Bacterial Peptidoglycan Polysaccharides in Sterile Human Spleen Induce Proinflammatory Cytokine Production by Human Blood Cells

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Peptidoglycan (PG) is the major component of the cell wall of gram-positive bacteria. In vitro, PG isolated from conventional bacterial cultures can induce secretion of proinflammatory cytokines by human monocytes, indicating that PG may be involved in immune responses against infections by gram-positive bacteria. To investigate the biologic activity of PG in human tissues, an improved method was developed to isolate significant amounts of PG from sterile human spleen tissue. Biochemical analysis demonstrated that PG isolated from human spleen is largely intact. Human whole blood cell cultures were able to produce the proinflammatory cytokines tumor necrosis factor-α and interleukin-1 and -6 after stimulation with PG isolated from human spleen. Cytokine induction was not sensitive to inhibition by polymyxin B, in contrast to lipopolysaccharide. Collectively, the data show that intact PG in sterile human tissue is biologically active and may induce local proinflammatory cytokine production.

Peptidoglycan (PG) is the major component of the cell wall of gram-positive bacteria. PG is composed of alternating *N*-acetyl glycosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc) long sugar chains that are interlinked by peptide bridges resulting in a large complex macromolecular structure [1]. During bacterial infection, PG and several other cell wall components are thought to be involved in the inflammatory reaction [2]. In gram-negative infections, lipopolysaccharide (LPS) is a well-known activator of the innate immune system [3, 4]. During gram-positive infections, when no endotoxin is present, PG can activate complement [5, 6] and granulocytes [7, 8] and also up-regulate expression of adhesion molecules on endothelial cells [8]. Of importance, PG can also induce production of proinflammatory cytokines such as interleukin (IL)-1 and -6 and tumor necrosis factor (TNF)-α by monocytes in vitro [9, 10]. Similar to LPS-induced cytokine production, inhibition can be achieved by preventing uptake of PG and/or activation of macrophages and monocytes by blocking the CD14 receptor [11, 12].

In addition to the role of PG in gram-positive infections, it has been hypothesized that PG is involved in the pathogenesis of chronic inflammation such as rheumatoid arthritis [13–15]. We are interested in the relationship between PG derived from the normal intestinal flora and the pathogenesis of rheumatoid arthritis [16, 17]. The presence of PG in relevant tissues is a prerequisite for this relationship. Most studies investigating the presence of PG in human tissues focus on the detection of muramic acid, the characteristic amino sugar in the glycan backbone of PG, which can be detected after release of the *N*-acetyl group of MurNAc by acid hydrolysis. Furthermore, radiolabeled PG has been used to study the localization of PG in vivo. With these different techniques, PG was detected in liver and spleen [18, 19], synovial tissues [18, 19], urine [20], synovial fluid [21], and peripheral blood leukocytes [22]. We detected PG in dendritic cells and macrophages of normal human spleen and synovial tissues of rheumatoid arthritis patients [23] by use of a monoclonal antibody (MAb) directed against bacterial flora–derived PG isolated from human feces [24]. We also isolated PG from normal human spleen with biochemical methods [25].

The presence of PG in human tissues other than the gastrointestinal tract suggests that the body is constantly exposed to bacterial products, even in the absence of clinically apparent infection. PG in human tissues is probably derived from the normal gut flora. Experiments in rats have shown that PG can be absorbed from the bowel wall [26].

All studies to determine the biologic activity of PG have focused on PG isolated from bacterial cultures. Thus far the biologic properties of PG in human tissues have never been studied. Therefore, the aim of this study was to determine whether PG in sterile human tissues has the capacity to induce production of proinflammatory cytokines by human peripheral blood mononuclear cells. To this end, we developed an improved method to isolate high amounts of structurally intact

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PG with minimal traces of contaminating protein from sterile human spleen tissue. Biological activity of this PG fraction was determined by analysis of its ability to induce production of proinflammatory cytokines by human blood cells.

Materials and Methods

Spleens used for isolation of PG. Seven unfixed human spleens were obtained from the pathology department immediately after surgery (SSDZ, Delft, The Netherlands) and kept frozen ($-20°C$) until use. Spleens were removed for surgical technical reasons from 3 patients with gastric carcinoma and from 4 patients because of splenomegaly due to hematologic diseases. Spleen samples were aerobically and anaerobically cultured on blood agar base (Oxoid, London) for 48 h at 37°C. The anaerobic plates were cultured in a jar with gaspack generator envelopes with palladium catalyst (Becton Dickinson, San Jose, CA). Bacterial growth was not observed, confirming that no bacteremia in vivo or contamination of the tissue after surgery had occurred.

Immunohistochemistry for detection of PG in spleen. To determine whether the spleens obtained to isolate PG contained PG in antigen-presenting cells, immunohistochemical staining of frozen sections was done according to the method of Kool et al. [24] for staining of rat spleen. We used the same MAb (2E9; mouse IgG3) recognizing human intestinal flora–derived PG.

PG isolation from human feces. PG from feces of a healthy subject was prepared as described previously [27]. In brief, feces were diluted in distilled water (40 g in 100 mL) and homogenized in a laboratory blender (model 400; Stomacher, Colworth, UK). After cambric gauze filtration, the suspension was centrifuged for 45 min at 5000 g. Four volumes of 96% ethanol were added to 1 vol of supernatant, and after 2 h at 4°C the precipitate was centrifuged for 15 min at 5000 g. The pellet was dissolved in and dialyzed against Milli-Q water (Millipore, Bedford, MA) for 48 h. Next, the suspension was centrifuged for 1 h at 100,000 g, and the clear supernatant was collected. Size-exclusion chromatography was done using dilutions of 15–60 mg/30 mL (depending on the viscosity of the solution) with a TSK HW75 column (Merck, Darmstadt, Germany; gel bed 700× eluent). After passage of 100 mL of void volume, fractions (8 mL/5 min) were collected and assayed for their protein and carbohydrate contents. High-molecular-weight fractions containing carbohydrates but no proteins were pooled, dialyzed, and lyophilized. From 100 g of feces, ~50 mg of PG could be retrieved by this procedure.

