Mammalian Peptidoglycan Recognition Protein Binds Peptidoglycan with High Affinity, Is Expressed in Neutrophils, and Inhibits Bacterial Growth *

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Running Title: Peptidoglycan Recognition Protein
SUMMARY

Peptidoglycan recognition protein (PGRP) is conserved from insects to mammals. In insects, PGRP recognizes bacterial cell wall peptidoglycan (PGN) and activates prophenoloxidase cascade, a part of insect antimicrobial defense system. Because mammals do not have the prophenoloxidase cascade, its function in mammals is unknown. However, it was suggested that an identical protein (Tag7) was a TNF-like cytokine. Therefore, the aim of this study was to identify the function of PGRP in mammals. Mouse PGRP bound to PGN with fast kinetics and nanomolar affinity (Kᵩ = 13 nM). The binding was specific for polymeric PGN or Gram-positive bacteria with unmodified PGN, and PGRP did not bind to other cell wall components or Gram-negative bacteria. PGRP mRNA and protein were expressed in neutrophils and bone marrow cells, but not in spleen cells, mononuclear cells, T or B lymphocytes, NK cells, thymocytes, monocytes, and macrophages. PGRP was not a PGN-lytic or a bacteriolytic enzyme, but it inhibited the growth of Gram-positive, but not Gram-negative bacteria. PGRP inhibited phagocytosis of Gram-positive bacteria by macrophages, induction of oxidative burst by Gram-positive bacteria in neutrophils, and induction of cytokine production by PGN in macrophages. PGRP had no TNF-like cytotoxicity for mammalian cells, and it was not chemotactic on its own or in combination with PGN. Therefore, mammalian PGRP binds to PGN and Gram-positive bacteria with nanomolar affinity, is expressed in neutrophils, and inhibits growth of bacteria.
INTRODUCTION

Innate immunity is the first line of defense directed against components common to microorganisms (1, 2). One of the antimicrobial mechanisms of innate immunity in insects is the prophenoloxidase cascade, which is present in hemolymph and cuticle (3). The prophenoloxidase cascade can be initiated by the binding of a 19 kDa protein, peptidoglycan-recognition protein (PGRP)\(^1\), to a bacterial cell wall component, peptidoglycan (PGN) (4).

Recently, insect PGRP as well as mouse and human PGRP homologs were cloned and shown to bind Gram-positive bacteria and PGN (5), thus demonstrating that this protein is highly conserved from insects to mammals, and providing another evidence for the highly conserved nature of innate immunity from insects to mammals. Very high expression of mammalian PGRP mRNA in the bone marrow, and lower expression in other lymphoid tissues (5) suggested a role for mammalian PGRP in immunity. However, the exact cell types expressing PGRP and the function of PGRP in mammals remained unknown. Because mammals do not have the prophenoloxidase cascade, mammalian PGRP must play another role.

PGN, an essential cell wall component of virtually all bacteria, is especially abundant in Gram-positive bacteria (6). PGN interacts with both humoral (PGRP, lysozyme, complement, soluble CD14) and cellular (hemocytes, macrophages, lymphocytes) components of the immune system in both vertebrates and invertebrates (7-9). In mammals, PGN activates macrophages through two pattern recognition receptors, CD14 (10-13) and Toll-like receptor-2 (TLR2) (14-16), and induces production of cytokines and chemokines (8-10, 13, 17, 18). Overproduction of these cytokines causes all major clinical manifestations of infections, including fever, inflammation, leukocytosis, hypotension, decreased peripheral perfusion, malaise, sleepiness, decreased appetite, arthritis, and in most severe cases, circulatory shock and multiple organ failure (7-9).

A novel protein, named Tag7, that was expressed in some tumor cell lines, was recently cloned and proposed to be a novel cytokine, a member of the tumor necrosis factor (TNF) family, with TNF-like cytotoxic activity (19). However, because Tag7 has the same sequence as mouse...
PGRP (5, 19), it is difficult to reconcile the proposed PGN-binding and TNF-like cytotoxic activities attributed to the same protein.

Therefore, the aims of this study were to determine: (i) the specificity of mammalian PGRP binding; (ii) which cells express PGRP mRNA and protein; (iii) whether PGRP has PGN-lytic, bacteriolytic, and/or bacteriostatic activities; (iv) whether PGRP has TNF-like cytotoxic activity; and (v) the effect of PGRP on the main functions of neutrophils and macrophages.
EXPERIMENTAL PROCEDURES

Materials. Recombinant full-length mouse PGRP (182 amino acids) was expressed in baculovirus-infected Sf-9 cells and purified by nickel affinity chromatography as described (5), except that FPLC was used for the purification, and was kept at 4°C in 10% glycerol. Affinity-purified recombinant PGRP gave one 20 kDa band on 12% SDS-PAGE stained with Coomassie blue (Fig. 1C). N-terminal amino acid microsequencing (on an Applied Biosystem 476A sequencer) of this PGRP band (after blotting to Immobilon P) revealed the following residues: XSFIVP (with X being most likely C, which cannot be determined by the sequencer), thus confirming the predicted protein sequence of mouse PGRP (5) and demonstrating that the purified recombinant preparation was a mature full-length protein devoid of the signal peptide. PGRP was negative for endotoxin in the Limulus assay (<3 pg endotoxin/µg PGRP).

Soluble PGN (sPGN), a polymeric uncross-linked PGN of approximate average M_r = 125,000, released from Staphylococcus aureus Rb or 845 grown in the presence of β-lactam antibiotics, was purified by vancomycin affinity chromatography (20, 21). Insoluble PGN was isolated from the cell walls of S. aureus 845 and sonicated as described (20). By quantitative chemical analysis, both sPGN and insoluble PGN had amino acid and amino sugar composition characteristic of S. aureus PGN (20, 21), and contained <24 pg endotoxin/mg determined by the Limulus assay (20).

Synthetic analogs of PGN fragments, PGN pentapeptide (L-Ala-D-isoglutaminyl-L-Lys-D-Ala-D-Ala) and muramyl dipeptide (MurNAc-L-Ala-D-isoglutamine, MDP) were from Sigma, and a disaccharide-dipeptide (GlcNAc-β1-4-MDP) was from Calbiochem (La Jolla, CA). No significant endotoxin contamination of these preparations was detected (<1 ng endotoxin/mg), determined by the Limulus assay (20). S. aureus ribitol teichoic acid (TA) and lipoteichoic acid (LTA) were the same as before (13).

