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Expression of a Long Pentraxin, PTX3, by Monocytes Exposed to the Mycobacterial Cell Wall Component Lipoarabinomannan

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PTX3 is a prototypic long pentraxin composed of a C-terminal domain similar to those of classical pentraxins (e.g., C reactive protein) and an unrelated N-terminal portion. PTX3 is expressed in a variety of cell types, notably mononuclear phagocytes and endothelial cells, after exposure to the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α). The present study was designed to assess whether mycobacterial components were able to induce expression and production of PTX3. Mycobacterial lipoarabinomannan (LAM) induced expression of PTX3 mRNA in human peripheral blood mononuclear cells. The non-mannose-capped version of lipoarabinomannan (AraLAM) was considerably more potent than the mannose-capped version ManLAM or the simpler version phosphatidylinositol mannoside. Among mononuclear cells, monocytes were responsible for LAM-induced PTX3 mRNA expression. Whole mycobacteria (*Mycobacterium bovis* BCG) strongly induced PTX3 expression. Pretreatment with actinomycin D abolished LAM-induced PTX3 expression, whereas cycloheximide only partially reduced the expression. LAM-induced PTX3 expression was associated with the production of immunoreactive PTX3. IL-10 and IL-13 did not inhibit the induction of PTX3 by LAM. Under the same conditions, these anti-inflammatory cytokines inhibited MCP-1 expression. In contrast, gamma interferon inhibited LAM-induced PTX3 expression. Thus, in addition to IL-1, TNF, and lipopolysaccharide, mycobacterial cell wall components also induce expression and production of the long pentraxin PTX3. The significance of PTX3 in the immunobiology of mycobacterial infection and its relevance in relation to clinical involvement remain to be determined.

Pentraxins (C reactive protein [CRP] and serum amyloid P component [SAP]) are acute-phase proteins conserved during evolution from *Limulus polyphemus* to humans (1, 9, 20, 21). CRP and SAP are made in the liver in response to inflammatory mediators, most prominently interleukin-6 (IL-6) (3, 30). A number of ligands have been identified for CRP and SAP, including phosphoethanolamine, phosphocholine, DNA, immune complexes, various sugars, and complement fragments (6, 11, 17, 22, 28, 33). Pentraxins represent a mechanism of innate resistance against microbes, tools to scavenge cellular debris, and components of the extracellular matrix as illustrated by amyloid deposits (21).

PTX3 is the prototypic long pentraxin, structurally related to, but distinct from, classical pentraxins. PTX3 was cloned as an IL-1-inducible gene in endothelial cells (5) and as a tumor necrosis factor (TNF)-inducible gene (TSG14) in fibroblasts (16). Inflammatory cytokines induce PTX3 expression in a variety of cell types, most prominently endothelial cells and mononuclear phagocytes (5, 15, 32). The COOH half-domain of PTX3 aligns with the full-length sequence of CRP and SAP, whereas the NH_2-terminal part of the protein does not show any significant homology with other known proteins, thus rendering PTX3 the prototype member of the long-pentraxin family. After the cloning of PTX3, other long pentraxins were identified, including apexin (19, 24), XL-PXN1 from *Xenopus laevis* (29), rat neuronal pentraxin (NP), human neuronal pentraxin (NPTX2), and Narp (12, 27, 31). In all these molecules, a C-terminal pentraxin domain is coupled with diverse, unrelated N-terminal portions. Recent evidence suggests that the prototypic long pentraxin, PTX3, may serve as a mechanism of amplification of innate immunity (4a).

Lipoarabinomannan (LAM) is a major cell wall-associated glycolipid produced by *Mycobacterium tuberculosis* which can be secreted in copious amounts. LAM, a complex heteropolysaccharide composed of a phosphatidylinositol unit and its nonreducing end, has immunoregulatory functions including stimulation of cytokine production by mononuclear phagocytes and chemotaxis (2, 4, 25, 34).

It was previously reported that lipopolysaccharide (LPS), the major component of the outer cell walls of gram-negative bacteria, induces PTX3 expression in vitro and in vivo (13, 16, 32). This observation prompted us to examine whether mycobacteria and their components induce expression and production of PTX3, which belongs to the pathophysiologically and diagnostically important family of pentraxins.

