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Human T-Cell Responses to Secreted Antigen Fractions of *Mycobacterium tuberculosis*

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The T-cell response of human donors to secreted antigen fractions of *Mycobacterium tuberculosis* was investigated. The donors were divided into five groups: active pulmonary tuberculosis (TB) patients with minimal and with advanced disease, *Mycobacterium bovis* BCG-vaccinated donors with and without contact with TB patients, and nonvaccinated individuals. We found that patients with active minimal TB responded powerfully to secreted antigens contained in a short-term culture filtrate. The response to secreted antigens was mediated by $CD4^+$ Th-1-like lymphocytes, and the gamma interferon release by these cells was markedly higher in patients with active minimal TB than in healthy BCG-vaccinated donors. Patients with active advanced disease exhibited depressed responses to all preparations tested. The specificity of the response to secreted antigens was investigated by stimulating lymphocytes with narrow-molecular-mass fractions of short-term culture filtrate obtained by the multielution technique. Considerable heterogeneity was found within the donor groups. Patients with active minimal TB recognized multiple secreted targets, but interestingly, six of eight patients demonstrated a predominant recognition of a low-mass (<10-kDa) protein fraction which induced high levels of gamma interferon release in vitro. Only a few of 12 previously characterized secreted antigens were recognized by T cells isolated from TB patients, suggesting the existence of a number of as yet undefined antigenic targets among secreted antigens.

Tuberculosis (TB) caused by the intracellular bacterium *Mycobacterium tuberculosis* remains a major worldwide health problem responsible for approximately 3 million deaths annually (41). An improved vaccine against the disease is needed and is the subject of ongoing efforts in a number of laboratories. T cells are known to play a crucial role in acquired resistance to mycobacterial infection (23, 30), and much work has accordingly been focused on the identification of mycobacterial antigens recognized by human T cells.

Studies in animal models have demonstrated that only live dividing mycobacteria efficiently induce protective immunity (31). This fundamental finding has been the basis for the hypothesis that proteins secreted from the multiplying and metabolizing bacilli at the early stage of infection are responsible for the recognition of infected host cells by protective T cells (6, 32). Protein secretion from *M. tuberculosis* has recently been the focus of studies both in this laboratory (5) and in others (2, 13). A defined short-term culture filtrate (ST-CF) enriched in secreted proteins has thus been demonstrated to hold the major targets for T cells generated in mice both at the height of infection (6) and in a state of memory immunity (8). Recently, experimental vaccines based on culture filtrate proteins have been demonstrated to induce high levels of protective immunity both in a guinea pig model and in a mouse model of TB (3, 34). These findings have strongly supported the hypothesis and led to the present study, undertaken to screen the T-cell recognition of secreted antigens by human donors.

Different strategies and methods have been so far used to identify T-cell antigens. Monoclonal antibodies (MAbs) have been widely used as tools to select mycobacterial proteins for purification or to isolate genes for cloning and recombinant production (15, 20, 26). This approach has an inherent bias, as antigens are selected by their antibody-inducing properties, and attempts have therefore been made to find alternative approaches to the identification of T-cell antigens in complex protein mixtures. One such approach has been the T-cell Western blot (immunoblot) method, in which proteins separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) are blotted onto nitrocellulose membranes, which are cut into molecular weight fractions, converted into particles, and added to cell cultures (1). Another method reported has been to isolate proteins separated by two-dimensional electrophoresis and use the protein solutes directly in cell culture (18). We have screened human T-cell responses to secreted proteins by a newly developed method in which complex protein mixtures separated by SDS-PAGE are electroeluted into fractions each containing a few protein bands in a physiological buffer (7). This technique provides the important advantage over the other screening techniques that isolated protein fractions can be analyzed and quantified, which makes comparison of the stimulatory properties possible. Our study demonstrate that the first phase of pulmonary TB in humans is characterized by the generation of gamma interferon (IFN-γ)-producing $CD4^+$ T cells recognizing secreted antigens. The results obtained suggest the existence of several as yet undefined key antigenic targets among culture filtrate proteins and suggest the existence of a promiscuous epitope in a low-mass (<10-kDa) secreted protein fraction.

MATERIALS AND METHODS

Donors. Seventeen TB patients and 40 healthy donors divided into *Mycobacterium bovis* BCG-vaccinated and nonvaccinated donors were tested in this study.