PG isolation from human spleen. Some 50–100 g of minced spleen tissue in 500–1000 mL Milli-Q water was homogenized in portions in a homogenizer (Virtis, New York) at 10,000 rpm for 30 s. The homogenate was sonicated 5 times for 1 min at maximum amplitude (soniprep 150; MSE, Crawley, UK). Acetic acid (96%) was added to a final concentration of 0.2 M. The extract was incubated at room temperature for 2 h under rotation and was subsequently heated gradually in a water bath by 100°C over 30 min. The extract was centrifuged at 10,000 g for 60 min (4°C). The volume of the supernatant was reduced by lyophilization to 25–50 mL, and the extract was then centrifuged at 100,000 g for 60 min (4°C). The supernatants were separated by gel filtration on a 275-mL gel bed (Sephadex G-25; Pharmacia, Uppsala, Sweden). Mur-

amic acid and protein content were determined in all fractions according to the methods of Hadzija [28] (with modifications [29]) and Bradford [30], respectively. The high-molecular-weight fraction containing muramic acid was collected, and size exclusion was done by loading 1-mL fractions on a Superdex 200 column (Pharmacia) connected to a fast performance liquid chromatograph (FPLC; Pharmacia). Protein was determined in all fractions according to Bradford [30], and PG was measured by ELISA. Fractions were pooled (see below), dialyzed against Milli-Q water, and lyophilized.

Analysis of PG Isolated from Human Spleen

PG detection by ELISA. To measure PG in the fractions after Superdex 200 (Pharmacia) gel filtration, an ELISA was performed. We coated 50 μL of the fractions overnight at 50°C in 96-well polystyrene microtiter plates. The plates were washed 3 times with PBS-0.02% Tween 20 (Fluka Chemie, Buchs, Switzerland), and 100 μL of MAb 2E9 (10 μg/mL in PBS-0.2%Tween) was added to the wells. After 1 h at 37°C, unbound antibody was removed by 3 wash steps. As detecting antibody, we used peroxidase-conjugated rabbit anti-mouse immunoglobulin (P260; Dako, Glostrup, Denmark) diluted 1:1000 in PBS-Tween 0.2%. After 3 washes, the colorimetric assay was developed at 37°C for 30–45 min after the addition of 100 μL of ortho-phenylenediamine/H_2O_2. The reaction was stopped by the addition of 50 μL of 4 M H_2SO_4, and the optical density was measured at a wavelength of 492 nm (Titertek Multiskan; Flow Laboratories, Irvine, UK). Results were expressed as optical density units.

Protein analysis according to the method of Lowry et al. [31]. The amount of protein in the pooled fractions was determined by the method of Lowry et al. [31], with modifications [32]. We added 1 mL of 2% Na_2CO_3 in 0.1 M NaOH to 200 μL of the pooled fractions and incubated it for 45 min at 70°C. After the samples were cooled, 20 μL of 0.5% $CuSO_4.5H_2O$ was added, and the mixture was incubated for 10 min at room temperature. Finally, 200 μL of Folin-Ciocalteen reagent was added during 45 min at 37°C. Absorbance was measured at 620 nm.

Muramic acid analysis by colorimetric muramic acid assay. In the pooled fractions, the amount of muramic acid was determined in accordance with the Hadzija method [28] with some modifications [29]. In short, 100 μL of the samples was hydrolyzed by heating for 2 h at 90°C with an equal volume of 5 M H_2SO_4, then neutralized with 100 μL 10 M NaOH. Hydrolyzed and unhydrolyzed samples (100 μL) were incubated with 50 μL 1 M NaOH at 37°C for 30 min. After the addition of 1 mL 18.8 M H_2SO_4, samples were heated for 3.5 min at 100°C, rapidly cooled on ice, and mixed with 10 μL 0.16 M $CuSO_4.5H_2O$ in H_2O and 20 μL 0.09 M ρ-hydroxydiphenyl in ethanol. After incubation for 30 min at 30°C, absorbance at 570 nm was determined by Titertek Multiskan. Solutions containing 0–100 μg of muramic acid (Sigma, St. Louis) per milliliter of H_2O were used as standards. The data are given as the difference in concentration between the hydrolyzed and nonhydrolyzed samples. In this way, only muramic acid linked to peptides in PG was determined, and contaminating sugars like rhamnose and lactic acid, which are determined in the nonhydrolyzed sample, were excluded.

Muramic acid assay by high performance liquid chromatography (HPLC). The presence of muramic acid in the isolated fraction

was also determined by amino sugars using HPLC reversed-phase techniques as described by Glauner [33]. In short, 300 μL of the isolated PG was hydrolyzed with 9 M HCl for 3 h at 90°C. The sample was frozen, lyophilized, and redissolved in 100 μL of H_2O. This sample was added to 100 μL 200 mM borate buffer, pH 8.8, and 100 μL 20 mM dansyl chloride (5-dimethylamino-naphthalene-1-sulfonyl chloride; Fluka Chemie) in acetone for 2 h at 37°C. The reaction was stopped with 35 μL 200 mM phosphoric acid.

