INFECTION AND IMMUNITY, Apr. 1994, p. 1444–1450
0019-9567/94/$04.00+0

Vol. 62, No. 4

Cytokine Gene Expression by Cultures of Human Lymphocytes with Autologous *Mycobacterium tuberculosis*-Infected Monocytes

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Received 24 August 1993/Returned for modification 22 November 1993/Accepted 26 January 1994

In order to better understand the immunoregulation following *Mycobacterium tuberculosis* infection, cytokine mRNA induction in response to in vitro infection of human monocytes with live virulent *M. tuberculosis* H37Rv cocultured with autologous lymphocytes was quantitated by reverse transcriptase-PCR. Induced levels of interleukin 1β (IL-1β), IL-2, tumor necrosis factor alpha, and gamma interferon (IFN-γ) were compared among groups of individuals representing three phases of immunity to infection with *M. tuberculosis*: naive normal control subjects, purified protein derivative (PPD)-reactive normal donors, and individuals with active tuberculosis (TB [diseased]). Levels of IL-1β and tumor necrosis factor alpha mRNA in cocultured cells from TB patients were 51 and 45%, respectively, of those obtained in cells from sensitized healthy volunteers and were comparable to those from naive normal donors. Lymphoproliferative responses to *M. tuberculosis* and induction of the T-cell cytokine IL-2 were predictably high in the cells of PPD-sensitized donors, low in normal naive individuals, and variable among TB patients. In contrast, the induced level of another lymphokine, IFN-γ, did not follow the pattern seen in IL-2 induction. Infection with live *M. tuberculosis* induced high levels of IFN-γ mRNA in lymphocytes of both PPD-sensitized and normal naive donors compared with those of TB patients. Interestingly, polyclonal stimulation with the mitogen concanavalin A induced similar IFN-γ levels in cells from all three donor groups. The high level of IFN-γ induced by the infection of monocytes from naive normal donors suggests a role for natural killer (NK) cells in the production of IFN-γ in this coculture system. This response appears independent of the role performed by T cells.

Tuberculosis (TB) remains an important cause of morbidity and mortality worldwide. Currently, the human immunodeficiency virus pandemic and the emergence of multiply-drug-resistant forms of *Mycobacterium tuberculosis* threaten to overwhelm control strategies (3). On exposure to the bacterium, most healthy individuals successfully contain the infection and go on to develop a vigorous cell-mediated immune response and a delayed-type hypersensitivity reaction to mycobacterial antigens. The protective immune response depends on the cooperation between infected macrophages, activated T lymphocytes, activated natural killer (NK) cells, and their induced cytokines (16). However, a disfunctional cell-mediated immune response to infection with *M. tuberculosis* may contribute to progressive primary infection or reactivation of endogenous foci of mycobacteria (5).

In vitro stimulation of leukocytes with mycobacteria or their products induces synthesis and release of several cytokines, including interleukin 1 (IL-1), IL-2, IL-6, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) (9, 17, 23, 24, 26, 29, 32). Among the T-cell cytokines produced within the granulomatous lesion, IL-2 and IFN-γ have important functions. Antigen stimulation of the T-cell receptor induces transcription of IL-2 and IL-2 receptor genes, which then signal clonal expansion of activated T cells (as well as NK and B cells) and the production of other cell-regulating factors (7). IL-2 also plays a key role in the generation of the cytokine cascade, with pleiotropic effects including lymphocyte proliferation. This lymphokine is also indirectly chemotactic for leukocytes, eliciting extravasation by inducing a change in the adhesive properties of endothelial cells (19). IFN-γ is responsible for activation of monocytes and stimulation of giant cell formation (10). IFN-γ has also been shown to inhibit intracellular growth of mycobacteria (8) and may exert its effects indirectly by inducing synthesis of the tuberculostatic 1,25-dihydroxyvitamin D_3 from its inactive precursor (15).

IL-1 probably plays a key role in the development of the hypersensitivity granuloma (18). Multiple biologic activities of this monokine include induction of IL-2 and IL-2 receptor in T cells and the promotion of IL-3 and IL-6 synthesis by monocytes. While IL-1 production is augmented during the early elicitation phase of granuloma development, production of TNF-α increases during the late recruitment and maintenance phases (10). Some of the activities ascribed to TNF-α include upregulated HLA expression, enhanced IFN-γ-induced Ia expression, and induction of IL-6 production (21).

