Inducible Expression of PTX3, a New Member of the Pentraxin Family, in Human Mononuclear Phagocytes

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The pentraxins C-reactive protein (CRP) and serum amyloid P component (SAP) are acute-phase proteins produced by liver epithelial cells. PTX3 was recently cloned as an interleukin-1 (IL-1)–inducible gene in endothelial cells, with structural similarities to pentaxins in the C-terminal half of the molecule. The present study was designed to investigate the expression of PTX3 in the human leukocyte populations. Human peripheral blood mononuclear cells exposed to lipopolysaccharide (LPS) or IL-1β expressed significant levels of PTX3 mRNA. Tumor necrosis factor-α (TNF-α) was a less-effective inducer of PTX3, whereas IL-6, monocyte chemotactic protein-1, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and interferon-γ were inactive. Among leukocytes, only monocytes exposed to inflammatory cytokines or LPS expressed the PTX3 transcript, which was undetectable in resting or stimulated polymorphonuclear cells, T- or B lymphocytes, and natural killer cells. PTX3 mRNA was also inducible in vitro monocyte-derived macrophages, in tumor-associated macrophages, and in the myelomonocytic cell lines HL60, U937, and THP1, but not in GDF8, with the latter possibly representative of earlier stages of myelomonocytic differentiation. T- and B-cell lines had no detectable PTX3. Inhibition of transcription by actinomycin D blocked induction of PTX3 in monocytes and nuclear run-on analysis showed that LPS induces the expression of the PTX3 gene at the transcriptional level in isolated monocytes. Cycloheximide had no effect on PTX3 induction in U937 cells, but was inhibitory on monocytes exposed to LPS or IL-1β. Monoclonal antibody against TNF and the IL-1 receptor antagonist did not inhibit induction of PTX3 in monocytes by LPS, thus excluding these cytokines as secondary stimulators of PTX3. IL-4, but not dexamethasone or transforming growth factor-β, inhibited PTX3 expression in monocytes. Using a PTX3-specific antisera, release of PTX3 protein was demonstrated for the first time in stimulated monocytes as well as in endothelial and fibroblastic cells. Thus, PTX3, unlike the classical pentraxins CRP and SAP, is expressed and released by cells of the monocyte-macrophage lineage exposed to inflammatory signals.

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In an attempt to better understand the molecular mechanisms underlying the metabolic response of human umbilical vein endothelial cells (HUVECs) to the cytokine interleukin-1β (IL-1β), we have recently cloned several "immediate early" genes from IL-1β-treated HUVECs by the use of a differential screening procedure. One of these genes, PTX3, is a novel member of the pentraxin gene family that includes C-reactive protein (CRP) and serum amyloid P component (SAP). PTX3 codes for a 381 amino acids-long protein whose C-terminal half is highly homologous with the entire CRP and SAP sequences and includes the "pentraxin" consensus motif and two cysteines conserved in all known members of the family. The PTX3 gene is organized in 3 exons and the C-terminal pentraxin domain is encoded exclusively by the third exon. No structural homology with other proteins has been found for the N-terminal half of PTX3.

Both CRP and SAP are acute-phase proteins extremely well conserved in evolution from horseshoe crab to humans. Although routinely used as reliable markers of inflammation, their exact in vivo function remains elusive. Their production is mainly restricted to the liver, although expression of CRP in natural killer (NK) cells has also been reported. We have previously shown that, on the contrary, PTX3 is induced by IL-1β and tumor necrosis factor-α (TNF-α), but not by IL-6, and in cells of different lineages, including endothelial cells, fibroblasts, and hepatocytes. In accordance with our data, the same gene, diverging from PTX3 by only two amino acids (at amino acid positions 48 and 202 of our published sequence), was cloned from TNF-stimulated fibroblasts and named TSG-14.