Rough LPS from Salmonella minnesota Re 595 (ReLPS, a minimal naturally occurring endotoxic structure of LPS, M_r = 2000-3000), obtained by phenol-chloroform-petroleum ether extraction (Sigma), was dissolved at 2.5 mg/ml in 0.2% triethylamine and its purity was analyzed as
described before (22). Purified smooth LPS, obtained from *Escherichia coli* O113 by phenol-water extraction (refined endotoxin standard, approximate average $M_r = 15,000$) was obtained from Ribi Immunochem Research (Hamilton, MT).

Recombinant human full-length (residues 1-323) soluble CD14 and recombinant human 1-152 CD14 N-terminal fragment were the same as before (13). Lysozyme (grade I from chicken egg), lysostaphin (affinity purified from *Staphylococcus staphyloccus*), cellulose (microgranular), chitin (poly-$[1 \rightarrow 4]$-$\beta$-N-acetyl-D-glucosamine from crab shells), zymosan (from *Saccharomyces cerevisiae* cell walls), *Micrococcus luteus* (ATCC 4698), *Bacillus subtilis* (ATCC 6633), and all other reagents, unless otherwise indicated, were from Sigma. *S. aureus* 845 and Rb cells were killed with gentamicin (18).

**Binding of $^{125}$I-PGRP to sPGN-agarose, cell walls, and bacteria.** PGRP (20 µg) was labeled with 2 mCi $^{125}$I using pre-coated iodogen tubes (Pierce, Rockford, IL) for 15 min at 22°C. Unbound $^{125}$I was removed by dialysis at 4°C against Dulbecco’s PBS without Ca$^{2+}$ and Mg$^{2+}$, and $^{125}$I-PGRP was stored in PBS with 10% glycerol at 4°C. The specific activity was 30-60 µCi/µg PGRP. $^{125}$I-PGRP yielded one 20 kDa band detected by autoradiography on 12% SDS-PAGE gels, that co-migrated with the Coomassie blue-stained band of unlabeled PGRP, confirming iodination of PGRP and not any potential otherwise undetectable contaminants (Fig. 1C).

sPGN, ReLPS, smooth LPS, GlcNAc-MDP, MDP, or PGN-pentapeptide were coupled to 40-165 µm agarose beads and the binding of iodinated PGRP to these agarose-immobilized preparations was performed as described for the binding of $^{32}$P-sCD14 (13). For a standard binding assay, $^{125}$I-PGRP (200 ng/ml) was incubated with 0.5 µl of sPGN-agarose (or agarose coupled to other compounds, or agarose alone, which had been subjected to mock coupling in a buffer alone) for 10 min at 37°C in a total volume of 60 µl. After adding PBS with 0.5 M NaCl, the agarose was centrifuged through 0.8 M sucrose, and the amount of $^{125}$I bound to the agarose pellet was measured (13). In some experiments, the amount of agarose, incubation time, or PGRP concentrations were varied. For inhibition with unlabeled PGRP, unlabeled PGRP was mixed with $^{125}$I-PGRP before addition to sPGN-agarose or control agarose. In other competitive inhibition
experiments, the competitors were first mixed with sPGN-agarose or control agarose, followed by addition of $^{125}$I-PGRP. The apparent dissociation constant ($K_d$) and the maximal binding at saturation ($B_{max}$) were calculated as before (13).

The binding of $^{125}$I-PGRP (200 ng/ml) to bacteria, insoluble PGN, and other insoluble preparations was performed as above with the amounts of bacteria or insoluble preparations (used instead of the agarose) indicated in the Results.

**Binding of native PGRP to sPGN and bacteria.** Mouse bone marrow cells (obtained as described below) were lysed in 0.2 M TRIS/HCl, pH 7.0, with 0.2 M NaCl, 4 mM EDTA, 10% glycerol, 1% NP-40, and a cocktail of protease inhibitors. Mouse recombinant PGRP (2 µg/group in the same lysis buffer with 2 mg/ml gelatin) or cell lysates (from 20 x $10^6$ cells/group) were incubated for 2 h at 4°C with 15 µl sPGN-agarose, 250 µg *M. luteus* cells, control agarose, or latex particles, and centrifuged at 10,000 x g. The sediments were suspended in PAGE sample buffer and 5x sample buffer was added to the supernatants (1% SDS and 1% 2-mercaptoethanol final concentrations), the samples were boiled and subjected to 12% SDS-PAGE and Western blotting with anti-PGRP antibodies as described below.

**Cells and cell lines.** Mice (female 6-16 wk-old BALB/c or ICR from Harlan-Sprague-Dawley, Indianapolis, IN) were anesthetized with ether, bled from the retroorbital venous plexus, the cells from their peritoneal cavities were washed out with RPMI-1640 with 5 U/ml heparin, and their spleens, thymuses, and femurs were removed. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by centrifugation through Histopaque (density 1.077 g/ml, Sigma), and were 90% pure. Polymorphonuclear leukocytes (PMNs) were separated by centrifugation through two Histopaque layers (density 1.083 g/ml and 1.119 g/l, Sigma) and were 90% pure as determined by morphology on Wright-stained smears. Peritoneal macrophages were separated from peritoneal washings by adherence to plastic (23, 24), and were >95% pure as determined by morphology on Wright-stained smears, non-specific esterase staining, and phagocytosis of Latex particles, and did not contain any detectable PMNs (<0.1%). Spleen cells and thymocytes were obtained by cutting the organs, releasing cells from the capsules, and
discarding connective tissues and capsules. Bone marrow cells were obtained by flushing isolated femurs with RPMI-1640 with 5 U/ml heparin. Purified B and T lymphocytes were obtained from spleen cells by labeling with R-phycoerythrin-conjugated anti-mouse IgG (whole molecule) or FITC-conjugated anti-mouse CD3 monoclonal Ab (Sigma), respectively, and sorting on the FACS-Star Plus cell sorter (Beckton-Dickinson, San Jose, CA). B and T lymphocytes were >97% pure as determined by FACS analysis, and did not contain any detectable PMNs (<0.1%).