It has been reported that peripheral blood mononuclear cells (PBMCs) from TB patients, when stimulated in vitro with purified protein derivative (PPD), release lower levels of certain cytokines, including IFN-γ and IL-2, than do cells from healthy donors (11, 24, 32, 33) and higher levels of other cytokines, including TNF-α and IL-1 (9).

We evaluated the cytokine response to in vitro infection with virulent *M. tuberculosis* at the gene expression level. An in vitro model of *M. tuberculosis*-infected human monocyte–T-cell interaction was used to analyze the kinetics and level of cytokine induction by measuring specific mRNA accumulation with reverse transcriptase (RT)-PCR technology. This enabled us to avoid some of the problems associated with cytokine detection in a biological assay such as lability, low concentration, and receptor-mediated uptake. Quantitation of these mRNA species allowed us to compare cytokine responses

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among groups of individuals representing three phases of immunity to *M. tuberculosis*.

(This work was presented in part at the 1992 Federation of American Societies for Experimental Biology meeting, Anaheim, Calif., 5 to 9 April 1992, and at the 92nd General Meeting of the American Society for Microbiology, New Orleans, La., 26 to 30 May 1992 [11a].)

MATERIALS AND METHODS

Human subjects. Venous blood samples were collected from PPD-reactive and nonreactive healthy volunteers and TB patients. TB patients from the Veterans Administration Hospitals in Temple and Houston, Tex., had been diagnosed on the basis of the demonstration of acid-fast bacilli in the sputum, and diagnosis of TB had been confirmed by a culture positive for *M. tuberculosis*. The patients were undergoing anti-TB drug treatment at the time blood samples were taken. All volunteers were human immunodeficiency virus type 1 and hepatitis B seronegative, and most had a history of tuberculin reactivity. In addition, they had no manifest coinfections or other conditions which might affect the immune response. They ranged in age from 43 to 66 years, and all were male. Normal control subjects were between the ages of 23 and 55 years and were not sex matched with regard to the TB patient group.

Preparation of leukocytes. Heparinized blood was separated aseptically and PBMCs were isolated as described previously (6) and were allowed to adhere for 1 h at 37°C in 5% CO_2 in a humidified atmosphere after which nonadherent (NA) cells were drawn off. Adherent (AD) cells were routinely in a >90% monocyte/macrophage ratio, as determined with the nonspecific esterase assay (14). Live, sonicated *M. tuberculosis* H37Rv (ATCC 27294) cells were added at approximately 1 bacterium per 10 AD cells in RPMI medium with 5% autologous serum and were incubated for 30 min. Medium was removed, the AD cells were washed vigorously three times to remove extracellular bacteria, and warm RPMI medium with 10% autologous serum was added back to the cultures. Replicate cultures in which the AD cells were not infected were set up. The presence of mycobacteria within AD cells was confirmed by acid-fast staining of cultures at intervals after infection. AD cells from the three different study populations (tuberculous, PPD reactive, and naive) phagocytosed *M. tuberculosis* with equivalent efficiency. After a second depletion of AD cells, the remaining NA cells were added to the AD cultures at an NA/AD ratio of 2:1 and incubated for 7 days.

Phenotypic analysis. For indirect immunofluorescent staining, NA cells were taken directly after the second adherence step or removed from coculture after 7 days and stained with a saturating amount of the following monoclonal antibodies: anti-CD3(Leu-4), anti-CD4(Leu-3a), anti-CD8(Leu-2a), anti-TCRαβ(TCR-1,αβWT31) (Becton-Dickinson, Mountain View, Calif.), anti-TCRγδ(TCRδ1) (T Cell Sciences, Cambridge, Mass.), and anti-CD28(9.3) (a generous gift from Bristol-Myers Squibb, Seattle, Wash.). Following incubation with a labeled secondary antibody [fluorescein isothiocyanate-conjugated sheep $F(ab')_2$ anti-mouse immunoglobulin (Sigma)], cells were analyzed with the EPICS V flow cytometer (Coulter Electronics, Hialeah, Fla.).