The previous report of expression of CRP detected in a subset of peripheral blood mononuclear cells (PBMCs) as well as the relatively widespread pattern of expression of PTX3 when compared with classical pentraxins prompted us to investigate whether cells of the hematopoietic system expressed and produced PTX3. We report here that inflammatory cytokines induce expression of PTX3 in mononuclear phagocytes, but not in other hematopoietic cells (lymphocytes, polymorphonuclear cells [PMNs], and NK cells), and for the first time show the release of the protein from stimulated cells from different lineages.

MATERIALS AND METHODS

Cell sources and culture conditions. The human myeloid cells lines HL60, U937, THP1, human B myeloma IM9, and the T cell leukemia Jurkat and Molt-4 were obtained from American Type Culture Collection (ATCC; Rockville, MD). The human Burkitt lymphoma Raji was a kind gift from Prof D.H. Crawford (London School of Hygiene and Tropical Medicine, London, UK). The human myeloid cell line GDF8 has been recently established and characterized.

The human fibrosarcoma cell line 8387 has been previously described.

Cell lines and freshly isolated leukocytes were cultured in RPMI 1640 (Seromed-Biochem KG, Berlin, Germany) supplemented with...
10% fetal calf serum (FCS; HyClone Steril System, Logan, UT) and 2 mmol/L glutamine ( Gibco, Glasgow, UK) at 37°C in the presence of 5% CO2. All reagents were tested for endotoxin contamination by limulus amebocyte lysate assay (Microbiological Associates, Walkersville, MD).

HUVECs were obtained and cultured as described previously22 and used at the third to seventh passage.

PBMCs were separated as described.23 Briefly, whole blood was fractionated by Ficoll Hypaque (Seromed-Biochem KG, Berlin, Germany) gradient centrifugation. The PBMCs were collected, washed twice with ice-cold phosphate-buffered saline (PBS), and then cultured in RPMI 1640 without FCS and used at 2 × 10^6 cell/mL. Monocytes were purified from the mononuclear cell fraction by a second centrifugation through a one-step discontinuous Percoll gradient (46%; Pharmacia Fine Chemicals, Uppsala, Sweden), as previously described.24 The percentage of monocytes in these populations was evaluated by nonspecific esterase staining and found consistently to be more than 90%.25 Monocyte-derived macrophages were obtained by a previously described culture of monocytes in RPMI 1640 with 10% autologous serum at the density of 3 × 10^6 cells/mL for 5 to 6 days at 37°C in the presence of 5% of CO2. Tumor-associated macrophages (TAM) were obtained from peritoneal effusions from advanced ovarian cancer patients after therapeutic paracentesis by centrifugation on discontinuous Ficoll-Hypaque gradients (75% to 100%) as described previously.25 Macrophages were collected at the 75% to 100% interface and subsequently separated from lymphocytes by centrifugation on a 42% Percoll gradient. The various phases of the separation procedure were monitored by morphologic examination of May-Grünwald-Giemsa-stained smears and by nonspecific esterase staining. When the separation was not satisfactory (less than 90% purity), the discontinuous Ficoll or Percoll gradients were repeated.

PMN isolation was performed as described.25 Briefly, whole blood was fractionated by Ficoll gradient centrifugation. PMNs were collected from the pellet, washed twice in PBS, resuspended in isosmotic (285 mosM) RPMI 1640, and layered on the top of a 62% Percoll solution. The PMNs were collected at the interface, washed in PBS, and resuspended at 2 × 10^6 cell/mL in RPMI 1640 without FCS. The purity (93% to 96%) of the cell preparations was verified by morphologic assessment of Giemsa-stained cytospin preparations.

Highly enriched NK cell populations were obtained by discontinuous Percoll gradient as previously described.26 Low-density cells were further depleted of contaminating T cells by panning on plastic dishes, as detailed elsewhere.27 The resulting NK populations were greater than 90% CD3 as assessed by FACS analysis (FACStar Plus; Becton Dickinson, Mountain View, CA) with the monoclonal antibody (MoAb) OKT3 (ATCC).