Separation of dansylated amino acids and amino sugars was accomplished by reversed-phase HPLC. Samples were analyzed using a single pump solvent delivery system (LAB 2248; Pharmacia) and a UV-VIS 2141 monitor (Pharmacia), both connected to a computer working with HPLC manager software to control the pump, gradient mixer, and UV-VIS detector operating at 330 nm. Integration and analysis of chromatograms was done using the same software (Pharmacia). Dansylated samples were separated by use of a 5-μm 4 × 250 nm column (SuperPac Sephacil C18; Pharmacia). The flow rate was 1 mL/min, and the two buffers were as follows: buffer A, 20 mM sodium phosphate, pH 5.25; and buffer B, 60% acetonitrile and 40% 50 mM sodium phosphate, pH 4.0. At 0, 10, 16, and 22 min, the concentration of buffer B was 30%, 50%, 100%, and 30%, respectively. Eluted products were detected at 330 nm, the wavelength that is maximally absorbed by the dansyl groups.

Induction of cytokine release in whole blood after stimulation with PG. Human peripheral blood was obtained from healthy donors and collected in sodium-heparin tubes (Vacutainer; Becton Dickinson). We added 12.5 μL of RPMI 1640 (Gibco, Breda, The Netherlands) containing isolated PG or LPS (Sigma) with or without the LPS antagonist polymyxin B (10 μg/mL; Sigma) to a polypropylene tube (Falcon; Becton Dickinson) containing 112.5 μL of blood. Inhibition assays were performed by incubation of human blood cells with 10 and 5 μg/mL anti-CD14 antibody (My-4, azide-free clone; Coulter, Hialeah, FL) or isotype control IgG2b antibody (MOPC-195 clone; Coulter) for 30 min at 37°C or by incubation of the isolated PG fractions and LPS with proteases (1%–0.001%: elastase, trypsin, α-chymotrypsin; Sigma) for 1 h at 37°C. The mixtures were incubated for 5 h at 37°C in a 5% CO_2 atmosphere, then 375 μL of RPMI was added to each tube, and the mixtures were centrifuged for 10 min at 400g. The supernatants were tested in bioassays for TNF-α, IL-1, and IL-6 activity.

TNF-α bioactivity was measured using the murine fibroblast cell line WEHI 164.13 in a cytotoxicity assay [34]. The cells were plated in flat-bottom tissue plates with 96-well plates (Costar, Cambridge, MA) at a concentration of 10^4 cells/well. The cells were allowed to adhere by incubation overnight at 37°C in a 5% CO_2 atmosphere. At day 2, 50 μL of actinomycin-D (4 μg/mL; Sigma) and 50 μL of diluted sample were added to the cells. Samples were tested in triplicate. After an overnight incubation (37°C, 5% CO_2), the MTT (Sigma) cytotoxicity test [35] was used to measure WEHI cell viability.

IL-1 bioactivity was measured by use of an IL-1–dependent subline of the murine T cell line D10.G4.1, designated D10(N4)M, by the method of Hopkins and Humphreys [36], with modifications [37]. One hundred microliters of sample was added to 10^4 cells/well, and the cells were cultured for 3 days. Proliferation of the cells was measured after 4 h of [³H]thymidine incorporation (0.2 μCi/well).

IL-6 activity was detected by use of the murine hybridoma cell line B9 according to Aarden et al. [38]. In the presence of 100 μL of sample, 5×10^3 cells/well were cultured for 3 days. Proliferation of IL-6–dependent B9 cells was measured after 4 h of [³H]thymidine incorporation (0.2 μCi/well).

Recombinant human IL-1β (UBI, Lake Placid, NY), IL-6 (gift of L.A. Aarden, CLB, Amsterdam), and TNF-α (Amersham, Amersham, UK) served as positive controls for the D10, B9, and WEHI assays. Cytokine activities of the samples were corrected for background activity of cells cultured in the absence of PG and were expressed as units per milliliter with 1 U corresponding to a half-maximum response obtained with a titration series of the recombinant cytokine.

TNF-α ELISA. TNF-α production in anti-CD14 and protease treatment experiments was measured by a capture ELISA that was done following the manufacturer's guidelines (Biosource, Fleurus, Belgium). In brief, polystyrene microtiter wells (Immuno maxisorp; Nunc, Roskilde, Denmark) were coated overnight at room temperature with human anti–TNF-α MAb, followed by a wash (0.9% NaCl) and 2 h of blocking (PBS/BSA 0.5%). Freshly thawed samples and a human TNF-α standard (calibrated against WHO international standard, TNF-α 87/650; National Institute for Biological Standards and Controls, Potters Bar, UK) were incubated for 2 h in the presence of a biotinylated second TNF-α antibody, followed by wash steps, polystreptavidine-HRP (CLB), and enzyme substrate (TMB peroxidase; KPL, Gaithersburg, MD). Optical density was measured at 450 nm.

Results

Immunohistochemical staining of human spleen. To confirm that antigen-presenting cells containing PG were present in human spleens to be used for purification of PG, immunohisto-
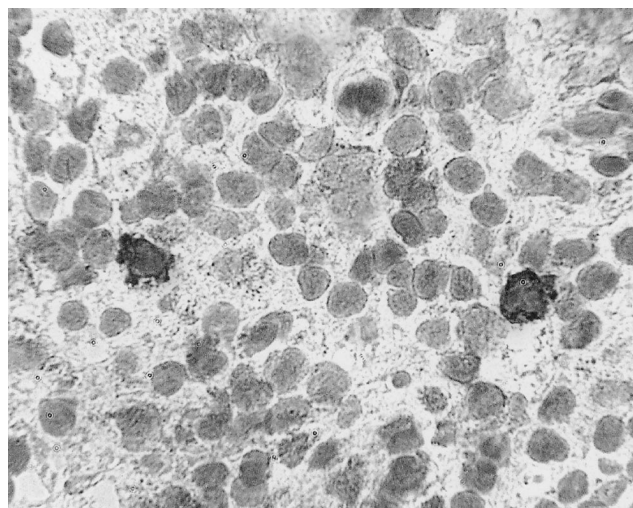

Figure 1. Detection of cells containing PG in human spleen. Immunohistochemical staining used 2E9. Morphology and anatomic localization of cells containing intracytoplasmatic PG is consistent with that of antigen-presenting cells. Magnification ×63.

chemical staining was done. With MAb 2E9 directed against PG derived from the normal intestinal flora, macrophages and dendritic cells in all 7 spleens stained positive (figure 1). With a negative control antibody of the same isotype (mouse IgG3) and with irrelevant specificity (anti-sheep red blood cells), no staining was found.