Proliferation assay. PBMCs were placed in 96-well flat-bottom tissue culture dishes (Becton-Dickinson) at 3×10^5 cells per well in RPMI medium with 10% autologous serum and 5 μg of concanavalin A (ConA [Sigma]) or 5 μg of PPD (PPD-RT46, Statens Seruminstitut, Copenhagen, Denmark) per ml for 6 days. On day 7, 5 μCi of [^{3}H]thymidine (ICN Radiochemicals, Irvine, Calif.) per ml was added for 6 h, after which cells were harvested onto glass wool fiber filters by means of a cell harvester (MASH II; MA Bioproducts, Walkersville, Md.). [^{3}H]thymidine incorporation into cellular DNA was measured by liquid scintillation spectroscopy. All samples were assayed in triplicate, and the results are expressed as mean counts per minute. Lymphoproliferation in response to PPD was considered positive when counts-per-minute values were more than 2 standard deviations above mean values obtained in healthy tuberculin-negative donors.

In a preliminary experiment, PPD-reactive donor PBMCs were tested over a range of cell densities (1×10^5, 3×10^5, and 5×10^5 cells per well) and concentrations of PPD (5, 10, 25, and 50 μg/ml). The optimal response was obtained with 3×10^5 cells per well and 5 μg of PPD per ml. These levels were used throughout the study.

RNA extraction. Cytoplasmic RNA was extracted from infected or uninfected cocultured cells at selected time points postinfection with RNAzol B RNA isolation solvent as described by the manufacturer (Cinna/Biotecx, Houston, Tex.). This method is a variation of the single-step acid guanidinium-thiocyanate–phenol-chloroform–RNA extraction method (4). Absorption spectroscopy was used to measure the purity and concentration of RNA, with an $A_{260/280}$ ratio of 2.0 indicating highly purified RNA.

Oligonucleotide primers and probes. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. The primers were RNA specific in that both the 5′ and 3′ primers span the junctions of 2 exons, thus precluding amplification of genomic DNA. Sequences for the internal 24-mer oligonucleotide probes used for hybridization were as follows: IL-1b, ccatggacaagctgaggaagatgc (2); IL-2, gccacagaactgaaacat cttcag (30); TNF-α, gttgtagcaaaccctcaagctgag (35); and IFN-γ, gaccagagcatccaaaagagtgtg. The PCR primer sequences for the cytokines IL-1β, IL-2, TNF-α, and IFN-γ and the control glyceraldehyde 3-phosphate dehydrogenase (GA3PD) were as follows: IL-1β, aaacagatgaagtgctccttccagg, tggagaacaccacttgtt gctcca (35); IL-2, gaatggaattaataattacaagaatccc, tgtttcagatccctt tagttccag (35); TNF-α, cagagggaagagttccccag, ccttggtctggtag gagacg (35); IFN-γ, gaaatattttaatgcaggtc, gtcagttaccgaataattag (22); and GA3PD, tggtatcgtggaaggactcatgac, atgccagtgagcttc ccgttcagc (34). Internal oligonucleotide probes were 3′ end labeled with [α-^{32}P]dATP and terminal transferase according to the supplier's protocol (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Specific activities ranged from 3×10^9 to 1×10^{10} cpm/μg.

Amplification. RNA was reverse transcribed into cDNA which was amplified with the GeneAmp RNA-PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.). The RT reaction mixture was incubated for 1 h at 42°C, heated to 99°C for 5 min, to inactivate the enzyme, and then quick-chilled on ice. PCR was performed with 30 cycles of 95°C for 1 min (denaturation) and 60°C for 1 min (primer annealing and extension). To normalize for the amount of input RNA, RT-PCR was performed with the constitutively expressed gene encoding GA3PD (34).

Southern transfer and hybridization. Equal portions of each PCR product were subjected to electrophoresis followed by capillary transfer to nylon membrane (ZetaBind; Cuno Inc., Meriden, Conn.) according to previously published procedures (20, 28). Following transfer, membranes were prehybridized in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–0.1% NaPPi–0.5% sodium dodecyl sulfate (SDS)–0.5% BLOTTO for 4 to 16 h at 65°C. Hybridization was performed in 6× SSPE containing 0.1% NaPPi–0.5% SDS, with 5×10^5 cpm of ^{32}P-labeled internal oligonucleotide probe per ml, for 8 to 12 h at 40°C. Filters were

