Cytokine Gene Expression Profile of Circulating CD4+ T Cells in Active Pulmonary Tuberculosis*

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T lymphocytes, particularly CD4+ cells, are thought to play an important role in the immune defense against Mycobacterium tuberculosis through the release of their wide array of cytokines. In vitro studies suggest that Mycobacterium-specific T-cell clones are of the TH1 subtype. Using the technique of reverse transcription-polymerase chain reaction, we have investigated the capacity for cytokine gene expression profile in ex vivo circulating CD4+ T cells from 20 patients with active pulmonary tuberculosis compared with that of 30 normal healthy tuberculin-positive volunteers. Venous blood samples were collected from the former prior to the initiation of chemotherapy. A significant increase in interleukin (IL-2) expression (p<0.001) and a significant decrease in IL-5 expression (p<0.0001) were observed in patients with tuberculosis but no differences were seen in the expression of IL-4 and interferon gamma between the two study groups. Our data support a TH1-like immune response in active tuberculosis.

(CHEST 1997; 111:606-11)

Key words: cytokines; helper T cells; interleukin 2; T lymphocytes; tuberculosis

Abbreviations: cDNA=complementary DNA; GM-CSF=granulocyte macrophage colony-stimulating factor; IFN=interferon; IL=interleukin; mRNA=messenger RNA; PBMC=peripheral blood mononuclear cells; PCR=polymerase chain reaction

The clinical manifestations of tuberculosis are dependent on the cellular immune responses to the tubercle bacilli, characterized by the accumulation of monocytes/macrophages, lymphocytes, and polymorphonuclear leukocytes in tuberculous lesions.1-3 These responses are initiated on sensitization of T lymphocytes by the bacterial antigen with the release of cytokines that regulate macrophage function. Activation of T lymphocytes in tuberculosis is supported by our previous findings that the serum concentration of soluble interleukin (IL)-2 receptor in patients with active disease was markedly raised and correlated significantly with the extent of disease.4 CD4+ T cells are likely to be the pivotal cells in orchestrating the immunologic defense against the mycobacteria as depletion of these cells is associated with increased susceptibility to tuberculous in both animals and humans.3,5,6

In mice, CD4+ T cells can be divided into TH1 and TH2 subsets depending on the profile of their secreted cytokines. Thus TH1 cells produce IL-2 and interferon-gamma (IFN-γ), but not IL-4 or IL-5, while TH2 cells release IL-4 and IL-5, but not IL-2 or IFN-γ.7 In vitro studies on human T-cell clones specific for Mycobacterium tuberculosis have yielded conflicting data on their profiles of cytokine production; thus, some investigators have shown a TH1-like pattern,8 while others have shown a mixed TH1 and TH2 pattern.9 An in vitro study on patients with active pulmonary tuberculosis revealed that the cells recovered from BAL were of TH1 phenotype with increased expression of IFN-γ.10 However, these BAL cells comprised both lymphocytes and macrophages. To date and to our knowledge, data on the cytokine profile of human CD4+ T cells in tuberculosis are still lacking, although the importance of these cells is well recognized.3

Using the sensitive technique of reverse transcription polymerase chain reaction (PCR), we have investigated the cytokine gene expression of CD4+ T cells, isolated from peripheral blood of patients with active pulmonary tuberculosis prior to the initiation of chemotherapy and compared them with those obtained from a group of tuberculin-positive healthy volunteers.

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**Materials and Methods**

**Subjects**

Twenty outpatients (14 males) with active pulmonary tuberculosis confined to upper lobe(s) participated in the study. Their mean age was 50 years (range, 16 to 50 years). On entry, all patients had positive smears for acid-fast bacilli in sputum or BAL specimens and subsequent cultures of these specimens yielded tubercle bacilli. None of the patients had any evidence of concomitant bacterial or viral infections as indicated by sputum and blood cultures and viral serologic study, including HIV. Clinically they all had mild disease and underwent uneventful recovery following chemotherapy. A control group of 30 healthy tuberculin-positive subjects (20 male) with a mean age of 48 years (range, 18 to 76 years) was also studied. Atopic status, assessed by skin prick tests to common allergens as previously described,11 was similar between the two groups of subjects (six atopic patients and 12 atopic healthy volunteers). Informed consent was obtained from all subjects and the study was approved by the hospital ethical committee.

