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Research Article

Native 30-kD antigen, also known as alpha antigen, is a fibronectin-binding protein that is secreted by live Mycobacterium tuberculosis. This antigen may play an important biological role in the host-parasite interaction since it elicits delayed type hypersensitivity response and protective immunity in vivo and T lymphocyte blastogenesis and IFN-gamma production in vitro. In the present study, we show that, TNF-alpha protein is produced in monocyte culture supernatants in response to 30-kD antigen and the level is as high as that to purified protein derivative of M. tuberculosis. This stimulatory effect was not due to contamination with either bacterial lipopolysaccharide or mycobacterial lipoarabinomannan. The preincubation of monocytes with plasma fibronectin significantly enhanced the release of TNF-alpha into the culture supernatants in response to 30-kD antigen. This effect was blocked by polygonal antibody to plasma fibronectin. In contrast, the monocytic cell line U937 failed to release TNF-alpha protein in the culture supernatants in response to 30-kD antigen with or without preincubation with plasma fibronectin. To determine whether this observation was due to differential binding of the 30-kD to fibronectin on these cells, a cell based ELISA was used. Pretreatment of monocytes with fibronectin enhanced their binding of the 30-kD antigen. U937 cells bound the 30-kD antigen weakly with or without fibronectin pretreatment. These results indicate that 30-kD antigen which is a known […]

Induction of Monocyte Expression of Tumor Necrosis Factor α by the 30-kD α Antigen of *Mycobacterium tuberculosis* and Synergism with Fibronectin

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Abstract

Native 30-kD antigen, also known as α antigen, is a fibronectin-binding protein that is secreted by live *Mycobacterium tuberculosis*. This antigen may play an important biological role in the host–parasite interaction since it elicits delayed type hypersensitivity response and protective immunity in vivo and T lymphocyte blastogenesis and IFN-γ production in vitro. In the present study, we show that, TNF-α protein is produced in monocyte culture supernatants in response to 30-kD antigen and the level is as high as that to purified protein derivative of *M. tuberculosis*. This stimulatory effect was not due to contamination with either bacterial lipopolysaccharide or mycobacterial lipoarabinomannan. The preincubation of monocytes with plasma fibronectin significantly enhanced the release of TNF-α into the culture supernatants in response to 30-kD antigen. This effect was blocked by polyclonal antibody to plasma fibronectin. In contrast, the monocytic cell line U937 failed to release TNF-α protein in the culture supernatants in response to 30-kD antigen with or without preincubation with plasma fibronectin. To determine whether this observation was due to differential binding of the 30-kD to fibronectin on these cells, a cell based ELISA was used. Pretreatment of monocytes with fibronectin enhanced their binding of the 30-kD antigen. U937 cells bound the 30-kD antigen weakly with or without fibronectin pretreatment. These results indicate that 30-kD antigen which is a known secretory antigen of *M. tuberculosis* is a stimulus for human monocytes to express TNF-α and that stimulatory effect may be mediated through plasma fibronectin. (*J. Clin. Invest*. 1996. 98:1261–1268.) Key words: monocytes • U937 cells • purified protein derivative • TNF-α • *M. tuberculosis*

Introduction

There has been increasing interest in the secreted antigens of growing mycobacteria as important for the development of protective cell-mediated immunity. This hypothesis derives from the finding that immunization with live *Mycobacterium tuberculosis* efficiently generates protective T lymphocyte responses, but killed preparations do not (1).

Proteins of the mycobacterial antigen 85 complex (85 A, B, and C) are major secretory products of actively proliferating *M. tuberculosis* and account for as much as 30% of culture filtrate proteins (2). The antigen 85 complex is comprised of genetically distinct proteins with known sequences, which are antigenically related (3).

The 85B (30-kD) antigen is also known as alpha antigen (4, 5) and antigen 6 (6). The gene and the amino acid sequences for alpha antigen are known for *M. bovis*, *M. kansasii*, and *M. avium* (5, 7, 8). The 30-kD antigen evokes delayed type hypersensitivity (DTH) skin test responses in sensitized guinea pigs with somewhat more specificity than purified protein derivative (PPD)[1] (9) and recently has been shown to induce protective immunity (10). It stimulates lymphocyte blastogenesis and interferon-γ production in healthy individuals, but not in patients with pulmonary tuberculosis (11, 12). In contrast, antibody to the 30-kD antigen is increased in patients with tuberculosis (13). This antigen has recently been shown to be a mycoyl transferase (14) and as such is important in *M. tuberculosis* cell wall synthesis. Interestingly guinea pigs immunized with 30 kD were protected against a challenging with *M. tuberculosis.* Therefore 30-kD antigen has assumed importance both in the biology of *M. tuberculosis* and in induction of host protective immunity.

Abou-Zeid et al. reported that 30-kD antigen from 3-d-old culture supernatant of *M. tuberculosis* binds fibronectin (15). Fibronectins (FN) are a family of high molecular weight glycoproteins found in plasma and tissues that are involved in cell motility and adhesion, regulation of cell morphology, phagocytic function, and wound healing (16). FN binding proteins such as the 30-kD antigen may play an important role in the pathogenesis of tuberculosis by facilitating the entry of mycobacteria into cells or acting as targets for cytotoxic mechanisms.

Mycobacterial culture filtrate and PPD stimulate human monocytes to produce cytokines such as interleukin-1 and TNF-α (17, 18, 19) and TGF-β (20). The mycobacterial constituents and the mechanisms that they activate the induction of cytokines by human monocytes remain undefined.

