Induction of Transforming Growth Factor β 1 by Purified Protein Derivative of *Mycobacterium tuberculosis*

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We examined the ability of purified protein derivative (PPD) of *Mycobacterium tuberculosis* to induce transforming growth factor β 1 (TGF- β 1), a potent immunosuppressive and macrophage-deactivating molecule, in blood monocytes from healthy individuals. TGF- β 1 activity in PPD-induced monocyte supernatants was identified by Western immunoblot analysis and was not inhibited by polymyxin B, an inhibitor of bacterial lipopolysaccharide (LPS). Furthermore, PPD at equivalent amounts in weight to LPS was as potent in stimulation of monocyte production of TGF- β 1 at 24 h of culture, as quantified by enzyme-linked immunosorbent assay. The inducing effect of PPD, in contrast to that of LPS, was sustained at later time points of culture (72 h). PPD enhanced the constitutive expression of TGF- β 1 steady-state mRNA in monocytes at 24 and 48 h of culture. In contrast, neither mycobacterial heat shock protein (64-kDa protein of *M. bovis*) nor LPS induced TGF- β 1 mRNA. Decay studies suggested a transcriptional rather than a posttranscriptional effect of PPD on TGF- β 1 gene expression.

Mycobacteria and their constituents are potent inducers of the production and release of proinflammatory cytokines by mononuclear phagocytes. Interleukin-1 (IL-1) and tumor necrosis factor α (TNF- α) are essential in further activation of the accessory and effector functions of the mononuclear phagocyte. Both IL-1 and TNF- α are induced by the culture filtrate and purified protein derivative (PPD) of *Mycobacterium tuberculosis* (36, 37). TNF- α has also been shown to be expressed by human monocytes and alveolar macrophages stimulated with whole mycobacteria (30) and by mouse macrophages stimulated with the mycobacterial glycolipid lipoarabinomannan (6). Furthermore, PPD and other mycobacterial antigens such as the 64-kDa heat shock protein (HSP) of *M. bovis* induce the expression of IL-2 receptors (IL-2R) on monocytes (27). Interaction of monocyte IL-2R with IL-2 enhances the antimicrobial activity of such cells against a number of pathogens through augmentation of reactive oxygen intermediates (35) and amplifies the effect of TNF- α against *M. avium* (3).

Another product of activated monocytes found abundantly at sites of inflammation is transforming growth factor β 1 (TGF- β 1), the prototype of the three isoforms of TGF- β (TGF- β 1, TGF- β 2, and TGF- β 3) found in mammalian tissues (32). TGF- β s are important in tissue repair and share a wide range of immunoregulatory activities. With regard to the inflammatory milieu in particular, TGF- β 1 is chemotactic to monocytes (33), induces the expression of receptors for the constant region of immunoglobulin (Fc γ RIII), which is important in immunophagocytosis (38), and regulates the production of IL-1, IL-6, and TNF- α (5, 20) and other growth factors, including TGF- β 1 itself (31). On the other hand, TGF- β 1 is anti-inflammatory in that it deactivates macrophage production of oxygen (29) and nitrogen intermediates (8) and

inhibits gamma interferon-induced HLA-DR expression by monocytes (7). Moreover, TGF- β 1 inhibits T-cell proliferation and interferes with the effector functions of cytotoxic T cells (19, 21) and natural killer cells (19, 26).

TGF- β 1 is secreted by cells in culture in a biologically latent form (110 kDa), (15, 17), which limits its potential bioactivity. Activation of latent TGF- β 1 by acidification, alkalization, or chaotropic agents in vitro (18) and possibly by the cell surface (23) or plasmin (15) in vivo releases mature TGF- β 1 (25 kDa), which is noncovalently associated with the remainder of its precursor.

Recently, expression of TGF- β 1 within a variety of chronic inflammatory lesions has been examined. Abundant TGF- β 1 activity has been demonstrated in the rat model of bleomycin-induced pulmonary fibrosis (12) and murine streptococcal cell wall-induced liver granuloma (16). Our recent studies have shown that TGF- β 1 is present in macrophages of granulomatous lesions of patients with active pulmonary tuberculosis. Furthermore, blood monocytes from patients with tuberculosis contain TGF- β 1 (27a). Whether this observation relates to exposure of lung macrophages or circulating monocytes to mycobacteria and its products is unknown. In the present study we examined the effect of PPD of *M. tuberculosis* on the expression of TGF- β 1 by human monocytes. We found that PPD was as potent as bacterial lipopolysaccharide (LPS) as an inducer of monocyte TGF- β 1 protein. Furthermore, and unlike LPS, the enhanced TGF- β 1 activity was partly due to an enhanced transcription of TGF- β gene in monocytes.

