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Characteristics of human T-cell clones from BCG and killed *M. leprae* vaccinated subjects and tuberculosis patients

Recognition of recombinant mycobacterial antigens

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Summary
A total of 121 human T-cell clones from 9 BCG vaccinated healthy subjects, 42 T-cell clones from 6 normal volunteers vaccinated with killed *M. leprae* and 52 T-cell clones from 4 tuberculosis patients were raised from peripheral blood mononuclear cells (PBMC). Irrespective of the group of subjects, all the proliferating clones were CD4+ CD8-. Some clones were specific to the antigens against which they were raised and others were limited to broadly crossreactive in proliferative assays. Antigen specificity of 3 *M. leprae* specific T-cell clones tested, was also manitained in lymphokine and cytotoxicity assays. Responses of the clones to BCG and *M. tuberculosis* H37Rv parallelled closely. Although, 68 % and 63 % of *M. leprae* raised T-cell clones responded to BCG and *M. tuberculosis,* respectively, only 9 % BCG raised T-cell clones and 23 % *M. tuberculosis* raised T-cell clones responded to *M. leprae.* Twenty four T-cell clones from BCG vaccinated subjects, 22 T-cell clones from *M. leprae* vaccinated volunteers and all the 52 T-cell clones from tuberculosis patients were tested for their reactivity to recombinant antigens of *M. leprae* and *M. tuberculosis* identified by monoclonal antibodies. Five specific clones from 2 *M. leprae* vaccinated subjects recognized an epitope on the *M. leprae* 18K protein, and one nonspecific clone from a third individual reacted to both the *M. leprae* and *M. tuberculosis* 65K proteins. From tuberculosis patients, one specific clone responded to the *M. tuberculosis* 65K protein while another limited cross-reactive clone proliferated to the *M. tuberculosis* 19K protein. None of the 24 BCG induced T-cell clones responded to the recombinant antigens.

Introduction
Leprosy and tuberculosis are the main mycobacterial infections affecting human being. In spite of the effective chemotherapy available, these diseases are among the major health problems in the developing world. The situation has been complicated because of nonavailability of efficient and specific diagnostic tests and effective vaccines. Lepromin and tuberculin skin tests have long been used but are only of prognostic value as these reagents are not specific for *M. leprae* and *M. tuberculosis.* Similarily, BCG vaccines against both diseases have been tried, but with variable effects (1-5). Whole bacilli are antigenically quite complex

and may have suppressor antigens/epitopes activating suppressor T cells (6, 7) which could suppress the function of helper/effector T cells (7, 8). It is, therefore, important to identify antigens/epitopes of *M. leprae* and *M. tuberculosis* recognized by helper T cells as a first step towards the development of new vaccines and specific diagnostic reagents. The recent developments in cloning of mycobacterial genomic DNA in foreign hosts and the isolation of recombinants expressing major mycobacterial protein antigens defined by monoclonal antibodies (9, 10) have made the above objective feasible. Here, we report the identification of recombinant *M. leprae* and *M. tuberculosis* antigens recognized by human CD4+ T-cell clones.

Materials and methods

MYCOBACTERIAL ANTIGEN INDUCED HUMAN T-CELL CLONES

One hundred and twenty one BCG induced T-cell clones from 9 BCG vaccinated healthy subjects (11), 42 *M. leprae* induced T-cell clones from 6 volunteers vaccinated with killed *M. leprae* (12) and 52 *M. tuberculosis* induced T-cell clones from 4 tuberculosis patients (13) were raised from peripheral blood mononuclear cells (PBMC). These clones were grown and maintained *in vitro* by weekly feeding of specific antigen and autologous feeder cells + 100 U recombinant interleukin-2 (IL-2) added twice a week.

MYCOBACTERIAL ANTIGENS

M. leprae was kindly supplied by Dr. R.J.W. Rees, Mill Hill, London, from the IMMLEP (WHO) bank. BCG and PPD were obtained from Statens Serum Institute, Copenhagen, Denmark. *M. tuberculosis* H37Rv and other mycobacteria were supplied by Dr. O. Closs, National Institute of Health, Oslo, Norway. The bacilli were killed by irradiation before use.

M. LEPRAE AND *M. TUBERCULOSIS* ANTIGENS EXPRESSED IN *E. COLI*

Crude *E. coli* lysates containing *M. leprae* and *M. tuberculosis* antigens recognized by monoclonal antibodies were prepared by inducing λgt11 recombinant lysogens of the strain Y1089 according to the method described earlier (12). Viscosity of the crude lysates was reduced by brief sonication (3 x 10 s) and protein content was determined by measurement of OD 280. Lysates were sterilized by millipore filtration and stored frozen at –70°C in aliquots.

