Differential Gene Expression in Response to Adjunctive Recombinant Human Interleukin-2 Immunotherapy in Multidrug-Resistant Tuberculosis Patients

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Administration of low-dose recombinant human interleukin 2 (rhuIL-2) in combination with multidrug chemotherapy to patients with multidrug-resistant tuberculosis (MDR TB) induces measurable changes in in vitro immune response parameters which are associated with changes in the clinical and bacteriologic status of the patients. To determine the molecular basis of these changes, we have used semiquantitative reverse transcriptase-initiated PCR (RT-PCR) and differential display technology. During rhuIL-2 treatment of MDR TB patients, decreased levels of gamma interferon (IFN-γ) mRNA in peripheral blood mononuclear cells (PBMC) relative to baseline levels were observed. However, at the site of a delayed-type hypersensitivity (DTH) response to purified protein derivative of tuberculin (PPD), the expression of cellular IFN-γ and IL-2 mRNAs was increased during rhuIL-2 therapy. Levels of other cytokine mRNAs were not significantly affected by rhuIL-2 administration. Using differential-display RT-PCR, we identified several genes expressed at the DTH skin test site which were up- or down-regulated during rhuIL-2 treatment. Cytochrome oxidase type I mRNA was increased in response to rhuIL-2 therapy relative to baseline levels, as was heterogeneous nuclear ribonuclear protein G mRNA. CD63, clathrin heavy chain, and β-adaptin mRNAs, all of which encode proteins associated with the endocytotic pathway of cells, were also differentially regulated by rhuIL-2 administration. The differential effects of IL-2 were confirmed in vitro by using PBMC obtained from PPD-positive individuals stimulated with Mycobacterium tuberculosis and IL-2. The differential expression of genes may provide a surrogate marker for leukocyte activation at a mycobacterial antigen-specific response site and for the development of an enhanced antimicrobial response which may result in improved outcomes in MDR TB patients.

Multidrug-resistant tuberculosis (MDR TB) is a devastating disease from which only 55% of immunocompetent patients recover after a prolonged course (a minimum of 24 months) of multidrug chemotherapy, while 22% ultimately die of TB (10). Patients who are coinfected with human immunodeficiency virus have a 50% response rate to antituberculosis chemotherapy, with 50% mortality due to TB (38). A potential means of improving patient response and survival is to combine optimal chemotherapy with pharmacologic augmentation of host immunity (immunomodulation). Recently, interleukin-2 (IL-2) has received increasing attention as an immunomodulatory drug in human infectious diseases. This cytokine is a pivotal regulator of cell-mediated immunity and has been shown to induce the proliferation and differentiation of lymphoid cells (36).

We have previously reported that patients with MDR TB treated with low-dose recombinant human IL-2 (rhuIL-2), in combination with optimized antituberculosis chemotherapy, show evidence of an enhanced antimicrobial response (21). In addition to decreased sputum bacillary load and decreased mycobacterial viability observed in response to rhuIL-2 adjunctive therapy, some of the patients experienced amelioration of symptoms and an improved lung radiologic picture (21, 22). These improvements were associated with an IL-2-induced increase in peripheral blood leukocyte CD25 and CD56 expression. In addition, we observed increased leukocyte proliferation in vitro in response to either purified protein derivative of tuberculin (PPD) or exogenously added IL-2, as well as an increase in lymphokine-activated killer cell-mediated cytotoxicity (21). However, the cellular and molecular events regulating these changes are as yet unclear.

The availability of MDR TB patients who are responding clinically and bacteriologically to treatment with an immune modulator such as rhuIL-2 provides a novel opportunity to investigate the molecular and cellular events involved in the Th1-type protective immune response. It is important to establish in vitro correlates of protective immunity so that the prognosis of infected individuals and the effectiveness of immune intervention strategies can be rapidly assessed; by investigating IL-2-induced changes in the immune status of patients, it might be possible to identify those changes which correlate with a favorable outcome.

In this study we have used a combination of semiquantitative reverse transcriptase PCR (RT-PCR) and differential-display technology to investigate the molecular basis of observed clinical and in vitro immunologic changes in MDR TB patients receiving adjunctive rhuIL-2 treatment to gain insight into how perturbations in immune activation might affect bacterial
clearance and clinical status. By investigating changes in gene expression in skin biopsies taken from sites of delayed-type hypersensitivity (DTH) reactions to mycobacterial antigens and from peripheral blood mononuclear cells (PBMC), we have begun to identify genes whose expression varies during rhU-2 therapy and which may contribute to the specific immune response to mycobacteria and to antimicrobial activity.

