Induction of interleukin 1 and tumor necrosis factor by mycobacterial proteins: The monocyte Western blot

(*Mycobacterium tuberculosis*)

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ABSTRACT Infection with *Mycobacterium tuberculosis* involves mononuclear phagocytic cells as hosts to intracellular parasites, accessory cells in the induction of the immune response, effector cells for mycobacterial killing, and targets of cytotoxic lymphocytes. When stimulated by whole mycobacteria or various mycobacterial preparations, monocytes and macrophages produce the cytokines interleukin 1 and tumor necrosis factor, which possess multiple functions, including immune induction, and may be responsible for the fever and cachexia prominent in tuberculosis. To identify mycobacterial proteins that may directly activate production of these cytokines, culture filtrate of *M. tuberculosis* that had been subjected to gel electrophoresis and transferred to nitrocellulose paper was used to stimulate monocyte production of cytokines. Fractions representing molecular weights of 46,000 and 20,000 consistently induced both interleukin 1 and tumor necrosis factor. The magnitude of the monocyte responses to these fractions was similar to that to intact mycobacteria or optimal concentrations of lipopolysaccharide. This stimulatory effect was not due to contamination with either bacterial lipopolysaccharide or mycobacterial lipoarabinomannan, as it was abolished by digestion with *Streptomyces griseus* protease but was unaffected by ammonium sulfate precipitation, preincubation with polymyxin B, or depletion of lipoarabinomannan by immunoaffinity chromatography. Proteins identified by this system may have considerable potential as immunogens, as the capacity to directly stimulate mononuclear phagocyte production of cytokines is an essential property of adjuvants.

Mononuclear phagocytes serve multiple functions in the pathophysiology of tuberculosis: host to an intracellular parasite, accessory cell for induction of the immune response, effector cell for mycobacterial killing, and target for killing by other cytotoxic cells. Activation of this complex network hinges on the expression of mycobacterial antigens on the surface of infected cells and the appropriate release by infected cells of the cytokines necessary for immune induction.

Mycobacteria are potent inducers of monocyte production of the cytokines interleukin 1 (IL-1) and tumor necrosis factor (TNF) (1, 2). This is not dependent on the presence of intact microbial organisms, as we have previously demonstrated cytokine induction by the dialyzed filtrate of *Mycobacterium tuberculosis* culture medium and by the protein-enriched fraction of that medium (purified protein derivative) (2). The capacity for induction of IL-1 by soluble factors of other bacteria has been thought mainly to reside in polysaccharides; indeed, *Escherichia coli* lipopolysaccharide (LPS), the prototypic agent used to induce IL-1 release *in vitro*, is active in this respect even in nanogram concentrations. The capacity of purified protein derivative to induce IL-1 is not medi-

ated by LPS, however, as mycobacteria lack this polysaccharide. Furthermore, purified protein derivative-induced production of IL-1 is unaffected by polymyxin, a cationic polypeptide antibiotic that binds to the lipid A moiety of LPS and inactivates most of its biologic activities (3, 4), thus also excluding a contaminating role for LPS. The specific components of mycobacteria responsible for induction of monocyte cytokine production are not known. In this series of experiments, we have adapted the technique of Western blot analysis to study monocyte activation and have identified protein fractions of *M. tuberculosis* at molecular weights of approximately 46,000 and 20,000 that result in monocyte expression of IL-1 and TNF. Corresponding fractions activated T lymphocytes from healthy donors. The unique capacity to stimulate both mononuclear phagocytes and T lymphocytes may define particularly immunogenic microbial products.

METHODS

Antigens. *M. tuberculosis* strain H37Rv was cultured in Proskauer-Beck medium. After 4–6 weeks, cells were removed by sedimentation followed by filtration with a 0.4- μ m membrane. The filtrate was dialyzed against water using a Spectra/Por 2 membrane (Spectrum Medical Industries), lyophilized, and resuspended in water. A protein-enriched fraction was prepared by precipitation in 50% saturated $(\text{NH}_4)_2\text{SO}_4$, followed by resuspension of the precipitate and dialysis against water. In some experiments, the culture filtrate was further purified by immunoabsorbent chromatography using monoclonal antibody to *M. tuberculosis* lipoarabinomannan (LAM) (kindly provided by Patrick Brennan, Colorado State University) coupled to Sepharose with cyanogen bromide. After repeated passage through the antibody column, the filtrate was concentrated over a Centricon filter. The protein content of each preparation was determined by a colorimetric assay (Bio-Rad). In some experiments, the anti-LAM treated *M. tuberculosis* filtrate was then digested with a 5-fold excess of nonspecific protease type XIV from *Streptomyces griseus* (Sigma) for 18 hr.