Isolation of PG from human spleen. The purification of PG from 7 individual human spleens was done as described. After homogenization and sonication, the relatively low-molecular-weight fraction was discarded by Sephadex G25 gel filtration. Figure 2 shows the elution pattern after Sephadex G25 gel filtration of 1 human spleen. Protein was first found after an elution volume of 120 mL of H_2O. Muramic acid was detected after an elution volume of 124 mL of H_2O until 156 mL. After an elution volume of 156 mL of H_2O, the signal for muramic acid increased sharply due to the presence of lactate, which interferes with the muramic acid assay. The fractions between 124 and 156 mL, which represented a relatively high-molecular-weight fraction, containing PG were pooled, and the relatively low-molecular-weight fraction was discarded.

The pooled fraction was loaded in 1-mL aliquots onto a Superdex 200 column to remove contaminating protein. The presence of PG in the eluted fractions was determined by ELISA. During the column passage, the aliquots were diluted 25 times, and therefore the colorimetric muramic acid assay was not sensitive enough to detect muramic acid. The protein content of all fractions was measured by the Bradford method [30]. Figure 3 shows the amount of protein and PG in all frac-

tions after Superdex 200 column gel filtration of 1 mL of the extract.

The fractions to be used for in vitro studies should contain minimal amounts of contaminating proteins. For this reason, fractions with comparable amounts of protein were pooled. This resulted in pooling of fractions 2–5 (P1), fractions 6–8 (P2), fractions 9–16 (P3), fraction 17 (P4), fractions 18–22 (P5), and fractions 23–25 (P6). Fraction 23–25 (P6) was used as a control, since this fraction did not contain PG or protein. After all runs of the 7 spleens, the corresponding fractions were pooled, dialyzed against Milli-Q water, lyophilized, and stored at −20°C.

Protein analysis. After Sephadex and Superdex 200 gel filtration, the level of protein in the fractions was measured according to Bradford [30]. With PG preparations of *Eubacterium aerofaciens* and *Brevibacterium divercatium,* we showed that the method according to Lowry et al. [31] was more sensitive to detect protein (data not shown). Because the dilutions of the pooled fractions used in some of the cytokine induction experiments were based on protein concentrations, we used the method of Lowry et al. (table 1). The results show that fractions P1 and P4 contain the lowest levels of protein.

Muramic acid analysis by colorimetric muramic acid assay. To determine the amount of PG in the pooled fractions, a colorimetric muramic acid assay was performed. Results are shown in table 1. Muramic acid could only be detected after acid hydrolysis to remove peptides from PG, indicating that PG present in human spleen is structurally mostly intact. In
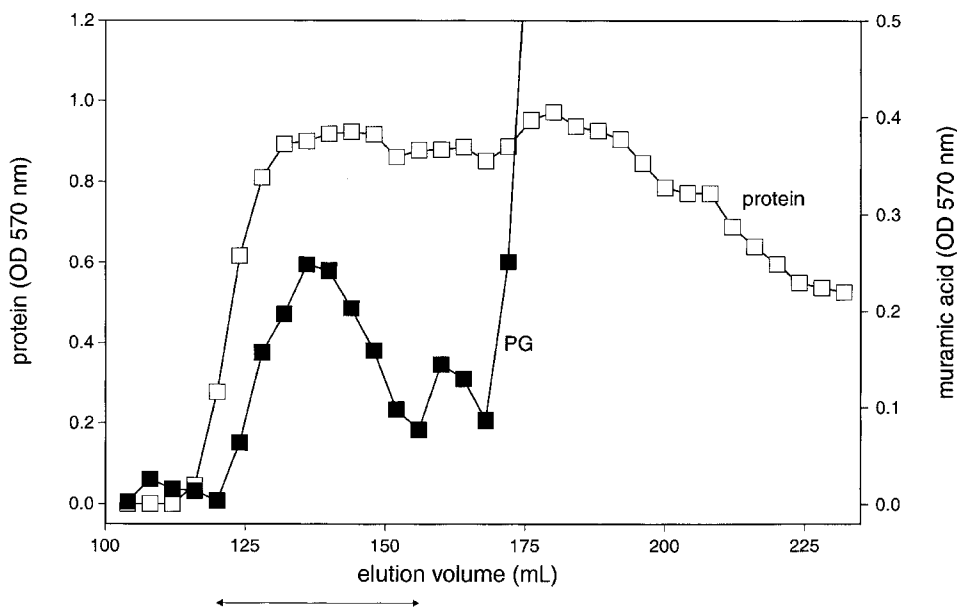