(i) TB patients. Patients were hospitalized at the Department of Pulmonary Medicine at Bispebjerg Hospital, Copenhagen, Denmark. A chest roentgenogram was obtained from each patient at the time of study, and the patients were graded for extent of TB according to National Tuberculosis and Respiratory Association criteria (29) and classified as either patients with active minimal TB,

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including patients with minimal lesions with slight to moderate density but without demonstrable cavitation, or patients with active advanced TB, including patients with marked cavitary lesions in the lungs. Active pulmonary TB was confirmed by culture of *M. tuberculosis* in sputum (or gastric secretion) of the lung. Both groups of patients were investigated at the onset of antibiotic treatment.

(ii) BCG-vaccinated donors. BCG-vaccinated donors were subdivided into donors with and without contact with TB patients (TB contacts and noncontacts). The TB contacts were staff members at the Department of Pulmonary Medicine at Bispebjerg Hospital.

(iii) Non-BCG-vaccinated donors. The group consisted of 11 donors who had not been BCG vaccinated and had no known contact with TB patients.

Mycobacterial antigens. (i) ST-CF. ST-CF was produced as described previously (5). In brief, *M. tuberculosis* bacteria (8×10^6 CFU/ml) were grown in modified Sauton medium without Tween 80 on an orbital shaker for 7 days. The culture supernatants were sterile filtered and concentrated on an Amicon YM3 membrane (Amicon, Danvers, Mass.).

(ii) *M. tuberculosis*. *M. tuberculosis* (H37Rv) was killed by incubation overnight in 2% glutaraldehyde. The suspension was washed three times in phosphate-buffered saline (PBS), and a rough estimate of bacterial numbers based on the optical density of the disperse suspension was obtained.

(iii) Purified mycobacterial antigens. The 18-kDa antigen and PhoS were purified at Statens Seruminstitut, Copenhagen, Denmark. The 85-kDa antigen (Ag85), GroES and the mycobacterium protein tuberculosis (MPT) antigen preparations tested were a kind gift of S. Nagai, Toyonaka, Japan. The recombinant 71-kDa antigen was made available through the World Health Organization Immunology of Mycobacteriology (IMMYC) Programme antigen bank. Tuberculin purified protein was prepared at the Tuberculin Department at Statens Seruminstitut. The antigens were all tested in cell cultures and found to be nontoxic in the concentrations used.

(iv) ST-CF fractions. ST-CF was fractionated as described by Andersen and Heron (7). Briefly, ST-CF in a quantity of 5 mg of protein was separated by SDS-PAGE (10 to 20% gel) overnight (11-cm-wide center well, 0.75-mm gel). Gels preequilibrated in elution buffer (2 mM phosphate buffer) were transferred to a Multi-Eluter (7) and electroeluted (40 V) for 20 min. The protein fractions were aspirated, adjusted to isotonia with concentrated PBS, and analyzed by separation on SDS–10 to 20% polyacrylamide gels followed by silver staining (11). The protein concentration in the fractions was estimated by the Micro BCA method (Pierce, Oud-Beijerland, The Netherlands). All fractions were stabilized with 2% human AB serum and kept frozen at −20°C until use.

Lymphocyte proliferation assays. Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood, diluted 1:1 in saline, and separated by sedimentation by Lymphoprep (Nycomed A/S, Oslo, Norway) density gradient centrifugation. The cells were collected at the interphase layer, washed twice, and cultured in flat-bottom microtiter plates (Nunc, Roskilde, Denmark) at 5×10^4 cells per well. Each well held 200 μl of RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 100 IU of penicillin per ml, 50 μg of streptomycin per ml, nonessential amino acids, 1 mM glutamine, and 10% human AB serum. On the basis of initial dose-response studies, antigens were added in the following concentrations: *M. tuberculosis* (H37Rv), 5×10^4 bacteria per well; ST-CF, 1 μg/ml; purified protein derivative, 2.5 μg/ml; ST-CF fractions, 1 to 2 μg/ml; and purified mycobacterial antigens, 2 μg/ml. Phytohemagglutinin (HA 17; Wellcome, Beckenham, United Kingdom) was used at a concentration of 1 μg/ml as a positive control for cell reactivity and viability.