In this study, we demonstrate that the 30-kD antigen induces release of TNF-α by human monocytes. Moreover, FN pretreated monocytes show increased binding of 30-kD antigen and release significantly higher concentrations of TNF-α than untreated monocytes in response to this signal. Modulation of cytokine production by binding of 30 kD to cell surface FN may be relevant to the immunopathogenesis of tuberculosis.

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1. *Abbreviations used in this paper:* FN, fibronectin; LAM, lipoarabinomannan; pFN, plasma FN; PPD, purified protein derivative.

Methods

Antigens and antibodies. Purified 30-kD antigen and polyclonal antibody to 30-kD antigen were prepared as described (9). Briefly, 30-kD antigen was prepared from culture filtrate of *M. tuberculosis* H37Ra by ion–exchange chromatography on diethylaminoethyl cellulose and further purified by HPLC. The characterization of 30-kD antigen was done by SDS-PAGE. Polyclonal antibody to 30-kD antigen had been prepared by immunization of an adult female goat with 30-kD antigen (21). PPD was a gift from Lederle Laboratories (Pearl River, NY). Human plasma FN and rabbit anti–human plasma FN antibody were purchased from Becton Dickinson (Bedford, MA). Limulus amebocyte lysate assay kits were purchased from BioWhittaker (QLC-1000; Walkersville, MD) and used to estimate endotoxin. The endotoxin contamination of 30 kD was 0.04 ng/μg; FN and PPD < 0.01 ng/μg; polyclonal antibody to FN 0.075 ng/μl; and polyclonal antibody to 30-kD antigen 0.01 ng/μl of antibody.

Cell preparations. Venous blood from healthy individuals was collected into heparin (20 U/ml) containing syringes and PBMC were isolated by density gradient sedimentation over Ficoll-Hypaque (Pharmacia LKB Biotechnology, Uppsala, Sweden) (22). PBMC were washed three times with RPMI-1640 (BioWhittaker) containing L-glutamine (200 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), amphotericin B (0.25 mg/ml), and Hepes (15 mM). PBMC were resuspended in RPMI-1640 supplemented with 5% (vol/vol) autologous serum and incubated at 37°C in 5% CO_2 air for 1 h on plastic petri dishes (100 × 20 mm; Falcon Labware, Lincoln Park, NJ) which had been precoated with autologous serum. After removing nonadherent cells, the adherent monolayers were dislodged by gentle scraping with a rubber policeman. Cells were 85–95% monocytes as determined by peroxidase activity (23) and were 99% viable by trypan-blue dye exclusion. Adherent cells were washed and resuspended in RPMI-1640 supplemented with 2% (vol/vol) autologous serum.

The monocytic cell line U937, originally derived from a patient with histiocytic lymphoma (24), was used as a source of homogenous monocytes in some experiments. The cell line was purchased from the American Type Culture Collection (Rockville, MD) and cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C in 5% CO_2 air.

Stimulation of TNF-α secretion by cultured monocytes. Blood monocytes or U937 cells were incubated in 24-well culture plates (3524; Costar Corp. Cambridge, MA) at 1×10^6/ml with or without the 30-kD antigen or PPD. In some experiments, various concentrations of lipoarabinomannan (LAM) (kindly provided by John T. Belisle, Colorado State University, Fort Collins, CO) or 30-kD antigen digested with a fivefold excess of nonspecific protease type XIV from *Streptomyces griseus* (Sigma Chemical Co., St. Louis, MO) for 18 h were added to the monocyte culture. Polymyxin B at 10 μg/ml (Sigma Chemical Co.) was added to all cultures to inactivate trace endotoxin contaminations (25). Polymyxin B at this concentration has no toxic or stimulatory effects on monocytes (26) and abrogated the induction of TNF-α by LPS at 1 μg/ml. Cell-free supernatant were obtained by centrifugation (10,000 *g* for 3 min) and supernatants were stored at −20°C until assayed.

Measurement of TNF-α in supernatants of cultured monocytes. Immunoreactive TNF-α in culture supernatants was determined by ELISA. Immulon 4 Microtiter plates (Dynatech Laboratories, Biotechnology Products, Chantilly, VA) were coated with murine monoclonal anti–human TNF-α (Clone F12; Olympus Corp., Lake Success, NY) at a concentration of 10 μg/ml overnight at 4°C. Subsequently, the antibody coated wells were blocked with 1% BSA in PBS (Bio Whittaker) for 1 h at 37°C. Culture supernatants and recombinant human TNF-α (Genzyme Corp., Cambridge, MA) in a range of concentrations were added to the wells and incubated for 2 h. After washing the plates, polyclonal rabbit anti–human TNF-α antibody (IP-300; Genzyme Corp.) was added to wells and plates were incubated for 2 h at room temperature. After washing, alkaline phosphatase conjugated anti–rabbit IgG (to detect FN) or anti–goat IgG (to detect 30 kD) (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to the wells for 1 h. Alkaline phosphatase substrate buffer (100 mM glycine, 1mM $MgCl_2$, pH 9.6) containing *p*-Nitrophenyl phosphate (1 mg/ml) was then added to each well. Absorbance was measured at 405 nm. This ELISA was sensitive to 10 pg/ml concentration of TNF-α and interassay variability was $< 10\%$.

Detection of FN binding to immobilized 30-kDa antigen. The binding of FN to 30-kD antigen was determined by ELISA. Depending upon the experimental design, the microtiter plates (Immulon 4) were coated with 200 μl of plasma fibronectin (pFN) (20 μg/ml), 30-kD antigen (5 μg/ml), or BSA (Sigma Chemical Co.) (250 μg/ml) for 1 h. Then the wells were rinsed three times with PBS and blocked with 1% BSA-PBS. After incubation (37°C, 1 h), 30-kD antigen–coated wells were further incubated with FN (20 μg/ml) (37°C, 1 h). After washing, rabbit polyclonal antibody to human FN (1:100 dilution) or goat polyclonal antibody to 30 kD (1:100) was added to the wells and incubated for 1 h at 37°C. After washing, an alkaline phosphatase conjugated anti–rabbit or anti–goat IgG (Boehringer Mannheim Biochemicals) was incubated in the wells (1 h). After washing, alkaline phosphatase substrate was added to wells and absorbance was measured at 405 nm.