**Blood Sampling**

Peripheral venous blood samples were taken from all patients prior to the initiation of antituberculosis chemotherapy and from all control subjects. All samples were randomly assigned code numbers and processed by an investigator who was blinded to the clinical status of the subjects.

**Isolation of CD4+ Cell Subset**

Peripheral blood mononuclear cells (PBMC) separated by Isopaque-Ficoll (Lymphoprep; Nycomed AS; Oslo, Norway) density gradient centrifugation were washed with phosphate-buffered saline solution, and then resuspended in culture medium consisting of RPMI 1640 (Gibco; Chargrin Falls, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), L-glutamine (2 mM/mL), penicillin (100 U/mL), and streptomycin (100 μg/mL). Magnetizable polystyrene beads coated with monoclonal antibody specific for CD4 (Dynal AS; Oslo, Norway) were used to select CD4+ T cells from PBMC as described previously.13 CD4+ T cells were 99.8±0.02% pure as assessed by flow cytometry (EPICS; Coulter Corp; Hialeah, Fla) using fluorescein conjugated anti-human Leu-3a monoclonal antibody (Becton Dickinson; San Jose, Calif). Immunofluorescence microscopy revealed these CD4+ cells were also CD3+ positive but were negative for CD11b. Cell viability was greater than 98% as determined by trypan blue dye exclusion test.

**Culture of CD4+ Cells**

Isolated CD4+ cells, adjusted to 1-mL aliquots of 1×106 cells, were dispensed into 48-well culture plates. The cells were incubated at 37°C for 6 h in humidified atmosphere with CO2 in the presence of phytohemagglutinin (10 μg/mL) and phorbol myristate acetate (0.5 ng/mL). As our preliminary experiments on unstimulated cells revealed that only small quantities of IL-2, IL-4, IL-5, and IFN-γ were detectable in normal subjects14 and in patients with tuberculosis (unpublished data), we have only studied stimulated CD4+ cells in the present experiment.

**Preparation of Total RNA and Oligonucleotide Primers**

A microadaptation of the guanidinium thiocyanate/caesium chloride procedure was used to prepare total RNA from CD4+ cells.15 All RNA samples prepared from 1×106 cells were dissolved in 20 μL DEPC-dH2O and were stored at −70°C until assay. The quality of RNA was checked by formaldehyde agarose gel electrophoresis and 2 μL of total RNA contained approximately 0.1 μg RNA. Specific primers of IL-2, IL-4, IL-5, IFN-γ, and β-actin were obtained (from Clontech; Palo Alto, Calif). Their sequences have been reported previously.15

**Detection of Cytokine Gene Expression by PCR**

Cytokine gene expression was studied in CD4+ cells using a kit (GeneAmp RNA PCR KIt; Perkin Elmer Cetus; Norwalk, Conn). Messenger RNAs (mRNAs) of each sample were first reversely transcribed into complementary DNAs (cDNAs), which in turn were subjected to PCR amplification using the specific primers mentioned above. In brief, first-strand DNA was synthesized in a final volume of 20 μL with the following components: 2 μL total RNA in DEPC-dH2O, 2.5 mM Tris HCl, 1 mM MgCl2, dNTP, and 10 U of RNase inhibitor. The mixture was incubated (in a Perkin-Elmer Cetus DNA Thermal Cycler) at 42°C for 30 min followed by 5 min at 94°C and flash cooling to 4°C. PCR was performed using a system (GeneAmp PCR System; Perkin Elmer, Cetus). cDNA (10 μL) was mixed with 2 mM MgCl2, 10 mM Tris-HCl buffer, 50 mM Tris HCl, 0.05 μM of specific PCR primers, 0.1 μM of?-actin primer, 1.25 U AmpliTaq DNA polymerase, and 2 μCi[α-32P]-dCTP in a final volume of 50 μL. Amplification was started with a 2-min denaturation at 94°C followed by 30 PCR cycles. Each cycle consisted of 60 s at 94°C for denaturation, 60 s at 62°C for annealing, and 1 min at 72°C for extension. The final extension lasted 10 min at 72°C in all instances. The experimental condition and number of cycles of PCR were predetermined to ensure that the amount of cytokine and “housekeeping” gene fragments were in the linear range of amplification. The efficiency of cDNA was controlled by using control cRNA (IL-1α RNA from plasmid pAW109 provided by Clontech) in every reaction.