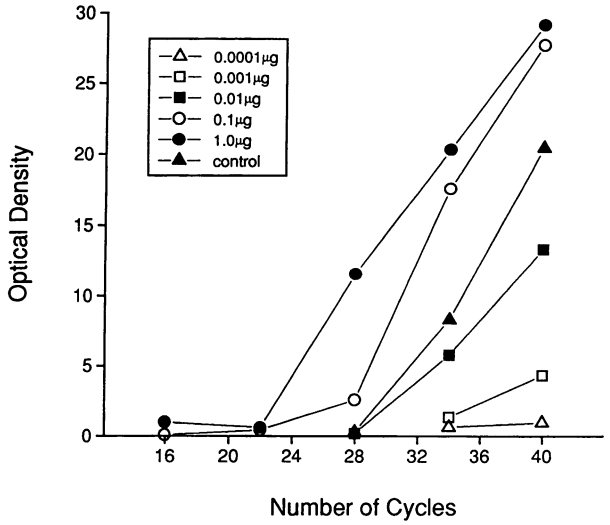

FIG. 1. Optimization of quantity of input RNA versus number of PCR cycles. Results from one of three replicate Southern blots of different amounts of input RNA (0.0001, 0.001, 0.01, 0.1, and 1 μg) assayed by RT-PCR for various numbers of PCR cycles (16, 22, 28, 34, and 40 in one experiment and 15, 20, 25, 30, 35, and 40 in two experiments) are shown. Radioactive bands were quantified by electrophoretic analysis imaging and were plotted for assay standardization.

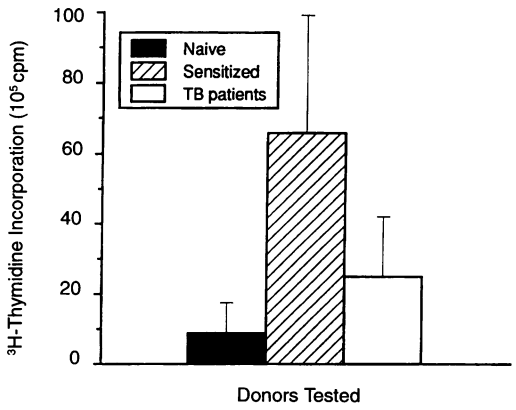

FIG. 2. PPD-induced lymphoproliferative responses from naive and sensitized donors and TB patients. PBMCs were cultured at 3×10^5 cells per well in the presence of PPD (5 μg/ml) for 6 days. [^{3}H]thymidine incorporation was assessed during the last 6 h of culture. Values are mean counts per minute ± standard error.

washed three times with 6× SSPE–0.1% SDS at room temperature for 15 min each, wrapped in cellophane wrap, and exposed to X-ray film (Hyperfilm-MP; Amersham Corporation, Arlington Heights, Ill.) at room temperature with an intensifier screen.

Quantitative analysis. Figure 1 is a graph of a Southern blot (one of three) analyzed by densitometry. These data were used to determine (i) the number of PCR cycles and (ii) the amount of input RNA such that PCR product formation remained within a linear range despite a potential 100-fold increase in the level of cytokine mRNA. To determine the validity of this approach, three separate experiments were run in which different amounts of input RNA from ConA-stimulated PBMCs (0.0001, 0.001, 0.01, 0.1, and 1 μg) were assayed by RT-PCR for IL-1β for various numbers of PCR cycles (16, 22, 28, 34, and 40 in one experiment and 15, 20, 25, 30, 35, and 40 in two experiments). The amount of product obtained was proportional to the amount of input RNA. Since a reference control was used as a standard against which values obtained from sample RNA were normalized, 0.01 μg of RNA was chosen as the quantity of input RNA whose efficiency of amplification most closely matched that of the control. From these data, it was determined that an initial quantity of 0.01 μg of total RNA per reaction mixture amplified for 30 cycles would represent a direct measure of cytokine mRNA over a 100-fold range of concentrations. Band intensity was determined with laser scanning densitometry (Visage 110; BioImage Products, Ann Arbor, Mich.). Alternatively, the radioactivity in each band was determined directly with the BetaScope 603 blot analyzer (Betagen, Waltham, Mass.), and counts per minute were extrapolated against those obtained from amplification of 10^4 copies of RNA construct. The RNA construct (referred to herein as the reference RNA) contained the 5′ and 3′ primers of 12 human cytokine genes connected in sequence with a downstream polyadenylated sequence and was included in the RNA-PCR kit as the positive control.