Human peripheral blood, obtained from healthy males (24-49 years old of Caucasian, Asian, or African-American origin) was separated by centrifugation through two Histopaque layers (density 1.077 g/ml and 1.119 g/ml, Sigma) into PBMC and into PMNs (98% pure as determined by morphology on Wright-stained smears). Monocytes were isolated from PBMC by adherence to plastic (25, 26), and were >95% pure as judged by morphology on Wright-stained smears, phagocytosis of Latex, and non-specific esterase, and did not contain any detectable PMNs (<0.1%). B lymphocytes, T lymphocytes, and NK cells were purified from PBMC by FACS cell sorting after labeling with phycoerythrin-conjugated anti-human CD19 monoclonal Ab (B cells), FITC-conjugated anti-human CD3 monoclonal Ab (T cells), or FITC-conjugated anti-human CD56 monoclonal Ab (NK cells) (all Abs from Beckton-Dickinson). In some experiments, T cells were removed by rosetting with neuraminidase-treated sheep red blood cells (27) before sorting for B cells and NK cells. The sorted cells were >99.8% pure as determined by FACS analysis and did not contain any detectable PMNs (<0.1%).

The following cell lines, all obtained from ATCC (Rockville, MD) and cultured as recommended by ATCC, were used: (a) mouse: B-lymphocytic cells: A20, 2PK-3, BCL-1, and WEHI-231; pre-B-cells: 70Z/3; hybridoma: FB2; T-lymphocytic cells: EL-4, R1.1, and YAK-1; macrophage cells: P388D1, J774A.1, and RAW264.7; and fibroblast: 929 (strain L); and (b) human: B lymphocytic cells: RAJI; T-lymphocytic cells: MOLT-4; monocytic cells: THP-1 and U-937; and fibroblast: HELA. Human umbilical vein endothelial cells (HUVEC) were obtained and cultured as described before (25).
Expression of PGRP mRNA by RT-PCR. Total RNA was isolated from mouse and human cells and cell lines with RNeasy Mini Kit (Qiagen, Valencia, CA) using mini-spin columns as recommended by the manufacturer. A single-tube, two-enzyme Access RT-PCR System (Promega, Madison, WI) was used as recommended by the manufacturer. Briefly, first strand cDNA was synthesized in 50 µl reaction mixture containing 1x AMV/Tfl reaction buffer, 0.2 mM dNTP, 1 mM MgSO₄, 0.1 U/µl Avian Myeloblastosis Virus (AMV) reverse transcriptase, 0.1 U/µl Tfl DNA polymerase, 1 µM upstream and downstream primers, and 1 µg of total RNA. Samples were amplified in a GeneAmp9600 thermocycler (Perkin Elemer, Norwalk, CT). Amplification cycles were preceded by a 2 min 94°C denaturation. Subsequent stages were at 94°C for 30 sec, 52°C for 60 sec, and 72°C for 90 sec, for the number of cycles indicated in Results. The primers for mouse and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as a house-keeping gene standard, were (28): sense, 5’ACC ACA GTC CAT GCC ATC AC3’, and antisense, 5’TCC ACC ACC CTG TTG CTG TA3’ which yields a 452 bp product. The primers for PGRP, designed based on the mouse and human PGRP sequences (5), were: mouse sense, 5’GCA ATG TGC AGC ATT ACC AC3’, mouse antisense, 5’TCT CAC TCT CGG TAG TGT TC3’ which yields a 358 bp product; human sense, 5 ATG TGG TGG TAT CGC ACA CG3, human antisense, 5’GTC CTT TGA GCA CAT AGT TG 3’ which yields a 342 bp product. PCR products were subjected to 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. 50 bp DNA step-ladder was used as the molecular weight standard. The sequences of all amplified PCR products were confirmed following extraction from the agarose gel and purification using QIAquick PCR purification kit (Qiagen) as recommended by the manufacturer, and then using automatic sequencing performed at Genemed Synthesis (South San Francisco, CA).

Generation of anti-PGRP antibodies. Mouse PGRP C-terminal (DQLYQVIQSWEHYRE) and N-sub-terminal (RALPSECSSRLGHC) peptides were synthesized and purified (>95% pure) by HPLC by Genemed Synthesis (South San Francisco, CA), and then coupled to Keyhole limpet hemocyanin with glutaraldehyde (C-terminal peptide), to bovine serum albumin (BSA) with m-maleimidobenzoyl-N-hydroxysuccinimide ester (N-sub-terminal peptide), or to ovalbumin with
glutaraldehyde (both peptides) as described (29). Female New Zealand white rabbits were immunized with an emulsion of both peptide-carrier conjugates with KLH and BSA in Complete Freund’s adjuvant, and boosted several times with an emulsion of the same peptide conjugates in incomplete adjuvant, followed by boosting with both peptides-ovalbumin conjugates in Complete and then in incomplete Freund’s adjuvants as described (30). The production of antibodies was monitored by Western Blots with purified mouse PGRP and anti-rabbit peroxidase-labeled second antibody and enhanced chemiluminescence reagent (ECL, Amersham) (17). The immune serum (1:4000 dilution) gave a strong reaction with 0.5 µg PGRP in 15 sec, and the pre-immune serum was completely negative on blots of 1 µg PGRP overexposed for 30 min. The immune serum was absorbed twice with BSA-agarose to remove strong cross-reactivity with mouse albumin.

**Detection of PGRP expression by Western blot.** Mouse or human cells (10⁶/group) were dissolved in 100 µl lysis buffer (0.1 M TRIS/HCl, pH 8.0, with 0.1 M NaCl, 4 mM EDTA, 30% glycerol, 1% NP-40, and a cocktail of protease inhibitors), boiled with PAGE sample buffer (2% SDS and 1% 2-mercaptoethanol), centrifuged at 12,000xg, and the supernatants were subjected to 12% SDS-PAGE, blotted onto PVDF membrane (Immobilon P, Millipore), and the membranes were blocked with 5% milk (17). The membranes were incubated with rabbit anti-PGRP serum (1:4000), and immunoreactive bands were detected with anti-rabbit IgG second antibody and ECL as above. For inhibition with peptides, 1 µl of anti-PGRP serum in 25 µl PBS with 2% gelatin was incubated at 4°C for 24 h with 25 µg of each PGRP peptide or with 50 µg of a control peptide (Sendai virus nucleoprotein fragment 321-336), and then diluted and used for detection of PGRP as described above.

