation of $CD4^+$ cells. One of these synthetic antigens, inoculated subcutaneously with adjuvant, was shown to specifically induce proliferation of the Th1 subset and provided immunoprotection against two species of *Leishmania* parasites.

Recent studies by Russell and Alexander have demonstrated that immunization of mice with the major cell surface glycoprotein gp63, results in cell-mediated immunity and protection from *Leishmania mexicana* infections (1). These findings suggested the possibility that the structures of the gp63 T cell epitopes might be predicted and chemically synthesized using the known *Leishmania major* primary structure (2) and the predictive algorithm proposed by Rothbard and Taylor (3). We report here that three synthetic T cell epitopes of the immunoprotective gp63 molecule stimulate proliferation of murine $CD4^+$ cells and that one specifically stimulates Th1 cells, the subset proposed to be responsible for immunoprotection in mice (4). The latter epitope was further shown to protect vaccinated mice from cutaneous leishmaniasis.

Materials and Methods

Animals. Mice were purchased from either The Jackson Laboratory (Bar Harbor, ME) or from the Charles River Breeding Laboratories (St. Constant, Quebec) and used at 8–10 wk of age. BALB/c mice for vaccine trials were bred in-house.

Synthetic Peptides. The following peptides were synthesized using Merrifield solid-phase methodologies on an Applied Biosystems Inc. (Foster City, CA) Model 430A peptide synthesizer and compositions confirmed by amino acid analysis: PT1, VRDVNWGALRIAVS; PT2, LTNEKRDILVKHLIP; PT3, YDQLVTRVVTHEMAHA; PT4, TRVVTHEMAHALGFSG; PT6, PFNVFSDAARCIDGAF; PT7, AARCIDGAFRPKATDG; PT8, RPKATDGIVKSYAGLC. The peptides represent gp63 residues 1–13, 48–61, 154–168, 158–173, 378–393, 385–401, and 394–409, respectively.

T Cell Proliferation Assays. Three animals were inoculated subcutaneously with 50 μg of peptide emulsified in 100 μl of CFA. Lymph nodes were removed after 9 d and 5×10^5 cells incubated for 3 d with synthetic peptides followed by further incubation for 20 h with 1 μCi [^{3}H]TdR. Controls show the incorporation of [^{3}H]TdR into unprimed lymph node cells incubated with peptides or 2 μg/ml Con A, as well as the incorporation into primed lymphocytes in the absence of challenge peptides. Control B cell proliferations used 20 μg/ml of *Escherichia coli* LPS. Lymphocyte stimulation was plotted as the average counts per minute (cpm) of triplicate experimental cultures minus the average of the relevant triplicate control values. Each of the proliferation experiments was replicated at least twice.

Complement-mediated Cell Lysis. Lysis was carried out using 5×10^5 lymph node cells incubated with either alloanti-Thy-1 antisera, anti-L3T4$^+$ or anti-Lyt-2.2$^+$ mAbs (Cedarlane Laboratories Ltd., Hornby, Ontario) for 60 min at 4°C, followed by addition of 10% rabbit complement for 60 min at 37°C.

Lymphokine Determinations. IL-2 and IL-4 were assayed using the IL-2– and IL-4–dependent CTLL-2 cell line (ATCC TIB 214). Specific neutralization was achieved with an anti-IL-2 antibody, S4B6 (17), or with the anti-IL-4 antibody 11B11 (ATCC HB). IL-3 was determined using the IL-3–dependent cell line DA-1. Results were expressed as units of lymphokine extrapolated from standard recombinant interleukin curves.

Immunoprotection Trial. Four groups of eight female BALB/c mice were used in this study. Each group was injected subcutaneously with 100 μg of test peptide in a temperature-dependent sol gel transition adjuvant, 8% Poloxamer 407 (BASF, Wayandotte, Ludwigshaven, FRG) with exception of the control group, which received only adjuvant. After 6 wk the mice were inoculated subcutaneously in the hind quarters with 2×10^4 stationary phase *L. major* promastigotes. A similar study used *L. mexicana* promastigotes and CBA mice. Progress of disease was monitored by measurement of lesion diameter beginning at 4 wk after inoculation.