Figure 2. Separation of relatively high- and low-molecular-weight fractions from human spleen extract. Twenty-five milliliters of human spleen extract was added to Sephadex G25 column. In all fractions, amount of protein (□) according to Bradford [30] and amount of muramic acid (■) were measured. Fractions between 120 and 156 mL of elution volume, which represented relatively high-molecular-weight fraction containing muramic acid, were pooled (↔).
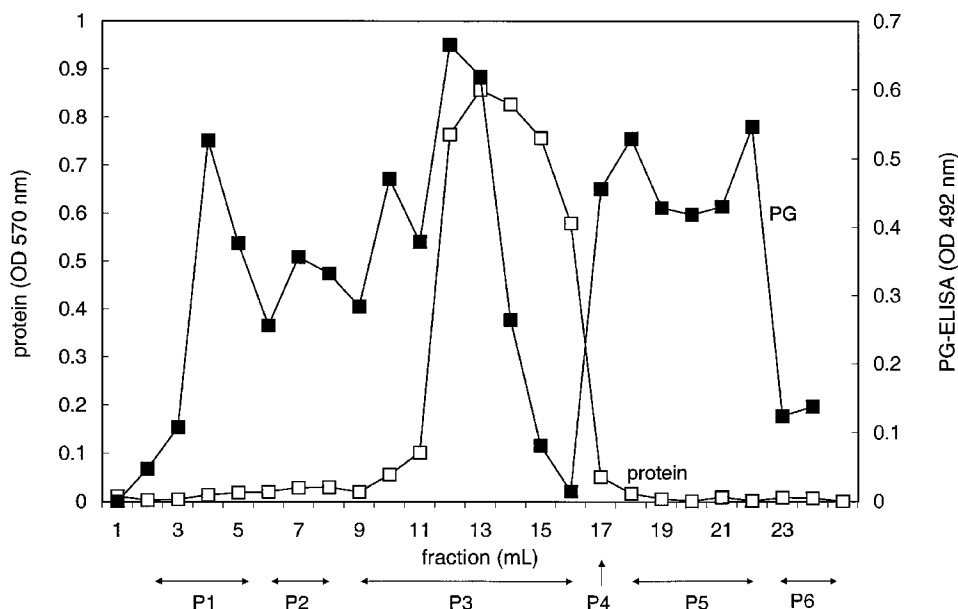
Figure 3. Separation of PG using Superdex 200 gel filtration. One milliliter of spleen extract after Sephadex gel filtration was added to Superdex 200 connected to fast-performance liquid chromatograph. Amount of protein (□) and PG (■) was measured in all fractions by Bradford method [30] and ELISA, respectively. Pooled fractions: F2–F5 (P1), F6–F8 (P2), F9–F16 (P3), F17 (P4), F18–F22 (P5), and F23–F24 (P6) (↔).

contrast, hydrolysis of PG isolated from human feces had no effect on the level of muramic acid measured by the colorimetric muramic acid assay. This indicates that PG in human feces contains no or minimal levels of peptide chains bridging the MurNac backbone. The colorimetric muramic acid assay could not be used to measure the amount of PG in P3 because the level of protein in the fraction was too high.

Muramic acid by HPLC analysis. To confirm the presence of PG in the fractions isolated from human spleen, P1 and P4 were analyzed for the presence of muramic acid using HPLC. These two fractions were used because protein levels were low. First, the fractions were applied to the column (figure 4A) and spiked by adding dansylated muramic acid to the fraction. The peak with a retention time of 12.8 min was covered by muramic acid added to the fraction (figure 4B). This is also the retention

time of muramic acid when it is loaded onto the column on its own. There are many peaks in the chromatogram, and therefore the peak with retention time 12.8 min was collected and reanalyzed to assess whether it had the same retention time as the standard dansyl muramic acid. The only peak in the chromatogram had the same retention time as dansyl muramic acid (figure 4C). This peak was spiked by adding muramic acid to the sample. The smaller peak with retention time 5.4 min belonged to the muramic acid added to the sample (figure 4D). In the PG fraction, we could not detect this peak because free dansyl groups were detected after this retention time. These results confirm the presence of muramic acid in both fractions. Because muramic acid is a unique component of PG, the HPLC analysis also confirmed the presence of PG in the fractions.

Induction of proinflammatory cytokines by PG. To investigate whether PG in human spleen tissue is biologically active, production of TNF-α, IL-1, and IL-6 was measured in vitro after incubation of human blood with PG isolated from human spleen. Because P1 contained the lowest amount of contaminating protein, this fraction was used. TNF-α, IL-1, and IL-6 were produced after stimulation with PG (figure 5). This production was dose-dependent, correlating with the concentration of P1 added to the blood cultures.

Polymyxin B is an efficient antagonist of LPS activity, thereby inhibiting LPS-induced cytokine production. To exclude the possibility that activity of PG isolated from human spleen is due to contaminating LPS, we analyzed whether cytokine induction by PG was blocked by polymyxin B. Production of

Table 1. Protein and muramic acid content in pooled fractions isolated from human spleen.

Fraction	Muramic acid (μg/mL)		Protein (μg/mL)	
	Before hydrolysis	After hydrolysis	Bradford method [30]	Lowry method [31]
P1	0	70	0	63
P2	0	62	44	170
P3	ND	ND	975	1300
P4	0	42	293	290
P5	0	56	34	75
P6	0	0	0	0

