Brief Definitive Report

THE RECOMBINANT 65-kD HEAT SHOCK PROTEIN OF *MYCOBACTERIUM BOVIS* BACILLUS CALMETTE-GUERIN/*M. TUBERCULOSIS* IS A TARGET MOLECULE FOR CD4+ CYTOTOXIC T LYMPHOCYTES THAT LYSE HUMAN MONOCYTES

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Protective immunity against mycobacteria is dependent on antigen-specific Th lymphocytes. It has been assumed that these Th upon recognition of antigen/MHC secrete macrophage-activating factors that enable effector macrophages to eliminate the intracellular organism (1).

Recently, however, we have found that mycobacteria are very potent inducers of antigen-specific $CD4^+$ and $CD8^+$ cytotoxic T (Tc) cells as well as antigen-nonspecific killer cells that lyse human monocytes (2). Also, in experimental animal models, evidence has recently accumulated in favor of an important role for Tc cells in the immune response to mycobacteria (reviewed in reference 3). In addition, *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG)-reactive human $CD4^+$ T cell clones have been reported to lyse PPD-pulsed target (antigen-presenting) cells (4). The identification of the cytotoxic effector cells as well as the antigens that they recognize are of obvious importance for the design of mycobacterial subunit vaccines.

Here we report that the recombinant 65-kD heat shock protein (HSP) (5) of *M. bovis* BCG, which is identical to the 65-kD protein of *M. tuberculosis*, shares >95% sequence homology with the *M. leprae* 65-kD gene (6) and shows extensive crossreactivity with the latter (reference 7) is an immunodominant antigen for $CD4^+$ BCG-specific Tc cells in a significant number ($\pm$ 20%) of BCG-responsive individuals. In addition, recombinant 65-kD HSP-stimulated effector cells also efficiently lysed autologous monocytes in the absence of antigen. This observation may shed new light on the role of the 65-kD HSP in autoimmunity.

Materials and Methods

Lymphocyte Transformation Test (LTT). Ficoll-isolated PBMC of 58 mainly Ethiopian individuals were cultured with BCG, purified protein derivative (PPD) (both from Statens Serum

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Inst., Denmark; 5 μg/ml), *M. leprae* (2.5×10^7 bacilli/ml; provided by Dr. R. J. W. Rees, Mill Hill, UK), recombinant 65-kD HSP of BCG (5 μg/ml), or control RPMI 1640 + 5% normal human serum, pulsed on day 6 with [^{3}H]thymidine, harvested 18 h later, and counted by liquid scintillation counting (Rackbeta II; LKB Instruments, Inc., Gaithersburg, MD).

Cytotoxicity Assay. As described in detail in reference 2, PBMC were stimulated during 7 d with PPD, BCG, or the recombinant 65-kD HSP of BCG in 24-well tissue culture plates (Linbro; Flow Laboratories, McLean, VA) (10^6 cells/ml).

Target cells were day 6 adherent cells of PBMC that had been plated in 96-well U-bottomed tissue culture plates (1.5×10^5 cells/well) (Titertek; Flow Laboratories). Approximately 10% of the cells adhered. This number was used for calculating E/T ratios. On day 6, the cells were pulsed with antigen (PPD, 25 μg/ml; BCG, 25 μg/ml; tetanus toxoid, 1:120 dilution; recombinant 65-kD HSP of BCG, 20 μg/ml) and labeled with 2 μCi of 51 sodium chromate (Amersham International, Amersham, UK) during 18 h and then washed three times with preheated medium. Effector cells were then added in triplicate to the target cells (150 μl/well). One triplicate with medium only was used for the determination of spontaneous release. 15 h later the total supernatant content of each well was transferred to a detachable counting tube (Linbro; Flow Laboratories) and 100 μl of triton-X was added to the original wells to lyse the remaining adherent cells. After 3 h the total volume of triton-X was transferred to similar tubes and the samples were counted (1270 Rack Gamma; LKB Instruments, Inc.). The percentage-specific killing for each individual well was calculated as follows: percent specific lysis = [test cpm/(test cpm + cpm after triton-X treatment of the same well)] × 100% − percent spontaneous release. The percentage spontaneous release was calculated as follows: cpm in spontaneous release well/(cpm in spontaneous release well + cpm after triton-X treatment of the same well) × 100%.