Detection of FN binding to cell-bound 30-kD antigen. The ability of the 30-kD antigen to bind to monocytes was examined by cellular ELISA. Blood monocytes suspended in serum-free medium in eppendorf tubes (1×10^6 cells/tube) were preincubated with FN (20 μg/ml) or medium for 1 h and then exposed to 30-kD antigen or medium. Subsequently, polyclonal antibody to FN (rabbit IgG) or polyclonal antibody to 30 kD (goat IgG) was added to the tube and incubated for 1 h. After incubation, depending on the species of the primary antibody, alkaline phosphatase conjugated anti–rabbit or anti–goat IgG, was added to the tubes and incubated for 1 h. After that, the cells were transferred to 96-well microtiter plates and alkaline phosphatase substrate was added into each well. The optical density due to reaction was measured by spectrometer at 405 nm. Each incubation was at 4°C and the cells were washed two times with serum-free medium after each step.

Protease digestion. In some experiments, 30-kD antigen was digested with nonspecific protease type XIV from *S. griseus* (Sigma Chemical Co.). Briefly, protease (2.5% of the amount of 30 kD in 0.1 M ammonium bicarbonate) was added with 30-kD antigen for 6 h (shaking) at 37°C. Then, the same amount of protease was added to the 30-kDa–protease mixture and incubated overnight (shaking) at 37°C. Protease digested 30-kD antigen was lyophilized and dissolved in medium and used in experiments.

Western blot. In some experiments, 30-kD antigen with or without digestion with protease, or LAM was used for Western blot according to the method described by Wallis (27). Briefly, 30-kD antigen before and after digestion with protease (10 μg each), or LAM (1.5 μg) underwent electrophoresis in 10% acrylamide gel. After transfer to nitrocellulose paper, nonspecific binding was blocked by incubation of the blots overnight with 1% BSA-PBS supplemented with 0.1% Tween 80. The blots then were washed three times with 0.1% BSA-PBS supplemented with 0.1% Tween 80 and incubated with monoclonal antibody to 30-kD antigen (mouse IgG) or polyclonal antibody to LAM (rabbit IgG) (1:1,600 dilution) (kindly provided by John T. Belisle, Colorado State University) in 1% BSA-PBS supplemented with 0.1% Tween 80 for 2 h at room temperature. After washing, the blots were incubated with alkaline phosphatase conjugated anti–mouse or anti–rabbit IgG (1:1,000 dilution) for 1 h at room temperature. The alkaline phosphatase activity was detected with nitroblue tetrazolium and 5 bromo-4-chloro-indolyl phosphate (Sigma Chemical Co.).

Radiolabeling of 30-kDa antigen. To iodinate 30-kD antigen, we repurified the antigen as previously described (9). The integrity of double purified 30-kD antigen was checked by immunoblotting. The 30-kD antigen was labeled with ^{125}I ($Na\ ^{125}I$; Ims 30; Amersham Corp, Arlington Heights, IL) and Iodobeads (Pierce Chemical Co., Rockford, IL) to a specific activity of $\sim$ 1.2–1.3 μCi/μg protein. Structural integrity of radiolabeled 30-kD antigen was assessed by SDS-PAGE. ^{125}I-30-kD antigen was kept at 4°C in 0.1% gelatin-PBS and remained stable for at least 1 mo under these conditions.

Binding of ^{125}I-30-kDa antigen to monocytes. The binding of radio-iodinated 30-kD antigen to monocytes was measured according to the method described by Enna (28). Briefly, 100 μl of the freshly prepared monocytes (1×10^6) was placed in 1.5 ml eppendorf tubes and incubated with various amount of ^{125}I-30-kD antigen for 1 h at 4°C. To assess nonspecific binding, in duplicate experiments, cells were preincubated with unlabeled 30 kD at 500 molar excess of labeled antigen for 1 h at 4°C before addition of the labeled protein. The cell suspension was centrifuged for 30 s at 10,000 *g* and resuspended in 100 μl of medium. The cell suspension was centrifuged through a 150-μl layer of a mixture of 84% silicone oil (550 fluids; Contour Chemical Co., North Reading, MA) and 16% paraffin oil (Fisher Scientific Co., Pittsburgh, PA) for 2 min at 10,000 *g*. The tips of the tubes containing the cell pellets were then cut and radioactivity was measured in a gamma counter (Gamma 5500; Beckman Instruments, Inc., Arlington Heights, IL).

Statistical analysis. Statistical analyses were performed by the student *t* test for paired data.

Results

Stimulation of TNF-α secretion by 30-kDa antigen. Initial experiments established that the 30-kD antigen stimulated TNF-α secretion and determined the optimal concentration. Blood monocytes were cultured with various concentrations (1–10 μg/ml) of 30-kD antigen for 18 h. The concentration of TNF-α in the culture supernatant was determined by ELISA. TNF-α secretion in response to the 30-kD antigen increased significantly with increments in the concentration of the 30-kD antigen and reached a plateau level when 5 μg/ml of the antigen was used (Fig. 1). Therefore, 5 μg/ml was used as the optimal concentration of 30 kD in subsequent experiments.