Negative controls for PCRs consisted of (1) samples in which the reverse transcriptase was omitted to detect any contamination by genomic cDNA; and (2) reagents control in which RNA was replaced by DEPC-dH2O. Positive control DNAs for IL-2, IL-4, IL-5, IFN-γ, and β-actin were obtained (from Clontech Laboratory).

**Agarose Gel Electrophoresis and Semiquantification of PCR Products**

The PCR mixture (20 μL) was added to 5 μL of loading dye mix and electrophoresed in a 9% constant-voltage field in 1.7% agarose (Sigma; St. Louis) containing ethidium bromide until the bromophenol blue dye front had migrated for a distance of 6 cm. αX174/Hae III digested DNA (Gibco) was used to determine the size of the PCR products. After electrophoresis, gel slides corresponding to the radioactive bands of PCR product were excised and counted by liquid scintillation. 32P incorporated into the PCR products of each of the cytokines was normalized with that of the amplified β-actin PCR products as described previously.16 The results were expressed as a ratio calculated from the counts per minute of amplified cytokine gene product over the counts per minute of amplified β-actin PCR product.

**Statistical Analysis**

All figures are mean±SEM unless otherwise stated. Mann-Whitney U test was used for comparisons between the two groups of subjects. Spearman’s rank correlation was used to examine the relationships between the expression of the individual cytokines and extent of disease on chest radiograph. All p values of <0.05 were considered significant.
RESULTS

IL-2 gene expression in CD4+ cells was significantly higher in patients with active pulmonary tuberculosis (0.54±0.02, n=20) than that in tuberculin-positive healthy control subjects (0.39±0.03, n=30, p<0.001, Fig 1). In contrast, IL-5 expression was significantly lower in the former group (0.47±0.04) than the latter (1.30±0.14, p<0.0001, Fig 2). However, analysis on data in patients revealed no significant correlation between the expression of these two cytokines.

There were no significant differences between the two groups of subjects in the expression of IFN-γ (Fig 3) or IL-4 (Fig 4). Furthermore, the ratio of expression for these two cytokines did not differ between the patients (0.94±0.14) and the control subjects (1.0±0.13; not significant). The expression of all four cytokines was similar between the atopic and nonatopic subjects (data not shown).

DISCUSSION

In this study, we have demonstrated a TH1-like immune response of circulating CD4+ T lymphocytes in patients with active tuberculosis whose capacity for IL-2 expression was increased, IL-5 expression was reduced, while that for IL-4 and IFN-γ was similar to normal control subjects. However, the expression of the individual cytokines demonstrated no significant correlation with each other.

The immune response of circulating CD4+ T cells may differ from their counterparts at the site of disease. In mice, granuloma induced by polymer beads coated with purified protein derivative of M. tuberculosis has a different cytokine profile from that of the draining lymph nodes in that the former produced IFN-γ while the latter produced both IL-2 and IFN-γ. Thus, it appears that the same type of cells may have a different cytokine profile according to their location. However, we have previously...
shown that serum concentration of soluble IL-2 receptor correlated with the extent and activity of tuberculosis in humans.\textsuperscript{4,15} Furthermore, we have demonstrated that gene expression for IL-3, IL-5, and granulocyte macrophage colony-stimulating factor (GM-CSF) in circulating CD4\textsuperscript{+} T cells could reflect disease activity in asthma.\textsuperscript{16,17} Taken together, these data suggest that peripheral blood may provide a useful and noninvasive means to study the immune response in tuberculosis, particularly in severely ill patients who may develop serious complications with invasive procedures such as tissue biopsy and BAL. Indeed, our data on circulating CD4\textsuperscript{+} T cells are in accord with those of bronchoalveolar T cells,\textsuperscript{10} both sets of data point to a TH\textsubscript{1}-like cytokine profile in tuberculosis.