Kinetics of mRNA induction. To determine the kinetics of induction of cytokine mRNA in this in vitro system, RNA was extracted from cells at 0, 6, 12, 24, and 48 h of coculture with either infected or uninfected macrophages from donors representing the three groups studied. RT-PCR was performed with 0.1 μg of total RNA per reaction mixture for the cytokines IL-1β, IL-2, TNF-α, IFN-γ, and the positive control GA3PD. We observed two peaks of mRNA accumulation during this time period which may result from two independent induction stimuli. In the uninfected cocultures, PCR product for IL-1β, TNF-α, and IFN-γ was visible at 0 through 24 h, with a peak intensity at 6 h; by 48 h of coculture, there was no visible product. In *M. tuberculosis*-infected cocultures, we observed a similar pattern, with product visible at 0 and 6 h, diminishing in intensity at 12 h, and then increasing again with a peak at 48 h (data not shown). Cytokine mRNA present from 0 through 12 h is believed to result from the physical process of cell separation, manipulation, and adherence. Cytokine mRNA at 48 h of coculture is thought to represent induction by the intracellular pathogen and was the time point at which RNA was evaluated for this study.

Statistics. Comparisons between groups were done by Student's *t* test for independent samples. Values of $P \geq 0.05$ were considered not significant.

RESULTS

Lymphoproliferation and phenotypic analysis. The lymphoproliferative responses to PPD-stimulated and *M. tuberculosis*-infected monocytes of normal naive donors, PPD-responsive donors, and patients with active TB were compared (Fig. 2). As expected, normal naive donors demonstrated no thymidine incorporation in response to PPD, while sensitized individuals demonstrated extensive proliferation. By comparison, cells from patients with active TB proliferated much less vigorously than did cells from healthy sensitized individuals. When NA cells from the three groups of donors were cocultured with *M. tuberculosis* H37Rv-infected AD cells, similar proliferative responses were observed (results not shown). The blastogenic responses induced by 5 μg of ConA, however, were similar in all three groups of donors (data not shown).

Phenotypic analysis of the cocultured infected cells from sensitized donors, patients with active TB, and normal control

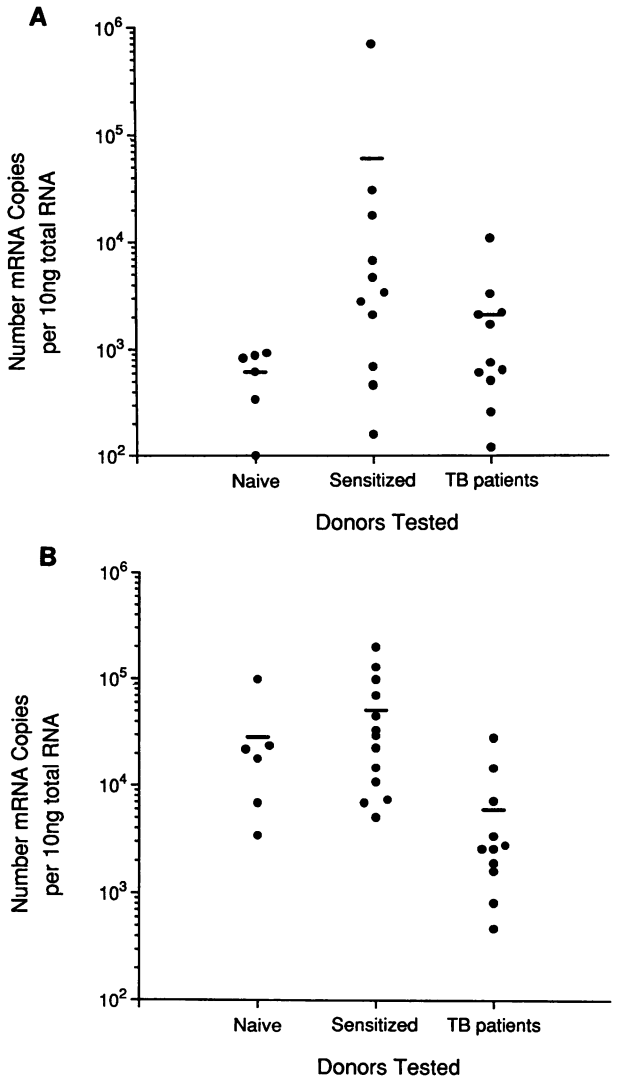

FIG. 3. IL-2 (A) and IFN-γ (B) mRNA induction in *M. tuberculosis*-infected cocultures of cells from naive and PPD-sensitized normal donors and TB patients. Counts per minute values were normalized to the reference standard RNA. Horizontal bars represent arithmetic mean level of transcription for each donor group.