MATERIALS AND METHODS

Patient population. Fourteen human immunodeficiency virus-seronegative hospitalized patients in Cape Town, South Africa, with culture-confirmed MDR TB and currently receiving optimized antituberculosis chemotherapy enrolled in this clinical study. One group of patients (n = 10) were given twice-daily intradermal injections of 12.5 μg (225,000 IU) of rhU-2 Aldesleukin Proleukin (Chiron Corporation, Emeryville, Calif.) for 30 days as described previously (21). A group of matched MDR TB patients (n = 4) who were treated with a placebo and not rhU-2 were included as controls (Table 1). The study was approved by the research institutional review board of the Rockefeller University Hospital, New York, N.Y., and by the ethics committee of the University of Cape Town, Cape Town, South Africa. All patients gave written informed consent.

Peripheral blood collection. Blood was collected from MDR TB patients at three time points during the study: before rhU-2 administration (prestudy), after completion of 14 days of rhU-2 administration (day 15; midstudy), and 7 days following the last rhU-2 injection (day 37; poststudy). For control patients, blood was collected at equivalent time points. Blood was also collected from four normal PPD-positive donors. PBMC were isolated by using Ficoll-Hypaque gradients as described below (21).

PBMC stimulation in vitro. (i) PBMC preparation. Human PBMC were obtained from heparinized blood by centrifugation through a Ficoll-Hypaque gradient. Freshly isolated PBMC (10^6/ml) were stimulated with 50 ng/ml of PPD (5 TU/ml) or with 50 U/ml of rhU-2 Aldesleukin Proleukin (Chiron Corporation, Emeryville, Calif.) for 18 h at 37°C in 5% CO2. Cultures were harvested at 0, 6, 24, 48, 72, and 96 h poststimulation. For proliferative assays, 10^5 stimulated cells/well were incubated overnight with 1 μCi of [3H]thymidine before harvest on glass fiber filters and scintillation counting. Culture supernatants were collected for evaluation of gamma interferon (IFN-γ) levels by enzyme-linked immunosorbent assay (Medgenix, Fleurus, Belgium) according to the manufacturer’s instructions.

(ii) Preparation of mycobacterial antigen. M. tuberculosis H37Ra (Difco) was used for stimulation of PBMC in vitro. Desiccated heat-killed mycobacteria (100 μg) was suspended in R10 medium to 10 μg/ml, vortexed twice for 1 min each time, and incubated at 4°C for 1 h. The bacterial suspension was sonicated three times for 20 s each at an output of 2 at 40% duty cycles with a probe sonicator (Heat Systems Ultrasound, Farmingdale, N.Y.) and then centrifuged at 800 × g for 3 min, aliquoted into 1 ml tubes, and frozen at −70°C until used.

DTH to mycobacterial antigens. Skin tests for DTH responses to PPD (5 tuberculin units/test; Connaught Laboratories Limited, Ontario, Canada) were injected intradermally onto the forearm at the start of the study (prestudy) and on day 28 of rhU-2 adjunctive therapy (or at equivalent time points for control patients). The local skin test response was evaluated at 24-h intervals, and 4-mm punch biopsies of the skin test site were obtained at 48 h postinjection. Punch biopsies were immediately frozen in liquid nitrogen.

RNA extraction and semiquantitative RT-PCR for cytokine mRNAs. RNA was extracted from PBMC and from homogenized skin biopsies by using RNAzol B (Tel-Test, Inc., Friendswood, Tex.). Total RNAs from six of the patients treated with rhU-2 and four patients with the placebo were reverse transcribed and PCR amplified for cytokine mRNA and control mRNA by using procedures and primers described previously (20, 21). Following Southern hybridization with radiolabeled probes, PCR-amplified cDNA was quantitated by using radioanalytic imaging and normalized to the constitutively expressed glyceraldehyde 3-phosphate dehydrogenase cDNA for RNA quantity control. mRNA (poly(A)+) was isolated from in vitro-stimulated PBMC obtained from the blood of normal PPD-positive donors by using the Quick Prep micro mRNA purification kit (Pharmacia Biotech, Piscataway, N.J.) according to the manufacturer’s instructions.