We have evaluated the cytokine gene expression in purified CD4\textsuperscript{+} T cells by PCR following reverse transcription using \beta-actin as a housekeeping gene, since its cellular concentration is independent of the functional status of the cell. This technique, however, provides only a semiquantitative estimate of the number of copies of mRNA per cell, but not the proportion of cells within the sample that transcribe cytokine genes. Currently there is no perfect method for the quantification of PCR products; even with the competitive PCR method used by other investigators for similar studies,\textsuperscript{18,19} one cannot be certain about the efficiency of the reverse transcription for each experiment. No matter which technique is adopted for quantifying PCR-amplified cDNA reversely transcribed from mRNA, it is impossible to ensure in practice that precisely equivalent starting amounts of RNA from the cells of each subject are obtained because there is inevitably a variable, albeit small, degree of contamination. However, this problem can be overcome by normalizing the PCR product to a “control” mRNA such as \beta-actin, provided that amplification of such a control can be demonstrated to occur within the exponential phase.\textsuperscript{20} Indeed, we have confirmed in our pilot study that PCR of \beta-actin, IL-2, IL-4, IL-5, and IFN-\gamma did lie in the exponential range of amplification under the experimental conditions described above. Furthermore, the accuracy and repeatability of reverse transcription were checked and control cRNA was used in every reaction. Our technique therefore provides a reasonably accurate means for comparing cytokine gene expression in different specimens on multiple occasions. The observed differences in the expression of IL-2 and IL-5 between our patients and healthy volunteers, although small, are statistically significant and are of similar magnitudes as those observed for IL-3, IL-5, and GM-CSF in our previous studies on asthma.\textsuperscript{16,17}

In two separate studies, we have previously shown that T-lymphocyte activation is a feature of active pulmonary tuberculosis and that the degree of activation lessens upon completion of a 6-month course of antituberculosis chemotherapy, though it still remains elevated in relation to the level seen in normal subjects.\textsuperscript{4,15} This present study provides a mechanism by which these activated cells can affect the immune response to the disease. The enhanced capacity to express IL-2, a T-cell growth factor, is likely, though not necessarily, to result in an increased production of this cytokine which in turn stimulates the clonal expansion of activated T cells and increases the cytolytic activity of natural killer cells and the generation of other cell-regulating factors, including other cytokines.\textsuperscript{21} It may also modify the adhesive properties of endothelial cells, thereby affecting leukocyte chemotaxis.\textsuperscript{22} That this cytokine is important in the immune defense against \textit{M tuberculosis} is further supported by the finding that its expression in PBMC is significantly lower in HIV-positive patients with tuberculosis than their HIV-negative counterparts.\textsuperscript{18}

In mice, production of the TH\textsubscript{1} cytokine IFN-\gamma is associated with protection against tuberculosis\textsuperscript{23-26} while the production of the TH\textsubscript{2} cytokine IL-5 is associated with increased susceptibility to the disease.\textsuperscript{27} In support of this, Zhang et al\textsuperscript{19} have shown that \textit{M tuberculosis}-stimulated PBMC from tuberculosis patients had reduced production and mRNA expression of TH\textsubscript{1} cytokines IFN-\gamma and IL-2, but with no change in the levels of TH\textsubscript{2} cytokines IL-4, IL-10, and IL-13 when compared with healthy tu-