**Assays for PGN-lytic, bacteriolytic, and bacteriostatic activities.** To test if PGPR had any PGN-lytic activity, biotin-labeled sPGN (13) (50 µg/ml in 10 mM HEPES, pH 7.0, with 0.85% NaCl, and with or without 1 mM ZnSO₄) was incubated for 24 h at 37°C without or with PGRP or control PGN-lytic enzymes (lysozyme or lysostaphin) at 0.1 to 100 µg/ml as described (13). The digests were subjected to SDS-PAGE, blotted onto Immobilon, and biotin-sPGN was detected with streptavidin-peroxidase and enhanced chemiluminescence as described (13). This method detects
the PGN-lytic activity of enzymes with either muramidase activity (such as lysozyme), which hydrolyze the glycan chain of PGN and convert high Mₐ sPGN into low Mₐ sPGN fragments, or with amidase or peptidase activity (such as lysostaphin), which remove biotin-labeled peptide from high Mₐ sPGN (13). The experiments were done without and with ZnSO₄, because T3 and T7 amidases, with which PGRP has some sequence homology (5), require Zn²⁺ for their activity (31).

To test if PGPR had any bacteriolytic activity, *M. luteus* (1 mg/ml in 10 mM HEPES, pH 7.0, with 0.85% NaCl, and with or without 1 mM ZnSO₄) was incubated in 96-well plates (100 µl/well) at 37°C without or with PGRP or a control bacteriolytic enzyme (lysozyme) at 1 to 25 µg/ml, and optical density at 540 nm (OD₅₄₀) was periodically measured with an ELISA plate reader.

To test if PGPR had any bacteriostatic activity, logarithmic-phase Gram positive (*Staphylococcus hemolyticus, Staphylococcus warneri, Staphylococcus capitis, and Bacillus megaterium*) or Gram-negative (*E. coli* and *Enterobacter cloacae*) bacteria (OD₆₆₀ = 0.4-0.5) were diluted with LB broth and incubated with PGRP (45 µg/ml) at 37°C with agitation. Samples were taken at regular intervals and the numbers of bacteria were determined by plating on agar plates.

**Phagocytosis.** *M. luteus* was labeled with fluorescein isothiocyanate (FITC) as described (32). PGRP was pre-incubated with FITC-*M. luteus* or fluorescent amide-modified latex particles (1 µm, Sigma) as a control for 30 min at 37°C. The mixtures were then added to mouse macrophage RAW264.7 cells (100 µl of 2.5 x 10⁶ cells in DMEM with 10 mM HEPES and 10% mouse serum) to yield 1 µg, 3.3 µg, or 10 µg of PGRP/ml, and 10:1 bacteria:cell ratio or latex particles:cell ratio (final concentrations), and slowly rotated (15 rotations/min) for 15 min at 37°C/5% CO₂ (32). The cells were washed 3 times with DMEM at 4°C, and the numbers of cells that phagocytized bacteria or latex particles, the numbers of phagocytized bacteria or latex particles, and the total number of cells were counted. The phagocytic index (PI) was calculated (32); PI = (percentage of cells containing at least one bacterium )x(mean number of bacteria per positive cell).

The ranges of phagocytic index without PGRP for *Micrococcus* and latex were 250-600 and 400-700, respectively. Serum was included in the medium because it was required for phagocytosis of...
bacteria, which were not phagocytized in the absence of serum, regardless of the presence of PGRP. Similar results were obtained with mouse serum and fetal bovine serum.

**Oxidative burst (NBT test).** Bacterially-induced oxidative burst in human neutrophils was measured by nitroblue tetrazolium (NBT) reduction method (33) using Sigma clinical diagnostic kit as recommended by Sigma. Briefly, stimulants (M. luteus, B. subtilis, E. coli, LPS, or phorbol dibutyrate [PDB] + ionomycin) were pre-incubated in siliconized glass vials for 10 min at 37°C in medium alone or with PGRP (10 μl), and then 50 μl of NBT solution and 50 μl of fresh human peripheral blood with 10 U/ml of heparin were added, to yield 40 μg/ml of bacteria, 1 μg/ml of LPS, 100 nM each of PDB and ionomycin, and 1 μg/ml, 3.3 μg/ml, or 10 μg/ml of PGRP (final concentrations). The vials were incubated for 10 min at 37°C and for additional 10 min at 22°C, blood smears were prepared, stained with the Wright stain, and the numbers of neutrophils that contained or did not contain the NBT granules were counted under the microscope.

**Induction of TNF-α and IL-6.** Mouse macrophage RAW264.7 cells grown conventionally in 10% fetal bovine serum or adapted to growth in serum-free HL-1 medium (BioWhittacker, Walkersville, MD) were cultured and stimulated as before (17) with medium alone (Nil), or with 0.1, 1, or 10 μg/ml sPGN, or 0.1 μg/ml (serum-free conditions) or 0.01 μg/ml (medium with serum) ReLPS, without or with 1 or 10 μg/ml PGRP. The culture supernatants were collected after 6 h of stimulation at 37°C/5% CO₂, and assayed for TNF-α concentrations using the L929 cytotoxicity assay (17) described below, or for IL-6 using the IL-6-dependent 7DT1 cells as before (25).

**Cytotoxicity for mammalian cells.** To test if PGRP has any TNF-like cytotoxic activity, we used the standard L929 cell cytotoxicity assay that is commonly used for measuring cytotoxic activity of TNF, performed as described (17). Briefly, medium alone or serial double dilutions of PGRP alone (20 pg/ml to 10 μg/ml), or recombinant mouse TNF-α standard alone (specific activity, 4 x 10⁷ U/mg, from Genzyme, Boston, MA), or of combinations of PGRP and TNF-α, were added to L929 cells and incubated for 18 h in the presence of 1.33 μg/ml actinomycin D. The cell morphology was evaluated under a phase-contrast microscope, the number of surviving cells
was measured spectrophotometrically after staining with crystal violet, and a standard curve for TNF-α cytotoxicity was fitted into a sigmoidal equation as before (17).