DDRT-PCR. Total RNAs from PBMC or homogenized skin biopsies of six of the patients treated with IL-2 and the four placebo-treated patients were used for differential-display RT-PCR (DDRT-PCR). RNA was treated with DNase (Promega, Madison, Wis.), after which 0.5 μg of RNA was reverse transcribed by using one of the anchored primers (DD1 [T12CC], DD2 [T12CA], or DD3 [T12GA]) to PCR-amplified cDNA. Replicate reactions with enzyme omitted or with only H2O instead of RNA were set up for negative controls.

Following reverse transcription, PCRs with both anchored primers and arbitrary primers (OPA14 [TCTGTGCTGG], OPA18 [AGGTGACCGT], OPA20 [GCTCTTTGTC], OPA21 [GGTCGATCC], OPA22 [GCTGCGATCC], OPA23 [GGTCGATCC], or OPA24 [GCCTTCTGCT]) were set up. The PCR conditions were 92°C for 50 s, 40°C for 1 min, and 72°C for 1 min, for 35 cycles.

Amplification products were electrophoresed on a 6% denaturing polyacrylamide gel, dried without fixation, and exposed for 1 to 4 days to BioMax MR scientific imaging film (Eastman Kodak Co., Rochester, N.Y.). Bands which appeared to be consistently differentially expressed in multiple patient RNA isolations were excised from the dried gel, eluted for 3 h at 65°C in Tris-EDTA, and reamplified with PCR with the appropriate primer sets, low-stringency conditions, and 30 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 1 min. PCR products were then electrophoresed on a single-strand conformation polymorphism gel for analysis of product purity (26).

Cloning and sequence analysis. The reamplified cDNA was subcloned into a TA pCRII or a pCR2.1 vector (Invitrogen, San Diego, Calif.) and sequenced (Sequenase version 2.0 DNA sequencing kit [U.S. Biochemicals] or DNA Stretch Sequencer [Perkin-Elmer/Amersham Biosystems], with SP6 and T7 or M13 and T7 promoter primers, respectively). The nucleotide sequences obtained were compared with known sequences by searching the GenBank, EMBL, and EST databases with the BLASTN search program of the National Center for Biotechnology Information Blast Network (26 March 1996) (1).

Northern blots. Total RNA at 20 μg/lane was poly(A)+ RNA at 1 μg/lane was subjected to electrophoresis by the standard formaldehyde protocol (34). RNA was transferred to a Zeta-Probe membrane (Bio-Rad, Hercules, Calif.) in 50 mM NaOH and UV cross-linked. Membranes were probed with 32P-labeled antisense RNA transcribed in vitro (Promega) after ligation of an optimized T7 promoter (Ambion, Austin, Tex.) to PCR-amplified cDNA.
RESULTS

Patients with MDR TB were enrolled in this study and assigned to rhuIL-2 treatment or control study groups while maintaining their antimycobacterial drug regimen (Table 1). To monitor the effects of IL-2 on leukocyte gene expression, PBMC were isolated from the blood of all patients at baseline upon study entry (prestudy), at day 15 of rhuIL-2 treatment (or the corresponding time point for controls) (midstudy), and 7 days following the last rhuIL-2 injection (or the corresponding time point for controls) (poststudy). In addition, PPD skin tests were performed for both groups of patients at the time of study entry and again after 28 days of rhuIL-2 injections (or at similar time points for control patients). A biopsy was taken from these tuberculin-reactive skin test sites at 48 h postinjection. Sets of two biopsies (baseline and 28 days) (of about the same size) were selected from six IL-2-treated patients and the four control patients not treated with IL-2. These biopsies and blood samples from the same 10 patients were used for cytokine mRNA analysis.

Cytokine gene expression in PBMC. Southern blotting of serially diluted RNAs from PBMC (baseline, 15 days, and 37 days) of four control patients was performed. Total RNA from the samples was amplified by RT-PCR and hybridized to radiolabeled probes specific for IFN-γ and IL-2 mRNAs. In PBMC obtained from all control patients tested, IFN-γ mRNA was readily measurable at all time points. IL-2 mRNA levels were lower and were detectable in PBMC only at the highest level of input RNA (50 ng of total RNA per reaction) (not shown).