To study the time course of secretion of TNF-α, blood monocytes from healthy individuals were cultured for various periods of time with or without 30 kD (5 μg/ml). TNF-α was detectable after 1 h of stimulation in culture supernatants and reached peak levels at 18 h of stimulation (Fig. 2).

Next, induction of TNF-α in monocyte culture supernatant in response to 30-kD antigen was compared to PPD. Monocytes from six different healthy donors were stimulated with either 30-kD antigen (5 μg/ml) or PPD (50 μg/ml) for 18 h and TNF-α concentrations in the supernatants were determined by ELISA. Both 30 kD and PPD significantly induced TNF-α (484±89 and 502±90 pg/ml, respectively) as compared to unstimulated culture (49±7 pg/ml). However, there was no significant difference in the relative amount of TNF-α production by monocytes in response to the 30-kD antigen compared to PPD.

To assure that the induction of TNF-α in monocytes by the preparation of 30 kD was due to 30-kD protein and not due to other possible lipid or polysaccharide contamination of the preparation, the 30-kD antigen was treated with protease as described in Methods. The proteolytic digestion of 30-kD antigen with protease was confirmed by SDS-PAGE analysis and Western blot analysis (Fig. 3). The 30-kD antigen was seen as a major band with negligible impurities (Fig. 3 *A*, lane *1*); the major band disappeared after digestion with protease (Fig. 3 *A*, lane *2*). Moreover, in the Western blot done in parallel (Fig. 3 *B*), monoclonal antibody to 30-kD antigen recognized only a single band at the 30-kD molecular weight region, which disappeared with protease digestion (Fig. 3 *B*, lane *1* and *2*, respectively). Furthermore, the protease treatment abrogated the capacity of 30-kD antigen to induce TNF-α by blood monocytes (Fig. 4).

Next, we assessed the LAM contamination of the 30-kD preparation in a Western blot using anti–LAM antibody (29). The amount of LAM in 30-kD antigen was estimated by comparison with known amounts of LAM assessed together with 30-kD antigen on the Western blot. Band densities were com-

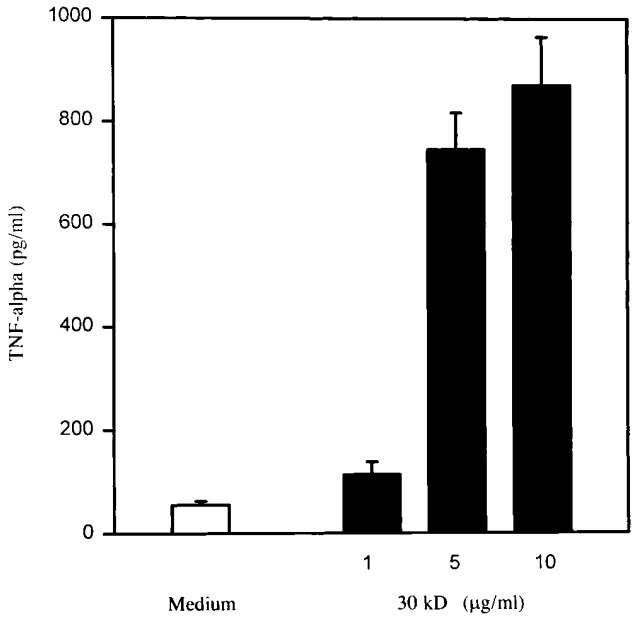

Figure 1. Dose–response analysis of TNF-α production. Blood monocytes obtained from two different healthy donors were cultured in the presence of the 30-kD antigen (1–10 μg/ml) and the concentration was measured by ELISA in 18-h culture supernatants. Each bar represents mean±SE, $n = 2$.

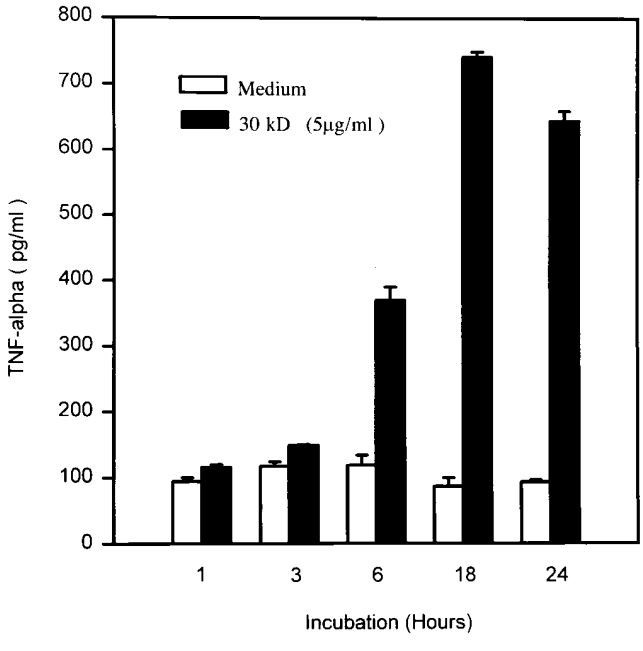

Figure 2. Kinetics of expression of TNF-α activity in monocyte culture supernatants. Monocytes obtained from each of two healthy individuals were cultured with (□) or without (■) 30-kD antigen (5 μg/ml). TNF-α concentration in the culture supernatants was measured by ELISA. Each bar represents mean±SE, $n = 2$.

