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The *Mycobacterium bovis* 32-Kilodalton Protein Antigen Induces Human Cytotoxic T-Cell Responses

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The 30-kDa protein (P32) is a mycobacterial secreted antigen which is homologous in *Mycobacterium bovis* and *M. tuberculosis*. In vitro, P32 induced T-cell proliferation. *M. tuberculosis*- or P32-stimulated T-cell lines lysed macrophages pulsed with P32 or *M. tuberculosis*, respectively. We conclude that P32 stimulates cytotoxic T cells specifically.

Tuberculosis is an infectious disease which afflicts 60 million individuals and is responsible for 3 million cases of death annually. Tuberculosis is no longer a major concern in developing countries only; incidences have dramatically increased in industrialized countries as well, primarily as a result of the AIDS epidemic. The emergence of multidrug-resistant *Mycobacterium tuberculosis* strains has exacerbated the situation. Thus, satisfactory control of the disease will depend on development of an effective new-generation vaccine.

Although protective immunity against tuberculosis crucially depends on helper T lymphocytes which activate tuberculostatic functions in macrophages, evidence has been presented that cytolytic T lymphocytes (CTL) are also required for optimum protection (8). The majority of antigens with potential relevance for future subunit vaccines against tuberculosis that have been identified thus far are of somatic origin, and only few secreted antigens have been identified. However, for none of them has a clear association with protection been formally proven.

Because *M. tuberculosis* is capable of replicating inside macrophages, somatic proteins should not be available for presentation to T cells early after infection. Rather, proteins secreted by metabolically active *M. tuberculosis* organisms within macrophages should represent the major source of T-cell antigens. The antigen 85 complex, comprising the 85A, 85B, and 85C proteins, represents a dominant group of secreted 30-kDa proteins from *M. tuberculosis*, *M. bovis*, and other mycobacteria, and the 30-kDa protein cognates of *M. bovis* (P32) and *M. tuberculosis* are highly homologous (1, 4–6, 12–14, 22). Evidence has been presented that P32 is expressed during human tuberculosis and leprosy infection and after *M. bovis* BCG vaccination, suggesting that it is a major target antigen of cellular and humoral immunity (9–11, 23, 24). We investigated whether P32 in vitro induces human CTL, which have been implicated in both resistance to and the pathogenesis of tuberculosis (8, 17). To this end, peripheral blood mononuclear cells (PBMC) were obtained from BCG-vaccinated healthy Caucasian donors (Blood Center Ulm) of unknown status of purified protein derivative reactivity. Nylon wool-enriched T cells (2×10^5/ml) were suspended in RPMI 1640 medium (supplemented with 10% heat-inactivated human A^+ serum; Biochrom KG, Berlin, Germany) together with autologous irradiated (3,000 R) accessory cells (4×10^5/ml) and stimulated with purified P32 (15 μg/ml), lysates of *M. tuberculosis* H37Ra (batch 3114-33-8; 15 μg/ml; Difco, Detroit, Mich.), concanavalin A (5 μg/ml; Sigma, St. Louis, Mo.), or heat-killed *Listeria monocytogenes* (incubation at 65°C for 120 min) (10^6/ml) for 2 days (mitogenic responses) or 7 days (antigen-specific responses) (5). Proliferation was measured by [^{3}H]thymidine (1 μCi/ml; Amersham International, Amersham, England) uptake during the last 12 h. T cells from all six donors tested responded to lysates of *M. tuberculosis* (median = 134,987 cpm), P32 (median = 23,406 cpm), and heat-killed *L. monocytogenes* (median = 54,790 cpm) ($P < 0.05$; Wilcoxon signed *U* test; Table 1). Nonspecific T-cell expansion caused by P32 was not detected after 2 days of culture (545 cpm). After 7 days of culture of PBMC with P32 or with *M. tuberculosis*, CD4 T-cell proportions were increased, as determined by fluorescence-activated cell sorting (data not shown). These results demonstrate that P32 specifically stimulates and expands T cells. Accordingly, others have shown that P32 induces lymphoproliferation and gamma interferon production in whole blood cell cultures from normal individuals (7).

Next, we expanded T-cell lines by bulk culture of PBMC (10^6/ml) with lysates of *M. tuberculosis* (50 μg/ml) and two to three cycles of 7-day antigen restimulation together with

TABLE 1. Specific proliferation of peripheral blood T cells from six normal individuals stimulated with P32 antigen

Cell population	Stimulus	Culture (days)	Proliferative response (median cpm [range])	SI[a]
T cells + accessory cells	P32 antigen	7	23,406[b] (14,356–72,079)	38
	M. tuberculosis	7	134,987[b] (65,476–182,945)	221
	L. monocytogenes	7	54,790[b] (25,424–110,659)	90
	None	7	610 (133–1,540)	1
T cells alone	P32 antigen	7	545 (253–1,182)	1
	P32 antigen	2	694 (174–1,009)	1
T cells + accessory cells	Concanavalin A	2	10,604[b] (2,414–14,059)	26
	None	2	401 (165–893)	1

[a] SI, stimulation index (experiment counts per minute/control counts per minute).
[b] $P < 0.05$ versus control (Wilcoxon signed *U* test).