To assess the effect of exogenous rhuIL-2 administration on the expression of cytokine mRNAs in patient PBMC, we used semiquantitative RT-PCR and primers specific for IFN-γ, IL-2, tumor necrosis factor alpha (TNF-α), IL-4, IL-10, IL-12, and the T-cell marker CD36. IFN-γ mRNA levels in PBMC decreased at the mid- and poststudy time points relative to the pre-IL-2 treatment time point (Table 2). Levels of the T-cell surface marker CD36 mRNA did not change significantly during rhuIL-2 adjunctive therapy (Table 2). Since T cells are a major source of IFN-γ, the ratio of IFN-γ mRNA to CD36 mRNA was calculated to evaluate the changes in IFN-γ mRNA expression on a per-T-cell basis. A decrease in the level of IFN-γ mRNA expression per T cell during and immediately following rhuIL-2 therapy was observed (Table 2). This change was not observed in the control patients not treated with rhuIL-2. Because the expression of IL-2 mRNA in patient PBMC was very low, we did not compare the effects of rhuIL-2 treatment on the expression of this cytokine mRNA in the two study groups.

TNF-α mRNA expression was undetectable in PBMC of three of six rhuIL-2-treated patients at all time points during the study and was unchanged by rhuIL-2 therapy in PBMC of four of six patients in whom low levels of TNF-α mRNA were detected at baseline (data not shown). Levels of IL-10 mRNA in patient PBMC at the pre-, mid-, and poststudy time points were low or below the limit of detection in all patients at all time points. IL-12 mRNA and IL-4 mRNA were not detected in PBMC of patients.

Cytokine gene activation in response to PPD administration in the skin. The importance of measuring cytokine gene activation at the site of mycobacterium-specific immune reactivity was underscored by the observation that mRNA expression for the Th1 cytokines IFN-γ and IL-2 is generally higher in this specific skin test site than in cells of the peripheral blood of the same patients. Also, IFN-γ mRNA expression is higher than IL-2 mRNA expression in the cells of the DTH response to PPD at 48 h (not shown). The expression of cytokine genes, including those for IL-2, IFN-γ, IL-1β, IL-4, IL-10, IL-12, and TNF-α at the PPD site, and the influence of rhuIL-2 administration on cytokine gene expression at this site were assessed for six MDR TB patients treated with rhuIL-2. In parallel, levels of IFN-γ and IL-2 mRNAs in PPD biopsies of four control patients were quantitated.

In contrast to the results obtained with PBMC, in the biopsy material mean mRNA expression levels for the T-cell cytokines IL-2 and IFN-γ were increased (1.3- and 2.5-fold, respectively, and 3.5- and 3.0-fold, respectively, on a per-T-cell basis) during rhuIL-2 therapy relative to the prestudy timepoint (Fig. 1). When calculated as the percent change from baseline levels of gene expression, IL-2 and IFN-γ mRNA levels increased to 175 and 200%, respectively, in response to rhuIL-2 treatment (Table 2). In control patients the expression of IL-2 and IFN-γ mRNAs was not increased (115 and 110%, respectively). IL-4 mRNA was not detected in the biopsies of five of six patients and was measurable at a very low level in RNA from only one patient biopsy (not shown).

<table>
<thead>
<tr>
<th>Site</th>
<th>Cytokine</th>
<th>Time point</th>
<th>Cytokine mRNA level (% activity) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IL-2-treated patients (n = 6)</td>
</tr>
<tr>
<td>PBMC</td>
<td>IFN-γ</td>
<td>Prestudy</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midstudy</td>
<td>52 ± 11*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poststudy</td>
<td>55 ± 23**</td>
</tr>
<tr>
<td></td>
<td>CD3</td>
<td>Prestudy</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midstudy</td>
<td>131 ± 23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poststudy</td>
<td>113 ± 19</td>
</tr>
<tr>
<td></td>
<td>IFN-γ/CD3</td>
<td>Prestudy</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midstudy</td>
<td>35 ± 7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poststudy</td>
<td>42 ± 9*</td>
</tr>
<tr>
<td>PPD biopsy (48 h)</td>
<td>IL-2</td>
<td>Prestudy</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midstudy</td>
<td>175 ± 32**</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>Prestudy</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midstudy</td>
<td>200 ± 106**</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>Prestudy</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midstudy</td>
<td>76 ± 69</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>Prestudy</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midstudy</td>
<td>372 ± 176</td>
</tr>
<tr>
<td></td>
<td>IL-12</td>
<td>Prestudy</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midstudy</td>
<td>143 ± 75</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>Prestudy</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midstudy</td>
<td>124 ± 45</td>
</tr>
</tbody>
</table>