NOTE. Protein was measured according to Bradford [30] and Lowry et al. [31]. Muramic acid was measured by colorimetric muramic acid assay. ND, not done.
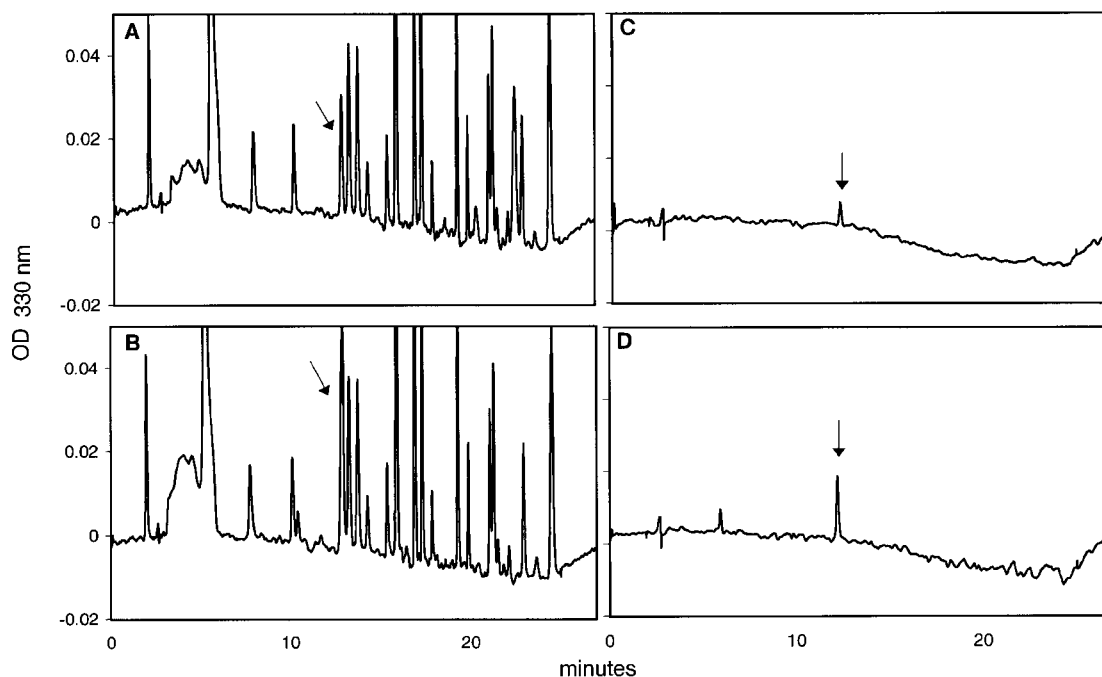
Figure 4. Identification of muramic acid in P5 isolated from human spleen. Reversed-phase high-performance liquid chromatography on C18 column (5 μm, 4 × 250 mm) in discontinuous gradient 0%–15% methanol over 27 min at 1.0 mL/min of *(A)* hydrolyzed and dansylated preparation of P5 of spleen from patient 1; *B* was like *A* but spiked with dansylated muramic acid. *C,* Collected peak with retention time 12.8 min from chromatogram *A. D* was like *C* but spiked with dansylated muramic acid. Absorbance was measured at 330 nm. Arrows indicate peak with retention time 12.8 min, identified as muramic acid. P1 (not shown) had same pattern.

TNF-α, IL-1, and IL-6 induced by P1 was not inhibited by polymyxin B. LPS served as a positive control in all experiments, and 1 ng/mL was able to induce the same level of cytokines induced by P1 (75 ng/mL muramic acid). As expected, LPS-driven induction of cytokines was inhibited by polymyxin B. PG isolated from human feces also induced TNF-α, but the concentration (based on the amount of muramic acid) needed to induce the same level of cytokine production as P1 was 150 times higher.

To exclude the possibility that contaminating proteins present in the P2–P6 fractions induced proinflammatory cytokine production, P1–P6 was analyzed on the basis of equal protein concentrations. The amount of protein was based on a 1:800 dilution of fraction P1 (75 ng/mL muramic acid). This fraction contained 78 ng/mL protein (according to the Lowry assay). All fractions were diluted, so the concentration of protein added to the blood cells was 78 ng/mL. As expected, P6, which contained no PG or protein, could not induce cytokine production by whole blood cells; however, P1, P2, P4, and P5 all induced cytokine production (table 2). P3 did not induce cytokine production. Since P3 contains very high amounts of proteins, a dilution to 78 ng of protein results in minimal traces of PG in the fraction. This clearly suggests that contaminating protein does not induce cytokine production. The levels of TNF-α, IL-1, and IL-6 induced by the distinct fractions differed strongly between fractions, indicating that protein present at equal concentrations in all fractions does not induce cytokine production. Consistent with this, the cytokine production directly correlated with the amount of PG in the fractions (correlation coefficients were 0.9, 0.8, and 0.9 for respective TNF-α, IL-1, and IL-6 production compared with the amount of PG in the fractions). This indicates that PG, but not protein, present in the fractions induces the production of TNF-α, IL-1, and IL-6 by human blood cells.

To confirm that PG and not contaminating proteins causes the cytokine production, human blood cells were incubated with anti-CD14 antibody for 30 min at 37°C. All fractions were diluted 80-fold, resulting in the following muramic acid concentrations: (P1) 88, (P2) 78, (P3) not done, (P4) 53, (P5) 70, and (P6) 0 ng/mL. Anti-CD14 antibody blocked cytokine production by the different fractions in a dose-dependent manner. TNF-α production was completely blocked in all fractions and LPS at a concentration of 10 μg/mL (figure 6). An isotype-matched control IgG2b antibody (MOPC-195) did not affect cytokine production.

Discussion

In the current study, we show that structurally intact PG isolated from sterile human spleen can induce production of
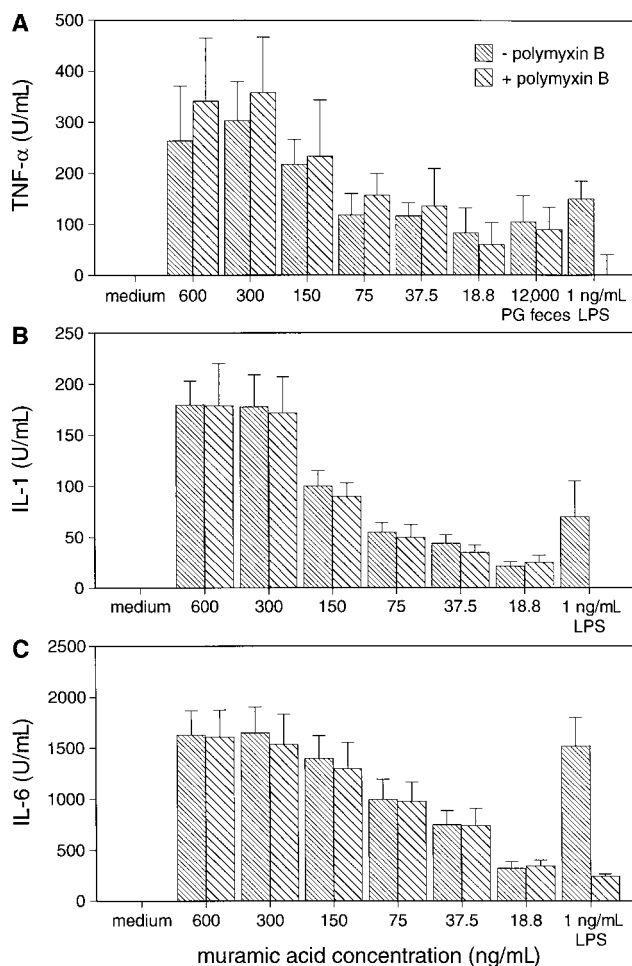
Figure 5. Production of proinflammatory cytokines by human blood cells after stimulation with PG isolated from human spleen. Human blood cells were incubated for 5 h in presence of P1 with or without polymyxin B. In supernatants, *(A)* tumor necrosis factor (TNF)-α production measured by WEHI 164.13 in cytotoxicity assay. *B,* Interleukin (IL)-1 production measured using IL-1–dependent subline of T cell line D10.G4.1. *C,* IL-6 production measured using IL-6–dependent murine hybridoma cell line B9. LPS, lipopolysaccharide.