Depletion of Lymphocyte Subsets with mAb-coated Magnetic Beads. Magnetic beads (Dynal A. S., Oslo, Norway) were coated with rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark) and then incubated with 50 μl of the appropriate second layer mAb (Leu-1 (CD5), Leu-2 (CD8), Leu-19 (HNK-1) (all from Becton Dickinson & Co., Mountain View, CA), BMA 044 (CD4) (Behringwerke AG, Marburg, Federal Republic of Germany) or normal mouse serum [control beads]). Cells and beads were mixed in a 1:4 ratio, centrifuged for 5 min at 4°C (1,000 rpm), and then incubated for another 15 min on ice. The pellet was gently resuspended and depleted for marker-positive cells with a magnetic particle concentrator (Dynal A. S.). The procedure was repeated two more times. Control bead-depleted cells were resuspended in complete medium to give a 30:1 E/T ratio in the highest concentration. The other cell suspensions were resuspended in the same volume as the control bead-depleted cells. Depletion was usually 85–100% effective as determined by immunofluorescence.

Results

Proliferative PBMC Responses of BCG Responder Individuals to the Recombinant 65-kD HSP of BCG. PBMC of 47 out of 58 individuals proliferated (i.e., ≥5,000 cpm) to BCG. Nine individuals (19%) also responded to the recombinant 65-kD purified HSP of BCG (Table I).

The Recombinant 65-kD HSP of BCG is an Immunodominant Target Molecule for PPD/BCG-induced Cytotoxic Effector Cells that Specifically Lyse Antigen-pulsed Monocytes. PPD- or BCG-stimulated effector cells from recombinant 65-kD responsive donors efficiently lysed PPD-pulsed, and to a much lower extent, nonpulsed or tetanus toxoid–pulsed autologous monocyte targets in a dose-dependent manner (Table II). PPD-induced effector cells of recombinant 65-kD (LTT) responsive donors also lysed recombinant 65-D–pulsed monocytes as efficiently as PPD-pulsed ones. PPD-stimulated effector cells of a recombinant 65-kD LTT nonresponder lysed PPD-pulsed but not recombinant 65-kD–pulsed monocytes (Table II, Exp. 4). The recombinant 65-kD HSP of BCG, therefore, appears to be an important target molecule for PPD- or BCG-stimulated cytotoxic effector cells of recombinant 65-kD LTT-responsive but not nonresponsive donors.

TABLE I

Proliferative Responses of PBMC from Different Individuals to M. bovis BCG, M. leprae, and the Recombinant 65-kD HSP of BCG Protein

No.	Clinical diagnosis	BCG/PPD	Recombinant 65-kD protein	*M. leprae*	Control
1	Normal	145,204 ± 8,733	53,710 ± 1,989	51,493 ± 4,797	821 ± 607
2	Normal	162,243 ± 19,895	12,229 ± 3,674	66,261 ± 6,333	2,208 ± 306
3	Normal	60,607 ± 11,584	9,989 ± 3,497	19,507 ± 2,381	540 ± 122
4	Normal	172,745 ± 14,839	20,585 ± 5,305	ND	1,116 ± 453
5	Normal	140,741 ± 4,314	5,906 ± 6	8,093 ± 2,955	1,633 ± 466
6	BT*	55,735 ± 7,758	10,504 ± 2,011	56,140 ± 11,884	2,369 ± 812
7	BT	61,260 ± 11,991	5,796 ± 508	23,711 ± 4,057	1,834 ± 811
8	BT	53,524 ± 22,786	22,535 ± 3,286	5,807 ± 1,264	2,863 ± 851
9	LL‡	59,357 ± 12,137	21,901 ± 5,175	955 ± 98	1,202 ± 224

The results are expressed as mean cpm ± SEM of [^{3}H]thymidine incorporation by triplicate cultures of 2×10^5 PBMC each. Results are only shown for the 9 out of 47 (21 healthy contacts, 13 BT, and 13 LL patients) BCG responsive individuals that responded to the recombinant 65-kD protein (see Materials and Methods).

* BT, borderline tuberculoid leprosy.

‡ LL, lepromatous leprosy.

The Recombinant 65-kD HSP of BCG is an Immunodominant Stimulatory Molecule for Cytotoxic Effector Cells that Lyse both PPD and Recombinant 65-kD HSP of BCG-pulsed Monocytes. To investigate if the recombinant 65-kD HSP of BCG also was immunodominant at the precursor rather than at the effector level, we raised recombinant 65-kD–stimulated effector cells from LTT-responsive donors. These effector cells efficiently lysed recombinant 65-kD–pulsed monocytes and, importantly, also PPD-pulsed monocytes (Table II). Nonpulsed or irrelevant antigen-pulsed monocytes were lysed to a lower extent than PPD- or recombinant 65-kD–pulsed ones, but recombinant 65-K-induced effector cells lysed nonpulsed monocytes much more (roughly twice as) efficiently than did PPD- or BCG-induced cytotoxic cells.