*Results are means ± standard errors of the mean. *, P < 0.05; **, P < 0.1.

Statistical analyses were carried out for the relative (percent) cytokine levels, comparing midtreatment to pretreatment results and posttreatment to pretreatment results.

ND, not done.
Levels of monocyte cytokine mRNAs were also evaluated. PPD biopsies obtained from all six rhuIL-2-treated patients had low but detectable levels of IL-1β, IL-10, IL-12, and TNF-α mRNAs (Table 2). An increase in the level of mRNA for IL-10 was observed in cells of the PPD site during rhuIL-2 therapy. IL-1β mRNA expression was not changed in the biopsies of most patients tested. Similarly, IL-12 mRNA and TNF-α mRNA expression at the site of the response to PPD administration did not change from the pretreatment to 28-day rhuIL-2 treatment time points. These results taken together indicate that administration of rhuIL2 resulted in changes in IL-2 and IFN-γ mRNA gene expression and that these changes differed at sites of specific antigen stimulation compared to responses in the peripheral blood.

Differential-display analysis of mRNAs isolated from biopsies of PPD skin test sites. In order to gain understanding of changes in expression of other genes which might occur at sites of antigenic stimulation, we used DDRT-PCR, a technique which permits the recognition of changes in expression of unselected genes (24, 25). PCR amplifications of RNA from PPD skin tests done before and during rhuIL-2 administration for six patients (or at the corresponding time points for four control patients not treated with rhuIL-2) were performed with 12 primer combinations on each RNA sample. A comparison of cDNA displays generated from RNAs isolated from patient biopsies obtained before and during rhuIL-2 administration (28 days) and from control patient biopsies obtained at similar time points is shown in Fig. 2. As can be seen from Fig. 2, several cDNA bands are differentially regulated in response to IL-2.

Table 3 summarizes information for 10 bands selected for cloning and sequence analysis. These bands were consistently differentially expressed in RT-PCR displays from a minimum of two but usually four patients. The sequencing information was compared with the combined databases from GenBank, EMBL, and EST, and the reported homologies span the entire length of the sequenced cDNA insert. cDNA band K26 was found to be 99% identical over 181 bp of the coding region of human CD63 mRNA (EMBL accession no. X62654) (19).

Band B1 was 95% homologous over 120 bp to human β-adaptin mRNA, also within the coding region (GenBank accession no. M34175) (32). cDNA B7a matched the sequence for human heterogeneous nuclear ribonuclear protein G (hnRNP-G) mRNA with 97% identity in the translated sequence over 137 bp (GenBank accession no. Z23064) (37). B7b was 99% identical over 135 bp within the coding region to human cytochrome oxidase subunit I mRNA (GenBank accession no. M10546) (35). B17 matched with 100% identity over 154 bp and cDNA B20 matched with 98% identity over 240 bp the sequence of GenBank accession no. D21260, human mRNA for the KIAA00334 gene, which is similar to rat clathrin heavy-chain mRNA (29). cDNA bands B23, B26, and B27 were found to be homologous to sequences deposited in the expressed sequence tag database created by the WashU-Merck EST Project (18). Band B23 was 97% identical over 151 bp to human cDNA clone 44827 (GB_EST H06717). Sequence B26 was 99% identical to human cDNA clone 246179 (GB_EST3: N55516). Band B27 was 98% identical over 146 bp to human cDNA clone 83610 (GB_EST6: T61071).

Differential-display analysis of mRNAs isolated from PBMC. DDRT-PCR was performed on RNA isolated from PBMC obtained from the same patients before IL-2 treatment,
detected at the post-rhuIL-2 treatment time point. Three sequences with homology to human cDNA genes, B23, B26, and B27, were similarly down-regulated in the blood and at the PPD response site. Band B24, which was 99% homologous over 140 bp to human cDNA clone 259934 (GB_EST N32924), was not identified at the PPD site but was found to be differentially regulated in PBMC (Table 3).