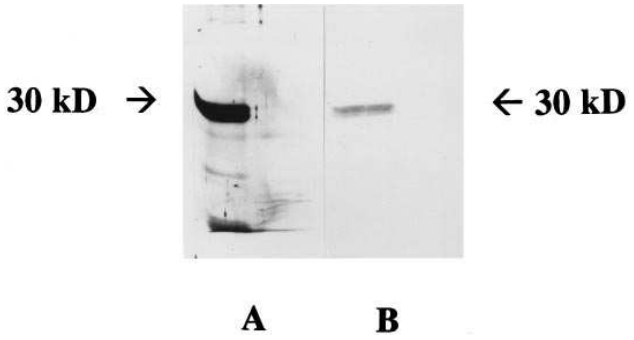

Figure 3. The SDS-PAGE gel electrophoresis with aura dye stain (*A*) and western immunoblot of 30-kD antigen (*B*) before and after digestion of 30-kD antigen with a fivefold excess of nonspecific protease type XIV from *Streptomyces griseus* for 18 h.

pared by densitometry. The amount of contamination of LAM in 30-kD antigen was < 1 μg/5 μg of 30-kD antigen. This concentration of LAM did not induce TNF-α production by monocytes (Fig. 4).

Binding of FN to 30-kD antigen. The binding of FN to purified 30-kD antigen was investigated by solid phase ELISA. To confirm that FN binds to 30-kD antigen and was not sticking nonspecifically to the ELISA plate, FN also was added to the wells which were precoated with BSA. After that, rabbit polyclonal antibody to human FN was added to the wells. Minimum amounts of FN bound to the wells precoated with BSA (Fig. 5 *A*). Both 30-kD antigen and FN bound with their respective capture antibodies and did not show cross-reaction with the alternate capture antibody (Fig. 5 *B*). Furthermore, as previously reported (15), 30-kD antigen was bound by FN as shown by the recognition of the 30-kDa–FN complex by the anti–FN polyclonal antibody (Fig. 5 *B*).

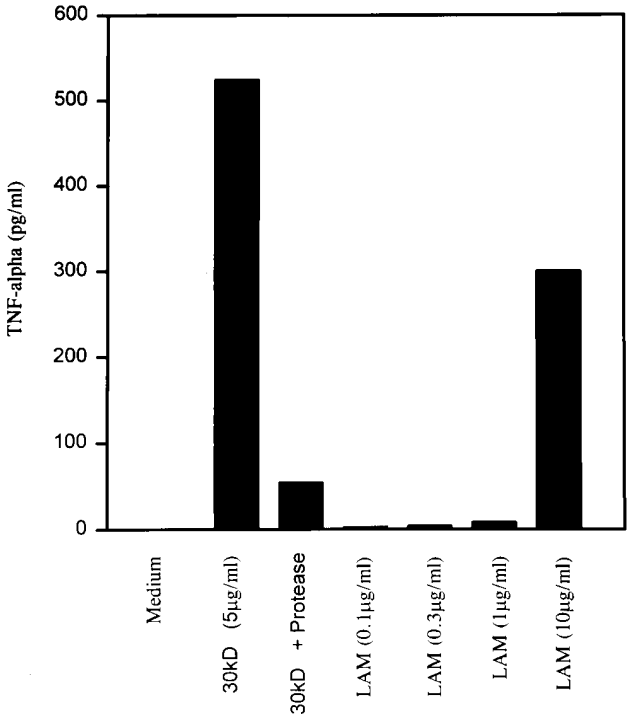

Figure 4. TNF-α expression by blood monocytes. The cells were stimulated with various concentrations of LAM (0.1 μg/ml to 10 μg/ml). The 30 kD antigen with and without digestion with protease were used to induce TNF-α by monocytes.

Generation of TNF-α by plasma FN preincubated monocytes. We next investigated the effect of 30-kDa–FN interaction on the release of TNF-α by human monocytes. Previously, it had been shown that, FN purified from plasma induced the expression of TNF-α mRNA but not production of the cytokine by monocytes (30). Monocytes or U937 cells were incubated with FN (20 μg/ml) on ice for 1 h. Then, the cells were washed two times with RPMI-1640. The cells were further incubated with or without rabbit polyclonal antibody to human pFN (1:100 dilution; Becton Dickinson) for 1 h on ice. Then, the cells were washed two times with RPMI-1640 and resuspended in serum-free medium. Cultures either received 30-kD antigen (5 μg/ml) or were left unstimulated. TNF-α in cell-free supernatant was assessed by ELISA. Preincubation with FN itself did not induce TNF-α production by monocytes (Fig. 6) in concentrations up to 100 μg/ml (data not shown). However, preincubation with FN resulted in production of higher concentrations of TNF-α upon stimulation with 30-kD antigen than by 30 kD alone ($P < 0.01$). This effect was blocked by polyclonal antibody to FN ($P < 0.05$).

Next, binding of the 30-kD antigen to monocytes was assessed by cellular ELISA. Preincubation of monocytes with FN enhanced binding of the 30-kD antigen (Fig. 7). The ability of FN to enhance the binding of 30-kD antigen to monocytes was blocked by anti–FN polyclonal antibody.

The U937 cell line binds FN (31) and was used to examine the interaction between the 30-kD antigen and FN. As compared to blood monocytes, TNF-α production by U937 cells in response to 30-kD antigen was negligible. Also FN treatment did not enhance 30-kD antigen induced TNF-α production (data not shown). Furthermore, by cellular ELISA U937 cells bound FN effectively, but only weakly bound 30-kD antigen, with or without pFN pretreatment (Fig. 8).