MATERIALS AND METHODS

Microorganisms, cytokines, and reagents. *Mycobacterium bovis* BCG (P3) was grown at 37°C in 5% CO_2 on a solid Löwenstein-Jensen medium (BioMérieux Italia) for 4 to 6 weeks. Bacteria were resuspended in Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA), and the concentration was measured by densitometry. We estimated that an A_{600} of 1 corresponds to 8×10^6 cells/ml. The mannose-capped version of LAM (ManLAM) and phosphatidylinositol mannoside (PIM; isolated from *M. tuberculosis* Erdman) and the non-mannose-capped version of LAM (AraLAM) and lipomannan (LM; isolated from a *Mycobacterium sp.*) are a kind gift from J. Belisle (College of Veterinary Medicine and Biomedical Sciences, Department of Microbiology, Colorado State University, Ft. Collins, Colo.) under National Institute for Allergy and Infectious Diseases contract N01-AI-25147. The evaluation of tuberculosis reagents for the presence of gram-negative bacterial endotoxin was done with the *Limulus* amebocyte lysate assay (BioWhittaker, Inc., Walkersville, Md.). It was found that ManLAM and AraLAM contained 17.7 and 12.7 ng of endotoxin, respectively, per mg, whereas LM and PIM contained less than 12.5 pg of endotoxin per mg. LPS from *Escherichia coli* 055:B5 (Difco, Detroit, Mich.) was used at 100 ng/ml. IL-10 was

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from Schering-Plough, and IL-13 was a kind gift of A. Minty (Sanofi Elf Bio Recherches, Labège, France). Both cytokines were used at 10 ng/ml. Gamma interferon (IFN-γ) was from Roussell Uclaf and was used at 500 U/ml. IL-1β was from Dompé, L'Aquilla, Italy and was used at 20 ng/ml. TNF-α, from BASF/Knoll, Ludwigshafen, Germany, was used at 500 U/ml. IL-1 receptor antagonist (IL-1ra) was from Cetus Corp., Emeryville, Calif. and was used at 5 μg/ml. The monoclonal antibody against TNF-α, B154.2, was a kind gift from G. Trinchieri (Wistar Institute, Philadelphia, Pa.) and was diluted at 1/2,000. All cytokines and compounds were routinely tested for endotoxin contamination by *Limulus* amebocyte assay (sensitivity, 1 pg/ml). Cycloheximide (CHX) and actinomycin D (Act D) (Sigma Chemical Co., St. Louis, Mo.) were used at 10 and 1 μg/ml, respectively.

Cells and culture conditions. Peripheral blood mononuclear cells (PBMC) were prepared as described previously (7). Briefly, whole blood was fractionated by Ficoll Hypaque (Seromed-Biochem, KG, Berlin, Germany) gradient centrifugation. PBMC were collected, washed twice with ice-cold saline solution (0.9% sodium chloride; DonBaxter Spa, Trieste, Italy), and then resuspended in RPMI 1640 (Seromed-Biochem KG) with glutamine (2 mmol/liter; Gibco, Glasgow, United Kingdom) and gentamicin (500 μg/ml). Monocytes were purified from the mononuclear cell fraction by a second centrifugation through a one-step discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) as described in reference 7. The percentage of monocytes in this population was evaluated by nonspecific esterase staining and was found to be more than 90%. Contaminating cells were mainly T cells and occasional NK cells. Monocytes, unlike mature macrophages, did not express the mannose receptor, as assessed with the PAM-1 antibody. The cells were washed with saline solution and incubated in endotoxin-free RPMI 1640 without serum at 10^7 cells/ml and 37°C in the presence of 5% CO_2. Usually, 30×10^6 PBMC and 15×10^6 monocytes were used for Northern analysis, whereas 5×10^6 monocytes were used for the enzyme-linked immunosorbent assay (ELISA). Cells were stimulated with the indicated stimuli for 5 h in the case of Northern analysis or 24 h in the case of ELISA.