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TABLE 2. Specific lysis by *M. tuberculosis*-activated CTL of targets pulsed with P32 or synthetic peptides thereof

Donor	Target priming[a]	% Specific lysis at effector/target ratio of:	
		45:1	15:1
1	Killed *M. tuberculosis*	23	17
	P32 antigen	16	13
	P32 peptide (1–24)	0	0
	P32 peptide Cys-103(104–120)	8	5
	P32 peptide Cys-225(226–238)	40	34
	Killed *L. monocytogenes*	1	0
	None	6	0
2	Killed *M. tuberculosis*	22	20
	P32 antigen	34	18
	P32 peptide (1–24)	19	16
	P32 peptide Cys-03(104–120)	21	12
	P32 peptide Cys-225(226–238)	19	5
	Killed *L. monocytogenes*	0	0
	None	0	0
3	Killed *M. tuberculosis*	45	35
	P32 antigen	16	7
	P32 peptide (1–24)	ND[b]	ND
	P32 peptide Cys-103(104–120)	21	16
	P32 peptide Cys-225(226–238)	40	13
	Killed *L. monocytogenes*	1	0
	None	3	0
4	Killed *M. tuberculosis*	37	2
	P32 antigen	14	1
	Killed *L. monocytogenes*	1	0
	None	4	1

[a] Amino acid sequences (in one-letter code) indicated in parentheses are as follows: *M. bovis* P32 (1–24), FSRPGLPVEYLQVPSPSMGRDIKV; Cys-103(104–120), CELPGWLQANRHVKPTGS; and Cys-225(226–238), CAKF LEGFVRTSNI.

[b] ND, not determined.

TABLE 3. Capacity of P32-activated CTL to recognize *M. bovis*-infected targets

Donor	Target priming	% Specific lysis at effector/target ratio of:	
		45:1	15:1
1	P32 antigen	16	6
	Killed *M. tuberculosis*	13	3
	Live *M. bovis* BCG[a]	44	9
	None	4	0
2	P32 antigen	18	7
	M. tuberculosis	10	8
	Live *M. bovis* BCG	13	4
	None	2	2
3	P32 antigen	10	7
	Killed *M. tuberculosis*	2	8
	Live *M. bovis* BCG	21	13
	None	4	2
4	P32 antigen	31	1
	Killed *M. tuberculosis*	55	42
	Live *M. bovis* BCG	41	16
	None	0	0
5	P32 antigen	8	2
	Killed *M. tuberculosis*	20	4
	Live *M. bovis* BCG	4	2
	None	5	0

[a] Infection ratio = 10:1; 24 h before assay.

recombinant interleukin-2 (30 U/ml; EuroCetus, Amsterdam, The Netherlands) (15). Afterwards, the cytolytic activities of T-cell lines were measured in a ^{51}Cr release assay. Results were expressed as percentage of ^{51}Cr release = (experiment control/high control) × 100 (15). *M. tuberculosis*-activated T-cell lines from four of four donors lysed target cells pulsed with *M. tuberculosis*, with P32, or with synthetic peptides from P32 (Table 2). In contrast, *M. tuberculosis*-activated CTL did not lyse *L. monocytogenes*-primed targets (16). Thus, we exclude CTL cross-reactive and nonspecific killer activities in this system. CTL activities toward the synthetic peptides of P32 (13 to 23 amino acids long) varied in the donors tested (5). Thus, our results indicate that *M. tuberculosis* stimulates a heterogeneous T-cell population with specificity for different P32 peptides. The high homology between the *M. bovis* and *M. tuberculosis* P32 antigen suggests that cross-reactive T cells participate in target cell destruction (3, 16, 20).

One major question for the development of subunit vaccines against tuberculosis is the identification of mycobacterial antigens of protective value. To approach this issue, we expanded P32-stimulated T-cell lines and investigated their cytolytic potential toward *M. bovis*-infected macrophages (infection at 24 h before assay at a ratio of 10:1) (Table 3). In four of five donors, significant lysis of *M. bovis*-infected target cells was detected. We note a reasonable variation of P32-activated CTL in lysis of targets primed with *M. bovis* BCG or *M. tuberculosis*. Although our data do not permit a conclusive explanation for this variation, we assume that the differential availability of P32 for antigen processing due to different amounts in the bacterial preparations and the distinct processing capacities of the macrophages may be responsible. In addition, the numbers of P32 T-cell precursors may vary among the donors studied. Since P32 is a component of the live attenuated BCG vaccine, vaccinated individuals express T-cell reactivity to this antigen. The P32 cognates are expressed both on the cell wall surface and in the cytosol of mycobacteria (2, 19–21). Consistent with our findings, T-cell clones from BCG-vaccinated individuals which respond to mycobacterial cell wall and culture filtrate antigens recognize BCG-infected monocytes (18).

It has been proposed that CTL are required for optimum protection (8). Therefore, our finding that P32-specific CTL recognize infected macrophages indicates that P32 is secreted and introduced into the macrophage processing and presentation machinery during infection. Although our study is limited to a small number of BCG-vaccinated individuals and needs to be extended to tuberculosis patients, our finding that P32 stimulated CTL of various fine epitope specificities suggests that P32 may be an important component of future subunit vaccines against mycobacterial infection.

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