All tests were carried out in triplicate. Cultures were incubated for 7 days at 37°C in an atmosphere of 5% CO_2 in humidified air. At 22 h prior to harvest, 0.25 μCi of [^{3}H]thymidine (TRA 120; Radiochemical Centre, Amersham, United Kingdom) was added to each well. The cells were harvested onto fiberglass paper, and the incorporated radioactivity was measured in a liquid scintillation counter. The proliferative responses were expressed in counts per minute.

Cell separation procedures. PBMCs were depleted of T cells by treatment with an anti-CD5 MAb (Becton Dickinson, Glostrup, Denmark) and rabbit complement (Cederlane, Hornby, Ontario, Canada). PBMCs were incubated with the MAb for 30 min on ice with gentle agitation, washed once, and resuspended in medium containing complement (1:5, complement-to-medium ratio) for 45 min at 37°C. The remaining cells were subsequently washed three times in medium. PBMCs were depleted of $CD4^+$ or $CD8^+$ T cells by treatment with Dynabeads M-450 CD4 or Dynabeads M-450 CD8, respectively (Dynal, Oslo, Norway). PBMCs and Dynabeads were washed in PBS containing 2% human AB serum before use. Cells and beads were mixed (1:10, cell-to-beads ratio) and incubated for 30 min on ice with gentle agitation. Cell depletions were performed on a magnetic particle concentrator (Dynal) for 2 min. The remaining cells were washed in medium. Cultures depleted of $CD4^+$ or $CD8^+$ T cells were supplemented with 10 U of interleukin-2 (IL-2) on day 4 of incubation. To enrich for T cells, PBMCs were passed through a nylon wool column as described previously (21). PBMCs were γ irradiated with 3,300 rad and used as antigen-presenting cells (APC).

The purity of the cell suspensions was assessed by flow cytometry. Purified cells were cultured at 2×10^4 to 3×10^4 cells per well.

Cytokine assays. The amount of cytokines present in culture supernatants was quantified by commercially available enzyme-linked immunosorbent assay kits. (IFN-γ kit from Holland Biotechnology, Leiden, The Netherlands; IL-4 kit from R & D Systems, Minneapolis, Minn.). Lymphocyte culture supernatants were harvested from parallel cultures at day 2 for the quantification of IL-4 and at day 5 for the quantification of IFN-γ. Cytokine levels below 25 pg/ml were considered negative.

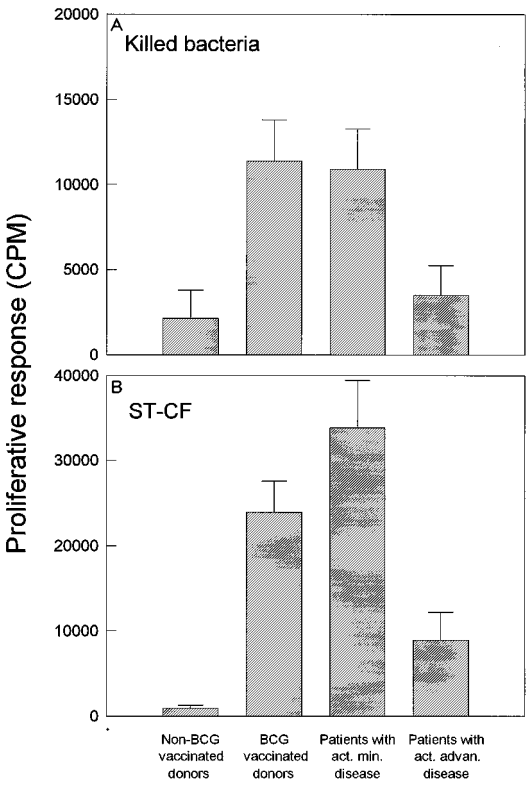

FIG. 1. Cellular reactivity to mycobacterial antigens. Proliferative responses of PBMCs isolated from non-BCG-vaccinated donors ($n = 11$), BCG-vaccinated donors (including BCG-vaccinated donors both with and without contact with TB patients; $n = 29$), patients with active minimal TB ($n = 10$), and patients with active advanced TB ($n = 7$). PBMCs were cultured with killed *M. tuberculosis* (A) or ST-CF (B). Values shown are means ± SEM. The proliferative response in unstimulated cultures was 261 ± 39 cpm.

Statistical methods. All data are means of results from triplicate wells. Comparison between groups of individuals was done by the Mann-Whitney two-tailed test; $P < 0.05$ was considered as significant.