donors revealed no major differences or changes relative to the starting population, with one exception. We observed an overall reduction in the percentage of $CD28^+$ T cells during coculture with infected AD cells in TB patients and PPD responders compared with their levels at the baseline analysis (15 and 12% reductions, respectively). These phenotypic changes were not significant compared with those of normal naive donors. However, when the percentage of $CD28^+$ cells and the level of the CD28 antigen expressed on cells of each individual were compared with the level of IL-2 mRNA expressed by these cells, an inverse correlation was observed (described below).

Cytokine mRNA induction in cocultured cells. The level of IL-2 mRNA induced in the cells by coculture with H37Rv-infected AD cells is demonstrated in Fig. 3A. As expected, cells from naive donors that did not proliferate in response to either PPD or mycobacterium-infected AD cells demonstrated the lowest levels of IL-2 mRNA. Cells from sensitized healthy donors expressed various levels of IL-2 mRNA, with a mean mRNA copy number about 30-fold higher than that observed in patients with active disease. In general, cells from patients with active TB expressed lower levels of IL-2 mRNA than did those from PPD-sensitized healthy donors: half of them were as low as those of normal naive donors, and half were within the range of those of the sensitized individuals. Within the group of sensitized normal and active TB patients, the T cells from the individuals with the lowest levels of IL-2 mRNA expression also demonstrated the highest levels of the CD28 antigen. As the amount of IL-2 mRNA expressed by the cells increased, a reduction in CD28 antigen expression was observed. The IL-2 mRNA levels observed in the cells correlated with the extent of thymidine incorporation observed in these groups of donors (Fig. 2).

In contrast, levels of IFN-γ mRNA (Fig. 3B) did not correlate with PPD responsiveness or disease status. Cells from normal naive individuals were induced by exposure to infected monocytes to produce levels of IFN-γ mRNA as high as those observed in sensitized healthy donors, while cells from TB patients demonstrated somewhat (10-fold) reduced levels of IFN-γ mRNA compared with those from PPD-sensitized healthy donors. The differences observed were not statistically significant by the binomial test (at $P > 0.1$) because of the broad range of values obtained. However, this general trend is suggestive of an IFN-γ production defect in patients with active disease.

Quantitation of mRNA expression of the monocyte cytokines IL-1β and TNF-α (Fig. 4) demonstrated a distribution similar to that observed with IL-2 mRNA. Cells from naive donors expressed the lowest levels of cytokine mRNA, while cells from healthy sensitized donors and TB patients expressed a wider range of levels, with normal sensitized individuals' cells expressing much higher mRNA levels than those from naive donors. As mentioned above, these results are of interest but are not statistically significant (at $P > 0.1$).

The cytokine mRNA responses to ConA stimulation of cells were similar in the three groups of donors and mirrored the extent of thymidine incorporation in ConA-stimulated cultures (Fig. 5).

DISCUSSION

The model presented here was designed to monitor subtle changes in T-cell subpopulation numbers and cytokine gene induction in response to infection with live, virulent *M. tuberculosis*. Therefore, we employed physiologically relevant parameters, including a low multiplicity of infection, autologous serum, the absence of exogenously added lymphokines, and a T-cell/monocyte ratio which allowed continued cellular differentiation over a 7-day coculture period. We then evaluated differences in immune response parameters to in vitro infection among cells from normal naive individuals, PPD-sensitized donors, or TB patients in this coculture model. We found that, despite the extensive mycobacterial antigenic load which is likely to occur in patients with active TB, T cells obtained from such individuals demonstrated reduced proliferative responses as well as relatively low levels of cytokine mRNA expression compared with those from normal healthy PPD-sensitized individuals. It has been reported that TB patients with newly diagnosed pulmonary disease have defective PPD-stimulated but normal streptococcal antigen-stimulated IL-2 production compared with healthy responders (1, 32). In the present study, similar observations were made with the cells of patients who had been on anti-TB drug therapy for relatively