Chemotaxis. Chemotaxis was evaluated as described (34). Medium alone (RPMI-1640 with 10 mM HEPES, pH 7.2, and 1 mg/ml or 10 mg/ml BSA), sPGN alone, PGRP alone, or sPGN and PGRP together (at 0.001 to 1 µg/ml), or control chemoattractants, 0.1 nM fromyl-methyl-leucyl-methionine (fMLP, Sigma), 20 ng/ml of monocyte chemoattractant protein-1 (MCP-1, sp. act. 1000 U/µg), or 200 ng/ml of GRO-α (sp. act. 100,000 U/µg, both human recombinant proteins produced in E. coli, and obtained from the National Cancer Institute, Frederick, MD), were placed in the lower chambers of the 96-well disposable ChmoTx chemotactic plates (Neuro Probe, Gaithesburg, MD). Human monocytes or PMNs were isolated as described above under Cells and cell lines. Monocytes were allowed to phagocytize latex particles (for 30 min at 37°C/5% CO₂), phagocytosis of latex had no adverse effect on the monocytes’ ability to migrate to chemotactic stimuli, but greatly aided their identification and differentiation from other mononuclear cells), were washed with medium, and 0.48 x 10⁶ cells (in 80 µl) were put on top of filters with 8 µm pores placed over the chambers containing the chemotactic stimuli. The chambers were incubated for 90 min at 37°C/5% CO₂, the filters were washed with PBS, dried, stained with Wright’s stain, and the numbers of monocytes that migrated into the filter were counted on equal numbers of random fields under oil immersion microscope (34). PMNs (0.18 x 10⁶ in 60 µl) were put on top of filters with 5 µm pores placed over the chambers containing the chemotactic stimuli. The chambers were incubated for 30 min at 37°C/5% CO₂, the cells were scraped off the filters (without separating the filters from the chambers), the filters were washed with PBS, incubated with 40 µl 10 nM EDTA for 30 min at 4°C, the plates were centrifuged at 1500 rpm for 10 min at 4°C, and PMNs in the lower chamber were counted.
RESULTS

PGRP binds peptidoglycan and Gram-positive bacteria with nanomolar affinity

To determine the specificity, kinetics, and affinity of interaction between mouse PGRP and PGN, we developed a binding assay, similar to the binding assay that was used to demonstrate the binding of PGN to CD14 (13), in which binding of $^{125}$I-labeled PGRP to soluble PGN (sPGN) immobilized on agarose beads was studied. Mouse PGRP bound to sPGN-agarose, but not to control agarose or ReLPS-agarose, in a dose-dependent manner (Fig. 1A), and maximum binding was reached in less than 10 min (Fig. 1B). The ratio of specific to nonspecific binding was 50 to 150 (Fig. 1).

PGRP binding was specific for polymeric sPGN (Fig. 2), as evidenced by: (a) binding of PGRP to sPGN-agarose and no binding to control agarose; (b) inhibition of binding by sPGN with an IC$_{50}$ = 0.2 µg/ml (2 nM); (c) no binding of PGRP to low M, PGN components (MDP, GlcNAc-MDP, or PGN pentapeptide) conjugated to agarose; (d) more than 10 to 20 times lower binding to ReLPS and smooth LPS; (d) no competitive inhibition by low M, PGN components (GlcNAc-MDP, and also by MDP, GlcNAc, and pentapeptide [not shown]); and (e) three to four orders of magnitude less inhibition by smooth LPS, ReLPS, lipoteichoic acid (LTA), or ribitol teichoic acid (TA). Of note, the same batches of agarose coupled to the above compounds showed different binding of $^{32}$P-soluble CD14, i.e., CD14 showed the highest binding to ReLPS, second highest binding to sPGN, third highest to MDP-agarose and GlcNAc-MDP-agarose, and lower binding to smooth LPS-agarose (ref. 13 and data not shown). Moreover, binding of sCD14 to sPGN-agarose was inhibited not only by sPGN (as was the PGRP binding), but also by ReLPS, LPS, and LTA (which did not inhibit PGRP binding) (ref. 13 and data not shown), thus showing different specificities of PGRP and CD14.

Binding of PGRP to sPGN-agarose was also not inhibited by up to 1 mg/ml of negatively charged sulfated polymers (dextran sulfate and heparin, data not shown), suggesting that the binding is not relying on negative charges. The binding was also not inhibited by lipophilic compounds (MDP-6-O-stearoyl and stearoyl-CoA, data not shown) at 100 µg/ml, a concentration
that completely inhibits binding of sCD14 to LPS\(^2\), suggesting that binding of PGRP to PGN does not involve lipophilic interactions.

At low concentrations (0.005 to 0.125 µg/ml), PGRP bound to sPGN with \(K_d = 13\) nM (Fig. 3). At higher concentrations (0.125 to 1 µg/ml), PGRP showed cooperative binding to sPGN, indicated by concave upwards Scatchard plot, with a second low affinity binding (\(K_d = 70\) nM). This cooperative binding suggests aggregation of PGRP at higher concentrations in the presence of PGN, or binding of multiple molecules of PGRP to each PGN molecule. This hypothesis of cooperative binding was also supported by the inability of unlabeled PGRP at higher concentrations to competitively inhibit binding of \(^{125}\)I-PGRP to sPGN and by the enhancing effect of unlabeled PGRP on the binding of \(^{125}\)I-PGRP to sPGN (see Fig. 4 below). Moreover, it was also suggested that Tag7 (PGRP) forms aggregates in solution (19).

At low concentrations (up to 0.1 µg/ml), unlabeled PGRP began to inhibit the binding of \(^{125}\)I-PGRP to sPGN (Fig. 4), but at higher concentrations unlabeled PGRP enhanced the binding of \(^{125}\)I-PGRP to sPGN (suggesting aggregation of PGRP at higher concentrations in the presence of PGN). These results are consistent with the cooperative binding at higher concentrations of PGRP revealed by the Scatchard plot (Fig. 3B).

PGRP binding to sPGN was not inhibited by sCD14 (Fig. 4) or by the 1-152 N-terminal amino acid fragment of sCD14, and binding of \(^{32}\)P-sCD14 to sPGN was not inhibited by PGRP (data not shown). These results suggest that PGRP and sCD14 bind to different epitopes on sPGN.