T lymphocytes were purified from the mononuclear cell fraction by rosetting with aminothiol oxidonium bromide hydrobromidetreated sheep red blood cells (AET-SRBC) and centrifugation through Ficoll-Hypaque as described previously.20 The purity of T-cell preparations was ascertained by staining with the anti-CD3 MoAb OKT3 by indirect immunofluorescence. B lymphocytes were purified by teasing out single cells from freshly excised tonsils and rosetting once with AET-SRBC.28 The nonrosetting cells were separated on a Ficoll gradient. They consisted of 93% to 96% B lymphocytes, as determined by indirect immunofluorescence with the anti-CD20 MoAb by indirect immunofluorescence. B lymphocytes were greater than 90% CD3- as assessed by FACS analysis (FACStar Plus; Becton Dickinson, Mountain View, CA) with the monoclonal antibody (MoAb) OKT3 (ATCC).

Cells were isolated from PBMCs by Percoll gradient centrifugation. They consisted of 93% to 96% B lymphocytes, as determined by indirect immunofluorescence with the anti-CD20 MoAb by indirect immunofluorescence. B lymphocytes were greater than 90% CD3- as assessed by FACS analysis (FACStar Plus; Becton Dickinson, Mountain View, CA) with the monoclonal antibody (MoAb) OKT3 (ATCC).

The cells at the interface between 40% and 50% Percoll were collected and will be called the "large" fraction; those at the interface between 55% and 75% Percoll will be called the "small" fraction. The "large" fraction contains a heterogeneous population of acti- vated and cycling B cells, whereas the "small" fraction contains virtually all resting B lymphocytes.21

**Cell stimulation conditions.** The cells were washed and incubated in endotoxin-free RPMI 1640 without FCS at 2 × 10^6 cell/mL with or without the stimulus, for different times at 37°C in the presence of 5% CO2. T lymphocytes were cultured for 3 days in RPMI 1640 supplemented with 10% FCS in the presence of phytohemagglutinin (PHA; Murex Diagnostic, LTD, Temple Hill, Darford, UK) at 1 µg/mL. IL-β (Dompé, L'Aquila, Italy) was used at 40 ng/mL final concentration, IL-2 (Roussel Uclaf, Paris, France) at 500 U/mL, IL-4 (InnuneX Corp, Seattle, WA) at 40 ng/mL, IL-6 at 50 U/mL (a kind gift of S. Gillis, InnuneX), interferon-γ (IFN-γ) at 100 U/mL (Roussel Uclaf), TNF-α at 500 U/mL (BASF/Knoll, Ludwigshafen, Germany), monocyte chemotactic protein-1 (MCP-1) at 10 ng/mL (Pepro-Tech Inc, Rocky Hill, NJ), IL-1 receptor antagonist (IRA) at 3 µg/mL (Cetus Corp, Emeryville, CA), transforming growth factor-β (TGF-β) at 1 ng/mL (Genentech, San Francisco, CA), granulocyte-macrophage colony-stimulating factor (GM-CSF) at 80 ng/mL (Behring, Marburg, Germany), macrophage-CSF (M-CSF) at 1,000 U/mL (Behring). It should be noted that these molecules have been routinely used in parallel experiments in the laboratory and found active in cell migration (MCP-1, M-CSF, and GM-CSF), macrophage or endothelial activation (IFN-γ, and proliferation (IL-6) assays.20,22,27,39 Furthermore, all cytokines were repeatedly tested for endotoxin contamination by limulus amebocyte lysate assay (sensitivity, 1 pg/mL). cycloheximide (CHX; Sigma Chemicals Co, St Louis, MO) was used at 10 µg/mL, actinomycin D (Act D; Sigma) at 1 µg/mL, and dexamethasone (DEX; Sigma) at 10^{-7} mol/L, lipopolysaccharide (LPS; Escherichia coli 055:B5; Difco, Detroit, MI) at 80 ng/mL. The MoAb against TNFα, B 154.2, was a kind gift from Dr G. Trinchieri (Wistar Institute, Philadelphia, PA); a 1,000 dilution of ascites neutralized 1,000 U/mL of recombinant TNF-α.