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\caption{IL-4/\beta-actin mRNA ratio in patients with active pulmonary tuberculosis (TB) and tuberculin-positive normal control subjects. Bar indicates mean values.}
\end{figure}
berculin reactors. Our data, in contrast, have shown that circulating CD4+ T cells have increased expression of IL-2 and reduced expression of IL-5, a TH2 cytokine. These discrepancies could be due to at least two factors. First, we used phytohemagglutinin and phorbol myristate to stimulate purified CD4+ cells, while the former studies used M tuberculosis to stimulate PBMC. Differences in the priming and culture conditions as well as the cell type studied may contribute to the conflicting results. Second, the severity of tuberculosis in our patients and those of Zhang et al.19 might differ. Our subjects with tuberculosis were outpatients with mild and early disease and they all underwent uneventful recovery following chemotherapy, but the severity and clinical course of the disease in the subjects of Zhang and coworkers is not known. Animal studies suggest that progressive tuberculosis may be more likely to display a reduced TH1 response or an enhanced TH2 response.23-27 Indeed, tuberculosis patients who are immunocompromised, such as those infected with HIV, have reduced expression of IL-2 in PBMC when compared with immunocompetent tuberculosis patients.18 It is plausible that the intensity of the TH1 response may determine the severity and extent of tuberculosis.

The absence of enhanced coexpression for the cytokines together with the finding of a lack of correlation between the increased IL-2 expression and the reduced IL-5 expression in our patients suggests that independent regulating mechanisms probably exist for the expression of the individual cytokines in tuberculosis. The original work by Mossman et al.7 on mice has led to the proposal that similar discrete subsets of T cells, TH1 and TH2, also exist in humans.28 However, this view has been challenged recently.29 Thus, although some studies have demonstrated TH1- and TH2-like responses in several disease states in humans, the cytokine profiles reported are diverse and show predominance of one cytokine rather than increased coexpression of a group of cytokines or a clear dissociation between the TH1 and TH2 cytokines. Furthermore, the demonstration that synthesis of an individual cytokine is continuously distributed among T cells and clones suggests that the detection of a particular cytokine is a function of the assay sensitivity.30 Our data, though derived from a population of CD4+ cells rather than from a single cell, support the notion that the TH1 and TH2 cells are not discrete cellular compartments of the immune system.

The observation that circulating CD4+ cells in tuberculosis have enhanced IL-2 expression and reduced IL-5 expression upon ex vivo stimulation suggests that these cells may be primed in vivo. One of the possible priming factors is IL-12 which has been shown to be important in upregulating the TH1 response and in downregulating the TH2 response from antigen-specific naive CD4+ T cells.30-32 However, a lack of IL-10 stimulation could also facilitate the development of TH1 cells as this cytokine is able to inhibit proliferation of TH1 clones.33,34 This hypothesis is supported by the observation that the M tuberculosis-induced type 1 response in PBMC from HIV-infected tuberculosis patients is enhanced both by neutralizing antibodies to IL-10 and by recombiant IL-12.18

In two separate studies on patients with acute severe asthma, we have previously found that the enhanced expression of both IL-5 and GM-CSF in circulating CD4+ T cells could be reduced significantly following clinical improvement with a 7-day course of systemic corticosteroid therapy.16,17 Whether the clinical improvement in tuberculosis with chemotherapy is associated with a change in cytokine expression pattern in CD4+ cells remains to be seen. Our previous data on the time course of serum soluble IL-2 receptor concentration in tuberculosis suggests that T-lymphocyte activation is still evident by the completion of short-course antituberculosis chemotherapy when clinical resolution of the disease is obvious.15

In conclusion, we have provided evidence of a TH1-like response in patients with active pulmonary tuberculosis, although the distinct pattern of enhanced coexpression of the TH1 cytokines and suppression of all the TH2 cytokines is not seen. Further studies to examine the factors determining the expression of these cytokines in relation to disease activity will enhance our understanding on the pathogenesis of tuberculosis and lead to its better treatment and prevention.

ACKNOWLEDGMENTS: We thank Irene Shum and P. K. Ma for their technical assistance.

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