The recombinant 65-kD HSP of BCG thus is an efficient stimulatory molecule for precursors of cytotoxic effector cells that specifically lyse PPD- and recombinant 65-kD–pulsed monocytes but also strongly lyse monocytes in the absence of antigen.

Membrane Phenotype of PPD and Recombinant 65-kD HSP of BCG-induced Cytotoxic Effector Cells. PPD or recombinant 65-kD HSP of BCG-induced effector cells predominantly consisted of $CD3^+/CD5^+CD4^+$ T cells (data not shown). The depletion of CD4 but not CD8 or Leu-19^+ cells from the effector population reduced or abrogated lytic activity against both PPD and recombinant 65-kD protein-pulsed monocytes (Fig. 1).

Discussion

The 65-kD HSP is a major immunogenic component of *M. bovis* BCG, *M. tuberculosis*, and *M. leprae*, and is frequently recognized by murine and human B cells and helper T cells (6–8). Little is known about the possible role of Tc cells in the human immune response to mycobacteria. We here show that the 65-kD HSP of *M. bovis* BCG/*M. tuberculosis* is an immunodominant target molecule for BCG/PPD-specific $CD4^+$ Tc lymphocytes that lyse human monocytes, and is an immunodominant

TABLE II

Antigen-specific and Antigen-nonspecific Lysis of Monocyte Targets by BCG/PPD or Recombinant 65-kD HSP of BCG-stimulated Cytotoxic Effector Lymphocytes

Exp.	Individual	Stimulating antigen	LTT index*	E/T ratio	Percent specific lysis of monocytes pulsed with: PPD	Recombinant 65-kD HSP BCG	Tetanus toxoid	No antigen
1	1	BCG	177	50:1	52	52	ND	19
				15:1	53	43	ND	11
				5:1	44	35	ND	11
		Recombinant 65-kD BCG	65	40:1	ND	58	ND	41
				12:1	ND	53	ND	37
				4:1	ND	40	ND	14
2	2	PPD	112	40:1	69	62	ND	29
				12:1	67	58	ND	22
				4:1	57	52	ND	14
		Recombinant 65-kD BCG	18	40:1	50	63	ND	41
				12:1	60	61	ND	35
				4:1	56	57	ND	25
3	3	PPD	155	50:1	45	29	21	20
				15:1	57	22	19	12
				5:1	38	16	0	5
		Recombinant 65-kD BCG	18	25:1	33	37	23	20
				8:1	19	26	12	10
				2:1	9	10	2	1
4	4	PPD	19/<1‡	30:1	54	5	21	17
				9:1	38	0	12	7
				3:1	25	0	7	3

Results are expressed as the mean percentage specific lysis of triplicate cultures calculated as described in Materials and Methods. SEM were mostly <10%.

* LTT index, cpm in antigen-stimulated cultures/cpm in nonstimulated cultures.

‡ LTT index for the recombinant 65-kD HSP of BCG.

triggering molecule for BCG/PPD-specific Tc cells during primary in vitro restimulation.

In mice, Tc cells are an important component of the immune response to mycobacteria (3). We have recently found that in humans, as well, mycobacteria are very potent inducers of antigen-specific CTL that lyse monocytes (2) and that these effector cells, in parallel with lysing infected target cells, also kill intracellularly located live mycobacteria (Kumararatne, D. S., B. Kale Ab, R. Kiessling, P. Converse, and T. Ottenhoff, unpublished results). This suggests that Tc cells may be pivotal effector cells in the actual protection against mycobacterial infections. The fact that the recombinant 65-kD HSP of BCG expresses immunodominant epitopes for peripheral Tc cells (this study) as well as B cells (6, 7) and Th (6–8) in a significant number of individuals renders this protein a seemingly interesting candidate for subunit vaccines.