RNA extraction and Northern blot analysis. RNA was extracted and purified by the classical guanidine isothiocyanate (Merck, Darmstadt, Germany) method as described previously (32). Ten micrograms of total RNA was run in standard formaldehyde agarose gel, blotted onto a GeneScreen Plus membrane (NEN Research Products, Boston, Mass.), and fixed at 80°C for 2 h. The membrane was routinely probed with 10^6 cpm/ml. Probes were labelled with the Megaprime DNA labelling kit (Amersham, Buckinghamshire, United Kingdom). Labelling and hybridization were done as suggested by the manufacturers. Briefly, prehybridization was done in a 50% formamide (deionized) solution containing 1% sodium dodecyl sulfate (SDS), 1 M sodium chloride, and 10% dextran sulfate at 42°C. The hybridization was done overnight with the probes indicated in the figure legends, and 100 μg of denaturated salmon sperm DNA per ml. The hybridized filters were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature and twice in 2× SSC–1% SDS at 60°C for 30 min with constant agitation. The blots were exposed to X-ray film (Eastman Kodak, Rochester, N.Y.), usually overnight at −80°C. The probe for human PTX3 was the full-length cDNA cloned in Bluescript vector (5). The plasmid containing the full-length cDNA for human MCP-1 (0.672-kb *Pst*I-*Pst*I fragment) was used as an MCP-1 probe (8). To quantify the signal, we have used a scanning densitometer (GS 300 transmittance/reflectance; Hoefer Scientific Instruments, San Francisco, Calif.). Results are given in units of relative fluorescence intensity.

ELISA. ELISA plates (96 well; Nunc-Immuno Plate, MaxiSorp; Nunc) were coated with 50 μl of monoclonal rat anti-PTX3 antibody MNB3 serum or rabbit anti-MCP-1 serum diluted 1/3,000 in coating buffer (phosphate-buffered saline plus 0.05% Tween 20) and were incubated overnight at 4°C. The plates were washed three times with the coating buffer, and then the wells were saturated for 2 h at room temperature with 5% dry milk diluted in coating buffer in order to block the nonspecific binding. One hundred microliters of either recombinant human PTX3 standards (purified from supernatant of the high-producing CHO clone [46]), MCP-1 recombinant protein (23), or cell supernatants was added, and the plates were then incubated for 2 h at 37°C. After three washes with the coating buffer, 100 μl of polyclonal rabbit anti-PTX3 or anti-MCP-1 antibodies was added for 2 h at 37°C. Wells were then washed and incubated with 100 μl of peroxidase-conjugated sheep anti-rabbit immunoglobulin G (Amersham) diluted 1/2,000 for 1 h at room temperature. After incubation the plates were washed, and 100 μl of chromogen substrate ABTS [2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid)] was added. Absorbance values were read at 405 nm in an automatic ELISA reader.

RESULTS

Induction of PTX3 expression by LAM. In order to evaluate whether different LAM preparations were able to stimulate PTX3 expression, we exposed PBMC to different glycerolipids for 5 h and evaluated PTX3 mRNA expression. Figure 1 shows a typical experiment in which different LAM preparations were tested, and Fig. 2 shows dose-response and kinetic analyses.

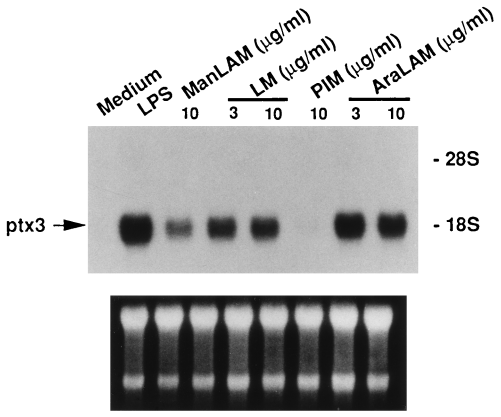

FIG. 1. Stimulation of PTX3 expression by mycobacterial cell wall components. PBMC were stimulated with LPS (100 ng/ml), ManLAM (10 μg/ml), LM (3 and 10 μg/ml), PIM (10 μg/ml), and AraLAM (3 and 10 μg/ml) for 5 h at 37°C. Total RNA was extracted and subjected to Northern analysis. The results of hybridization with a PTX3 probe are shown. The photograph of the ethidium bromide-stained nitrocellulose membrane is also shown to document that a comparable amount of RNA in each lane had been loaded and transferred. 18S and 28S, rRNA.

LAM induced a substantial level of PTX3 transcripts in human PBMC. AraLAM and LM stimulated PTX3 expression to the same extent as LPS, whereas ManLAM was less effective (Fig. 1). In order to better evaluate the difference, a dose-response analysis was performed as shown in Fig. 2A. AraLAM was more potent than ManLAM for inducing the expression of the transcript. Indeed, 0.01 μg of AraLAM per ml was sufficient to induce gene expression, whereas 1 μg of ManLAM per ml was required to produce an observable effect. The relative potency of AraLAM versus ManLAM varied considerably in different donors. When cells were stimulated with PIM (Fig. 1), we observed a very low level of induction of gene expression, detectable after a longer exposure of the membrane (data not shown). Induction of PTX3 was rapid and transient, with a maximum at 5 h and a return to baseline after 23 h (Fig. 2B).