Gel Electrophoresis. Antigen (500 μ g of protein or, in the case of the protease-treated preparation, the products of digestion of 500 μ g of protein) was mixed with an equal volume of reducing sample buffer [4% SDS/20% (vol/vol) glycerol/10% 2-mercaptoethanol/1.5% Tris-HCl, pH 6.75], heated to 100°C for 2 min, and applied to a 7 $\times$ 10 cm 9% acrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose paper, washed in phosphate-buffered saline (PBS), and stained with Aurodyne (Janssen). The nitrocellulose paper was cut into 2-mm horizontal strips, transferred to individual glass tubes, allowed to dry, and dissolved in 1 ml of dimethyl sulfoxide (Sigma) (5); 3 ml of 0.05 M

Na_2CO_3 (pH 9.6) was added dropwise with continuous agitation. The precipitate was pelleted, washed once, resuspended in 0.5 ml of RPMI 1640 medium, and frozen at -30°C .

Western Blotting. Nonspecific binding was inhibited by incubation of nitrocellulose transfers in RPMI 1640 medium with 10% fetal calf serum (FCS) for 2 hr at 37°C . Monoclonal antibody to LAM was diluted 1:1000 in 1% bovine serum albumin in PBS. After overnight incubation at room temperature, the nitrocellulose paper was washed in PBS/0.01% Tween 80, and incubated overnight in alkaline phosphatase-coupled anti-mouse immunoglobulin (Sigma) diluted 1:1000 in bovine serum albumin (1% in PBS). Alkaline phosphatase activity was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Cell Culture. Sixty microliters of each fraction of nitrocellulose particles was placed in individual 2-ml tissue culture wells, and incubated for 1 hr in 0.5 ml of complete medium [RPMI 1640 medium (M.A. Bioproducts) with 2 mM L-glutamine/gentamicin (100 $\mu\text{g}/\text{ml}$)/15 mM Hepes] with 4% heat-inactivated pooled human serum (PHS) and polymyxin B (20 $\mu\text{g}/\text{ml}$). LPS (1 $\mu\text{g}/\text{ml}$) and purified *M. tuberculosis* LAM (provided by Patrick Brennan), both with and without polymyxin B, and whole irradiated (4×10^6 rad; 1 rad = 0.01 Gy) *M. tuberculosis* (50 $\mu\text{g}/\text{ml}$) with polymyxin B served as controls. Blood mononuclear cells from tuberculin-negative donors were obtained by density sedimentation over Ficoll/Hypaque (Pharmacia). Tissue culture grade 100-mm Petri dishes were coated with PHS and incubated at 37°C for 15 min; 4×10^7 mononuclear cells were cultured in each dish in 5 ml of complete medium with 5% heat-inactivated FCS (HyClone) and 5% PHS for 1 hr. Nonadherent cells were removed by washing with 5% FCS in medium. Adherent cells (>90% monocytes) were covered with Hanks' balanced salt solution without calcium or magnesium, removed with a rubber policeman, pelleted, and resuspended in medium at a density of 3×10^6 cells per ml. One-half milliliter of the cell suspension was added to each well containing the nitrocellulose particles and incubated at 37°C in 5% CO_2 /95% air for 20 hr. The cells were then removed by centrifugation, and the supernatant was frozen at -30°C .

Lymphocyte Blastogenesis. Blood mononuclear cells from tuberculin skin test reactors (2×10^5 cells) were cultured in microtiter wells in 100 μl of complete RPMI 1640 medium with 10% PHS and 10 μl of each nitrocellulose fraction in replicates of three. [^3H]Thymidine (1 μCi ; 1 Ci = 37 GBq) was added during the final 24 hr of a 120-hr culture. Cells were harvested with a semi-automated harvester, and [^3H]thymidine content was assessed by scintillation photometry.