**TABLE 3. cDNA fragments identified by differential display in the biopsy of the PPD site**

<table>
<thead>
<tr>
<th>cDNA band</th>
<th>Regulation with IL-2</th>
<th>Primers</th>
<th>Sequence homology</th>
<th>Regulation with IL-2$^c$</th>
<th>Patient PBMC in vitro$^a$</th>
<th>Control PBMC in vitro$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K26</td>
<td>Up</td>
<td>DD2, OPA20</td>
<td>CD63</td>
<td>No</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>B1</td>
<td>Down</td>
<td>DD1, OPA18</td>
<td>β-Adaptin</td>
<td>No</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>B7a</td>
<td>Up</td>
<td>DD2, OPA18</td>
<td>hnRNP G protein</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>B7b</td>
<td>Up</td>
<td>DD2, OPA18</td>
<td>Cytochrome oxidase I</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>B17/B20$^d$</td>
<td>Up</td>
<td>DD3, DD1, OPA18</td>
<td>Clathrin heavy chain</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>B23</td>
<td>Down</td>
<td>DD1, OPA20</td>
<td>Human cDNA</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>B26</td>
<td>Down</td>
<td>DD2, OPA18</td>
<td>Human cDNA</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>B27</td>
<td>Down</td>
<td>DD2, AP4</td>
<td>Human cDNA</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>B24$^e$</td>
<td>No</td>
<td>DD2, OPA14</td>
<td>Human cDNA</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ PBMC not stimulated in vitro.
$^b$ PBMC stimulated in vitro; genes induced by *M. tuberculosis* and further up-regulated by rhuIL-2.
$^c$ Gene identified independently in the patient PPD skin test site, where it is up-regulated in response to rhuIL-2 administration, and in patient PBMC, where it is down-regulated in response to rhuIL-2 administration.
$^d$ Gene not identified by differential display at the PPD skin test site but down-regulated in PBMC in response to rhuIL-2 treatment.

**FIG. 3.** Example of cDNA display gels generated by using RNA isolated from PBMC obtained from the blood of an MDR TB patient before rhuIL-2 treatment (prestudy), on day 14 of rhuIL-2 treatment (IL-2 mid), and 7 days after rhuIL-2 adjunctive therapy (post IL-2). The arrows mark cDNA fragments potentially up- or down-regulated in response to IL-2 administration.

**TABLE 4. Effect of rhuIL-2 on antigen-induced gene expression in vitro**

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>IFN-γ (pg/ml)</th>
<th>Area under the curve (fold increase) for cDNA fragment$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B7a</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>0</td>
<td>1.057 (1)</td>
</tr>
<tr>
<td></td>
<td><em>M. tuberculosis</em></td>
<td>434</td>
<td>1.240 (1)</td>
</tr>
<tr>
<td></td>
<td><em>M. tuberculosis</em> + IL-2</td>
<td>2,169</td>
<td>1,832 (1.7)</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>0</td>
<td>713 (1)</td>
</tr>
<tr>
<td></td>
<td><em>M. tuberculosis</em></td>
<td>245</td>
<td>1,589 (2.2)</td>
</tr>
<tr>
<td></td>
<td><em>M. tuberculosis</em> + IL-2</td>
<td>5,477</td>
<td>2,086 (3)</td>
</tr>
</tbody>
</table>

$^a$ B7a, hnRNP-G; B7b, cytochrome oxidase I; B17, clathrin heavy chain. B7a and B17 were evaluated from total RNA; B7b was evaluated from mRNA. Fold increases are normalized to baseline values. Results are from one representative experiment of three such experiments and are means of triplicate samples.
clathrin heavy chain, both expressed at relatively high levels in resting cells, were evaluated from total cellular RNA. However, the gene for cytochrome oxidase I was not expressed at high enough levels in total RNA and required isolation of mRNA for quantitation of differential expression. The gene for cytochrome oxidase I (B7b) appeared to be most sensitive to differential regulation in the presence of IL-2 (Table 4 and Fig. 4). A similar pattern was reproducibly observed in cells of the same donor, and in different donors, although the degree of expression varied from donor to donor.