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Results and Discussion

The primary structure of *L. major* gp63, as described by Button and McMaster (2) and corrected by Miller et al. (5), was used along with the predictive algorithm of Rothbard and Taylor (3) to select seven peptide sequences as potential T cell epitopes (PT1–4 and PT6–8). The ability of these peptides to stimulate lymphocyte proliferation was tested by immunization of mice, followed by in vitro challenge of isolated lymph node cells with the test peptides. Fig. 1 shows the dose-response curves after challenge of primed lymph node cells with the immunizing peptides. Three peptides were found to be stimulatory. The overlapping peptides PT3 and PT4, which cover a highly conserved region believed to be responsible for zinc binding (6, 7), stimulated proliferation in cells derived from BALB/c (H-2^d), A/J (H-2^a), and CBA (H-2^k) mice but not in C57BL/6 (H-2^b) lymphocytes. Peptide PT6 was pan-specific for this collection of mouse haplotypes, stimulating lymphocytes from all strains. Verification that [^{3}H]TdR incorporation was associated with T cell proliferation was provided by T cell–specific depletion using anti-Thy-1 antisera (data not shown) or anti-L3T4$^+$ mAb and complement (Fig. 2). While only data for the peptide PT6 are shown, similar proliferation data were obtained for peptides PT3 and PT4 and in experiments with lymphocytes from the other responding mouse strains. These data show that the proliferating cells are primarily of the CD4$^+$ subset. Evidence was also obtained that the B cell population was unaffected by the depletion procedure by using the B cell mitogen LPS (Fig. 2).

Recent findings have suggested that proliferation of either the CD4$^+$ Th1 or Th2 subsets determines whether an immunoprotective or disease exacerbating condition is found after infection with *Leishmania major* (4). A study of lymphokine production, after challenge of lymph node cells with the stimulatory peptides, showed that only PT3 was capable of stimulating IL-2 (Table 1) and that none of the epitopes produced measurable IL-4. It could be concluded that under the immunization protocol used, PT3 specifically induced proliferation of the CD4$^+$ Th1 subset (8). These findings, together with the fact that gp63 was known to elicit protective cell-mediated immunity in mice (1), strongly supported the candidacy of the PT3 epitope as a potential vaccine for cutaneous leishmaniasis.

Results of immunoprotection experiments using these synthetic epitopes showed that a molecularly defined vaccine was

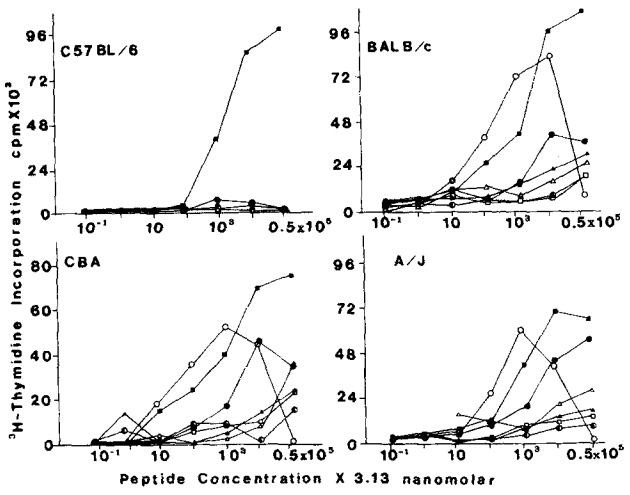

Figure 1. T cell proliferation dose-response curves for seven *L. major* gp63 synthetic peptides screened against four mouse strains with differing MHC haplotypes. Data represent averages of triplicate proliferation experiments after subtraction of average control proliferations. The average control values were: C57BL/6, 6.8 × 10^3 cpm; BALB/c, 11.6 × 10^3 cpm; CBA, 21.8 × 10^3 cpm, and A/J, 8.4 × 10^3 cpm. PT1 (△), PT2 (▲), PT3 (○), PT4 (●), PT6 (■), PT7 (□), PT8 (◐).

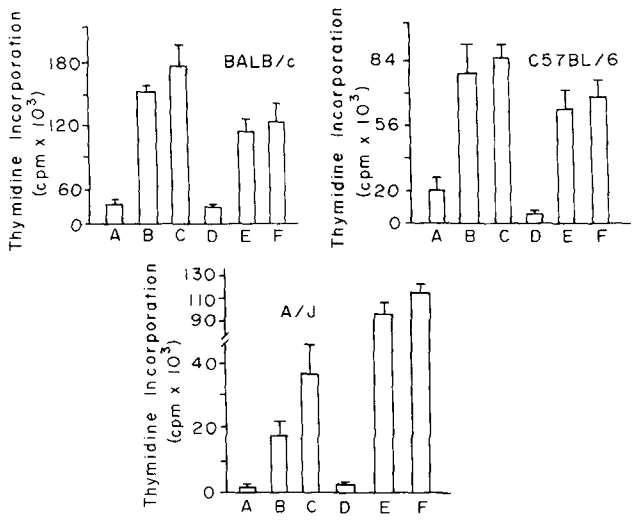

Figure 2. Effect of anti-L3T4$^+$ (CD4$^+$) and anti-Lyt-2.2$^+$ (CD8$^+$) mAbs and rabbit complement on PT6 stimulation of primed lymphocytes. (*A* and *B*) Lymph node cells from immunized mice incubated with anti-L3T4$^+$, or anti-Lyt-2.2$^+$ mAb and rabbit complement followed by PT6 (156 μM). (*C*) Complement alone followed by peptide. (*D*) No additions. (*E*) LPS-induced proliferation. (*F*) LPS proliferation after exposure to anti-L3T4$^+$ antibody and complement.