TNF Assay. Murine L929 cells were removed from culture flasks with trypsin, pelleted, and resuspended in Eagle's minimal essential medium (M.A. Bioproducts) with 2 mM L-glutamine/15 mM Hepes/1% nonessential amino acids/0.2% penicillin/streptomycin/15% FCS at a density of 0.25×10^6 cells per ml. One hundred microliters of the cell suspension was added to each well of 96-well culture plates and incubated overnight. The following day, 10 μl of actinomycin D (20 $\mu\text{g}/\text{ml}$) (Sigma) was added to each well, followed by 100 μl of monocyte supernatant serially diluted in complete RPMI 1640 medium in replicates of three. After 20 hr of incubation, 50 μl of neutral red (0.1% in PBS) was added to each well. After 20 min, the wells were emptied by inversion and rinsed once with warm PBS. The remaining stained cells were dissolved in 100 μl of 0.1 M sodium phosphate (monobasic) (50% in ethanol). The optical density at 570 nm was determined by using an automated ELISA reader (Dynatech). TNF activity in half-maximal units/ml was determined by probit analysis, using computer software developed by one of the authors (R.S.W.).

IL-1 Assay. Monocyte supernatants were serially diluted in complete RPMI 1640 medium. One hundred microliters of

each dilution was placed in microtiter wells in replicates of three. Female C3H/HeJ mice (8–10 weeks old) were sacrificed by cervical dislocation. Thymic tissue was passed through stainless steel mesh and washed in complete RPMI 1640 medium. Aggregates were removed by brief 1-g sedimentation. Cells were suspended in complete RPMI 1640 medium with 20% FCS, 50 μM 2-mercaptoethanol, and phytohemagglutinin (2 $\mu\text{g}/\text{ml}$) (Burroughs Wellcome) at a density of 15×10^6 cells per ml. One hundred microliters of the cell suspension was added to each well. [^3H]Thymidine (1 μCi) was added during the final 8 hr of a 72-hr culture. Cells were harvested with a semiautomated harvester, and [^3H]thymidine content was assessed by scintillation photometry. IL-1 activity in half-maximal units/ml was determined by probit analysis.

RESULTS

Culture filtrate of *M. tuberculosis* that had been subjected to SDS/PAGE, transferred to nitrocellulose paper, cut into horizontal strips, dissolved in dimethyl sulfoxide, and precipitated in an aqueous buffer to produce a fine particle suspension was used to stimulate monocytes in culture, in an adaptation of the T-cell Western blot technique developed by Abou-Zeid *et al.* (5) and Young and Lamb (6). The quantity of filtrate applied to the gel was selected to allow adequate representation of proteins without overloading and loss of resolution. The volume of nitrocellulose particles (10 μl) was selected to allow adherence of >80% of the monocytes to the particles rather than the plastic culture well. Tuberculin-negative donors were used to minimize the effect of any contaminating T cells. Prior to culture, the particles were incubated with polymyxin B to eliminate the effect of contaminating bacterial LPS. Fig. 1 shows the results of TNF bioassay of the supernatant of monocytes stimulated with each of the fractions, which we have termed a "monocyte Western blot." Two peaks of TNF production were evident at M_r 47,000 and 20,000. Repeated experiments using 20% acrylamide gels confirmed the presence of these peaks and failed to identify additional ones, although it is possible that proteins poorly represented in culture filtrate might induce monocyte cytokine expression if present in higher concentration. The magnitude of induced TNF activity was comparable to that of intact mycobacteria or *E. coli* LPS. Unlike LPS, the effect of the mycobacterial preparations was unaffected by polymyxin. Many other protein bands identified in *M. tuberculosis* filtrate by gold staining of nitrocellulose transfers (see Fig. 5, lane 1) failed to induce production of

FIG. 1. Induction of monocyte production of TNF by nitrocellulose-bound fractions of *M. tuberculosis* filtrate. Internal numbers represent $M_r \times 10^{-3}$. With the exception of one LPS control (Δ), all samples (including whole mycobacteria) were incubated with polymyxin B prior to addition to the monocyte culture. $\square$, Paper alone; Δ , LPS + polymyxin B; $\bullet$, *M. tuberculosis*.

FIG. 2. IL-1 content of the samples in Fig. 1.

TNF. Nitrocellulose paper alone also failed to induce TNF activity.

IL-1 assay of these same fractions is shown in Fig. 2. The TNF-containing fractions had IL-1 activity, as did additional fractions at M_r 90,000 and 35,000. This may reflect increased sensitivity of monocytes to the mycobacterial constituents for induction of IL-1 as compared to TNF, or it may reflect increased sensitivity of the IL-1 assay.

While mycobacteria do not possess bacterial LPS, they do contain LAM, a polysaccharide with many of the same physicochemical properties as LPS. LAM has an apparent M_r of $\approx 30,000$ on SDS/PAGE, but it can form larger complexes with proteins and thus have variable migration on gel electrophoresis. Purified LAM may have the capacity to stimulate monocyte production of TNF, although with a potency of 2–3 orders of magnitude less than that of LPS (Fig. 3). This effect was partially inhibited by preincubation with polymyxin B.