ANTIGEN INDUCED PROLIFERATION OF T-CELL CLONES

1 x 10^4 cloned T cells were added to the wells of 96 well flat bottom plates with adherent cells from 1 x 10^5 irradiated autologous PBMC. Antigens were added in triplicate. The plates were incubated at 37°C in a humidified atmosphere of 5 % CO2 and 95 % air. 0.045 MBq ^{3}H-thymidine was added to the cultures during the last 4 h of a 72 h incubation period. Cultures were harvested and radioactivity incorporated was determined by standard methods (14). Median CPM values from triplicates have been used to express the results. A clone was considered responder to a given antigen when △ CPM was ⩾ 1000 and T / C > 2, where

$$\triangle \text{ CPM} = \text{(CPM in cultures with T-cell clone + antigen + APC) - (CPM in cultures with T-cell clone + APC)}$$

$$\text{and, T / C} = \frac{\text{(CPM in cultures with T-cell clone + antigen + APC)}}{\text{(CPM in cultures with T-cell clone + APC)}}$$

INTERLEUKIN-2 AND GAMMA INTERFERON PRODUCTION AND ASSAY

1 x 10^5 cloned T cells were added into the wells of 24 well costar plates with adherent cells from 1 x 10^6 irradiated PBMC and either *M. leprae* (5 x 10^7 bacilli/ml) or BCG (20 μg/ml, wet weight) in 1 ml RPMI-1640 + 15 % AB serum + 1 % Penicillin- Streptomycin. The plates were incubated in a humidified atmosphere of 5 % CO2 and 95 % air at 37°C. Cell free supernatants were collected after 18 h for IL-2 assay and after 48 h for interferon assay by methods described earlier (11). The bulk of interferon activity in the supernatants from T-cell clones + antigen + adherent cells, was eliminated by treatment at pH-2 and antigamma interferon antibodies, indicating that it was due to gamma interferon (INF-γ).

CYTOTOXICITY ASSAY

Adherent cells were obtained from 1 x 10^6 PBMC in 24 well costar plates. BCG at 20 μg/ml and *M. leprae* at 5 x 10^7 bacilli/ml were added into each well together with 2 x 10^5 cloned cells. Control wells did not have T-cell clones. The plates were incubated at 37°C for 6 days. Nonadherent cells were removed and adherent cells were further incubated at 37°C for 30 min in 0.01 % solution of neutral red in saline + 10 % FBS. The wells were washed 3 times with saline and neutral red taken up by viable macrophages was released and percentage cytotoxicity was calculated according to the method of Parish and Mullbacher (15).

Results

GENERAL CHARACTERISTICS OF T-CELL CLONES

All the T-cell clones raised and maintained from the three groups of people were CD4+ and CD8-, except one clone from a BCG vaccinated subject which was CD8+ CD4-. Testing with a battery of mycobacterial antigens revealed that clones from all three groups recognized epitopes of varying specificity (Table 1). Among 118 clones tested, 31 were specific to the antigens against which they were raised, and others were limited to broadly crossreactive in proliferative assays (Table 1).

Antigen specificity of 3 *M. leprae* raised T-cell clones tested was also maintained in other test systems. For example, only *M. leprae* antigens but not PPD could induce IL-2 and IFN-γ production from these clones (Table 2). Similarly the clones exhibited cytotoxic activity against antigen presenting cells in the presence of *M. leprae,* whereas cytotoxicity was not observed in the presence of BCG (Table 2).

Responses of the T-cell clones from all 3 groups of subjects to BCG and *M. tuberculosis* parallelled closely. Although, the majority of *M. leprae* raised T-cell clones from all individuals responded to BCG (68 %) and to *M. tuberculosis* (63 %), only few clones (9 %) from 3 of 7 BCG vaccinated subjects and 23 % clones from 2 of the 3 tuberculosis patients responded to *M. leprae* (Table 3).

IDENTIFICATION OF RECOMBINANT MYCOBACTERIAL ANTIGENS BY T-CELL CLONES

A total of ninety eight T-cell clones (24 T-cell clones from BCG vaccinated subjects, 22 T-cell clones from killed *M. leprae* vaccinated volunteers and 52 T-cell clones from tuberculosis patients) were tested for their reactivity to the major protein antigens of *M. leprae* and *M. tuberculosis* expressed from recombinant DNA and identified by monoclonal antibodies. Five specific T cell clones from 2 *M. leprae* vaccinated subjects responded to the *E. coli* lysate containing the 18K protein of *M. leprae* (12). One crossreactive T-cell clone from another *M. leprae* vaccinated subject responded to the *M. leprae* as well as to the *M. tuberculosis* 65K proteins (Table 4). From tuberculosis patients, one specific T-cell clone proli-

Table 1. Antigen specificity and crossreactivity of T-cell clones from different donor groups.