Study of the expression of plasma membrane receptors for 30-kD antigen on monocytes. Our observations indicated that FN enhanced binding of 30-kD antigen to monocytes and stimulated monocytes to release TNF-α. To determine whether human blood monocytes expressed specific receptors for 30-kD antigen, the protein was repurified and confirmed for homogeneity by immunobloting (See Methods), radiolabeled with ^{125}I-Na, and used in receptor binding assays. The structural integrity of 30-kD antigen had not changed after repurification and labeling with radioactive ^{125}I as confirmed by Western blot and SDS gel electrophoresis (data not shown). Cell-bound radioactivity was plotted versus the concentration of ^{125}I-30 kD and specific binding activity to the cells was calculated. A Scatchard analysis was performed by plotting the ratio of monocytes bound to free (B/F) versus the number of monocytes bound ^{125}I-30-kD molecules as previously described (32). The human monocytes bound labeled 30-kD antigen nonspecifically and in a nonsaturable manner (data not shown).

Discussion

In this study, we demonstrate a new biological function of 30-kD antigen, namely direct stimulation of TNF-α secretion by blood

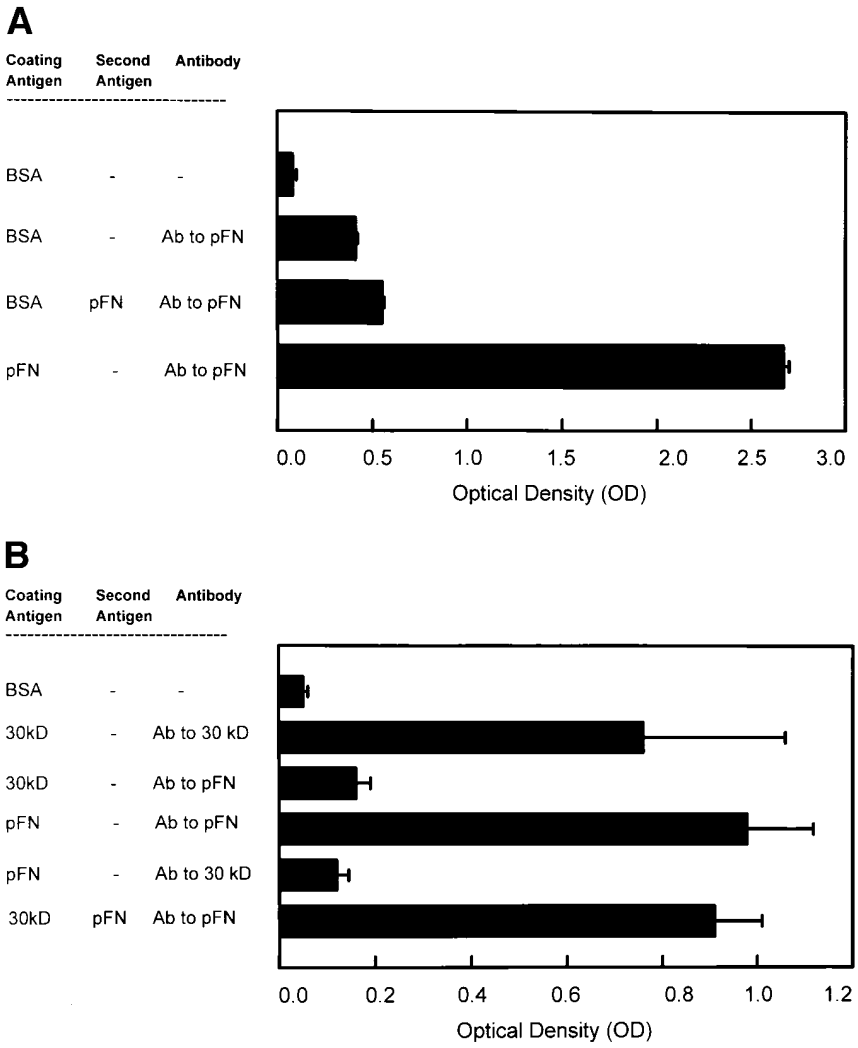

Figure 5. (*A*) ELISA analysis of the binding of the fibronectin to BSA. The wells coated with either BSA (250 μg/ml) or human plasma fibronectin (20 μg/ml) were captured by rabbit antifibronectin polyclonal antibody. Each bar represents mean±SE, $n = 3$. (*B*) ELISA analysis of the binding of 30-kD antigen to fibronectin. The wells coated with 30 kD (5 μg/ml) or fibronectin (20 μg/ml) were captured by goat anti–30-kD polyclonal antibody or rabbit antifibronectin polyclonal antibody. Each bar represents mean±SE, $n = 3$.

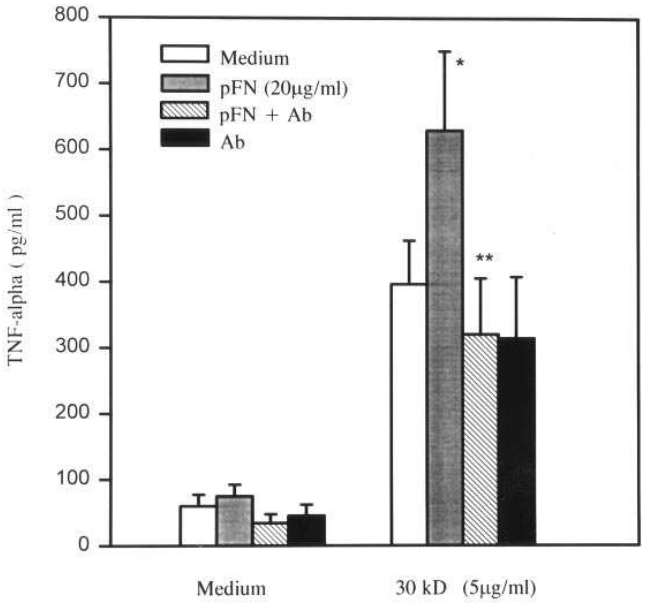

Figure 6. Effect of fibronectin on 30-kD antigen stimulated expression of TNF-α. Monocytes were pretreated with fibronectin (20 μg/ml) for 1 h before subsequent incubation with rabbit antifibronectin polyclonal antibody for 1 h. Monocytes with or without pretreatment with fibronectin were cultured in the presence of medium alone or the 30-kD antigen (5 μg/ml) for 18 h. TNF-α activity in the culture supernatants was measured by ELISA. Each bar represents mean±SE, $n = 5$. *$P < 0.01$ compared to the monocytes cultured with 30-kD antigen without pretreatment with fibronectin. **$P < 0.05$ compared to monocytes pretreated with fibronectin and cultured with 30-kD antigen.