PGRP binding to sPGN was inhibited by lysozyme, which binds to and hydrolyzes a bond in the (GlcNAc-MurNAc)\(_3\) fragment of the PGN glycan chain, but not by lysostaphin, which binds to and hydrolyzes the peptide portion of PGN (Fig. 4). These results suggest that PGRP binds to the similar epitope on the glycan chain of PGN as lysozyme.

Mouse PGRP bound to some Gram-positive bacteria (Micrococcus and Bacillus), and this binding was 4 to 8 times higher than to S. aureus (another Gram-positive bacterium) or E. coli (a Gram-negative bacterium) (Fig. 5A). Based on the amount of PGN in Micrococcus and Bacillus
cells, binding of PGRP to these bacteria was approximately 10 times lower than the binding to sPGN-agarose (there is 1.5 µg sPGN bound per 1 µl of sPGN-agarose, and approximately 1 µg PGN/10-20 µg of Gram-positive bacteria, ref. 35). This lower binding of PGRP to PGN in intact bacteria may be related to more limited accessibility for PGRP binding of the peptide-cross-linked PGN incorporated into the cell wall. Still lower binding of PGRP to *E. coli* and *S. aureus* suggest even poorer availability of the form of PGN that binds PGRP on the surface of *E. coli*, in which PGN is hidden below the outer membrane, and *S. aureus*, in which PGN is highly cross-linked and heavily modified by covalently linked ribitol teichoic acid, proteins, and O-acetylation (36).

PGRP also bound to insoluble *S. aureus* cell wall PGN, zymosan (*Saccharomyces* cell wall), and chitin, but did not bind to cellulose (Fig. 5B). However, this binding was rather poor, since 300-600 times less PGRP bound to insoluble cell wall PGN than to soluble agarose-bound PGN from the same *S. aureus* strain. The binding to zymosan and chitin was even lower than to insoluble PGN. The two main differences between the soluble and insoluble PGN are extensive peptide-cross-linking in insoluble PGN (which could make PGRP binding sites unavailable) and extensive O-acetylation in insoluble cell wall PGN, in contrast to sPGN, which is not cross-linked and not O-acetylated. If PGRP shows preference for non-O-acetylated PGN, this specificity would be similar to the specificity of lysozyme, which only acts on non-O-acetylated PGN (which makes *S. aureus* resistant to lysozyme, ref. 36). These results are consistent with the binding to intact bacteria (Fig. 5A), because *Micrococcus* and *Bacillus*, which bind PGRP much better than *S. aureus*, are, in contrast to *S. aureus*, also highly sensitive to lysozyme, because they have non-O-acetylated PGN (36). Therefore, all these data together suggest that PGRP has a similar specificity to lysozyme, but 200 to 300 times higher affinity (37).

Native mouse PGRP had a similar binding potential as the recombinant PGRP, as shown by similar binding of virtually all native PGRP from the lysates of mouse bone marrow cells and recombinant PGRP to sPGN-agarose and *Micrococcus*, and no binding of either PGRP to control agarose or latex particles, as detected by Western blotting (Fig. 6). These results confirm that the recombinant PGRP has the proper conformation for the binding to PGN and bacteria, and that the
C-terminal 6xHis tag present in the recombinant PGRP has no major influence on the binding of PGRP to PGN and bacteria.

**PGRP is selectively expressed in polymorphonuclear leukocytes**

Previously, high expression of PGRP mRNA was found in the bone marrow (5), and much lower expression was found in the spleen and other organs with high content of immune cells, such as lungs, intestine, and blood (5, 19), suggesting that PGRP may play a role in immunity. To gain some insight into the function of PGRP, our next experiments were performed to identify the cell type(s) that express PGRP mRNA and PGRP protein.

Using RT-PCR we demonstrated that in the mouse, PGRP mRNA was highly expressed in PMNs and in bone marrow cells (Fig. 7A). PGRP mRNA was very weakly expressed in spleen cells and peripheral blood mononuclear cells (PBMC), and was not expressed in thymocytes, B and T lymphocytes, and peritoneal macrophages (Fig. 7A). PGRP mRNA was not expressed in any of the mouse cell lines (B cell, T cell, and macrophages, data not shown), when the same number of PCR cycles was run that revealed high expression of PGRP mRNA in neutrophils and bone marrow (Fig. 7A). However, when the number of PCR cycles was increased from 20 to 48, PGRP mRNA was expressed to various extent in 3 out of 4 mouse B-lymphocyte cell lines, whereas, even after 48 PCR cycles expression of PGRP mRNA was still not detectable in two T-lymphocyte, three macrophage, one pre-B-lymphocyte, and one plasma cell (hybridoma) cell lines (Fig. 7B).

This low level expression of PGRP mRNA in B cell tumors is not surprising, because Tag7 (identical to PGRP) was expressed in some tumors and was cloned from a tumor cell line (19). PGRP mRNA expression in B cell tumors may be related to its chromosomal location in band 7A3, because this band is genetically linked with lupus nephritis in MRL and New Zealand mice, which are characterized by overactivation of B cells (19).

Stimulation of T or B lymphocytes, macrophages, B-lymphocytic, pre-B cell, or macrophage cell lines with PGN, LPS, or Gram-positive bacteria for 6 to 72 h did not induce PGRP expression in these cells (data not shown). Weak expression of PGRP mRNA in mouse spleen cells and PBMC was likely due to low level of contamination of these cells with PMNs (because, in contrast...
to humans, the density of mouse PMNs is very similar to the density of mononuclear cells, and
differential centrifugation through ficoll is not as effective in separating mouse PMNs from
mononuclear cells 3).

In humans, PGRP mRNA was also strongly expressed in PMNs, and was not expressed in
PBMC, monocytes, B lymphocytes, T lymphocytes, and NK cells (Fig. 7C). PGRP mRNA was
also not expressed in human T-lymphocyte (MOLT-4), B-lymphocyte (RAJI), and monocyctic (U-
937 and THP-1) cell lines (Fig. 7C).

Although the expression of most proteins is regulated at the transcriptional level, expression
of some proteins is regulated at the translational level, and the presence of PGRP mRNA does not
necessarily mean the expression of PGRP protein. Moreover, the high sensitivity of PCR-based
methods makes them susceptible to false-positive results from contamination of non-expressing
cells by small numbers of expressing cells. For these reasons, we next determined the expression
of PGRP protein in mouse cells using Western Blots and anti-mouse PGRP antibodies.