To ascertain whether LAM might be responsible for our observations, *M. tuberculosis* culture filtrate was depleted of LAM by ammonium sulfate precipitation followed by immunoaffinity chromatography using monoclonal anti-LAM antibody. Western blot analysis with anti-LAM antibody

FIG. 3. Induction of TNF by LPS and LAM in the presence (●) and absence (○) of polymyxin B.

FIG. 4. Western blot of *M. tuberculosis* filtrate using monoclonal anti-LAM antibody. Lane 1, untreated filtrate. Lanes 2–4, samples sequentially treated by ammonium sulfate precipitation (lane 2) and then anti-LAM affinity chromatography once (lane 3) and twice (lane 4).

showed progressive loss of LAM (Fig. 4). Approximately 5% of the initial LAM remained, with an apparent M_r of 53,000. Protein staining of these preparations showed similar overall protein content and persistence of all major protein bands (Fig. 5). Ammonium sulfate-precipitated and LAM-depleted *M. tuberculosis* filtrate was subjected to gel electrophoresis and was used to stimulate monocyte production of cytokines as in the previous experiments. This preparation retained its capacity to induce TNF production, as shown in Fig. 6, with the main peak of reactivity at M_r 42,000. One additional peak at M_r 88,000 may correspond to a M_r 90,000 protein identified by IL-1 assay of untreated *M. tuberculosis* culture filtrate. The relationship of the M_r 57,000 and 27,000 peaks to the proteins identified in other blots is uncertain. It is possible that this variation was due to alterations in protein electrophoretic mobility after removal of LAM and other polysaccharides. Comparisons between preparations were hindered by the width of the fractions and the relatively poor resolution of one-dimensional gel electrophoresis. The peaks were present in both of two donors studied, however.

To confirm that proteins were in fact responsible for the cytokine induction, LAM-depleted *M. tuberculosis* filtrate was subjected to protease digestion. Protein degradation was confirmed by gold staining of the nitrocellulose transfer (Fig. 7, lane 2), a technique with a sensitivity comparable to that of silver staining of a gel. Western blot analysis of this digest with anti-LAM monoclonal antibody revealed a single band at $M_r \approx 60,000$ (lane 1). The protease digest failed to induce monocyte production of TNF (Fig. 8).

To determine whether the proteins capable of monocyte activation were also targets of T-cell recognition, T-cell

FIG. 5. Protein stain of the *M. tuberculosis* preparations in Fig. 4.

FIG. 6. Induction of monocyte production of TNF by LAM-depleted *M. tuberculosis* filtrate, following ammonium sulfate precipitation and anti-LAM affinity chromatography (lane 4 of Figs. 4 and 5). Internal numbers represent $M_r \times 10^{-3}$. Symbols are the same as in Fig. 1.

Western blot analysis of two healthy tuberculin reactors was performed. In this assay, *M. tuberculosis* filtrate fractions were incubated with blood mononuclear cells, and blastogenic responses were measured by [3 H]thymidine incorporation. Five major peaks of T-cell reactivity could be detected (Fig. 9). These correspond precisely to fractions that stimulate monocytes, as summarized in Table 1.

DISCUSSION

Fever and cachexia are prominent in tuberculosis. These manifestations of the nonspecific host response to infection are likely mediated by the products of mononuclear phagocytic cells, particularly the cytokines IL-1 and TNF. Indeed, monocytes from patients with tuberculosis are primed *in vivo* to produce increased amounts of IL-1 when stimulated *in vitro* with either mycobacteria or bacterial LPS (7).

In addition to mediating the nonspecific or acute-phase response, IL-1 plays an essential role in immune induction, facilitating T-lymphocyte expression of interleukin 2 (IL-2) receptors and IL-2 release (8, 9). This process is critical to the expansion of antigen-specific T lymphocytes, and the subsequent elaboration of other lymphokines with monocyte activating factor activity, such as interferon γ , which may be important for mycobacterial killing. Indeed, mice injected with antibody to TNF fail to develop granulomas in response to bacillus Calmette-Guérin administration and are unable to contain the mycobacterial infection (10). All commonly used adjuvants are potent inducers of IL-1 and TNF; it is likely that their immune-inducing properties are due to their monocyte activating effects.