Since LPS induced PTX3 expression in vitro and in vivo, it was important to assess whether contaminant LPS could account for LAM-induced PTX3 expression. AraLAM and ManLAM preparations had similar endotoxin contaminations (around 10 ng/ml), which were unlikely to account for induction since they showed a 30- to 100-fold difference in potency (Fig. 2A). Moreover, as shown in Fig. 3, polymyxin B (which binds stoichiometrically to the lipid A moiety of LPS and inhibits its activity) used at 2.5 μg/ml did not affect the capacity of AraLAM to induce PTX3 expression, whereas it inhibits nearly completely LPS-induced PTX3 expression.

A previous study has shown that among leukocytes only mononuclear phagocytes express PTX3 (32). In agreement with this previous report, we found that LAM induced PTX3 expression in purified monocytes. As shown in Fig. 4, ManLAM induced a stronger signal in monocytes than in PBMC, presumably as a consequence of a higher frequency of responding cells. Having established that the mycobacterial cell wall component LAM has the capacity to induce PTX3 expression in monocytes, we considered it important to evaluate whether whole mycobacteria had this property. As shown in Fig. 5, 10^5 *M. bovis* BCG cells are sufficient to stimulate PTX3 expression. We observed that with 10^7 cells gene expression is comparable to that obtained when cells are stimulated by LPS (100 ng/ml).

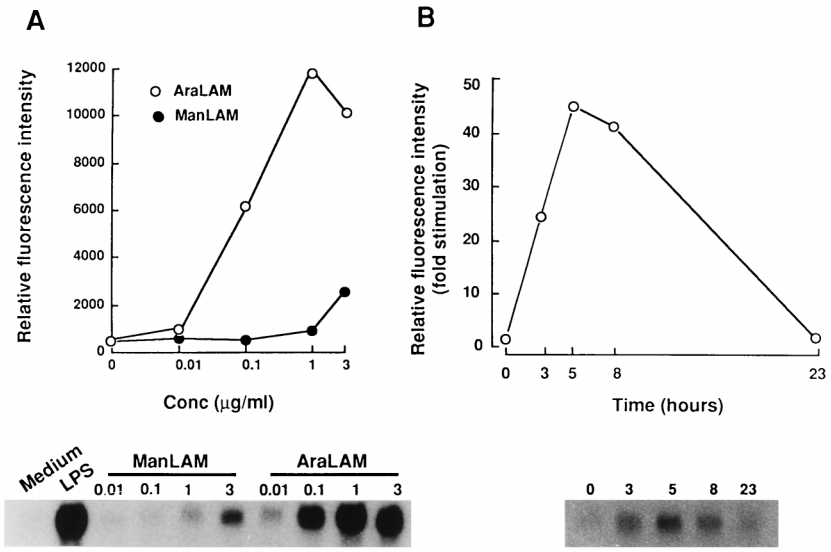

FIG. 2. Expression of PTX3 in PBMC: dose-response relationship and kinetics. (A) PBMC were stimulated with increasing concentrations of ManLAM and AraLAM for 5 h at 37°C, and then total RNA was extracted and subjected to Northern analysis. In the upper part, the quantification of mRNA expression, done with a scanning densitometer, is presented as relative fluorescence intensity. The lower part shows the results obtained when the blot was hybridized with PTX3 probe and the film was exposed for 48 h at −80°C. The experiment shown is representative of two performed. (B) PBMC were stimulated with 1 μg of AraLAM per ml for the indicated times at 37°C, and total RNA was extracted and subjected to Northern analysis (lower part). The quantification of mRNA expression, done with a scanning densitometer, is presented as relative fluorescence intensity (upper part). The experiment shown is representative of two performed.