PGRP protein was exclusively expressed in mouse PMNs and bone marrow cells, and was
not expressed in spleen cells, PBMC, purified B lymphocytes, purified T lymphocytes, and purified
peritoneal macrophages (Fig. 8A). The anti-PGRP Abs used for detection of PGRP were specific
for PGRP, because the appearance of only the PGRP band (but not other non-specific bands) on
the Western blots was inhibited by the PGRP peptides (Fig. 8B). The appearance of the PGRP
band was not inhibited by an unrelated control peptide (not shown). The amount of PGRP in
neutrophils was approximately 1-2 µg PGRP/10^6 PMNs, as estimated on Western Blots by
comparing serial dilutions of cell lysates and purified PGRP (data not shown). PGRP protein was
also not detectable in any of the mouse B lymphocytic, T lymphocytic, pre-B cell, hybridoma, or
macrophage cell lines (data not shown). These results indicate exclusive expression of PGRP
protein in PMNs. The expression of PGRP in the bone marrow is likely due to the high content of
PMNs in the bone marrow, which is the site of PMN production and maturation and contains the
reserve pool of PMNs.
**PGRP is not a PGN-lytic enzyme, but it inhibits growth of Gram-positive bacteria**

We next tested whether mammalian PGRP had a PGN-lytic or bacteriolytic activity because of three reasons. First, because PGRP sequence has some homology to the T3 and T7 bacteriophage lysozymes (5), which are amidases (i.e., they hydrolyze the bond between muramic acid and the peptide, unlike the vertebrate lysozyme, which is a muramidase, i.e., it hydrolyzes the glycosidic bond between GlcNAc and MurNAc of the glycan chain). Second, because binding specificity of PGRP to PGN is similar to vertebrate lysozyme. And third, because PGRP is expressed in PMNs, and one of the functions of PMNs is digestion of phagocytized bacteria.

However, mouse PGRP that bound to PGN with nanomolar affinity did not have any detectable PGN-lytic activity even at 100 µg/ml, when incubated for 48 h at 37°C. By contrast, under the same conditions, complete lysis of sPGN with control enzymes, lysozyme and lysostaphin (determined by Western blots), was detectable at 0.5 µg/ml of lysozyme and 5 µg/ml of lysostaphin, and 50% digestion was detectable at 0.05 µg/ml and 0.5 µg/ml, respectively.

PGRP also had no detectable bacteriolytic activity for *M. luteus* at concentrations up to 25 µg/ml when incubated for up to 24 h. By contrast, lysozyme caused virtually complete lysis of the bacteria (reduction of OD540 from 0.75 to 0.05) in 30 min at 10 µg/ml and in 90 min at 1 µg/ml of lysozyme.

We next considered the possibility that PGRP may have an antibacterial effect (bacteriostatic or bactericidal), because PGRP is present at high concentrations in PMNs, and because the main function of PMNs is phagocytosis and killing of bacteria. Indeed, PGRP significantly inhibited the growth of Gram-positive bacteria (*S. hemolyticus* and *B. megaterium*, Fig. 9 B-D, and also *S. warneri* and *S. capitis*, not shown), but had no effect on the growth rate of Gram-negative bacteria (*E. coli*, Fig. 9A, and also *E. cloacae*, not shown). PGRP by itself, however, was not bactericidal, and it only inhibited the growth rate of bacteria by about 50%, i.e., it took approximately twice as long to generate similar numbers of bacteria in the cultures with PGRP compared to cultures without PGRP (Fig. 9D and data not shown). Upon prolonged incubation, both cultures eventually reached similar numbers of bacteria (Fig. 9D and data not shown).
**PGRP inhibits phagocytosis and oxidative burst in leukocytes**

We next tested the effect of PGRP on phagocytosis of bacteria and on bacterially-induced oxidative burst in leukocytes, because PGRP could be present in serum at low concentrations and/or it could be released from PMNs at the inflammatory sites (the concentration of PGRP in mouse serum was lower than 5 µg/ml, determined on Western blots). Because of its nanomolar affinity of binding to PGN and bacteria, PGRP (released from PMNs) even at low concentrations could modulate the host responses to PGN and bacteria by analogy to complement components or mannose-binding protein that act as opsonins (1, 2), or to LPS-binding protein (LBP) that enhances cell responses to LPS (38, 39).

PGRP inhibited phagocytosis of *Micrococcus* bacteria, but not of Latex particles, by mouse macrophage RAW264.7 cells (Fig. 10A), and the inhibition was dose-dependent (not shown). PGRP also inhibited oxidative burst in human PMNs generated by PGRP-binding bacteria (*Micrococcus* and *Bacillus*), but it did not inhibit generation of oxidative burst by PGRP-non-binding bacteria (*E. coli*) and other PGRP-non-binding stimuli (LPS, or PDB + ionomycin) (Fig. 10B). This inhibition was also dose-dependent (not shown).

**PGRP inhibits cytokine induction in macrophages**

Because bacteria and their PGN component are potent inducers of cytokine production in macrophages (8, 9), we next considered a possibility that PGRP might have an effect on the macrophage activation by PGN and bacteria. This effect could be either inhibitory, by analogy to the LPS-binding bactericidal permeability-inducing protein (BPI), a protein present in neutrophils (40, 41), or enhancing, by analogy to LBP, an acute-phase protein present in plasma (38, 39).

First, we evaluated the effect of PGRP on the induction of TNF-α and IL-6 production by a mouse macrophage cell line, RAW264.7, adapted to growth in serum-free medium. Serum-free conditions were selected to avoid any possible interference of the native PGRP that could be present at low concentration in the serum. PGRP, at 10 µg/ml, inhibited sPGN-induced TNF-α and IL-6 production by 62% and 80%, respectively, but did not significantly inhibit ReLPS-induced production of TNF-α and IL-6 (Fig. 11). At 1 µg/ml, PGRP still inhibited sPGN-induced
production of IL-6 by 65%, but it did not significantly inhibit the production of TNF-α (not shown).