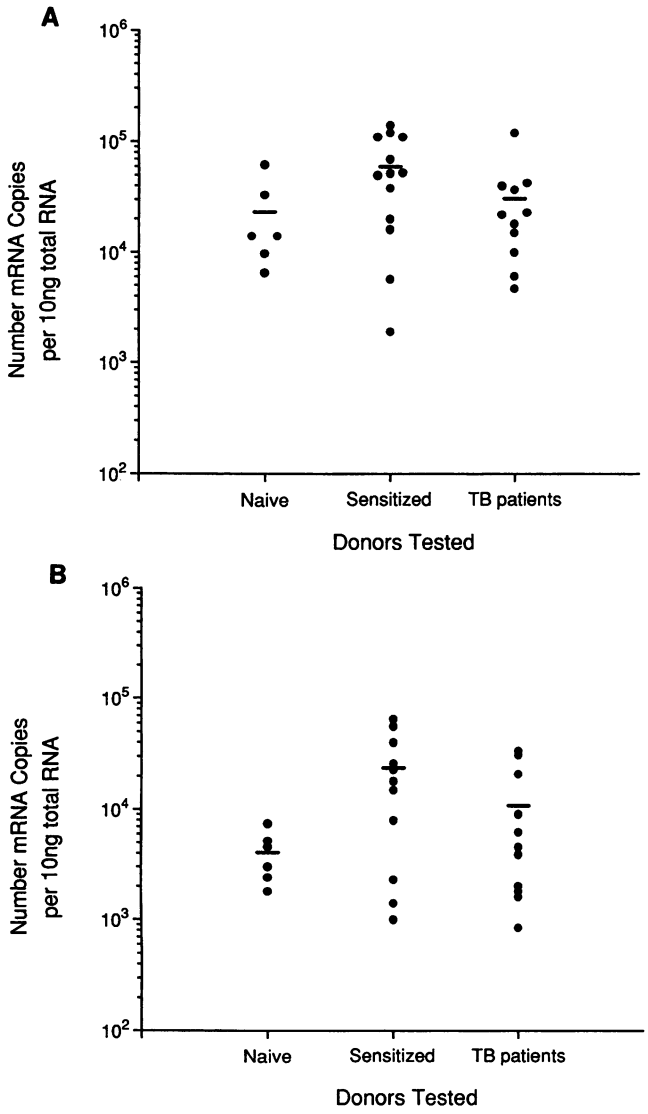

FIG. 4. IL-1β (A) and TNF-α (B) transcription levels in *M. tuberculosis*-infected cocultures of cells from naive and PPD-sensitized donors and TB patients. Horizontal bars represent arithmetic mean level of mRNA transcripts for each donor group.

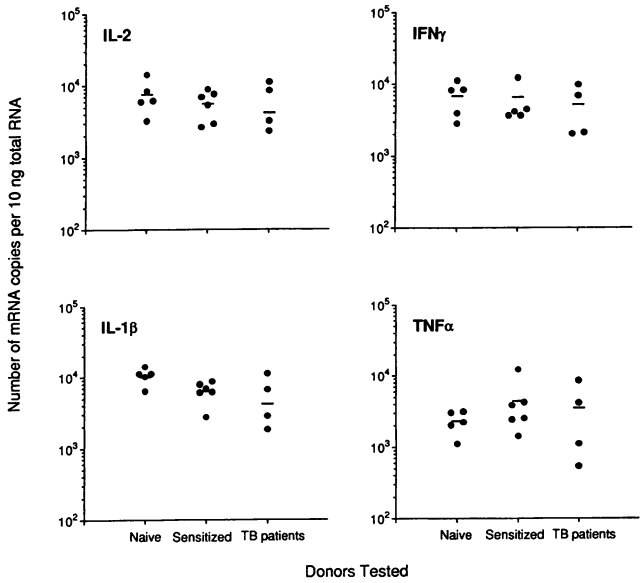

FIG. 5. Mitogen-induced cytokine mRNA levels. PBMCs from the three donor groups were stimulated for 6 h with 10 μg of ConA per ml followed by RNA extraction and RT-PCR analysis for IL-2, IFN-γ, IL-1β, and TNF-α. Horizontal bars represent arithmetic mean value for each donor group.

long periods of time. Five of 11 patients studied here were chronic TB patients exhibiting positive sputum smears despite a mean duration of therapy of 12 months. This suggests that T-cell unresponsiveness occurs in chronically treated patients as well as in newly diagnosed patients and is not alleviated by long-term drug therapy.