**RNA extraction, Northern blots, and run-on analysis.** RNA was extracted and purified using the guanidine isothiocyanate (Merek, Darmstadt, Germany) method as described previously.25,40 Twelve micrograms of total cellular RNA was run in standard formaldehyde-agarose gel, blotted onto nitrocellulose membranes (Schleicher and Schuell. Dassel, Germany), and fixed under vacuum at 80°C for 2 hours. Probes, labeling, and hybridizations were performed as previously described.40 The hybridized filters were washed twice in 2 × SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature and once in 0.5% SSC, 0.1% SDS at 55°C. The blots were exposed to Kodak X-AR x-ray film (Eastman Kodach, Rochester, NY) generally overnight at −70°C. The probe for the detection of PTX3-specific RNA was the full-length human cDNA cloned in Bluescript vector as reported. The β-actin probe has been described previously.41

Nuclei were isolated from unstimulated monocytes after 1 hour of exposure to LPS (80 ng/mL) and from unstimulated 8387 fibroblasts after 1 hour of exposure to TNF (500 U/mL) and used for in vitro transcription as reported previously. The labeled samples were heated for 5 minutes at 95°C and then hybridized to the immobilized template DNAs at 42°C in 45% deionized formamide, 0.2 mol/L sodium phosphate buffer, pH 7.2, 1 mmol/L EDTA, 7% SDS, and 250 µg/mL E coli RNA for 48 hours. The probes were the empty pBlueBac III vector (Stratagene, La Jolla, CA) as negative control and the full-length cDNAs for PTX3,1 human IL-1β, human IL-6, and β actin.42 Washing conditions were as previously reported.42

Production of polyclonal antiserum. Immunization of rabbits to obtain anti-PTX3 antibody was performed by repeated subcutaneous (SC) injection with the recombinant protein produced in BL21 (DE3) bacteria. Briefly, a 1,222-bp fragment of PTX3 (from nucleotide 36 to nucleotide 1258 according to our published sequence) was subcloned into the BamHI site of pET3c vector41 and used to transform competent BL21 cells.42 Colonies were expanded in NZCYM
medium for 4 to 5 hours at 37°C and then induced with 0.6 mmol/L IPTG (Sigma) for 3 hours. At the end of the incubation the bacteria were pelleted, dissolved in sonication buffer (300 mmol/L KCl, 20 mmol/L HEPES, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.05% NP-40), sonicated for 10 minutes on ice, and then ultracentrifuged at 35,000 rpm for 30 minutes. The sonication and ultracentrifugation steps were repeated twice and the pellets were dissolved in 8 mol/L urea. The solubilized proteins were separated in a 10% polyacrylamide gel under reducing conditions. The gel slice containing recombinant PTX3 was excised, mechanically disrupted in saline, and injected SC into a 28-day-old rabbit (Charles River, Calco, Italy). Boosts were administered at 2, 4, and 9 weeks and serum was collected 7 days after the last injection.

Detection of PTX3 by Western analysis. The full-length PTX3 cDNA was subcloned into the BamHI site of the pSG5 vector (Stratagene) in both orientations. Ten micrograms of the recombinant plasmids was transfected into COS cells grown at 60% confluence in 100-mm Petri dishes using the calcium phosphate method. After 18 hours, medium was replaced by fresh Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% FCS for an additional 24 hours of culture. The dishes were then washed extensively with saline and incubated for an additional 18 hours with 4 mL of DMEM without FCS. Supernatants were then collected for Western analyses.