Antigens	Proliferation of T-cell clones (CPM X 10^{-3})								
	Antigen specific			Limited crossreactive			Broad crossreactive		
	BK-13	TK-6	LK-5	BK-115	TK-3	LK-3	BK-2	TK-8	LK-4
Control	0.7	1.1	0.1	0.1	1.1	0.3	4.4	0.5	0.3
BCG	*44.0*	*5.0*	0.2	*18.9*	*7.4*	0.8	*46.5*	*21.1*	*4.2*
M. tuberculosis /PPD	*49.5*	*32.9*	0.2	*13.4*	*8.1*	1.0	*35.4*	*34.6*	*21.5*
M. africanum	*30.4*	*11.0*	NT	*6.7*	*10.1*	0.6	*47.3*	*27.8*	*4.4*
M. leprae	0.2	1.0	*22.0*	0.2	*7.2*	*4.5*	*19.2*	*16.6*	*1.8*
M. avium	0.8	1.2	0.3	*19.0*	1.2	*11.7*	*36.2*	*34.7*	*6.0*
M. smegmatis	0.1	1.9	NT	0.7	1.2	0.2	*37.8*	*36.6*	*11.3*
M. asiaticum	0.5	1.6	NT	0.1	0.2	0.1	*50.4*	*12.1*	*3.0*
M. xenopi	0.1	1.4	0.3	0.5	0.6	*1.6*	*33.9*	0.9	0.6
M. phlei	0.2	0.5	NT	0.2	0.5	0.1	*27.0*	*28.3*	*1.5*
M. vaccae	0.1	1.1	0.4	0.2	0.4	0.1	*28.7*	*38.2*	*2.9*
M. gordonae	0.1	1.0	NT	0.1	*9.5*	0.1	*47.2*	*33.6*	1.2
M. fortuitum	0.2	0.4	NT	0.4	*13.2*	0.1	*40.0*	*34.6*	*14.5*
M. kansasii	NT	1.9	0.1	NT	0.2	0.1	Nt	*15.2*	0.3

The clones with initials BK, TK and LK were raised from BCG vaccinated subjects, tuberculosis patient and *M. leprae* vaccinated subjects, respectively.
The values showing significant proliferation ($\triangle$CPM $\geqslant$ 1000 and T/C c 2) are in italics.

Table 2. Multiple lymphokine production and macrophage killing by M. leprae *specific T-cell clones.*

T-cell clones	Antigens	Interleukin-2 activity U/ml	Interferon activity T / C*	Proliferation CPM X 10^{-3}	Cytotoxicity (%)
2/4F	*M. leprae*	2.0	*2.8*	*5.3*	100
	PPD/BCG	0.0	1.4	0.2	0.0
1E7	*M. leprae*	2.5	*3.9*	*3.6*	100
	PPD/BCG	0.0	1.8	0.2	14
6C8	*M. leprae*	3.0	*3.1*	*5.5*	NT
	PPD/BCG	0.0	1.2	0.1	NT

$$* \text{ T / C} = \frac{\text{Interferon activity in culures with T-cell clones + antigen + antigen presenting cells}}{\text{Interferon activity in cultures with antigen + antigen presenting cells}}$$

The values showing signicant interferon activity (T/C $>$ 2) and proliferation are in italics.

Table 3. Comparison of the response of T-cell clones from different groups to the antigens of BCG, M. tuberculosis *and* M. leprae.

T-cell clones raised from	Responsiveness to antigens		
	BCG	*M. tuberculosis*/ PPD	*M. leprae*
		Responder / tested (% positive)	
BCG vaccinated subjects	44 / 44 (100 %)	42 / 44 (95 %)	4 /44 (9 %)
M. leprae vaccinated subjects	21 / 31 (68 %)	21 / 33 (63 %)	41 / 41 (100 %)
Tuberculosis patients	22 / 22 (100 %)	22 / 22 (100 %)	5 / 22 (23 %)

ferated to the *M. tuberculosis* 65K protein and another limited crossreactive T-cell clone recognized an epitope on the 19K protein of *M. tuberculosis.* However, these clones did not respond to recombinant *M. leprae* proteins of corresponding molecular weights (13).

Table 4. Proliferation of a crossreactive T-cell clone from a killed M. leprae *vaccinated subject to the* E. coli *lysates containing either* M. leprae *or* M. tuberculosis *65K protein.*

Antigens	Proliferation	
	CPM X 10^{-3}	T /C
Control	1.2	1.0
M. leprae	34.8	29.0
BCG	28.1	23.4
Lysate containing *M. leprae* 65K protein	18.3	15.2
Lysate containing *M. tuberculosis* 65K protein	35.3	29.4
Control lysate	0.9	0.7

The majority of T-cell clones from killed *M. leprae* vaccinated subjects and from tuberculosis patients and all of the 24 clones from BCG vaccinated subjects did not respond to the *M. leprae* or M. tuberculosis recombinant proteins identified by monoclonal antibodies (Table 5).