RESULTS

Patients with active minimal pulmonary TB are characterized by increased responsiveness to secreted antigens of *M. tuberculosis*. The T-cell responses of different groups of human donors to two major antigen preparations were tested. ST-CF represents a mixture of secreted antigens, whereas killed *M. tuberculosis* is highly enriched in antigens of somatic origin. These two preparations were chosen to cover the whole antigen repertoire of *M. tuberculosis*, and the preparations were used to stimulate PBMCs from TB patients and from BCG- and non-BCG-vaccinated healthy donors in vitro.

TB patients were divided according to clinical status (see Materials and Methods for details) into patients with active minimal TB and patients with active advanced TB. Both BCG-vaccinated donors and patients with active minimal TB demonstrated a powerful proliferative response to the mycobacterial antigens, whereas non-BCG-vaccinated donors gave exceedingly low responses (Fig. 1). Comparing the responses of BCG-vaccinated donors and patients, we found patients with active minimal TB to be characterized by somewhat in-

TABLE 1. Characterization of the proliferative response of PBMCs to ST-CF

Expt	Proliferative response[a] (mean cpm [10^3]; $n = 3$)				
	PBMCs	PBMCs depleted of T cells[b]	T cells + APC	T cells[c]	APC
1	27.08	0.34	28.41	3.71	0.13
2	16.09	0.23	14.36	1.51	0.05

[a] Values for nonstimulated cell cultures never exceeded 0.21. The pooled SEM for the experiments is 19% of the mean.
[b] Depletion of T cells was done by complement-mediated cytotoxicity.
[c] T cells were purified by passage of PBMCs through nylon wool columns.

creased responses to ST-CF, although this difference was found to be statistically insignificant ($P = 0.14$) because of the large variation among individual donors. Killed bacteria yielded responses at the same level in the two groups. Patients with active advanced TB had a depressed response to both antigen preparations compared with the response of patients with active minimal TB.

Of importance, the response to ST-CF was associated with a pronounced release of IFN-γ in the group of patients with active minimal TB (3,283 ± 874 [standard error of the mean {SEM} cpm) compared with the group of BCG-vaccinated donors (1,381 ± 278 cpm). This difference was found highly significant ($P = 0.02$). The responses to killed bacteria, in contrast, were similar in the two groups of donors (1,154 ± 412 and 862 ± 248 cpm, respectively). Detectable levels of IL-4 were not found in any of these cultures.

The cellular response to ST-CF is mediated by specific $CD4^+$ T cells. The cellular response to ST-CF was characterized by a combination of T-cell enrichment and subset depletion. To distinguish the response from a B- or T-cell mitogenic response, the identities of the responding cell populations were established by in vitro culture with T-cell-depleted cultures and purified T cells with or without APC (Table 1). The response to ST-CF was found to be dependent on the presence of both T cells and APC and was therefore concluded to be an antigen-specific T-cell proliferative response.

To identify the subpopulation of T cells recognizing ST-CF, PBMCs were depleted of $CD4^+$ or $CD8^+$ T cells. The response to ST-CF was abrogated by depletion of the $CD4^+$ population, whereas depletion of the $CD8^+$ population had no effect (Table 2). To circumvent the depending of endogenously produced IL-2, all cultures were enriched with 10 U of IL-2 per ml.

Human donors recognize multiple secreted antigens. The specificity of the T-cell response in different donor groups was investigated by stimulating PBMCs with protein fractions obtained by the multielution technique (7). The technique was used on ST-CF and resulted in narrow fractions with a minimal overlap between neighboring fractions (Fig. 2).

TABLE 2. Definition of the T-cell subset recognizing ST-CF

Expt	Proliferative response[a] (mean cpm [10^3]; $n = 3$)		
	PBMCs	PBMCs depleted of[b]:	
		$CD8^+$ cells	$CD4^+$ cells
1	54.26	42.48	0.54
2	27.12	25.85	1.05

[a] Values for nonstimulated cell cultures never exceeded 1.12. The pooled SEM for the experiments is 8% of the mean.
[b] PBMCs were depleted of $CD4^+$ or $CD8^+$ cells by magnetic beads. The depletion had no effect on the response to phytohemagglutinin.