Human fibrosarcoma cells (8387) were grown in minimal essential medium (MEM; Gibco) with 10% FCS. Subconfluent cells were stimulated with 500 U/mL of human recombinant TNF-α for 48 hours in MEM without FCS. Confluent HUVECs were washed and incubated for 18 hours in serum-free RPMI containing 20 ng/ml human recombinant IL-1β. Human peripheral blood monocytes were incubated for 42 hours in serum-free RPMI 1640 with 80 ng/ml LPS. At the end of incubation, the supernatants were collected, phenylmethylsulfonyl fluoride (PMSF; Sigma) was added to a 1 mmol/L final concentration, and they were centrifuged at 2,000 rpm for 10 minutes and concentrated 10-fold with a Centriprep 10 (Amicon, Inc. Beverly, MA). The proteins separated on 10% polyacrylamide gels were electroblotted onto nitrocellulose filters (Schleicher and Schuell) at 70 V for 4 hours. Filters were incubated for 1 hour at room temperature in PBS containing 5% dry milk in PBS (blocking buffer) and then for 18 hours at 4°C with the rabbit polyclonal anti-PTX3 antiserum diluted 1:2,000 in blocking buffer. After washing with PBS containing 0.5% NP40 (Sigma), filters were incubated for 1 hour at room temperature with horseradish peroxidase-labeled goat antirabbit IgG (Amersham, Amersham, UK), diluted 1:5,000, and washed extensively with PBS containing 0.05% NP40. Labeled proteins were detected by enhanced chemiluminescence (ECL system; Amersham) in accordance with the manufacturer’s instructions.

RESULTS

PTX3 expression in human PBMCs. To determine whether PBMCs were able to express the PTX3 transcript, we isolated total cytoplasmic RNA from freshly prepared PBMCs after incubation for 6 hours in the absence or presence of LPS or IL-1β. As shown in Fig 1, PTX3 mRNA is undetectable in unstimulated cells, but is strongly induced by both signals. Similar results have been consistently observed with 10 different donors (data not shown and figures below). As a positive control for this and subsequent experiments, we used total cytoplasmic RNA isolated from HUVECs stimulated for 1 hour with IL-1β as previously reported.1 Unfractionated PBMCs were exposed to IL-1β or LPS and RNA was isolated at various times to study the kinetics of PTX3 expression. As can be seen in Fig 2, PTX3 expression is rapid and transient, as previously reported for other cell types,1 peaking at 4 hours for IL-1β and at 8 hours for LPS.

In the previous experiment, a relatively high concentration of LPS (80 ng/mL) was used. When different doses were tested, it became clear that PTX3 mRNA induction is readily detectable with concentrations as low as 1.56 ng/mL (data not shown).

Our previous observations had shown that PTX3 could be induced by exposure to IL-1β and TNF-α in different cell types, but not by IL-6.1 We therefore exposed PBMCs to different cytokines, as illustrated in Fig 3. The RNA was extracted after 6 hours and studied for PTX3 expression. The results show that LPS and IL-1β are by far the most effective inducing agents, whereas TNF-α is a very weak inducer; in this case, PTX3 transcript is detectable only after long exposure of the filters (4 to 5 days; data not shown). On the contrary, all the other signals tested, including M-CSF, GM-CSF, INF-γ, and IL-6, were repeatedly negative for PTX3 induction (Fig 3). Boiling and IRA blocked induction of PTX3 by IL-1, thus excluding any role of contaminating LPS in induction by this cytokine (data not shown and see below).

Mononuclear phagocytes account for PTX3 expression in leukocytes. We went on to characterize the PBMC type responsible for the observed expression of PTX3. Purified populations of T and B lymphocytes, monocytes, NK cells, and PMNs were cultured in the presence of different stimuli. Total cellular RNA was isolated at various times and analyzed for gene expression. As can be seen in Fig 4, PTX3 was

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never detectable in untreated cells and only in the purified monocytic population was an abundant message for PTX3 evident upon exposure to LPS (Fig 4). T or B lymphocytes, NK cells, and PMNs did not express substantial amounts of PTX3 mRNA before or after stimulation with LPS (not shown) or with prototypic activation signals (eg, PHA for T cells, IL-2 for NK cells). A faint hybridization band was seen occasionally with PMNs (Fig 4). We concluded that monocytes are the only leukocyte population expressing appreciable amounts of PTX3 mRNA and do so only after activation.

