

A RECOMBINANT 64 KILODALTON PROTEIN OF
MYCOBACTERIUM BOVIS BACILLUS CALMETTE-GUERIN
SPECIFICALLY STIMULATES HUMAN T4 CLONES
REACTIVE TO MYCOBACTERIAL ANTIGENS

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Tuberculosis and leprosy are chronic infectious diseases that presently affect more than 65 million people (1, 2). Apparently the majority of individuals exposed to or infected with pathogenic mycobacteria develops effective immunity that is exclusively based on the cellular immune response, and recent evidence suggests that T helper/inducer (T4) lymphocytes are of particular importance for protection (3–6). In previous studies whole bacteria or crude bacterial extracts have been used for stimulation of heterogeneous T cell populations. Thus, the bacterial proteins recognized by mycobacteria-reactive T lymphocytes are completely unknown. Recently, a 64 kD protein antigen of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) has been cloned in *Escherichia coli* (7), and T cell clones (TC) with reactivity to mycobacterial antigens have been established (8). It has therefore become possible to characterize defined mycobacterial antigens by their potential to stimulate distinct TC. We have used this approach to characterize the reactivity against a recombinant *M. bovis* BCG antigen (antigen A) of human TC that were derived from a leprosy patient by in vitro stimulation with *M. leprae* and from three normal PPD-reactive individuals by stimulation with BCG. This is of particular interest for vaccine development, and for the design of better-defined and more specific diagnostic skin test reagents.

Materials and Methods

Bacterial and Antigen Preparations. PPD was obtained from the Statens Serum Institute, Copenhagen, Denmark. *M. leprae* proteins (batch CD24) and irradiated *M. leprae* organisms (batch CD20) were kindly supplied by Dr. R. J. W. Rees through the World Health Organization–Immunology of Leprosy (WHO-IMMLEP) program and had been prepared according to protocol 1/79, WHO document TDR, IMMLEP-SWG (S) 80.3. *M. leprae* cell wall preparation (batch IV) had been extracted from lyophilized *M. leprae* with chloroform/methanol, and was kindly provided by Dr. P. Brennan, Colorado State Univ., Fort Collins, CO. *M. bovis* BCG was originally obtained from Dr. R. J. North, Trudeau Institute, Saranac Lake, NY; *M. intracellulare* and *M. avium* were kindly provided

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by Dr. W. Brehmer, Robert-Koch Institut, Berlin, Federal Republic of Germany; and *M. tuberculosis* was a kind gift of Dr. J. K. Seydel, Forschungsinstitut Borstel, Borstel, Federal Republic of Germany. Cultivable mycobacteria were grown in Bacto Dubos Broth (Difco Laboratories, Detroit, MI) supplemented with BSA and Tween 80 at 37°C under shaking conditions. Washed mycobacteria were heat-killed and ultrasonicated at 60 W (three times, 3 min each) using a Branson Sonifier B12 with microtip. For crude preparations of cell wall and cytoplasmic fractions, the sonicated material was centrifuged at 20,000 *g* for 20 min and separated pellets (cell wall-enriched) and supernatants (mainly cytoplasmic proteins) were used.

Detection of *M. bovis* BCG Protein by SDS-PAGE. 20 µg protein of *E. coli* lysates and 6 µg protein of *M. bovis* BCG lysate were applied to SDS-polyacrylamide gels (9) of 7.5% acrylamide. After electrophoresis (3 h at 120 V, constant voltage) the separated proteins were silver stained (NEF-982; New England Nuclear, Boston, MA).

Production of Antigen A by *E. coli* K12. For the production of antigen A, we used *E. coli* K12 host strain 1046 carrying the plasmids pCI857 plus pRIB1000. Plasmid pRIB1000 is a recombinant DNA plasmid composed of the expression plasmid pPLc236 (10) and a 4.9 kb *M. bovis* BCG DNA fragment. The latter DNA fragment encodes for the antigen A (7). The expression of antigen A is low when cells are grown at 28°C due to the presence of the plasmid pCI857-encoded lambda repressor, which prevents transcription of the lambda promoter on plasmid pRIB1000. Overproduction occurs at 42°C due to inactivation of the thermolabile repressor. *E. coli* cells were grown to exponential phase in NZYM medium (11) at 28°C and incubated overnight at 42°C. After washing with saline, the cells were broken in a French press and centrifuged for 10 min at 10,000 *g*. The supernatant was used as a crude preparation of antigen A. As a control, similar cell extracts were made from *E. coli* K12 1046, carrying only plasmid pCI857.