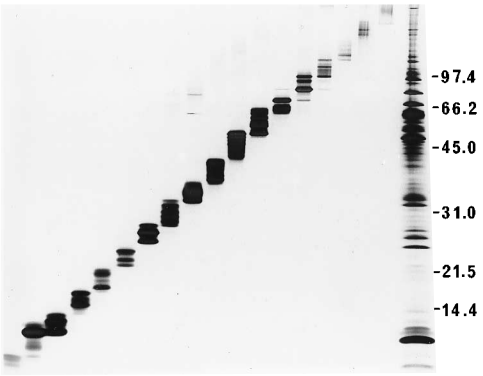

FIG. 2. Secreted protein fractions of *M. tuberculosis*. ST-CF was separated into narrow-molecular-mass fractions by the multielution technique. The fractions were analyzed by SDS-PAGE and silver staining. ST-CF is shown in last lane. The migration of molecular weight markers is shown at the right in kilodaltons.

Such a panel of fractions was used to screen the antigen recognition patterns of different human donor groups. BCG-vaccinated healthy donors were subdivided into TB contacts and noncontacts, and patients with active minimal TB were compared with patients with active advanced TB at the time of diagnosis. Non-BCG-vaccinated donors were not found to respond to the fractions, and the results have therefore been omitted.

The cellular immune responses of both patients and healthy BCG-vaccinated donor groups showed a marked heterogeneity when stimulated with ST-CF fractions, and in each case the response was directed to multiple secreted antigens (Fig. 3). All groups of donors included donors who responded markedly to ST-CF fractions and donors at a low level.

The average proliferative response to ST-CF fractions was high for the group of patients with active minimal TB, whereas the patients with active advanced TB demonstrated a markedly lower reactivity (Fig. 4A). The two groups of BCG-vaccinated donors both responded powerfully to the fractions although at a lower level than patients with active minimal TB. Significant differences in neither response level nor specificity were found in the two vaccinated donors groups.

IFN-γ release to culture supernatant does not always correlate with cellular proliferation, and we therefore evaluated T-cell responses further by screening culture supernatants for the presence of IFN-γ. Lymphocytes isolated from both BCG-vaccinated donors and patients with active advanced TB were found to produce rather low levels of IFN-γ, whereas lymphocytes isolated from patients with active minimal TB produced high amounts of IFN-γ when stimulated with ST-CF fractions. Secreted proteins contained in fraction 1, holding molecules of molecular mass below 10 kDa, were found to possess superior IFN-γ-inducing capability in patients with active minimal TB and elicited an average release of about 2,500 pg/ml. The fraction which comigrated with the Ag85 family also elicited a very potent release of IFN-γ, reaching about 2,000 pg/ml (Fig. 4B). In six of eight patients, however, the low-molecular-mass fraction 1 was the most powerful inducer of IFN-γ.

The strong T-cell reactivity to ST-CF is due to several novel stimulatory secreted antigens. ST-CF represents a mixture of previously characterized secreted antigens and several as yet undefined molecules. We therefore continued our study by investigating if the high reactivity to ST-CF could be explained by reactivity to some of the previously characterized antigens.