MATERIALS AND METHODS

Preparation of cells. Peripheral blood mononuclear cells were isolated by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation from heparinized blood of healthy individuals whose PPD skin test was negative (4). The peripheral blood mononuclear cells (5×10^6 /ml) were resuspended in RPMI 1640 (BioWhittaker Inc., Walkersville, Mass.) supplemented with 5% (vol/vol) heat-inactivated human AB serum and 5% (vol/vol) heat-inactivated low-endotoxin (0.1 ng/ml) fetal calf serum (Hyclone Laboratories, Logan, Utah) and incubated at 37°C for 1 h on plastic petri dishes (100 by 20 mm; Falcon Labware, Oxnard, Calif.) which had been precoated with AB

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serum. After removal of nonadherent cells, the adherent monolayers were dislodged by gentle scraping with a rubber policeman. Adherent cells thus obtained were 90% monocytes as determined by peroxidase staining (10) and were 99% viable by trypan blue staining.

Generation of monocyte conditioned supernatants. Adherent cells were suspended in RPMI 1640 supplemented with 2 mM L-glutamine, penicillin and streptomycin (100 μ /ml each), and 0.5% fetal calf serum at 10^6 cells per ml and added to 24-well plates. PPD (a gift of Lederle Laboratories, American Cyanamid Co., Wayne, N.J.), or LPS of *Escherichia coli* F583 (Sigma Chemical Co., St. Louis, Mo.) was added to cell cultures at the indicated concentrations. After 24 and 72 h, culture supernatants were harvested by centrifugation at $175 \times g$ for 5 min. Cell-free supernatants were stored at -70°C in aliquots until use. Each aliquot was thawed once prior to use. In control experiments, freeze-thaw treatment lowered the activity of both recombinant TGF- β 1 (Genentech, South San Francisco, Calif.) and purified TGF- β 1 (Rand D systems, Minneapolis, Minn.) insignificantly (about 5%). The PPD used in this study contained 16 ng of endotoxin per mg of protein as measured in the chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker). LPS at 1.6 ng/ml, which corresponds to the amount of endotoxin contamination of PPD at the highest concentration used (100 μ g/ml), did not induce TGF- β 1 in monocyte cultures. Optimal induction of TGF- β 1 was seen at 10 μ g of LPS per ml.

Western immunoblot for TGF- β 1. Supernatants were dialyzed against two changes of $0.1 \times$ Dulbecco's phosphate-buffered saline (PBS; BioWhittaker) and then freeze-dried. The residues were reconstituted with 100 μ l of 4 mM HCl plus 1 mg of bovine serum albumin (BSA; Sigma) per ml. Samples (15 μ l) were electrophoresed in a 12% polyacrylamide gel under dissociating (0.1% sodium dodecyl sulfate [SDS]) but nonreducing conditions by the method of Laemmli (13) and then transferred to nitrocellulose paper (Sigma) by the method of Towbin et al. (28). After excess protein-binding sites were blocked with 3% BSA–20% fetal calf serum–PBS solution, the membranes were incubated with a polyclonal chicken anti-human TGF- β 1 antibody (AB-101; R and D Systems, Minneapolis, Minn.), which shows a low (<5%) cross-reactivity with TGF- β 2 and TGF- β 3, or chicken immunoglobulin G (R and D Systems) at a 1:1,000 dilution overnight at room temperature. Membranes were washed three times with PBS and incubated with alkaline phosphatase-conjugated goat anti-chicken immunoglobulin G (Zymed) (1:1,000) for 4 h. After being washed again, membranes were exposed to substrate solution. The reaction was stopped with distilled water, and the immunoblots were dried and photographed. Membranes incubated with chicken immunoglobulin G did not show any reactivity.

Quantitation of TGF- β 1. An enzyme-linked immunosorbent assay (ELISA) for TGF- β 1 was used for quantitation of TGF- β 1. Immulon 4 plates (Dynatech Laboratories, Chantilly, Va.) were coated with a mouse monoclonal antibody to human TGF- β 1 (12H5; a gift from Genentech). This antibody has been previously demonstrated to recognize TGF- β 1 but not TGF- β 2 or TGF- β 3 (14). The polyclonal chicken anti-human TGF- β 1 antibody (R and D Systems) was used as a capping antibody. All samples were treated with 1 N HCl acid for 30 min and neutralized with 1 N NaOH–N-2-hydroxyethylpiperazine–N'-2-ethanesulfonic acid (HEPES). A standard curve was generated with recombinant TGF- β 1 over a concentration range of 0.01 to 10 ng/ml. This assay is sensitive to 50 pg of TGF- β 1 activity per ml.