Generation of Human T Cell Clones. PBL of an Indian patient with tuberculoid leprosy classified on the basis of clinical and histopathological criteria described by Ridley and Jopling (12), and of three healthy PPD⁺ donors were prepared by flotation on Ficoll/metrizoate. Human T lymphocytes specific for mycobacterial proteins were stimulated in cultures of 1.5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% screened human A or AB serum, 2 mM L-glutamine, 25 mM Hepes buffer, 2×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin in the presence of 10 µg/ml of the respective soluble antigen or 10^7 cells/ml heat-killed and sonicated microorganisms. PBL bulk cultures from the leprosy patient were transferred from Bombay to Freiburg for further culturing and cloning. This patient was selected from others because of his considerable T cell reactivity in vitro and his HLA-DR phenotype (DR3), which also occurred frequently in a panel of pretested volunteers in Freiburg who provided APC for maintaining antigen-specific T cells. After culture for 7 d in 24-well flat-bottomed microtiter trays (Nunc, Roskilde, Denmark), IL-2 was added (15 U/ml, Biotest, Frankfurt, Federal Republic of Germany) for 4 d. Subsequently, viable cells were separated on Ficoll/metrizoate and plated at one cell per microculture well of 96-well round-bottom trays (Nunc) in medium containing 60 U/ml IL-2 together with 5×10^4 autologous irradiated (4,000 rad) PBL, or plated at five cells/well with IL-2 (60 U/ml) and HLA-D-matched irradiated (4,000 rad) PBL as APC. Both cultures also contained 10 µg/ml mycobacterial protein in a total volume of 100 µl. T cells from PPD-reactive donors were immediately cloned after the initial stimulation. After 10–14 d, growing clones could be transferred. Subclones were obtained by recloning. Every 3–4 d, fresh IL-2 was added, and irradiated pooled feeders were added every 7 d. Before use in assay systems, TC were rested 6–7 d after the last addition of feeder cells. Phenotype analysis was performed cytofluorometrically with the mAb OKT3, OKT4, OKT8, OKT8, (Ortho Diagnostics, Westwood, MA).

Proliferative Assay. 10^4 TC were incubated in 200-µl triplicate cultures together with 5×10^4 irradiated (4,000 rad) HLA-D-matched PBL as APC, plus antigen, in round-bottom microtiter wells (Nunc). Antigen concentrations are indicated in the legend to Table I. After 72 h of in vitro incubation at 37°C, 5% CO₂, humidified atmosphere, cultures were labeled for 16 h with 1 µCi of [³H]TdR per well, and harvested into glass fiber filters. [³H]TdR incorporation was measured in a liquid scintillation spectrometer.

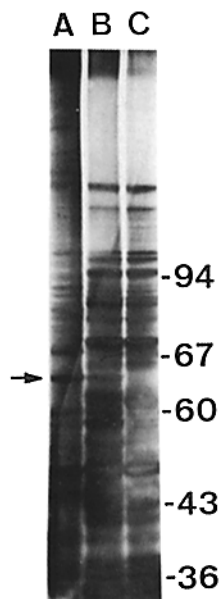


FIGURE 1. SDS-PAGE (7.5% polyacrylamide) of BCG lysate (A) compared to *E. coli* lysates with (B) or without (C) the antigen A insert. Antigen A is marked by an arrow.

Results and Discussion

A gene bank of *M. bovis* BCG has been constructed (7) in *E. coli* by cloning mycobacterium DNA fragments into a lambda cloning vehicle. Clones of this library have been screened for the production of mycobacterial antigens by using a polyclonal anti-*M. bovis* BCG antiserum. ~50% of the antigen-positive clones produced one particular mycobacterial protein antigen, designated antigen A. This protein has an M_r of 64 kD on SDS-PAGE, and a variety of mycobacterial species as well as PPD preparations contain (7) proteins crossreacting with antigen A. In Fig. 1, at 64 kD, a prominent band can be seen in lysates of both BCG and *E. coli* expressing antigen A. Densitometric determinations showed that antigen A is ~2% of total protein in these crude extracts.