Mechanisms of LAM-induced PTX3 expression. To obtain information as to the mechanisms involved in the regulation of LAM-induced PTX3 expression in PBMC, we performed experiments with Act D, a transcription inhibitor, and CHX, a protein synthesis inhibitor. As shown in Fig. 6, upper panel, Act D or CHX alone did not stimulate PTX3 expression. Preincubation of cells with Act D resulted in a complete inhibition of LAM-induced PTX3 expression, while preincubation with CHX only partially reduced PTX3 expression. However, and as previously demonstrated (32), Act D and CHX completely inhibited induction of PTX3 stimulated by LPS. These results were consistently observed with four different donors. LAM induced IL-1 and TNF (2, 25, 34), and these induced PTX3 (15, 16). IL-1ra and anti-TNF did not inhibit LAM-induced PTX3 expression (Fig. 6, lower panel). These results suggest that LAM does not induce PTX3 via IL-1 and TNF.

Regulation of LAM-induced PTX3 expression by cytokines. An extensive search revealed that among cytokines, only IL-1 and TNF induce PTX3 expression in monocytes and endothelial cells (32). It was of interest to investigate whether anti-inflammatory cytokines (IL-10 and IL-13) could affect PTX3 induction by LAM in human monocytes. As shown in Fig. 7A, IL-10 and IL-13 alone did not induce PTX3 expression and did not appreciably modulate LPS- or AraLAM-induced PTX3 expression. To validate these observations further, we hybridized the same filters with the MCP-1 cDNA probe (Fig. 7B). As previously reported, both IL-10 and IL-13 induce a strong inhibition of the LPS-induced MCP-1 expression. Interestingly, AraLAM also induced MCP-1 expression, and this expression

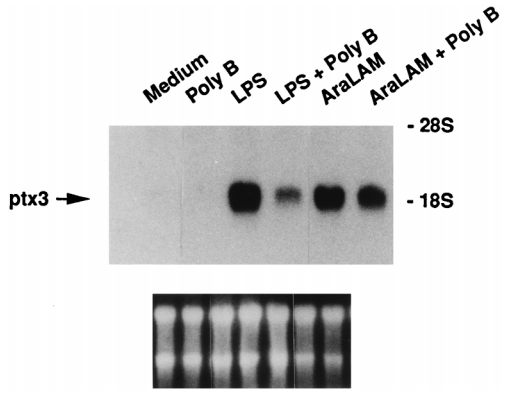

FIG. 3. Effect of polymyxin B on LAM-induced PTX3 expression. PBMC were stimulated with the indicated agonists in the presence or absence of 2.5 μg of polymyxin B (Poly B) per ml for 5 h at 37°C. Total RNA was extracted and subjected to Northern analysis. The blot was hybridized with PTX3 probe. The ethidium bromide-stained membrane is shown. Densitometric analysis gave the following values in arbitrary units: medium, 1; polymyxin B, 1; LPS, 8,143; LPS plus polymyxin B, 1,223; AraLAM, 4,515; AraLAM plus polymyxin B, 3,867. 18S and 28S, rRNA.

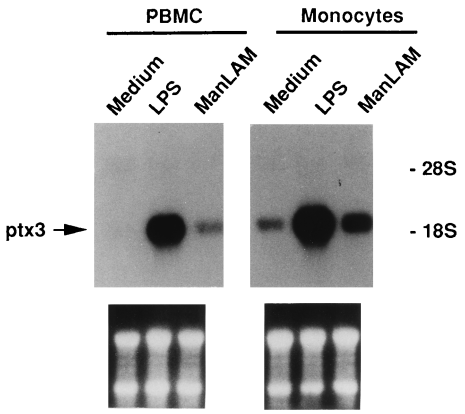

FIG. 4. Expression of PTX3 in LAM-stimulated monocytes. PBMC (left panel) and monocytes (right panel) were stimulated with LPS (100 ng/ml) and ManLAM (3 μg/ml) for 5 h at 37°C. Total RNA was extracted and subjected to Northern analysis. The blot was hybridized with the PTX3 probe. The ethidium bromide-stained membrane is shown. 18S and 28S, rRNA.