FIG. 7. Western blot analysis of protease-treated *M. tuberculosis* filtrate using anti-LAM antibody (lane 1), protein stain (lane 2), and anti-LAM Western blot of control protease alone (lane 3).

FIG. 8. Failure of induction of TNF by LAM-depleted *M. tuberculosis* filtrate that had been subjected to protease digestion. Symbols are the same as in Fig. 1.

Synthesis of these cytokines is induced by a variety of microbial stimuli, including polysaccharides (11), phorbols (12), inert particles (13), intact Gram-positive and -negative bacteria (14), spirochetes (15), and mycobacteria (1). Although mycobacteria do not possess Gram-negative LPS, mycobacterial cell walls contain a related polysaccharide, LAM, which is heavily acylated by lactate, succinate, palmitate, and 10-methyloctadecanoate, and contains glycerol and a polyol phosphate in addition to arabinose and mannose (16). LAM and LPS share many physicochemical properties, including hydrophobicity, and tend to copurify. The LAM used in this investigation had been passed through a commercial endotoxin-removing column, which uses polymyxin B. This cationic polypeptide antibiotic binds the lipid A moiety of LPS and neutralizes most of its biologic properties (3, 4). The effect of inadvertent contamination of samples with LPS can often be further minimized by preincubation with polymyxin B in soluble form. As LAM does not contain the lipid A group, differential sensitivity to preincubation may be used to identify contamination with LPS. Moreno and coworkers (17) have suggested that LAM is a major stimulus for production of TNF by pleural fluid cells *in vivo* in human tuberculosis and that this production is dependent on intact acyl groups. Our observations partly concur, in that we found modest induction of TNF by LAM *in vitro* that was only partially reversible by polymyxin B. While this observation could have been due to the modest LPS contamination, the possibility that other mechanisms could account for partial neutralization of LAM by polymyxin cannot be excluded. Nonetheless, several findings suggest that mycobacterial proteins and not LAM account for most of the induction of cytokine production in monocytes by *M. tuberculosis* filtrate. We demonstrated persistence of induction of TNF despite depletion of most immunoreactive LAM by sequential am-

FIG. 9. T-cell Western blot analysis of *M. tuberculosis* filtrate of two healthy tuberculin skin test reactors. Internal numbers represent $M_r \times 10^{-3}$. $\square$ and $\circ$, responses of donors 1 and 2, respectively, to nitrocellulose paper alone.

Table 1. Molecular weights ($\times 10^{-3}$) of fractions of *M. tuberculosis* filtrate identified by lymphocyte blastogenesis and monocyte cytokine production

T-cell blastogenesis	Monocyte	
	TNF	IL-1
19	20.5	20.5
34	—	35
47	46	46
68	—	—
85	—	90

monium sulfate precipitation and column chromatography. Fractions other than those containing the remaining LAM retained the capacity to induce TNF. Digestion with protease abolished the stimulation of TNF production in the monocyte Western blot, although it did not interfere with detection of LAM by conventional Western blot. Finally, we found induction of monokines in the same fractions that induced T-cell activation; stimulation of T cells is generally thought to be a property of proteins.

There is considerable interest in the identification of immunodominant and potentially protective protein antigens of *M. tuberculosis*, given the current increases in tuberculosis worldwide, and the apparent inadequate level of protection afforded by vaccination with bacillus Calmette-Guérin (18). The form of this research has been adapted from that in other bacterial systems: the identification of recombinant antigens through the immunization of laboratory animals, the development of monoclonal antibodies, and the screening of DNA expression libraries (19, 20). However, the prominent differences that exist between murine and human immune repertoires, B- and T-cell epitopes, and responses of immunized rather than infected hosts (21–23) raise fundamental questions regarding the use of immunized laboratory animals to identify mycobacterial antigens of significance in human disease. These questions have provided the impetus for the development of systems to identify potential vaccine candidates. We have demonstrated the use of monocytes to identify antigens of *M. tuberculosis* with the capacity to stimulate production of cytokines with multiple biologic activities, including immune induction, and have found that the M_r 46,000 and 20,000 fractions with this capacity were stimulatory to T lymphocytes from healthy tuberculin-reactive donors. While the resolving power of this technique as applied to one-dimensional gel electrophoresis is limited, it is likely that with two-dimensional isoelectric focusing gel electrophoresis, individual proteins could be identified. The technique therefore holds great promise in the identification of immunogens with adjuvant-like properties. The vaccine potential of these proteins of *M. tuberculosis* must then be

examined in experimental models for induction of protective immunity against mycobacterial infection.

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