Table 1. *Lymphokine Production after Stimulation of T Cells from BALB/c and CBA mice with Synthetic gp63 Peptides*

Challenge peptide	Lymphokine*					
	IL-2		IL-3		IL-4	
	BALB/c	CBA	BALB/c	CBA	BALB/c	CBA
	U/ml					
PT3	0.8	1.9	3.8	ND	0	0
PT4	0	0	0.3	ND	0	0
PT6	0	0	7.0	ND	0	0
No peptide	0	0	0	0	0	0

* Zero values indicate no detectable lymphokine.

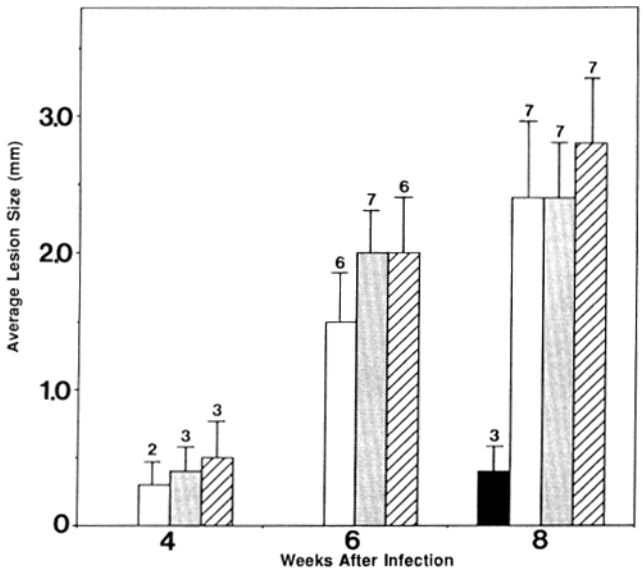

Figure 3. Development of *L. major* infection in groups of eight BALB/c mice after immunization with synthetic peptides. Lesion diameters are shown above each bar along with the SEM. Controls receiving adjuvant only (*hatched bar*); PT3 (*solid bar*); PT4 (*open bar*); PT6 (*shaded bar*).

a strong possibility. BALB/c mice were injected with a single dose of either peptide PT3, PT4, or PT6 in adjuvant, or with adjuvant alone, and after a 6 wk rest were infected with 2 $\times$ 10^4 stationary phase. *L. major* promastigotes. Fig 3 shows results of the first study using the adjuvant Poloxamer 407. Lesion development was evident at 4 wk in control animals, as well as in animals inoculated with PT4 and PT6 but not in the group immunized with PT3. At 6 wk the number of infected animals in previously infected groups increased, along with the average lesion size, while no evidence of infection could be found in the group immunized with PT3 plus adjuvant. Not until 2 mo after inoculation was there evidence of lesion development in the latter group. This appeared to be the end of a period of premunition induced by the single subcutaneous injection of antigen. A second experiment, using the same protocol but with a slightly higher adjuvant concentration (12.5%), provided further verification of the immunoprotective capacity of PT3. Not only was this peptide once again shown to limit the disease, but half the animals vaccinated with PT3 cured of disease 12–16 wk after inoculation with parasites. Results using *Leishmania mexicana* and the previous protocol were even more convincing, with complete lack of disease in the vaccinated group 3 mo after parasite challenge.

That these findings were not entirely related to the Poloxamer 407 adjuvant was shown by conducting another *L. major* experiment but replacing Poloxamer with CFA. No sign of disease was found in vaccinated animals 5 wk after parasite inoculation, while all controls showed lesion development. Nonetheless an interesting result from the second *L. major* experiment with Poloxamer indicated that the use of adjuvant was an important determinant in the outcome of these subcutaneous vaccinations. When PT3 was injected in the absence of adjuvant, an exacerbation in lesion growth was observed (data not shown). This result mimicked a previously described disease exacerbation produced in BALB/c mice after subcutaneous vaccination with irradiated *L. major* promastigotes (9, 10). A possible explanation for these results has recently been proposed suggesting that adjuvant could affect the physiological state of APCs, stimulating production of costimulatory factors appropriate for Th1, rather than Th2 cell proliferation (11).

While long-term immunity was not attained with a single injection, these results are very promising and experiments to further characterize the immunoprotective utility of the synthetic peptides are now in progress. It should be feasible to assess whether the same epitope can induce protection or exacerbation dependent upon the route or mode of administration, and conversely, whether several types of epitopes can exist within the gp63 structure. Indeed preliminary evidence has indicated that using the Poloxamer adjuvant both forms of epitope are found in gp63, arguing against the general utility of large, undefined, recombinant antigens. Experimentation can now proceed with single epitopes, rather than complex structures containing both an array of undefined T cell epitopes as well as oligosaccharides. The findings reported here show that a molecularly defined approach to a solution of these problems is not only feasible but critical for the development of vaccines against cutaneous leishmaniasis.

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