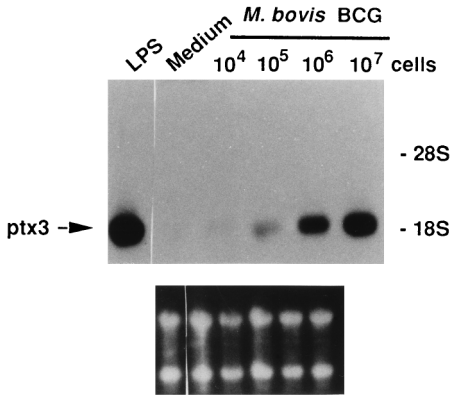

FIG. 5. Induction of PTX3 expression by *M. bovis* BCG. PBMC were stimulated with the indicated numbers of *M. bovis* BCG cells for 5 h at 37°C. PBMC were then lysed in a guanidium buffer. RNA extraction and Northern analysis were performed as described in Materials and Methods. The results of hybridization with the PTX3 probe and the ethidium bromide-stained nitrocellulose membrane are shown. The experiment shown is representative of two performed.

is inhibited by IL-10 and IL-13 exposure (18). In general, IFN-γ synergizes with bacterial products for macrophage activation (10, 25). Surprisingly, a dramatic inhibition of the AraLAM-induced PTX3 message was seen with a clear dose-response effect (Fig. 8A). As shown in Fig. 8B, IFN-γ synergized with AraLAM for MCP-1 expression as expected.

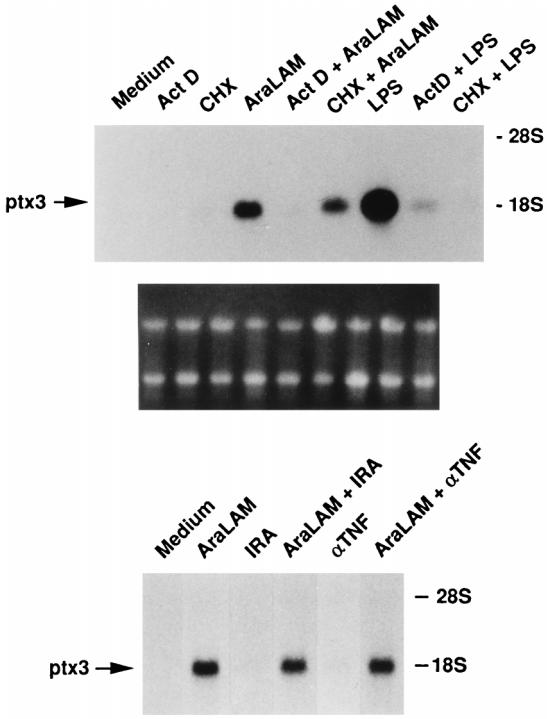

FIG. 6. Mechanisms of LAM-induced PTX3 expression in PBMC. (Upper panel) Effect of CHX and Act D. PBMC were stimulated with LPS (100 ng/ml) and AraLAM (1 μg/ml) for 5 h at 37°C in the presence or absence of CHX (10 μg/ml) or Act D (1 μg/ml). Total RNA was extracted and subjected to Northern analysis. The results of hybridization with the PTX3 probe and the ethidium bromide-stained membrane are shown. The experiment shown is representative of two performed. (Lower panel) Effect of IL-1ra and anti-TNF. The protocol was as above except for the presence of IL-1ra (IRA) and anti-TNF. The anti-TNF B154.2 antibody was used at a 1:2,000 final dilution, and IL-1ra was used at a concentration of 5 μg/ml.

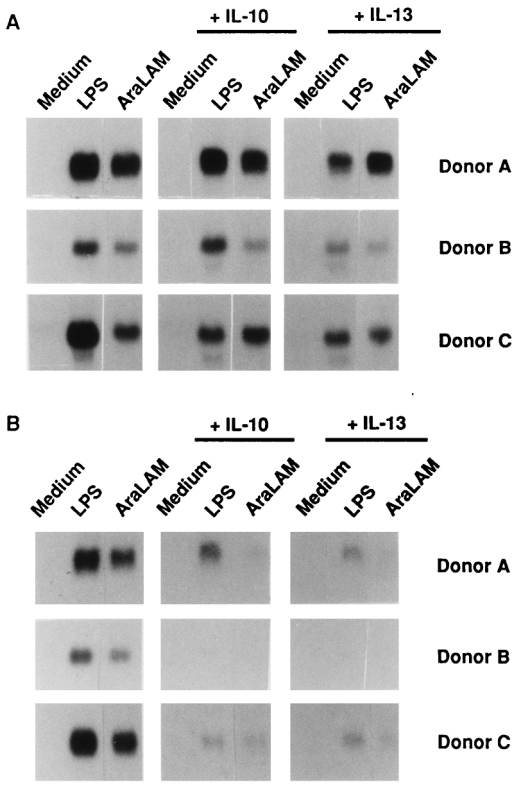

FIG. 7. Regulation of LAM-induced PTX3 expression by cytokines. PBMC isolated from three different donors were stimulated with LPS (100 ng/ml) and AraLAM (1 μg/ml) for 5 h at 37°C in the absence (left blots) or presence of IL-10 or IL-13 used at a concentration of 10 ng/ml. Total RNA was extracted and subjected to Northern analysis. The results of hybridization with PTX3 (A) and MCP-1 (B) probes are shown.