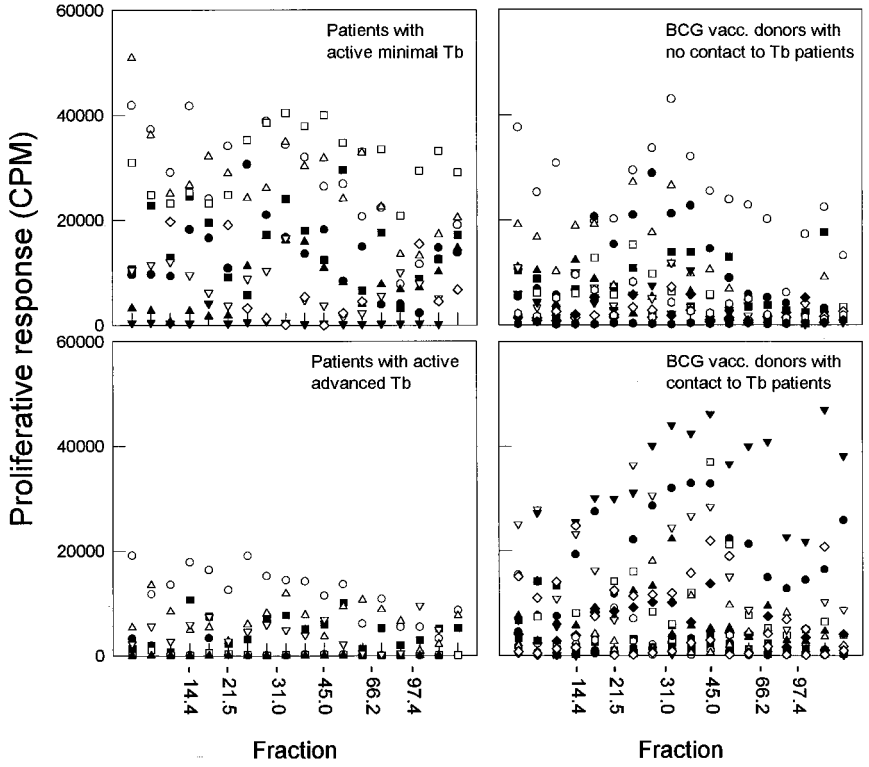

FIG. 3. Human T-cell recognition of secreted protein fractions. Lymphocytes isolated from TB patients and vaccinated donors were stimulated with ST-CF fractions in vitro, and the proliferative response was monitored. TB patients were divided into those with active minimal TB ($n = 9$) and those with active advanced TB ($n = 7$). BCG-vaccinated donors were divided into TB contacts ($n = 9$) and noncontacts ($n = 17$). Each marker type represents one individual. The migration of molecular mass markers (as shown in Fig. 2) is indicated in kilodaltons at the bottom.

Lymphocytes isolated from four TB patients were stimulated with a panel of purified antigen preparations, all previously demonstrated to be prominent culture filtrate components (2, 5, 28). The localization of these proteins in ST-CF has recently been described (3). ST-CF was found to initiate a pronounced proliferation in all donors, whereas most of the purified antigens were not recognized (Table 3). The 18-kDa antigen induced a limited cellular proliferation in both donor 1 and donor 3, whereas Ag85B and MPT32 induced limited responses in donors 1 and 3, respectively.

We considered the possibility that the broad reactivity pattern observed upon stimulation with ST-CF fractions reflected the response to a wide distribution of peptides originating from a few highly stimulatory fragmented antigens. This question was addressed by testing the reactivity of a MAb with specificity for the 70-kDa antigen, previously demonstrated to be sensitive to proteolytic cleavage (unpublished data). Western blot analyses demonstrated the 70-kDa antigen to be extensively degraded (a ladder-like reactivity pattern) in a long-term culture filtrate (7-week cultures), whereas the MAb probed a single band in ST-CF.

DISCUSSION

Studies aimed at the identification of mycobacterial molecules relevant for human immunity to TB have to rely on hypothetical correlates of protective immunity, lacking the direct demonstration of acquired resistance provided by immunization experiments in experimental animals. The T-cell responses of purified protein derivative-reactive healthy donors with contact to TB patients have in this way been suggested as a model of protective immunity against TB (19, 37). Antigens responded to by this group but not recognized by T cells from TB patients should, according to this hypothesis, be implicated in protective immunity. Opposed to this hypothesis is the marked influence of factors related to nonspecific or innate resistance to infection. In mice, resistance and susceptibility to a number of intracellular pathogens (leishmaniae, salmonellae, and mycobacteria) are controlled by a single, dominant autosomal gene named *Bcg/Lsh/Ity* (10, 38). This gene regulates macrophage activation and thereby early antimicrobial activity. Genetic linkage analysis of human populations has provided evidence for a human homolog of this gene (10), and recently this homolog was mapped to human chromosome 2q (40). In addition, the multifactorial nature of TB infection adds a number of socioeconomic causes to the list of predisposing factors. Nutrient deficiency has thus convincingly been demonstrated to be a factor of major importance for the outcome of infection in the guinea pig model of the disease (12). Such differences have no relationship to specific immunity and thereby contrast with the above-mentioned "hole in the T-cell repertoire"-based explanation of susceptibility. In the present study, we therefore applied an alternative strategy and chose to focus our search for immunologically relevant antigens on key antigenic targets recognized during the first phase of active infection and therefore presumably involved in the early recognition of the infected macrophage by host T lymphocytes.