**DISCUSSION**

In our previously reported studies, patients with MDR TB received low-dose rhuIL-2 in combination with optimized multidrug antituberculous therapy. We observed immunologic responses to this adjunctive treatment which were associated with clinical, bacteriologic, and radiologic changes. Changes included decreased sputum bacterial load and viability, improved chest X-ray results, and decreased symptoms in approximately 60% of patients (21, 22). During rhuIL-2 administration, there was enhanced in vitro lymphocyte proliferation to mycobacterial antigens, augmented leukocyte proliferative activity to IL-2 in limiting-dilution assay in vitro, increased lymphokine-activated killer activity, and expanded numbers of CD25 \(^+\) (low-affinity IL-2R) and CD56 \(^+\) (NK) cells in the peripheral blood. In order to define the underlying cellular and molecular bases of these changes, we have assayed the changes in transcriptional activity of mononuclear cells of the blood and in the biopsies of PPD-reactive skin test sites at time points during and following rhuIL-2 adjunctive therapy for comparison with levels measured at the pre-IL-2 time point.

As observed in our previous study (21), we report here that levels of IFN-\(\gamma\) mRNA in PBMC generally decreased during rhuIL-2 administration coordinately with improvement in patient symptoms. Other cytokine mRNA levels in the cells of the peripheral blood of these patients, including IL-1\(\beta\), IL-2, IL-4, TNF-\(\alpha\), IL-10, and IL-12 mRNA levels, remained unaffected by rhuIL-2 administration. A different pattern was observed in the PPD skin test sites, where we observed increased expression of IL-2 and IFN-\(\gamma\) mRNAs during rhuIL-2 therapy. This effect was selective, since the expression of other cytokines, including IL-1\(\beta\), IL-12, and TNF-\(\alpha\), was not significantly affected. In our pilot study (21) we had reported that cytokine mRNA expression levels in cells at the PPD site were not significantly influenced by 14 days of adjunctive rhuIL-2 therapy. In our current study we performed the second PPD injection after 28 days of rhuIL-2 therapy and took a biopsy from the site on day 30 of treatment. Thus, the effects of IL-2 were evaluated in the present study following a longer period of rhuIL-2 treatment. It is possible that the additional 14 days of daily rhuIL-2 administration may have significantly modified the host response to antigen stimulation in the skin.

IL-2 and IFN-\(\gamma\) have been demonstrated to play major roles in the development of cell-mediated immunity (CMI) to mycobacterial infection. In patients infected with *Mycobacterium leprae*, expression of IL-2 and IFN-\(\gamma\) predominates in the skin lesions of tuberculoid leprosy patients with an active CMI response, whereas these cytokines are not expressed in the skin of lepromatous leprosy patients, who do not have protective CMI to the infecting bacilli (41). Moreover, IFN-\(\gamma\) production is enhanced at the site of disease in the pleural fluid in patients with tuberculous pleuritis (2). Since this manifestation of tuberculosis is usually self-resolving concomitant with the emergence of CMI (15), these observations provide evidence for the contribution of the Th1-type cytokine IFN-\(\gamma\) in protective immunity to mycobacterial infections.

The reduced levels of IFN-\(\gamma\) mRNA seen in PBMC, in contrast to the increase seen at the PPD site, suggest that in response to rhuIL-2 administration, mycobacterium-specific T cells home to sites of antigenic stimulation, such as the infected lung or the PPD skin test site, where they become primed or activated to produce elevated levels of Th1 cytokines. These results reinforce the importance of investigating immune responses at the sites of infection, particularly when attempting to identify correlates of protective immunity, since responses in the peripheral blood may not truly reflect antigen-specific responses that are occurring elsewhere.

IFN-\(\gamma\) is a major contributor to macrophage activation (16, 17) and to the induction of two important antimycobacterial pathways, leading to the generation of reactive oxygen intermediates and l-arginine-derived nitric oxide (NO) (7, 8, 11, 30, 33, 40). Despite the evasive mechanisms employed by mycobacteria to block the production and resist the toxic effects of oxygen radicals as demonstrated in vitro (4, 8, 12, 13), the respiratory burst generated by IFN-\(\gamma\)-activated phagocytes may play an important role in the control of *M. tuberculosis* in vivo. This pathway might contribute to the rhuIL-2-induced bacteriologic effects seen in this study.