Table 5. Numbers of T-cell clones from different groups of subjects responding to recombinant mycobacterial antigens.

T-cell clones from	Response to recombinant antigens Responder / Tested
M. leprae vaccinated subjects	6 / 22
BCG vaccinated subjects	0 / 24
Tuberculosis patients	2 / 52
Total	8 / 98

Discussion

In this and earlier reports we have described the characteristics of human T-cell clones from BCG vaccinated subjects (11), *M. leprae* vaccinated volunteers (12) and tuberculosis patients (13). The T-cell clones from all three groups had identical requirements of antigen and antigen presenting cells for their continuous growth *in vitro*. They were CD4+ CD8- and recognized epitopes of varied specificity ranging from highly specific to broadly crossreactive. Almost all of the BCG raised T cell clones responded to *M. tuberculosis* H37Rv and vice versa. The majority of T-cell clones raised against *M. leprae* also responded to BCG and *M. tuberculosis* H37Rv, but only few clones raised against BCG and *M. tuberculosis* H37Rv responded to *M. leprae*. Could this be explained on genetic basis? In this context van Eden et al. (16) have suggested a role of HLA-DR3 in protecting against skin test nonresponsiveness to the mycobacterial antigens in healthy British individuals and Ottenhoff et al. (17) have shown HLA-DR4 association with high responsiveness to *M. tuberculosis* specific antigens among spanish leprosy patients.

Predominance of mycobacterial antigen reactive CD4+ T cell clones from the PBMC of either normal subjects or diseased people have been reported by other investigators as well (18-28). This could be due to the intrinsic property of mycobacterial antigens. Severity of the disease might also be a reason. The T-cell clones from tuberculoid leprosy patients were exclusively CD4+ (19, 20), whereas T-cell clones from a borderline lepromatous leprosy patient were both CD4+ and CD8+ (7), and the 2 suppressor clones raised from lepromatous leprosy skin lesions were CD8+ (8). Alternatively, CD8+ cells might have special requirements for the long term propagation *in vitro* (21).

The mycobacterial antigen induced T-cell clones were also producing multiple biologically important lymphokines like IL-2, IFN-γ and granulocyte-macrophage colony stimulating factor (11 and table 3). These lymphokines may have a direct or indirect role in protection against infections by recruiting and activating macrophages, which are the hosts for intracellular parasites like mycobacteria. However, the same T-cell clones were also cytotoxic for such macrophages in the presence of specific mycobacteria (22 and Table 4). It is possible that such cytotoxic mechanisms may help the destruction of invading organisms by killing those macrophages which have become incompetent to kill the mycobacteria. Such macrophages will otherwise become a fertile soil for bacterial growth and thus may provide an escape mechanism for the infection. However, the immune system may possibly foil such attempts by killing these macrophages via specific cytotoxic cells.

Our studies and a report from others (23) have demonstrated the potential use of T-cell clones in identifying recombinant mycobacterial antigens from crude *E. coli* lysates which is not possible using peripheral blood lymphocytes due to toxicity of the crude lysates. This toxic effect is less severe on the reactivity of T-cell clones because of shorter incubation per-

iod to assess their reactivity as compared to peripheral blood lymphocytes. Using this system we have identified T-cell clones exclusively reacting either with an 18K protein of *M. leprae* or with a 19K protein of *M. tuberculosis*. These two proteins of comparable molecular weight are specific to the respective mycobacteria as their DNA do not hybridize (RA Young, personal communication). One specific clone from a tuberculosis patient responded to the 65K protein of *M. tuberculosis* but not to the 65K protein of *M. leprae*. DNA encoding these two proteins have been shown to hybridize and their amino acid sequences are quite similar except for a small region (RA Young, personal communication). However, the epitope seen by the T-cell clone in our system seems to be specific for the *M. tuberculosis* complex, as this clone did not respond to other mycobacteria tested. Crossreactive epitopes are also present on the 65K protein of *M. tuberculosis* as suggested from our work (Table 4) and the work from other laboratories (23). Studies are in progress to determine the nature of the epitopes seen by the T-cell clones so that corresponding peptides can be synthesized and their clinical relevance, if any, evaluated.

The mouse monoclonal antibodies raised against *M. leprae* and *M. tuberculosis* in different laboratories react to a limited number of proteins when tested either against the lysates of these mycobacteria (24) or the recombinant proteins made in foreign hosts (10,24). However, the majority of human T- cell clones did not respond to these recombinant proteins. This could either be due to the reason that epitopes seen by T cells reside on proteins not recognized by antibodies, or that the repertoire of mouse B cells with respect to response to the mycobacterial proteins is quite limited. Alternatively, our T-cell clones may recognize epitopes/ antigens nonprotein in nature.

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