In an effort to further demonstrate the ability of cells of the myelomonocytic pathway to express PTX3 and to define its pattern of expression in these cells, cell populations representative of mature tissue macrophages and in vitro established cell lines “frozen” at different stages of maturation were studied by Northern analysis. As shown in Fig 5, PTX3 gene expression is not detectable in the very immature myeloid GFD8 cell line (recently established from an M1 leukemia). It is induced by IL-1β and TNF-α in the “intermediate” HL60 line (recently reclassified as M2 leukemia) and is induced by TNF more effectively than by IL-1β in the monoblastic U937 cell line (Fig 5). The THP1 cell line, which is more differentiated toward monocytes, responds to the same signals much like normal mature monocytes, in that IL-1β is a more potent activator of PTX3 expression.
Fig 4. Expression of PTXB-specific RNA in different human leukocytes. Leukocyte populations of different lineages were purified and stimulated as described in Materials and Methods. The different times of incubation of T lymphocytes with PHA are indicated. All other signals were administered for 6 hours. Total RNA was hybridized with the PTX3 probe. The same blot was subsequently hybridized with a β-actin probe to verify that equal amount of RNA had been loaded into the gel. 18S indicates the position of the 18S ribosomal RNA. This experiment is representative of three performed.

Fig 5. Expression of PTXB in different hematopoietic cell lines. The indicated cell lines were cultured in the presence or absence of IL-1β (40 ng/mL) or TNF-α (500 U/mL) for 6 hours before RNA extraction. The results of hybridization with the PTX3 probe are shown and below is the photograph of the ethidium bromide-stained nitrocellulose membrane. This experiment is representative of three performed.

than TNF-α (Fig 5). The high background level of the PTX3 transcript evident in the THP1 and HL60 cell lines has been observed repeatedly and is limited to these two cell lines out of the many cell types tested so far. Similarly, cell lines from other lineages, including B- and T-cell lines (Raji [Fig 5], IM9, Jurkat, and Molt-4 [data not shown]), were studied. In agreement with the data on mature circulating cells, these do not express PTX3 mRNA and are not responsive to IL-1β and TNF-α.

We also explored the response of human macrophages at an advanced stage of differentiation. Mature macrophages derived by in vitro culture of purified monocytes as well as freshly isolated TAM were tested for PTX3 production upon stimulation with LPS or LPS and IFN-γ, respectively, two stimulation regimens known to be effective in activating macrophages. For the TAM portion of the experiments, we used RNA prepared for a different study, which did not include cells treated with IFN-γ and LPS alone. In both cases, the PTX3 transcript was clearly detected in the activated but not in the untreated populations (Fig 6).

Regulation of PTX3 expression in monocytes. To obtain indications as to the mechanisms of PTX3 regulation in mononuclear phagocytes, we studied the effects of the transcription inhibitor Act D and of the protein synthesis inhibitor CHX on PTX3 induction. As shown in Fig 7A and B, Act D inhibited induction of PTX3 by LPS and IL-1β in monocytes and U937 cells. Furthermore, when we investigated the de novo transcription of the PTX3 gene by nuclear run-
on analysis, PTX3 was induced by 1 hour of exposure to LPS in monocytes, although at a lesser extent than IL-1β and IL-6 (Fig 8). We have also observed de novo induced transcription in the 8387 fibroblastic cell line by 1 hour of exposure to TNF (Fig 8), in agreement with similar observations performed on the TSG-14 gene.21 CHX had divergent effects on PTX3 expression in monocytes as opposed to U937 cells. In the U937 cell line, PTX3 mRNA induction by IL-1β, although relatively weak, does not require protein synthesis and, if anything, is stronger in the presence of CHX (Fig 7A). On the contrary (and surprisingly), the IL-1β response of normal monocytes is dramatically reduced by CHX (Fig 7B). A similar effect of CHX is also observed upon LPS stimulation of U937 or monocytes (Fig 7A and B), in that CHX inhibits the induction of PTX3 mRNA in LPS-treated monocytes but not in U937 cells (Fig 7). These results were consistently observed in three separate experiments (data not shown).