It was surprising that cells from naive donors that did not proliferate in response to mycobacterial antigens and did not express the T-cell-specific cytokine IL-2 did express significant levels of IFN-γ mRNA. IFN-γ is a cytokine known to be produced by activated αβT cells as well as activated γδT cells and NK cells (25). Our observations suggest that NK cells or possibly γδT cells from naive donors are the likely source of IFN-γ mRNA in the cocultured peripheral blood cells. The relatively lower amount of IFN-γ mRNA expressed by cells from TB patients corresponds to the reduced proliferative response and could result from reduced T-cell activation and/or reduced NK cell activation.

The inverse relationship between CD28 expression and the amount of IL-2 mRNA induction in these primary cell cultures corroborates previous observations by Rotteveel et al. (27) who demonstrated that low levels of CD28 expression of T-cell clones coincided with expression of the TH1 lymphokines IL-2 and IFN-γ and anti-CD3-mediated cytotoxicity. Conversely, $CD4^+$ clones with high levels of CD28 expression produced low levels of lymphokines and were not cytotoxic. The CD28 surface molecule is involved in the very complex signal transduction pathway which modulates lymphokine production. Stimulation of T cells via CD3 plus CD28 leads to high levels of cytokine mRNA and prolonged secretion of inflammatory cytokines, including IL-2, IFN-γ, TNF-α, granulocyte macrophage–colony-stimulating factor, and colony-stimulating factor 1. This is due to stabilization of certain mRNA transcripts and regulation of transcriptional initiation through CD28 stimulation (12).

Using this in vitro model of macrophage-lymphocyte interaction employing AD cells infected with virulent *M. tuberculosis* H37Rv, we have presented results which concur with those obtained in previous studies which used animal models of disease and/or avirulent organisms or mycobacterial antigens. Because of the carefully defined and controlled conditions of the coculture model, very subtle changes in T-cell subpopulation numbers and levels of cytokine induction were observed. Furthermore, with the sensitive PCR technique, we report the decreased induction of cytokines by TB patient cells in response to mycobacterial infection as expressed at the level of mRNA transcription. This assay allows the detection of small differences in mRNA quantity between individuals who differ in their anti-mycobacterial immune status. The cytokine profiles presented here suggest that patients with active TB manifest reduced activity at the T-cell, monocyte, and NK cell level compared with presensitized normal individuals. Decreased IFN-γ induction in response to *M. tuberculosis* infection may lead to diminished macrophage activation and a more

permissive environment for the bacterium. It was recently reported that PBMCs from patients with active TB, as well as those from lepromatous leprosy- and human immunodeficiency virus type 1-infected individuals, display decreased NK cell numbers and activity compared with those from normal control subjects (13, 31). Following low-dose IL-2 treatment, the leprosy patient NK cell numbers in PBMCs increased sixfold; IL-2 therapy of human immunodeficiency virus patients resulted in enhanced NK and lymphocyte-activated killer cell activity in vitro, without significant changes in the number or percentage of $CD56^+$ or $IL\text{-}2R^+$ cells in PBMCs. Since NK cells produce a number of cytokines, including IFN-γ, IL-2, and TNF-α, they may make a significant contribution to the development of a successful immune response within the infected tissue milieu. Since we amplified cytokine mRNA from a mixture of cells, it is not possible to assign a contribution to each cell type. The next step in this study will be to sort cells and determine the role of each leukocyte subpopulation in the cytokine response to viable *M. tuberculosis*. Investigations focusing on NK cell function and cytokine regulation in patients with active tuberculosis are under way.

ACKNOWLEDGMENTS

We thank Jill E. Clarridge for help with the acquisition of clinical specimens, Betty Rosenbaum for technical assistance with flow cytofluorometry, Scott Bearden for technical help on hybridization procedures, Judy Adams for preparation of figures, and Thomas A. Ficht, Gilla Kaplan, and Zanvil Cohn for helpful discussions and manuscript review.

This study was supported in part by the Public Health Service (NIH grant RO1-AI27204).

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