that PG in human tissues other than the gut is derived from normal intestinal flora. That we can isolate 50 mg of PG per 100 g of feces shows that high amounts of PG are continuously present in the intestine.

We hypothesize that PG is able to traverse the intestinal wall by unknown mechanisms and gain access to other sites of the body through the bloodstream or lymph circulation. PG is located in antigen-presenting cells in tissues like human spleen and lymph nodes and also in synovial tissues. This was shown by immunohistochemical staining with a MAb against PG isolated from human feces [23]. To investigate the relevance of PG in human tissues, we studied the biologic activity of PG isolated from human spleen. To obtain a high amount of PG, it was necessary to modify the method described earlier [25]. Because the passage of the spleen extract after Sephadex G25 gel filtration through a DEAE and Dowex column causes a considerable loss of PG, we developed another method to extract the protein. This was performed by size exclusion using Sephadex G25 gel filtration followed by Superdex 200 gel filtration. Because the PG-containing fraction is diluted 25-fold by the Superdex 200 column passage, the colorimetric muramic acid assay is not sensitive enough to detect PG in each fraction. Therefore, we performed an ELISA using a MAb against PG that is a more sensitive method to detect PG.

We used two methods to detect PG in pools P1–P6. The HPLC analysis was used to confirm the presence of muramic acid in the fractions, leading to the conclusion that PG was present. The colorimetric muramic acid assay was used to measure the amount of PG in the fractions. This method was also used to determine whether PG is intact in terms of the presence of peptide interlinks. This is important because earlier studies show that the structure of PG is very crucial for its biologic activity. For example, muramyl dipeptide is the minimal structure of PG that is biologically active, but a much higher amount is needed compared with large molecules of PG that have a repetitive nature [12]. Furthermore, PG isolated from the ileum, which is mostly intact, can induce arthritis in rats. PG isolated from feces that are not intact cannot induce arthritis in rats [39]. This suggests that the structural integrity of PG is crucial

the proinflammatory cytokines TNF-α, IL-1, and IL-6 by human blood cells. Other studies have demonstrated the capacity of cytokine induction by PG derived from bacterial cultures [9, 10], but the ability of PG present in human tissues to induce cytokine production has not been reported.

PG is thought to play an important role in the immunologic response against gram-positive infections, but it has also been hypothesized to be involved in the pathogenesis of chronic inflammations like rheumatoid arthritis. Our group investigated the role of PG derived from the normal intestinal flora in the pathogenesis of rheumatoid arthritis. The presence of PG in several human tissues [18–23] suggests that the human body is also constantly exposed to PG outside the gut. It is possible

Table 2. Production of tumor necrosis factor (TNF)-α and interleukin (IL)-1 and -6 by human blood cells after stimulation by the different fractions pooled after Superdex 200 gel filtration.

Fraction	Muramic acid	Protein	TNF-α	IL-1	IL-6
P1	75	78	290 ± 25	35 ± 4	853 ± 80
P5	64	78	140 ± 20	55 ± 10	476 ± 55
P2	28	78	64 ± 7	15 ± 2	123 ± 12
P4	11	78	38 ± 4	14 ± 1	89 ± 9
P3	ND	78	0	0	0
P6	0	0	0	0	0
Medium	0	0	0	0	0

NOTE.   Human blood cells were incubated for 5 h with all pooled fractions (P1–P6) diluted to protein concentration of 78 ng/mL. TNF-α, IL-1, and IL-6, measured in supernatants by bioassays, are shown in U/mL; muramic acid and protein are in ng/mL. ND, not done.
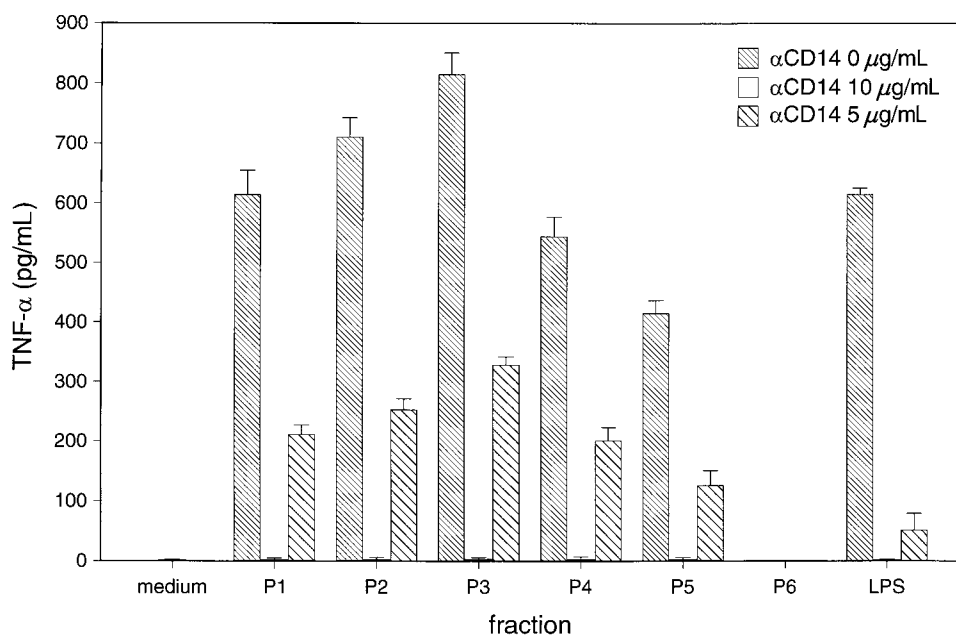
Figure 6. Inhibition of tumor necrosis factor (TNF)-α production by human blood cells after incubation with anti-CD14 antibody. Human blood cells were incubated with anti-CD14 antibody (5 and 10 μg/mL) for 30 min then stimulated with P1–P6 and lipopolysaccharide (LPS) for 5 h. TNF-α production measured by capture ELISA.