PTX3 protein production. Having established that LAM induces PTX3 mRNA expression in monocytes, we considered it important to evaluate whether PTX3 protein was indeed released and measurable under these experimental conditions. Exposure to LAM or *M. bovis* BCG markedly augmented the production of immunoreactive PTX3, as assessed by ELISA (Fig. 9A). As expected, at the protein level IFN-γ inhibited AraLAM-induced PTX3 production (Fig. 9B).

DISCUSSION

The results presented here show that the mycobacterial cell wall component LAM induces expression of the prototypic long pentraxin PTX3 in human PBMC. LAM induced PTX3 expression in monocytes, which, as indicated in a previous report, constitute the only leukocyte population capable of expressing this molecule (32). Transcript expression was associated with protein production and release, as assessed by a specific ELISA.

Stimulation of PTX3 expression by LAM was blocked by Act D, indicating that the induction is dependent upon gene transcription. Inhibition of protein synthesis by CHX did not completely impair the induction of PTX3 mRNA by LAM. LAM has been shown to induce expression of inflammatory cytokines including TNF and IL-1 (34). These are known inducers of PTX3 (5). The observation that CHX does not totally block LAM-induced PTX3 expression suggests that secondary medi-

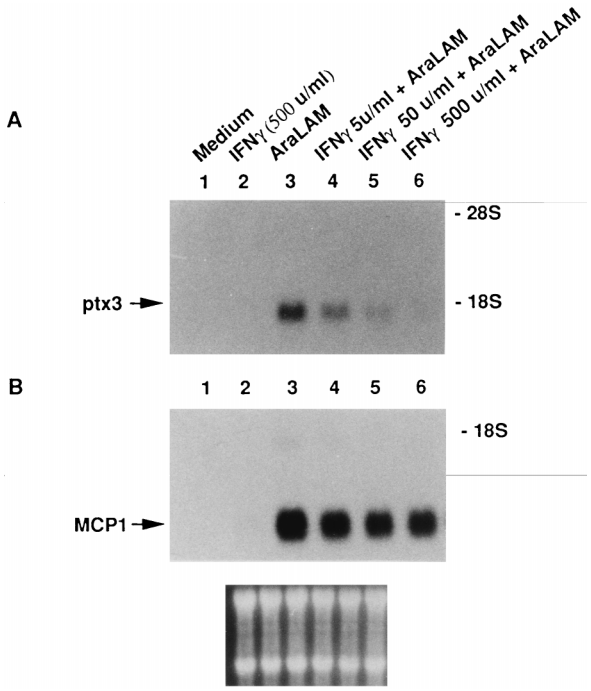

FIG. 8. Inhibition of AraLAM-induced PTX3 expression by IFN-γ in PBMC. PBMC were stimulated with AraLAM (1 μg/ml) for 5 h at 37°C in the presence of the indicated concentrations of IFN-γ or in the absence of IFN-γ. Total RNA was extracted and subjected to Northern analysis. The results of hybridization with PTX3 (A) and MCP-1 (B) probes are shown. The ethidium bromide-stained membrane is shown. The experiment shown is representative of two performed.

ators, including IL-1 and TNF, could be required for induction of PTX3 expression. To explore this hypothesis, we pretreated PBMC with IL-1ra or an anti-TNF antibody and induced the expression of PTX3 with LAM. The results clearly indicate that these two cytokines are not required for PTX3 gene expression.