We have tested the ability of antigen A to stimulate human T4 clones using this crude lysate. 105 TC from a leprosy patient in the tuberculoid phase of the disease have been established initially by stimulation with a mixture of *M. leprae* proteins and PPD (8). 11 T4 clones specific for either PPD (2 of 11), *M. leprae* protein (5 of 11), or crossreactive to both preparations (4 of 11) were selected because of their considerable growth after specific stimulation. All of them had the phenotype T3⁺, T8⁻, T4⁺. The four crossreactive TC could also be stimulated by BCG. In Table I, proliferative responses of five representative TC are shown. 2G2 and 2D1, which expressed selective reactivity towards either *M. leprae* protein or PPD, could not be restimulated by antigen A. However, two of the three crossreactive TC (shown in Table I) that reacted with both PPD and *M. leprae* antigen were restimulated by antigen A. The response of clones 1H11 and 4F1 to 10 μ g/ml PPD, 10 μ g/ml *M. leprae* protein, or 20 μ g/ml of the *E. coli* lysate (containing ~0.4 μ g/ml protein A), respectively, was comparable, suggesting that the antigen recognized by these TC was identical in the different

TABLE I
Stimulation of Human T4 Clones with Reactivity to Mycobacterial Proteins by Antigen A

Antigen	[³ H]TdR uptake by T cell clones [mean cpm (±SD)]				
	2C11	1H11	4F1	2G2	2D1
Control	254 (162)	60 (11)	48 (2)	129 (31)	64 (2)
OVA	198 (36)	73 (19)	64 (16)	83 (26)	77 (5)
PPD	35,272 (188)	7,959 (18)	1,360 (13)	85 (13)	11,086 (195)
<i>M. leprae</i> protein	84,500 (9,625)	5,949 (443)	1,024 (43)	13,760 (93)	91 (10)
K12	141 (57)	74 (12)	70 (22)	42 (13)	65 (30)
K12 + antigen A	146 (70)	4,489 (642)	1,496 (27)	58 (26)	104 (31)

10⁴ T cells from TC were stimulated with 10 µg/ml PPD, 10 µg/ml of a protein preparation from *M. leprae*, 20 µg/ml of *E. coli* protein (*E. coli* K12 lysate), and antigen A produced by *E. coli* K12 (K12 + antigen A) together with 5 × 10⁴ irradiated (4,000 rad) HLA-D-matched PBL. No stimulation was observed by the APC alone, as can be seen from the comparison of TC alone (control) and TC cultured with PBL together with an irrelevant protein (OVA). Stimulation was assessed in triplicates by the incorporation of [³H]TdR in a 72-h assay; SD in parentheses.