to its biologic activities. PG isolated from human spleen was largely intact, because no muramic acid could be detected before hydrolysis. Analysis of PG isolated from human feces revealed that the PG is not intact. With the improved purification of PG, we were able to isolate 1.9 μmol muramic acid per 100 g of spleen tissue. The amount of muramic acid isolated is similar to that by the earlier described method [25] (2.3 μmol/ 100 g spleen tissue), but the amount of protein in the fraction is 20-fold lower, which means that the fraction is more suitable for in vitro studies.

The cytokine induction assays show that PG isolated from human spleen can induce induction of TNF-α, IL-1, and IL-6. Kinetics of cytokine induction by PG was the same as of LPS, but the amount of PG needed was 100 times higher than with LPS. The fact that low amounts of PG isolated from human spleen were required in comparison with PG from human feces indicates that intact PG is more active than a more degraded form.

To exclude contamination of the isolated fraction, the sample was analyzed using blood agar plates after every purification step. The whole procedure was also done with PBS. With this procedure, we were unable to induce cytokine production (data not shown). Contamination by LPS was excluded by incubating the fractions with polymyxin B before the various PG preparations to the blood cells. No inhibition of the cytokine production was found in contrast with cytokine induction by LPS. The results show also that the levels of cytokine produced correlated with PG concentrations and not with protein concen-

trations, confirming that PG is responsible for the cytokine induction. This was confirmed with fraction P3 that contained very high concentrations of protein and unknown levels of PG. After the sample was diluted to 78 ng/mL protein, no cytokine production could be measured.

There are three other indications that the cytokines produced by the different fractions are elicited by PG and not by contaminating proteins. First, the incubation of the spleen extract in acetic acid at 100°C for 2 h will cause a loss of the tertiary and secondary structure of the proteins. This will lead to a reduction, if not a complete loss, of function of the proteins. Second, the fact that TNF-α measured by ELISA could be completely blocked in all fractions by incubating the human blood cells with αCD14 also indicates that the proteins in the fraction are not responsible for the cytokine production. CD14, which is known as the LPS receptor, also binds PG and activates cytokine production [11, 12]. Binding of proteins to CD14 mediating cytokine production has not been described. The third indication that PG is responsible for the cytokine production is that treatment of fraction P1–P6 with proteases (elastase, trypsin, and α-chymoptrysin) does not affect cytokine production. By using different concentrations of proteases (0.1–0.001% vol/vol), cytokine induction was equal with and without treatment (data not shown). The use of higher protease concentrations was not possible because the proteases then induced cytokine production directly. The inactivation of protein could not be monitored by measuring the loss of proteins because the

protein content of the proteases added to fractions was much higher than of the original low level of contaminating proteins.

Various studies, including the present study, have shown that PG is not restricted to the gut, even in the absence of infection. In all 15 spleens we have obtained thus far, PG-containing cells could be detected by use of immunohistochemistry. All spleens used were removed during surgery, because this is the only way to obtain sterile human spleens. These spleens were removed for various reasons (including when the spleen was not diseased), indicating that healthy people contain PG in their spleens. It has been hypothesized that these bacterial products are derived from the intestinal flora. Because the present study showed that PG in the human spleen is mostly intact, it is likely that PG will pass the bowel wall before it is degraded by enzymes [39].

A prime question is whether the presence of PG in sterile human spleen tissues has any biologic significance. To answer this question, it is important to know whether PG in human tissues has biologic activity. This study shows that in vitro PG has the capability to induce cytokine production by human blood cells. If this activity also occurs in vivo, antigen-presenting cells containing PG might be activated by PG to produce proinflammatory cytokines. The function of this cytokine production is possibly the triggering of a continuous immune response for controlling normal homeostasis. It was recently argued that continuous exposure to bacterial antigens is important in regulation of Th1 versus Th2 CD4-positive T cells [40]. Disturbance of this Th1-Th2 balance may lead to autoimmune diseases. A possible way by which this balance can be disturbed may be the presence of too much PG in tissues because of intestinal injury. The presence of PG in tissues where the clearance of antigens is very difficult, for example synovial tissue, could induce local inflammatory processes.

In conclusion, PG isolated from human spleen is largely intact and can induce cytokine production by human blood cells at very low concentrations. To further investigate whether PG-containing cells in vivo can also induce proinflammatory cytokine production in situ, these cells will be analyzed by immunohistochemistry. In addition, immunization of mice with PG will allow kinetic analysis of local cytokine induction by splenic antigen-presenting cells containing PG.

Acknowledgments

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