The use of DDRT-PCR to identify novel genes which may be IL-2 regulated at the site of an antigen-specific response (the PPD skin test site) has provided some intriguing findings. Three of the differentially expressed genes, those for CD63, clathrin heavy chain, and \(\beta\)-adaptin, are associated with the endocytic vacuolar pathway of cells. CD63 is a lysosomal membrane glycoprotein and has been shown to be present on the membranes of macrophage phagosomes containing dead *M. tuberculosis* bacilli and not present on phagosomes containing live organisms (9). Thus, the molecule appears to be associated with a more mature vacuole. Clathrin heavy chain is a structural component of coated pits and vesicles which are responsible for selective endocytosis of ligand-bound plasma membrane proteins, including receptors for various extracellular ligands. Clathrin-coated vesicles are also associated with the trans-Golgi network and facilitate the delivery of newly synthesized lysosomal enzymes to a prelysosomal compartment (6). \(\beta\)-Adaptin is a subunit of the plasma membrane adapter complex which functions in binding to the cytosolic domains of certain receptors, leading to the assembly of clathrin (31). Differential regulation of genes encoding proteins which are involved in vacuolar maturation and trafficking, either directly by IL-2 or indirectly as a downstream consequence of changes
in expression of other cytokines such as IFN-γ, suggests that the intracellular fate of mycobacteria might be modulated by administration of rhuIL-2.

The observation that cytochrome oxidase type I is up-regulated in response to IL-2 is also interesting in that it suggests that changes in oxidative metabolism may be a feature of the increased antigen-specific activation following administration of rhuIL-2. Cytochrome oxidase is located in the mitochondrial membrane and is the last component in the chain of electron transport resulting in production of ATP (5). The differential expression of this mRNA species induced by rhuIL-2 administration may indicate an increased level of mitochondrial respiration associated with enhanced leukocyte activation and/or increased phagocyte oxidative metabolism in response to increased production of IFN-γ by T cells. Cytokine-induced changes in the expression of genes encoding various oxidase components were recently described for another system. Patients with chronic granulomatous disease treated with IFN-γ improved clinically, with a reduction in the incidence of infections (39). IFN-γ administration in these patients, as well as in vitro stimulation of monocytes and neutrophils with IFN-γ, resulted in a significant increase in the level of a cytosolic NADPH oxidase mRNA. The observation that cytochrome oxidase type I is differentially regulated in this system may be important in the control of mycobacterial infection for another reason. Increases in cytochrome oxidase would result in increases in ATP levels in the macrophages. Since ATP has been shown to induce apoptosis of mycobacterium-infected macrophages and decreased viability of intracellular mycobacteria, up-regulation of this pathway may also be involved in the clearance of bacilli in the patients treated with rhuIL-2 (23, 27).

The different pattern of differential regulation of genes in the peripheral blood leukocytes compared to that in the cells migrating into the site of PPD deposition in the skin is of interest. The fact that genes are not found to be differentially expressed does not prove conclusively that they are not affected by rhuIL-2 treatment. Rather, our observations suggest that these genes are not obviously modulated in PBMC in response to rhuIL-2 treatment of patients. Thus, we may have confirmation for selective effects of rhuIL-2 treatment on cells migrating to an antigen-specific response site compared to effects in cells in the circulation.

Our results indicate that differential-display technology can be used to identify subtle changes in transcriptional expression of genes in cells of tissues or blood samples obtained during rhuIL-2 (or other immunomodulatory) adjunctive therapy. Because of the very limited quantity of patient RNA available for these assays, it is not possible to expand the analysis of differential regulation of putatively identified transcripts by using the most quantitative assay, i.e., Northern blotting. We therefore used PBMC from normal PPD-positive donors to confirm that the putatively identified transcripts are indeed differentially regulated following exposure to rhuIL-2. When cells stimulated by Mycobacterium were treated with rhuIL-2 in vitro, we observed up-regulation of the same genes, this time by utilizing a quantitative assay, i.e., Northern blotting. Thus, our studies have identified a number of novel candidate genes whose expression can now be studied in detail in order to evaluate their role in the antituberculous response which is enhanced by the administration of rhuIL-2.

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