LAMs are complex structural constituents of mycobacteria endowed with immunomodulatory properties (14, 23–26). For instance, the core unit of LAM is formed by a phosphatidylinositol unit and a nonreducing end which can be capped with mannose residues (ManLAM) or can be noncapped (AraLAM). LM and PIM are simpler versions of LAM. LM lacks arabinan, whereas PIM lacks both arabinan and most mannan residues. LAM is likely to play an important role in the immunobiology of mycobacteria, possibly representing a target for immune responses and a determinant of bacterial pathogenicity and of septic host responses, such as fever and wasting. In the present study we found that AraLAM and the simpler version, LM, activate the gene to the same extent. ManLAM is less effective than AraLAM for inducing PTX3 expression. This result is consistent with previous observations on the relative potency of these molecules for modulating mononuclear phagocyte functions (2, 4, 14). When we used PIM, we obtained a modest PTX3 expression. In fact, it has already been shown that PIM is less effective in inducing cytokine production (2). LAMs have been reported to activate mononuclear phagocytes via CD14 and an unidentified coreceptor(s) (26, 34). The action of IFN-γ on AraLAM-induced PTX3 expression was independent of changes in receptor expression since IFN-γ did not inhibit CD14 expression. Moreover, IFN-γ inhibited PTX3 expression when applied as late as 24 h after the stimulus (data not shown). The elucidation of the mode of action of IFN-γ will eventually require an analysis of signal transduction and the PTX3 promoter; this work is under way.

PTX3 is a prototypic long pentraxin consisting of a C-terminal pentraxin domain encoded by the third exon of the gene coupled with an unrelated N-terminal portion (5, 32). The same organization is observed in other long pentraxins (12, 19, 24, 27). The major source of classical short pentraxins (CRP and SAP) in serum is the liver. Even though the precise roles of CRP or SAP are not yet known, diverse functions such as opsonization and scavenging of cellular debris have been described for these molecules (21). The function of PTX3 has not yet been defined. PTX3 differs from classical pentraxins in ligand specificity, but shares the ability to bind the C1q protein of the complement cascade (4a). The classical pentraxin CRP is a useful diagnostic tool for monitoring infection and inflammatory diseases. In preliminary assays we found that at least some patients with tuberculosis infection have elevated PTX3

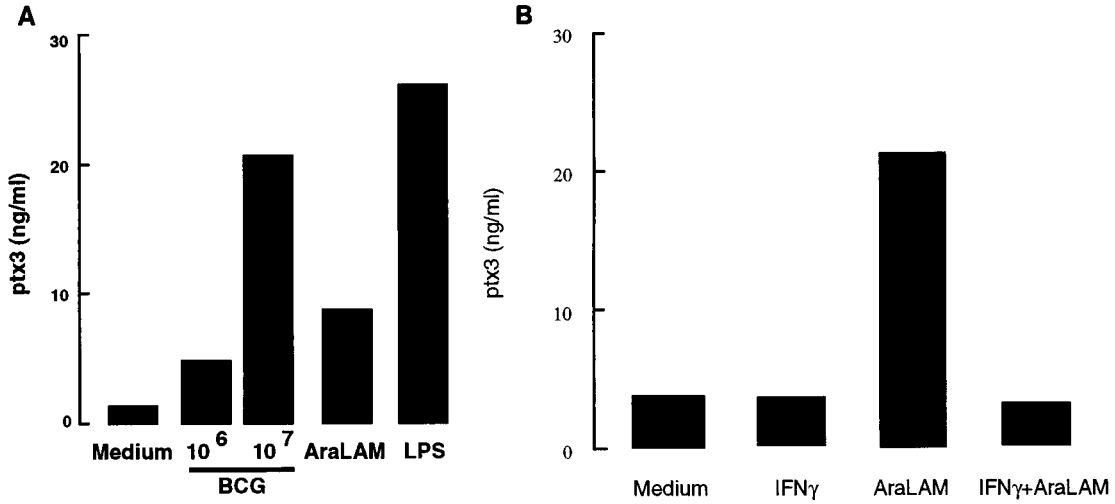

FIG. 9. Production of immunoreactive PTX3 by LAM-stimulated monocytes. (A) Monocytes were stimulated with *M. bovis* BCG, AraLAM (10 μg/ml), and LPS (100 ng/ml) for 24 h at 37°C (A) or were stimulated with AraLAM in the presence or absence of IFN-γ (500 U/ml) for 24 h at 37°C (B). The supernatant was then collected and subjected to ELISA as described in Materials and Methods. The experiment shown is representative of three performed.

levels (data not shown). We also have tested other microorganisms such as inactivated *Streptococcus* spp. and *Candida albicans*. The results (data not shown) indicate that these microorganisms are able to induce PTX3 expression. Therefore, expression of PTX3 is induced in monocytes following interactions with diverse infectious agents and their products. The results reported here, as well as the properties of classical pentraxins, suggest that the role and significance of PTX3 in mycobacterial infections deserve further study.

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