Isolation and analysis of RNA. Total RNA was extracted from monocytes (7×10^6 to 10×10^6) by the guanidinium-cesium method as previously described (27). For Northern (RNA) blot analysis, 5 to 10 μ g of RNA was electrophoretically separated through a 1% agarose–2.2 M formaldehyde gel containing ethidium bromide (0.5 μ g/ml). RNA was then transferred to nylon membranes and cross-linked to the membranes with UV light (Stratagene). The membranes were prehybridized (1 M sodium phosphate, 15% formamide, 0.1% BSA, 0.5 M EDTA, 20% SDS) for 4 h at 42°C and then hybridized in a solution similar to prehybridization solution that contained the *Eco*RI insert of human TGF- β 1 cDNA (a gift from J. Bell, University of Illinois) which was ^{32}P labeled by random priming. Filters were washed in $1 \times \text{SSC}$ –0.01% SDS ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate) at 55°C for 15 min and exposed to XAR-5 film (Kodak Laboratories, Rochester, N.Y.) at -70°C with an intensifying screen. To ensure equal loading of RNA, each blot was stripped with $0.5 \times \text{SSC}$ –0.1% SDS–50% formamide at 65°C for 30 min and then reprobed with a ^{32}P -labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe (American Type Culture Collection).

Statistical analysis. Data were analyzed by the paired *t* test. Significant values are considered to occur at $P < 0.05$.

RESULTS

Production of TGF- β by PPD-stimulated monocytes. We first analyzed PPD-stimulated monocyte culture supernatants for TGF- β activity by the Western blot technique. For this purpose, culture supernatants were dialyzed, lyophilized, and resuspended in 4 mM HCl before being subjected to SDS-polyacrylamide gel electrophoresis. The denaturing gel conditions (SDS) of this technique have been previously shown to be sufficient for activation of most of the latent TGF- β (18). PPD

FIG. 1. Western blot analysis of PPD induced TGF- β 1 activity. Monocytes were cultured with PPD (10 μ g/ μ l) or medium alone for 24 h and concentrated cell-free supernatants were subjected to Western blotting (see Materials and Methods). Purified TGF- β 1 (R and D Systems) at 10 and 2 ng was analyzed simultaneously. Molecular weight markers (MW) (in thousands) are shown on the left. The positions of fully activated (25-kDa) and partially activated (50-kDa) TGF- β 1 are shown by arrows on the right.

at 10 μ g/ml induced TGF- β 1 (Fig. 1), and whereas most of the activity was visible as a 25-kDa band, some reactivity was also present around 50 kDa, which probably represents partial activation of TGF- β 1. The purified TGF- β 1 prepared from platelets (purchased from R and D Systems) run simultaneously with the monocyte supernatants contained an identical faint band of this higher molecular mass in addition to the major activity visible at 25 kDa. Supernatants were generated over a concentration range of PPD (0, 1, 10, and 100 μ g/ml) in the presence and absence of polymyxin B (10 μ g/ml) and analyzed by Western blotting. Polymyxin B at 10 μ g/ml completely abolishes cytokine induction by LPS at 1 μ g/ml. Induction of TGF- β 1 was seen at both 10 and 100 μ g of PPD per ml and was unaffected by polymyxin B (data not shown).

Next, the concentration of TGF- β 1 in monocyte supernatants were assessed by ELISA. Monocytes were cultured in the presence of PPD at 10 μ g/ml, LPS at 10 μ g/ml, or medium alone, and cell-free supernatants were collected after 24 h. Supernatants were acid activated as described in Materials and Methods. As compared with unstimulated cultures, PPD at 10 μ g/ml induced 1.5- to 11-fold (mean, 4-fold)-higher concentrations of TGF- β 1 in the nine donors tested ($P < 0.01$) (Fig. 2). The induction of TGF- β 1 by PPD and LPS was comparable at 24 h. In three donors, PPD- and LPS-induced TGF- β 1 was

FIG. 2. Induction of TGF- β 1 by PPD and LPS in monocytes. Monocytes were cultured with PPD (10 μ g/ml), LPS (10 μ g/ml), or medium alone for 24 h. Cell-free supernatants were assessed for TGF- β 1 by ELISA. Results are the mean $\pm$ standard error of the mean for nine experiments.