monocytes from healthy individuals, as well as synergy between 30-kD antigen and pFN in stimulation of TNF-α. The 30-kD antigen was selected for study for two reasons. First, it is a major extracellular product of *M. tuberculosis* and was the first to be purified and molecularly characterized. Second, it is a major target of the cellular immune response in healthy PPD-positive donors, whereas patients with active tuberculosis fail to respond to it. Additional interest in this antigen has been recently generated by the finding that it elicits protective immunity in guinea pigs (10) and, therefore, has become a leading vaccine candidate. The induction of cytokines by 30-kD antigen may relate to its role as an immunogen. Further, the FN-binding property of the 30-kD antigen may have a role in invasion of host cells and phagocytosis or in boosting the TNF-α inducing response.

The 30-kD antigen used in our experiments was purified from the culture filtrate of the H37Ra strain of *M. tuberculosis* by ion exchange chromatography and HPLC (9). By SDS-PAGE analysis, 30 kD was pure with no other protein contam-

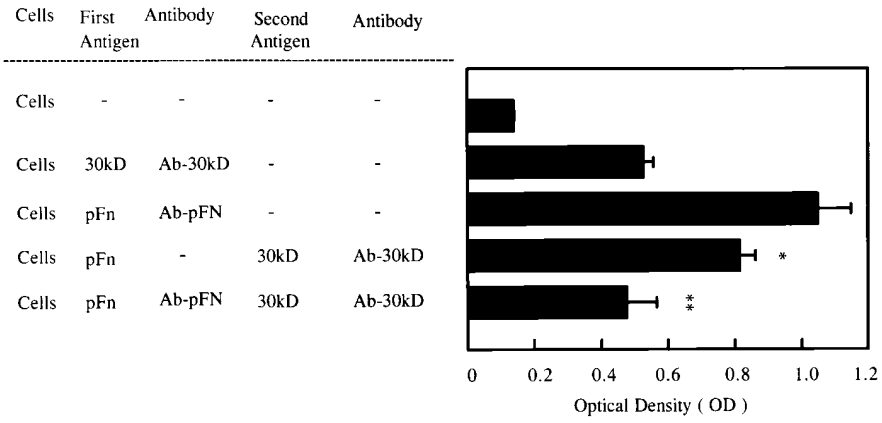

Figure 7. ELISA analysis of the binding of 30-kD antigen (5 μg/ml) to fibronectin (20 μg/ml) pretreated monocytes. Each bar represents mean±SE, $n = 3$. Binding of 30-kD antigen to fibronectin was significantly decreased after inhibition with rabbit antifibronectin polyclonal antibody. $*P <$ 0.01 compared to the monocytes without pretreated with pFN. $**P < 0.05$ compared to pFN pretreated (without antibody to pFN) monocytes.

ination. The low endotoxin contamination of 30-kD antigen (0.04 ng/μg) was further neutralized by addition of polymyxin B to the monocyte cultures. Also, in the absence of polymyxin B, LPS at 30 ng/ml neither induced nor synergized with 30-kD antigen in production of TNF-α by monocytes (data not shown). Therefore it is unlikely that induction of TNF-α was due to endotoxin contamination of 30 kD.

Mycobacteria contain LAM, a polysaccharide with many of the same biologic properties as LPS. However, the amount of LAM contamination of 30-kD antigen (< 1 μg/5 μg) at most contributed in < 1 μg/ml of LAM in culture which is insufficient to induce TNF-α by monocytes (Fig. 3 *B*). Previously, Wallis et al. also reported that concentrations of LAM below 10 μg/ml induced negligible amounts of TNF-α by blood monocytes (27). Furthermore, protease digested 30-kD antigen failed to induce TNF-α by monocytes (Fig. 4) supporting the contention that the 30-kD antigen rather than LAM or other nonprotein contaminants was responsible for induction of TNF-α in monocytes.

Previous studies have indicated that *M. bovis* BCG, PPD, and crude culture filtrate of *M. tuberculosis* induce TNF-α secretion by blood monocytes (18, 27, 33). Moreover, mycobacterial proteins appear to be the most active culture filtrate constituent in terms of induction of cytokines (27). Our previous characterization of the relative activity of culture filtrate proteins relied on one- and two-dimensional immunoblotting (27). This technique requires boiling culture filtrate in 2-mercaptoethanol and transfer to nitrocellulose of sufficient quantities to stimulate monocytes. The activity of the 30-kD antigen was not detected, possibly indicating that bioactivity requires tertiary structure of the molecule or that insufficient amounts of 30-kD antigen were transferred to nitrocellulose. In fact other culture filtrate proteins including a 58-kD protein stimulated monocyte production of cytokines (34). It remains to be determined whether the 58-kD protein binds FN or shares biologically active epitopes with the 30-kD antigen. Nonetheless, the activity of the 30-kD antigen in this regard is of special relevance because it is among the most abundant culture filtrate constituents; elicits a protective immune response (32); is a vital enzyme in cell wall synthesis, and, binds FN.