The finding that CHX inhibited the induction of PTX3 in monocytes (but not in U937) raised the interesting possibility that LPS- or IL-1β–induced cytokines acted as ultimate inducers of PTX3 in this cell population. Based on the panel of cytokines tested on PBMC (see above, Fig 3), attention was focused on IL-1β and TNF-α. As shown in Fig 9, the addition of IRA abolished the effect of IL-1β, whereas the anti–TNF-α (at active concentrations; see Materials and Methods) did not. More interestingly, PTX3 induction by LPS was not abrogated neither by IRA nor by anti–TNF-α nor by the contemporary presence of both, suggesting that during the response to LPS, IL-1β and TNF-α are not the major secondary mediators of PTX3 induction. In this same experiment, we have also exposed PBMCs to the combination of IRA and anti–TNF-α to verify that they do not induce PTX3 expression per se (Fig 9), as when tested independently (data not shown).

Mediators with anti-inflammatory activity (eg, glucocorticoids and some cytokines) regulate both the production of inflammatory molecules by monocytes and the acute-phase response. It was therefore of interest to determine whether DEX, TGF-β, or IL-4 were able to alter the induced PTX3 gene expression in PBMCs (Fig 10). Interestingly, although totally inactive when tested alone, IL-4 was able to switch off the IL-1β–induced PTX3 expression, whereas TGF-β did not induce expression and did not interfere with the inducibility of the message by IL-1β. On the same line, we tested the effect of adding the classical anti-inflammatory agent DEX on LPS-treated PBMCs. As indicated in Fig 10B, DEX was unable to induce PTX3 expression and did not interfere with the inducibility of the message by LPS when administered in association with LPS 30 minutes before or 30 minutes after LPS (Fig 10B). To control for the biologic activity of DEX in our experiments, the
Production of the PTX3 protein. Although the protein had been predicted from its amino acid sequence to be secreted, we wished to verify this and confirm its inducibility. A fragment of the PTX3 cDNA was subcloned into a prokaryotic expression vector and the partially purified recombinant protein was repeatedly injected into a rabbit to raise antisera for PTX3 showing that in all cases a specific signal for PTX3 protein is detectable only upon stimulation, although in these cells the higher molecular weight form (43 kD) is much more prominent. These data clearly demonstrate that PTX3 is a secreted molecule produced by several cell types after exposure to inflammatory cytokines.

**DISCUSSION**

The work presented here was aimed at determining the capacity of human leukocytes from a variety of lineages to express PTX3 in response to different stimulatory agents. PTX3 is a new member of the pentraxin gene family, that includes CRP and SAP in many different species. The data primarily show that normal human mononuclear phagocytes respond to IL-1β, LPS, and, to a lesser extent, TNF-α stimulation with the induction of the PTX3 mRNA, whereas cells from other lineages, ie, T or B lymphocytes, NK cells, and PMNs, do not express PTX3 message in any of the conditions used.

The induction of the PTX3 message in monocytes is rapid and transient with a peak at about 4 to 6 hours and can be observed even upon exposure to low doses of LPS (1.5 ng/mL). Furthermore, we show that PTX3 gene transcription is clearly induced in this cell population by LPS stimulation. Other cytokines, including IL-6, MCP-1, M-CSF, GM-CSF, and IFN-γ, that activate different functions in monocytes, as well as macrophages, do not induce PTX3 transcription.

Expression of PTX3 is dependent on active transcription in human monocytes (as it is in fibroblasts) and that its message is directly induced by LPS and IL-1β in U937 mononoblasts, whereas it seems to be regulated differently in normal monocytes, in this last case requiring the synthesis of some protein component.