FIG. 3. TGF- β mRNA expression in monocytes. Cells were incubated with PPD (10 μ g/ml), LPS (10 μ g/ml), or medium alone (-), and total RNA was assessed for TGF- β 1 mRNA (2.5 kb) at 3 h (5 μ g of RNA), 24 h (10 μ g of RNA), and 48 h (8 μ g of RNA). Stripped blots were assessed for GAPDH expression. Note the higher GAPDH levels of RNA expressed in unstimulated monocytes at 48 h, indicating extra loading.

assessed both at 24 and at 72 h. There was an almost-twofold augmentation in the amount of PPD-induced TGF- β 1 in monocyte cultures at 72 h compared with 24 h (2.128 ± 0.31 and 1.163 ± 0.08 μ g/ml, respectively; $P < 0.05$) whereas LPS-induced cytokine activity remained the same (1.577 ± 0.29 and 1.377 ± 0.145 μ g/ml respectively). In two experiments, the mycobacterial HSPs of *M. bovis* (64 kDa) and *M. tuberculosis* (70 kDa) were used at several doses (1, 10, and 25 μ g/ml) to induce TGF- β 1. Cytokine activity was optimally generated by these reagents at 10 μ g/ml; however, the induced activity was about 1/10 of that of LPS- or PPD-induced TGF- β 1 (data not shown).

Induction of TGF- β 1 mRNA by PPD. The expression of TGF- β 1 mRNA in blood monocytes, similar to other cell types, is constitutive (1). However, to examine if increased TGF- β 1 activity in culture supernatants of PPD-stimulated monocytes was associated with transcription of TGF- β 1 mRNA, Northern blot analysis for TGF- β 1 was performed. Monocytes were cultured in the presence of PPD at 10 μ g/ml, LPS at 10 μ g/ml, or medium alone, and total-cell RNA was assessed for TGF- β 1 activity after 3, 24, and 48 h of culture. Stripped blots were reprobed with a GAPDH riboprobe, and densitometric analysis was performed. Whereas similar levels of TGF- β 1 mRNA were seen at 3 h, a significant induction of TGF- β 1 transcription was seen in PPD-stimulated monocytes compared with unstimulated monocytes (3.2-fold higher) at 24 h (Fig. 3). On the other hand, a down regulation of TGF- β 1 mRNA at 24 h was apparent in LPS-stimulated monocytes compared with unstimulated monocytes (2.4-fold lower). At 48 h, PPD-induced levels remained higher than LPS-induced levels. Next, TGF- β 1 mRNA was compared in monocytes stimulated with PPD, the 64-kDa HSP of *M. bovis*, the 70-kDa HSP of *M. tuberculosis*, and LPS after 24 h of culture. All stimuli were used at 10 μ g/ml. Again, PPD induced higher mRNA levels than did LPS (3.5-fold), the 64-kDa HSP (3.8-fold), and the 70-kDa HSP (1.5-fold) (Fig. 4). Some induction of TGF- β 1 mRNA by the *M. tuberculosis* 70-kDa HSP was also seen.

mRNA decay studies. To assess if the increase in steady-state TGF- β 1 mRNA levels seen in PPD-induced monocytes was due to stabilization of TGF- β 1 mRNA, decay studies were performed. Monocytes were cultured in the presence of PPD (10 μ g/ml), LPS (10 μ g/ml), or medium alone for 1 h and then received actinomycin D at 5 μ g/ml. Cultures were terminated at 1, 3, and 6 h, and total-cell RNA was assessed for TGF- β 1 expression by Northern blot analysis (Fig. 5). The mRNA decay rate of PPD-stimulated monocytes was only slightly higher than that of unstimulated monocytes. On the other hand, monocyte cultures containing LPS demonstrated a rapid decay of TGF- β 1 mRNA, over twice that of unstimulated cultures. In

FIG. 4. Induction of TGF- β mRNA by PPD. Monocytes were cultured in medium alone (lane 1) or with PPD (lane 2), the 64-kDa HSP of *M. bovis* (lane 3), the 70-kDa HSP of *M. tuberculosis* (lane 4), or LPS (lane 5). Each stimulant was used at a concentration of 10 μ g/ml. Northern blot analysis was performed on total RNA extracted at 24 h for TGF- β 1. Stripped blots were assessed for GAPDH expression.

two other similar experiments, decay rates of PPD-stimulated and unstimulated cells were comparable to each other. The half-lives of TGF- β 1 mRNA in PPD-stimulated and unstimulated monocytes were 7.4 and 8.6 h, respectively.

DISCUSSION

The findings in this study indicate that PPD of *M. tuberculosis* strongly induces monocytes to produce TGF- β 1. Induction of TGF- β 1 by PPD was neither due to LPS contamination nor due to the presence of mycobacterial HSPs. PPD was more potent in induction of TGF- β 1 than was LPS at equivalent concentrations, since at later time points of culture (72 h) the cytokine activity was higher than for LPS-stimulated monocyte cultures. Furthermore, PPD increased steady-state TGF- β 1 mRNA levels in monocytes. Since the stability of TGF- β 1 mRNA was not increased by PPD, transcriptional activation of TGF- β 1 gene by PPD is suggested.