Binding of 30-kD antigen by FN as shown previously (15) and confirmed in this study (Fig. 5 *B*) is potentially important for several reasons. FN binds to several strains of mycobacteria (35) including BCG. Attachment of BCG to bladder epithelial cells was abrogated by anti–FN antibody (36). Also, exogenous FN enhanced the attachment of BCG to the bladder transitional cell carcinoma cell line, T24 (37). On the other hand, blood monocytes express receptors for FN (38), and show an enhanced ability to ingest IgG-coated sheep erythrocytes in the presence of FN (39, 40). Furthermore, the phagocytosis-enhancing effects of FN on neonatal monocytes was augmented in the presence of opsonizing antibody against group B streptococci (41). Moreover, binding of FN to human monocytes enhances the phagocytic function of monocytes for bacilli (16). These data suggest that an interaction between FN, possibly bound to its receptor on mononuclear phagocytes, and microorganisms may be important in host defenses.

Whether interaction of 30-kD antigen expressed in the cell wall of *M. tuberculosis* and FN serves as a phagocytic signal for *M. tuberculosis* needs to be established. In this regard, phagocytosis of *M. leprae* was markedly reduced in FN-depleted serum

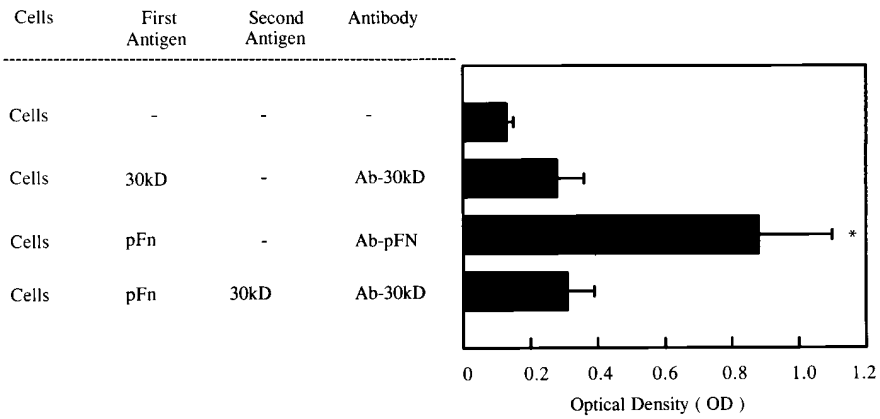

Figure 8. ELISA analysis of the binding of 30-kD antigen (5 μg/ml) to the fibronectin (20 μg/ml) pretreated U937 cells. Binding of 30-kD antigen to U937 cells or fibronectin pretreated U937 cells was not significantly different. Each bar represents mean±SE, $n = 6$. $*P < 0.05$ compared to U937 cells alone.

(42). The previously characterized 55-kD protein of *M. tuberculosis* described by Abou-Zeid et al. is known for FN-binding capacity (43). Similarly, other FN-binding mycobacterial proteins have been characterized including a group of antigens (57, 58, and 60 kD) from the culture filtrate of *M. tuberculosis* that may be important in invasion (15). The TNF-α stimulatory properties of these proteins have not been described.

TNF-α induces mycobactericidal granuloma in mice (44) and enhances the intracellular growth inhibition of *M. tuberculosis* by macrophages (45). The increased production of TNF-α by FN preincubated monocytes in response to 30-kD antigen and increased binding of 30-kD antigen after preincubation with FN (and reversal of both by antibody to FN) indicate that binding of the 30-kD antigen to FN on the monocyte surface induces TNF-α production. The pFN used in our study did not alone induce TNF-α (Fig. 6) in concentrations up to 100 μg/ml (data not shown). This is in agreement with data from Eierman et al. (30) that adherence of human monocytes to plastic or FN enhances the expression of interleukin-1 and TNF-α mRNA but does not result in secretion of these cytokines. In contrast, Beezhold et al. have found that intact FN purified from plasma or FN fragment (120-kD fragment) induced TNF-α by blood monocytes (46). Differences in the source of FN and the state of activation of monocytes may account for these differing results. In our study, monocytes may have been less activated since we used autologous serum instead of FBS.

Peake et al. have shown that interaction of 30-kD antigen of *M. bovis* (AN5) with FN involves the binding of multiple regions of the 30-kD antigen to the collagen binding domain of FN (47). Plasma membrane receptors of the monocytes might bind FN through the RGDS (Arg-Gly-Asp-Ser) amino acid sequence of the cell-binding domain (48). Ferreira et al. have shown that, FN bound to U937 cells both through the RGDS and 38-kD heparin-binding domain which are located within the IIICS region of FN (31). Despite FN binding by U937 cells as shown in this study (Fig. 8) and elsewhere (49), TNF-α was not produced by these cells in the presence of 30-kD antigen nor was binding of the 30-kD antigen enhanced. Whether differences in regions of FN bound by U937 cells and monocytes would account for differences in binding of the 30-kD protein as well as induction of TNF-α is not clear.

Specific saturable plasma membrane receptors for 30-kD antigen were not detectable on human monocytes. The structural integrity of 30-kD antigen had not changed after repurification and labeling with radioactive ^{125}I as confirmed by Western blot and SDS gel electrophoresis (data not shown). It seems likely, therefore, that the 30-kD antigen directly binds to the FN present on the surface of monocytes and thus stimulates the production of TNF-α.

In summary, these observations support an expanded central role for the 30-kD antigen in the pathogenesis of tuberculosis. The direct induction of the cytokine TNF-α and modulation of same by FN indicate a potential role in clinical features (fever, sweats, weight loss), granuloma formation, and bacterial clearing. An immune response directed at the 30-kD antigen, therefore, will have many and complex effects on the interaction between *M. tuberculosis* and the human host.

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