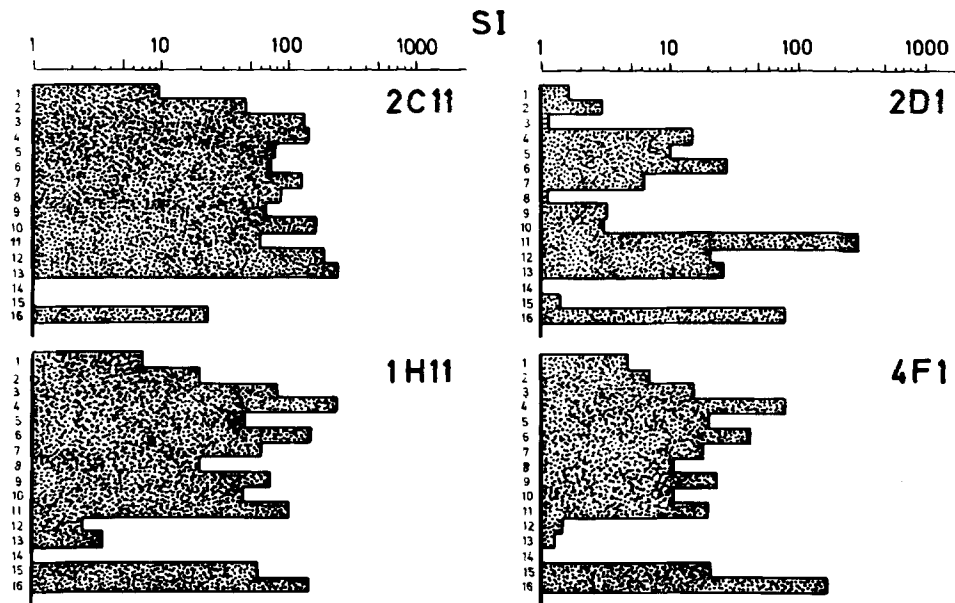


FIGURE 2. Stimulation pattern of four TC from a leprosy patient using a panel of mycobacterial antigen preparations. Four independent clones with reactivity to mycobacterial antigens were cultured with APC and different antigen preparations. Protein concentrations or numbers of mycobacterial organisms per culture are: (1) *M. leprae*, 10⁶; (2) sonicated *M. leprae*, 10⁶; (3) *M. leprae* protein, 10 µg/ml; (4) *M. leprae* cell wall fraction, 10 µg/ml; (5) sonicated BCG, 10⁶; (6) BCG cell wall fraction, 10 µg/ml; (7) BCG cytoplasm fraction, 10 µg/ml; (8) sonicated *M. tuberculosis*, 10⁶; (9) *M. tuberculosis* cell wall fraction, 10 µg/ml; (10) *M. tuberculosis* cytoplasm fraction, 10 µg/ml; (11) PPD, 10 µg/ml; (12) sonicated *M. intracellulare*, 10⁶; (13) sonicated *M. avium*, 10⁶; (14) *E. coli* K12, 20 µg/ml; (15) *E. coli* K12 expressing antigen A, 20 µg/ml; (16) IL-2, 8 U/ml. Stimulation was assessed by [³H]TdR incorporation, and stimulation indices were calculated as test cpm divided by control cpm (see Table I; cpm values with OVA). Note that stimulation indices are shown in logarithmic scale.

antigen preparations. The control *E. coli* extract had no mitogenic effect for these TC (Table I). TC were not stimulated by antigen alone, indicating dependence on presentation by APC (data not shown). In Fig. 2, proliferation profiles

of TC 1H11, 4F1, 2C11, and 2D1 in response to a variety of mycobacterial antigens are shown in a logarithmic scale. Clone 2C11 expressed a broad reactivity pattern to several mycobacterial antigens including *M. avium* and *M. intracellulare*, whereas clone 2D1 showed a distinct and more restricted pattern. A third type of stimulation profile was shared by TC 1H11 and 4F1, both reactive to antigen A. These similarities in their proliferation profiles indicate that the same antigen was recognized by TC 1H11 and 4F1.

Panels of BCG-reactive TC were established from three healthy PPD⁺ donors to estimate the proportion of antigen A-reactive T cells among them. The initial stimulation with BCG was followed immediately by cloning under limiting-dilution conditions to minimize in vitro selection. The frequency of antigen A-reactive TC varied. Out of those TC that could be restimulated significantly by BCG, in three distinct donors, 45 of 66, 3 of 12, and none of 8, respectively, were reactive with antigen A, suggesting that this antigen for some individuals may be an important T cell antigen.

Recently, a recombinant protein of *M. bovis* BCG has been produced (7), and this offered the possibility of defining T cell antigens by means of mycobacteria-reactive TC (8). It was encouraging to find that whole *E. coli* lysates containing the relevant antigen did not exert suppressive effects for specific stimulation of TC (see Table I), thus facilitating screening assays with transfected proteins without need for prior purification.

The individual stimulation patterns of TC 2H11 and 4F1 in the presence of a variety of crude mycobacterial extracts were similar, indicating that these TC recognized the same antigen. This approach may be useful for defining major T cell antigens by comparing stimulation profiles of a large number of TC established under minimal in vitro selection pressure, with undefined antigen extracts. TC with the most frequent stimulation pattern should recognize a major T cell antigen, and representative TC could be used for identification of the homologous antigen in different preparations during the course of purification.

Although it seems suggestive, the observation that many T cells recognize the same antigen does not necessarily imply a protective role for this antigen. Therefore, additional criteria are needed. Acquired resistance against mycobacteria depends on specific T lymphocytes (1, 2), and present evidence suggests that these are T4⁺, T8⁻ cells that produce IFN- γ and/or macrophage activating factor (3-6). Therefore, identification of protective antigens might be possible with TC that meet these requirements. Our antigen A-reactive TC, 2H11 and 4F1, express the T4⁺, T8⁻ phenotype, and after stimulation with antigen secrete IFN- γ (8), and hence may exert antibacterial activity. Besides this deductive characterization, at present there is no other way for defining T cell antigens as protective at the preclinical level. Fortunately, a considerable number of BCG-reactive TC from healthy individuals and from a leprosy patient could be stimulated. However, further investigations on frequencies of antigen A-reactive TC in patients with different mycobacterial diseases are needed to estimate the role and the potential use of antigen A as T cell antigen in these diseases.

Summary

A recombinant 64 kD protein of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) (antigen A), which amounted to ~2% of an *E. coli* lysate, was tested for

its capacity to stimulate human T4 clones reactive to mycobacterial proteins. Two out of four crossreactive clones, established from a patient with tuberculoid leprosy, which could be stimulated by protein preparations of *M. leprae* and *M. tuberculosis*, and by particulate *M. bovis* BCG were also reactive to antigen A without further enrichment from *E. coli* lysate. In addition, BCG-reactive T cell clones from two of three healthy PPD⁺ donors reacted with antigen A. This finding shows that human T cell clones may be useful for probing gene-cloned proteins of potential value for vaccination against diseases where protection is mediated exclusively by T cells.

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