The recombinant 65-kD HSP of BCG was only immunodominant in ±20% of the individuals tested and we are presently trying to define the antigens of BCG

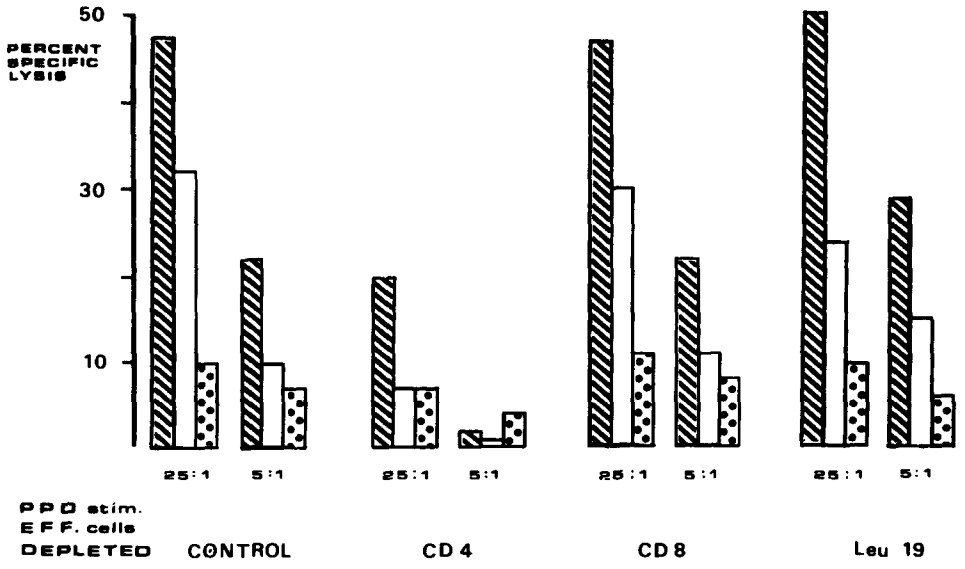

FIGURE 1. Effect of depletion of cytolytic effectors for different cell surface markers with marker-specific mAb-coated magnetic beads on specific lysis of PPD pulsed (*hatched bars*), recombinant 65-kD HSP of BCG-pulsed (*open bars*), or -nonpulsed (*dotted bars*) monocytes.

that can induce cytotoxic cells in those BCG responders that do not respond to the recombinant 65-kD HSP.

An interesting observation was the remarkably high level of killing of autologous nonpulsed monocytes by recombinant 65-kD–induced cytotoxic cells. The former effector cells also strongly expressed the Leu-19 (HNK-1) marker as well as strong lytic activity against tumor cell lines (unpublished observations). An important and clinically relevant side effect of an immune response primarily focussed to the recombinant 65-kD HSP might thus be self (specific or nonspecific) reactivity that could result in tissue destruction, an essential feature of tuberculosis and tuberculoid leprosy. Several other studies support a pathological role for the recombinant 65-kD HSP: adjuvant arthritis can be induced in Lewis rats by recombinant 65-kD HSP-reactive T cell clones (9). T cells from rheumatoid arthritis patients recognize an *M. tuberculosis* fraction (10) that is probably enriched for the recombinant 65-kD HSP. T cell clones from tuberculoid (high responder) leprosy patients also frequently recognize the same HSP (7).

Future studies will have to determine whether the 65-kD determinants that are recognized by protective Tc cells and Th can be dissociated from the determinants that induce autoreactive T cell and non-T cell cytotoxic effector cells.

Summary

Since little is known about Tc cells in the human immune response to intracellular parasites, we have studied the role of Tc cells in response to *M. bovis* Bacillus Calmette-Guerin (BCG). Donors whose PBMC responded to BCG, purified protein derivative (PPD), and the recombinant 65-kD heat shock protein (HSP) of BCG gener-

ated BCG/PPD-specific $CD4^+$ effector T lymphocytes that lysed PPD as well as recombinant 65-kD–pulsed monocytes. Nonpulsed or irrelevant antigen-pulsed target cells were lysed to a much lower but still significant extent. PPD-stimulated effector lymphocytes of a recombinant 65-kD nonresponder lysed PPD but not recombinant 65-kD–pulsed monocytes.

Recombinant 65-kD–educated effector lymphocytes lysed both recombinant 65-kD– and PPD-pulsed monocytes. In addition, these effector cells efficiently lysed nonpulsed target cells. These results demonstrate that in recombinant 65-kD responders, the recombinant 65-kD HSP of BCG is an immunodominant target as well as a triggering molecule for BCG/PPD-specific $CD4^+$ cytotoxic T cells that lyse autologous monocytes. The implications of these findings with respect to the role of the 65-kD HSP in autoimmunity are discussed.

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