Finally, we demonstrated that PTX3 protein is indeed pro-
duced and released in the culture supernatant upon stimulation of normal monocytes, HUVECs, or fibroblasts.

From several points of view, PTX3 behaves similarly to other "immediate-early" genes induced in monocytes-macrophages. In particular, a rapid and transient induction in both monocytes and endothelial cells (perhaps reflecting the common ontogenetic origin of these different cell types) after exposure to the same stimulus has been previously described for other genes including c-jun, c-fos, MCP-1, its murine equivalent, and IL-8, among others. Furthermore, the inhibitory effect of IL-4 has been reported in monocytes for the induction of the expression of other cytokines, including IL-1, IL-6, TNF-α, MCP-1, its murine equivalent, and IL-8. Interestingly, although PTX3 was cloned as an "immediate-early" gene in HUVEC stimulated by IL-1β in the presence of CHX, its "direct" expression seems somehow differently regulated along the myelomonocytic differentiation pathway, because it requires active protein synthesis in normal monocytes but not in their immature precursors. We have at the moment no explanation of what the "protein intermediate(s)" may be, but experiments have documented that, at least in the case of the LPS response, mediators others than the production and release of IL-1 and TNF by monocytes are implicated. Whether they are new released molecules or, rather, intracellular messengers remains to be investigated. These data are reminiscent of the pattern of expression of the MCP-1 chemokine that is also dependent on active protein synthesis in human monocytes stimulated by IL-1β, LPS, and thrombin, but not in stimulated HUVECs (unpublished data).

PTX3 shares similarities with CRP and SAP that include an extensive sequence homology (extending throughout the third exon of PTX3 that corresponds to the C-terminal half of PTX3 protein), the induction by IL-1 and TNF, and the production by hepatocytes. On the other hand, differences between PTX3 and the other pentraxins include the completely divergent N-terminal half of the molecule, the nonresponsiveness of PTX3 to IL-6, and the wider range of cell types that are able to produce PTX3, including fibroblasts, endothelial cells, and monocytes-macrophages. Indeed, a gene called TSG-14, which differs only by two amino-acids relative to PTX3, has been cloned from TNF-α-stimulated fibroblasts, thus confirming the more promiscuous cell type responsiveness of this new pentraxin. Furthermore, we have also been able to confirm in the course of this study that all leukocyte subpopulations tested do not express detectable levels of CRP mRNA by Northern analysis (data not shown), whereas its message is abundantly detectable only in hepatocytes.

Although known for many years, the in vivo role of CRP and SAP is still unclear. However, several observations suggest that they may play a role within the natural immune system, eg, as opsonins for macrophages and neutrophils, as inhibitors of neutrophil chemotaxis, by increasing tumor cytotoxicity mediated by macrophages, and by inducing the release of several cytokines by macrophages, and more recently, by inducing the synthesis of tissue factor by monocytes. Furthermore, CRP has been found on the surface of NK cells and may be produced by these cells, although the transcript was detectable only after PCR amplification. In all cases, rather high concentrations of CRP or SAP have been used, perhaps mimicking the high circulating levels reached by these molecules during the acute-phase response. It will be of great interest to test the ability of PTX3 to perform similar functions, particularly because PTX3 is produced and released directly by monocytes-macrophages, fibroblasts, and endothelial cells and may therefore take part in localized inflammatory processes, during which cells of natural immunity play a major role.

In perspective, it is particularly intriguing that PTX3, on the one hand, shares with the classic acute-phase reactants (CRP in humans and SAP in mice) the characteristic of being produced and released in response to inflammatory cytokines and, on the other hand, it is the first pentraxin produced and released in vitro by cells lining the blood vessels and by circulating monocytes themselves. Preliminary data suggest...
that its murine equivalent is produced in vivo upon intravenous LPS injection (unpublished observations). It will therefore be important to conduct further work to define the relevance of PTX3 as a new mediator and marker for inflammatory conditions.

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