Our study demonstrates that patients with an active minimal pulmonary TB infection respond powerfully to secreted antigens of *M. tuberculosis*. The antigens are recognized by IFN-γ-producing Th-1-like cells stimulated during the first phase of infection and therefore highly responsive in patients with a

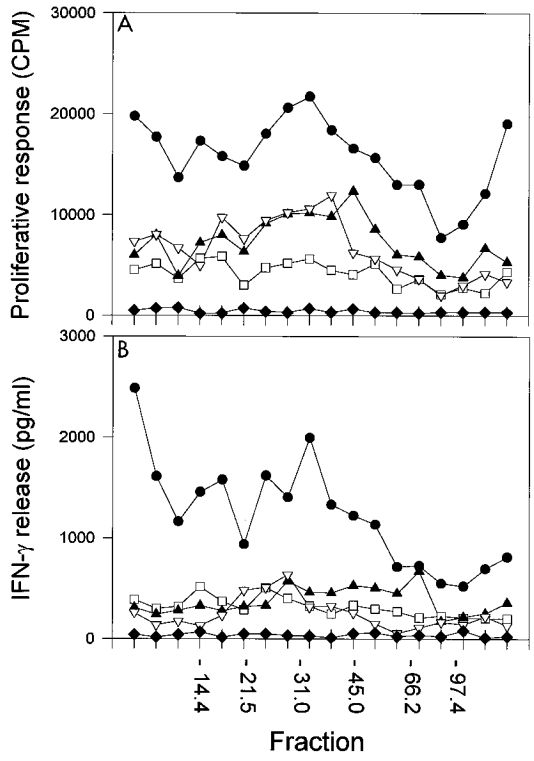

FIG. 4. Patterns of T-cell recognition in different donor groups. The average responses to ST-CF fractions are depicted for each group of donors. (A) Proliferative responses; (B) IFN-γ release to culture supernatants. Symbols represent patients with active minimal TB (●), patients with active advanced TB (□), BCG-vaccinated TB contacts (▲), BCG-vaccinated with TB noncontacts (▽), and non-BCG-vaccinated donors (◆). SEM is not indicated, but the variation within each group can be seen in Fig. 3. The migration of molecular mass markers (as shown in Fig. 2) is indicated in kilodaltons at the bottom.

newly diagnosed active infection compared with the group of BCG-vaccinated healthy donors. Somatic antigens in a preparation of killed *M. tuberculosis*, on the other hand, provoked the same level of reactivity in these two donor groups. This result suggest a preferential induction of human T-cell responses to the secreted part of the antigenic repertoire early during infection, in agreement with the specificity of T-cell responses observed in mouse models of TB infection (6, 8, 33). Our findings therefore provide evidence in support of the recently emerging notion that secreted antigens from *M. tuberculosis* are the target molecules recognized on infected macrophages early during the course of infection (6, 33). Decisive for the outcome of infection may be the speed with which infected host cells are located by effector T cells recognizing peptides derived from such secreted antigens. The recall of protective immunity is the subject of ongoing studies in this laboratory. A very recent study using the mouse model of TB infection has in this way provided evidence that immunity is expressed as an accelerated recruitment of memory effector cells recognizing secreted antigens of *M. tuberculosis* (4). It therefore seems reasonable to hypothesize that it is critically important to induce T-cell memory to secreted antigens during a vaccination to obtain an efficient immune surveillance. The soundness of this hypothesis has recently been emphasized by the demonstration of a very efficient protective immune response in mice, induced by an experimental vaccine based on ST-CF and a synthetic adjuvant (3). In the present study, patients with active advanced TB were found to exhibit a depressed immune response to mycobacterial antigens. Such a depression is in agreement with other studies, and both release of suppressive factors by monocytes and lymphocytes (22, 24) and preferential sequestration of antigen-specific T cells into the infected areas (36) have been proposed as responsible for this immunosuppressive status. Our study does not provide evidence to support any of these hypotheses but emphasizes the importance of a thorough clinical classification and discrimination between different patient categories to obtain relevant results. In this study, the specificity of the response to ST-CF was investigated by the recently developed multielution technique (7). In agreement with previous screenings of human lymphocyte responses by using separated antigens (14, 19, 37), we find that T cells from both healthy donors and TB patients recognize multiple targets and that a considerable heterogeneity exists within donor groups. Of importance, however, six of eight patients with active minimal TB demonstrated a predominant recognition of a low-molecular-mass fraction containing secreted proteins below 10 kDa. This result is encouraging and suggests that although extensive variation of responses is found within a human population, promiscuous epitopes recognized by a high proportion of donors may exist. It is intriguing to speculate that the identification of such critical epitopes may allow the construction of subunit vaccines effective in individuals of various HLA types.