TGF- β 1 is one of five homodimeric polypeptides forming the TGF β family; these peptides share 70 to 80% homology and several biological activities. Of the three isoforms found in mammalian tissues, TGF- β 1 is the predominant isoform produced by activated mononuclear phagocytes (1), and in view of its potent and at times biomodal effects on immunomodulation

FIG. 5. Degradation of TGF- β 1 mRNA. Monocytes were cultured in medium alone (—), or with PPD (---) or LPS (---). Cultures then received actinomycin D (5 μ g/ml) and terminated at 0, 1.5, 3, and 6 h after addition of actinomycin D. Total RNA was assessed for TGF- β 1 by Northern blot analysis. Stripped blots were assessed for GAPDH expression. Demistometry was performed on the autoradiographs, and TGF- β 1 readings were corrected for GAPDH levels. Data are expressed as the percentage of TGF- β 1 mRNA remaining, calculated as (corrected density at each time point/corrected density at time 0) $\times$ 100.

and macrophage function, its role in pathogenesis of diseases is likely to be critical. Whereas TGF- β 1 enhances the replication of a number of intracellular pathogens (25) including *M. avium* (2) and *M. tuberculosis* (9a), its role in the immunopathogenesis of infectious processes is not fully understood. However, murine streptococcal cell wall-induced granulomas (34) and lung granulomatous lesions from patients with untreated tuberculosis contain TGF- β 1 (27a). It is therefore interesting that PPD prepared from autoclaved culture filtrate of *M. tuberculosis* induces the expression of TGF- β 1 mRNA and protein in monocytes.

We have recently observed that lipoarabinomannan of *M. tuberculosis* is in general a poor inducer of many cytokines in human monocytes; levels of TGF- β 1 were less than 50% of levels of LPS-induced cytokine activity (7a). Furthermore, as observed in this study and in contrast to induction of IL-2R (27) and TNF- α (30), mycobacterial HSPs, including the 64-kDa HSP of *M. bovis* and the 70-kDa HSP of *M. tuberculosis*, were not potent in induction of TGF- β 1. Therefore, the capacity to induce TGF- β 1 by PPD is probably due to the non-HSP peptides contained in PPD rather than to the HSP peptides or lipoarabinomannan of PPD.

Increased expression of steady-state TGF- β 1 mRNA by PPD was of interest in view of the constitutive expression of TGF- β 1 in all cell types (22) and the evidence indicating that regulation of TGF- β 1 activity occurs mainly at the level of translation and activation of the latent molecule (17, 22). In particular, bacterial LPS did not induce TGF- β 1 transcription in monocytes or monocytic cell lines (1). Our data confirm this finding and further indicate that LPS-treated monocytes have shorter TGF- β 1 transcript half-lives than do untreated monocytes. Furthermore, TGF- β 1 mRNA survival was not up regulated by PPD. Therefore, the enhanced steady-state expression of TGF- β 1 in PPD-treated monocytes may presumably be due to an enhanced transcriptional rate or to increased transport of TGF- β 1 mRNA into the cytoplasm, or both. Transcriptional activation of TGF- β 1 has been shown in streptococcal cell wall-induced granulomas in mice (16) and in in vivo models of myocardial infarction, bone fracture healing, and liver regeneration (22). We have observed that freshly isolated monocytes from some but not all patients with active pulmonary tuberculosis have enhanced expression of TGF- β 1 mRNA compared with monocytes from healthy individuals (27a). Additionally, unstimulated monocytes from patients contained and secreted TGF- β 1. Whether these observations are secondary to priming of blood monocytes by mycobacterial products in vivo is conjectural, although the present data would support this possibility since mycobacterial PPD induced TGF- β 1.

Activation of TGF- β 1 should also be interpreted in the context of other cytokines, such as TNF- α and IL-1 β , that are induced by PPD in human monocytes. Since TGF- β 1 modulates the expression and activity of these cytokines (5, 9) and additionally down regulates T-cell responses (11, 24), activation of TGF- β 1 may be critical in the ability of the host to control an activated immune response in the setting of tuberculosis. In fact, mycobacterial protein-induced TGF- β 1 in monocytes may be responsible, at least in part, for the well-established immunosuppression by active tuberculosis (27). Further studies are needed to establish the relevance of TGF- β 1 in the pathogenesis of disease due to *M. tuberculosis*.

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