TABLE 3. Proliferative responses by lymphocytes isolated from TB patients

Antigen[b]	Approximate mass (kDa)	Proliferative response[a] (mean cpm [10^3]; $n = 3$)			
		Donor 1	Donor 2	Donor 3	Donor 4
ST-CF		41.54	11.66	13.72	5.03
GroES	10	0.07	0.11	0.13	0.04
MPT46	14	0.26	0.05	0.12	0.05
MPT53	15	2.16	0.09	0.07	0.08
18 kDa	18	6.75	0.11	4.48	0.25
MPT64	26	2.52	0.11	0.05	0.04
MPT51	27	1.68	0.05	0.44	0.07
Ag85B	30	3.37	0.16	0.23	0.04
Ag85A	31	0.16	0.10	0.25	ND
Ag85C	31	2.27	0.05	0.09	ND
PhoS	38	2.00	0.03	2.69	0.20
MPT32	41	0.20	0.11	5.10	ND
71 kDa	71	0.12	0.14	0.08	0.23

[a] The pooled SEM for the experiments is 27% of the mean. Donors 1 and 2, patients with active minimal TB; donors 3 and 4, donors after antibiotic treatment. ND, not determined.

[b] The purified antigen preparations were used in noninhibitory concentrations of 1 to 2 μg/ml in the cell cultures.

Various attempts have been made to identify key antigens of importance for protective immunity against *M. tuberculosis*, and several antigens have been reported to elicit a powerful human T-cell response (15, 20, 25, 26). In this study, the recognition of an extensive panel of previously characterized secreted single antigens was tested in four TB patients. Surprisingly, only a few of these preparations elicited any detectable proliferative response although all of the patients tested responded to ST-CF. This result emphasizes the existence of several as yet undefined target molecules among the antigens secreted by *M. tuberculosis* during growth.

A previously uncharacterized 6-kDa secreted protein has recently been identified as the major antigenic target for T cells mediating long-lived immunological memory in a mouse model of TB infection (4). This antigen is a major component of the low-molecular-mass fraction identified in the present study as responsible for powerful T-cell proliferative responses and

high IFN-γ release in most of the TB patients tested. The coding gene encodes a low-mass polypeptide of 90 amino acids, a finding which emphasizes the presence of unique antigens in the low-mass fraction and not only a collection of peptides derived from degraded proteins of higher molecular mass. Recently separations in nondenatured polyacrylamide gels suggested that this molecule in its native configuration exists as a tetramer with a molecular mass of about 24 kDa (39). It is tempting to speculate that this antigen may be responsible for some of the reactivity found within a cluster of highly stimulatory secreted antigens located in the region from 30 to 100 kDa and with a pI of 4 to 5 (14). The precise identity of this antigen is the subject of ongoing efforts in our laboratory.

BCG-vaccinated donors were found to maintain a brisk in vitro T-cell proliferative response to secreted proteins although sensitized 20 to 40 years earlier. This finding indicates the establishment of a highly responsive and long-lived mycobacterium-specific memory T-cell repertoire. Immunological memory can be maintained either as long-lived resting progeny of T cells stimulated during the primary response or, as suggested by Beverly (9), as memory T-cell clones maintained for long periods of time through constant antigenic restimulation either by cross-reacting antigens or by persisting antigen present on specialized cells. The dependence of antigen for the maintenance of long-term immunological memory is a subject of much debate. Recently Müllbacher used adoptive transfer experiments to demonstrate that cytotoxic T-cell memory is independent of the presence of antigen (27). Gray et al., however, reported memory within both the T- and B-cell compartments to require the persistence of antigen (16, 17). Vaccination with BCG may provide a life-long antigen stimulus, as this vaccine has been demonstrated to persist in the vaccinated donors for prolonged periods of time (35). This prolonged exposure may trigger the high reactivity needed to protect against exogenous reinfection. In view of this observation a live recombinant BCG carrier expressing promiscuous epitopes recognized early during the natural infection in the human host may be a feasible approach for the development of a future vaccine against TB.

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