Next we tested the influence of serum on the effect of PGRP on the sPGN-induced cytokine production, because serum could contain other components that could either enhance or inhibit the effect of PGRP. The sPGN- and ReLPS-induced production of TNF-α and IL-6 in RAW264.7 cells cultured in the presence of 10% serum was not significantly changed by the presence of 1 or 10 µg/ml of PGRP, thus demonstrating that serum abolished the inhibitory effect of PGRP on sPGN-induced cytokine production. This may be due to the already diminished responses of cells to sPGN in the presence of serum that we have observed before (14), although the components in the serum responsible for this decreased responsiveness to sPGN are still unknown.

*PGRP has no TNF-like cytotoxicity*

We next tested whether PGRP was cytotoxic for tumor cell lines, because it was suggested that Tag7, which has identical sequence to PGRP (5), is a new cytokine with a TNF-like cytotoxicity (19). However, none of our mouse PGRP preparations (which all bound PGN with nanomolar affinity, see above), at 20 pg/ml to 10 µg/ml, had any cytotoxicity for L929 cells under the conditions at which TNF-α induced 50% cytotoxicity at 10 pg/ml and 95% cytotoxicity at 100 pg/ml, as evaluated microscopically and measured spectrophotometrically (Fig. 12). Moreover, PGRP had no effect on TNF-α-induced cytotoxicity for L929 cells (Fig. 12), and it also was not cytotoxic for three other tumor cell lines and for primary cells (mouse and human leukocytes, human fibroblasts, and primary human endothelial cells). This lack of PGRP cytotoxicity was confirmed independently in two laboratories.

*PGRP and PGRP:sPGN complexes are not chemotactic*

Because chemotaxis and production of chemotactic chemokines are the most prominent host responses to bacteria and bacterial products, including PGN (18), we also tested if PGRP by itself or in combination with sPGN had any chemotactic activity. However, neither PGRP nor sPGN by themselves or together (at 0.001 to 1 µg/ml) were chemotactic for human monocytes or
human PMNs, whereas, in the same experiments, control chemotactic agents induced 8 to 14 fold increases in directional migration of monocytes (0.1 nM fMLP and 20 ng/ml MCP-1) and neutrophils (0.1 nM fMLP and 200 ng/ml GRO-α).
DISCUSSION

Our results demonstrate that mammalian PGRP binds to PGN and Gram-positive bacteria (with unmodified PGN) with nanomolar affinity, is expressed in neutrophils, inhibits growth of Gram-positive bacteria, and inhibits some of the bacterially-induced functions of neutrophils and macrophages. These results support the notion (5) that mammalian PGRP has analogous function to insect PGRP (4, 5, 42), i.e., it most likely plays a role in antibacterial innate immunity. However, the exact function of PGRP in mammalian and insect immunity may be different, because in mammals, PGRP may function as an antibacterial intracellular protein present in neutrophils, whereas, in insects PGRP is likely to function in a humoral defense mechanism (4, 42).

It was suggested that PGRP is a pattern-recognition protein for PGN (5, 42), as it can recognize PGN, a cell wall component common to all bacteria. CD14 is another pattern-recognition protein that also functions to recognize PGN. However, the specificities, the structures, and the functions of PGRP and CD14 are quite different. The specificity of PGRP is much narrower than of CD14: PGRP binds with high affinity only to soluble, uncross-linked, and unmodified polymeric PGN, and binds much poorer to highly peptide-cross-linked O-acetylated PGN, and does not bind to low M, soluble or solid-phase-bound PGN fragments and other bacterial cell wall components, such as LTA or LPS. By contrast, sCD14 binds to all of these compounds (except for soluble low M, PGN subunits) with similar affinity (13). Also, PGRP binds to PGN with a faster kinetics and a higher affinity than CD14. Moreover, PGRP and CD14 seem to be specific for different epitopes on PGN, because PGRP and CD14 do not compete with each other for binding to PGN. PGRP is expressed exclusively in PMNs, where it is present in granules3 and probably functions as an intracellular antibacterial protein, whereas, CD14 is a cell-surface receptor expressed primarily on monocytes and macrophages that triggers pro-inflammatory responses of these cells (8-13). In addition, CD14 is present in a soluble form in plasma, where it functions to activate CD14-negative cells and to enhance the responses of CD14-positive cells (8-13). By contrast, PGRP inhibits some of the cell-activating effects of PGN and Gram-positive bacteria. Therefore, given all these differences in the specificities and functions of PGRP and CD14, it is not surprising
that they do not share any structural or sequence homology. PGRP also has no structural and sequence homology to TLR2, another pattern-recognition receptor that recognizes PGN (14-16).

The binding specificity of PGRP is similar to the specificity of lysozyme (43). They both have preferential specificity for unmodified PGN present in non-pathogenic Gram-positive bacteria, such as *Micrococcus* and *Bacillus*, and bind poorly to more pathogenic bacteria, such as *S. aureus*, which have extensively modified PGN (by O-acetylation, and by teichoic acid and other molecules bound to it). Perhaps the non-pathogenic bacteria are not pathogenic because their cell walls are easily and with high affinity recognized by the components of the mammalian innate immune system (such as PGRP and lysozyme), which leads to their effective elimination by the innate immune system. By contrast, more pathogenic bacteria have modified their PGN and cell walls in such a way, that they are not as effectively recognized by the innate immune system, and thus are not as easily eliminated as non-pathogenic bacteria by the innate immune system.

We have confirmed that mouse PGRP (5) and Tag7 (19) is the same protein, because they have the same cDNA sequence (5, 19) and gene sequence (19 and 4). However, two aspects of our results on the expression and function of PGRP differ from the results reported for Tag7 (19).

First, we found that PGRP is primarily expressed in neutrophils, is only very weakly expressed in spleen cells, and is not expressed in purified macrophages, T and B lymphocytes, and NK cells, that are free of neutrophils. Tag7 mRNA was shown to be present in peritoneal macrophages, spleen, and lung (19), however, its presence in neutrophils or highly purified lymphocyte subpopulations was not studied (19). We have found that contamination of tissues with blood-derived neutrophils makes these tissues positive on PGRP mRNA expression tests, which could be one possible explanation of the positive results of Tag7 expression in peritoneal macrophages, spleen, and lung (19). Also, in contrast to the results reported for Tag7 (19), we could not detect any increase in PGRP expression in lymphocytes or macrophages following stimulation with PGN, LPS, or bacteria. Thus, our current results are consistent with previously reported (5) very high expression of PGRP in the bone marrow (the site of neutrophil production and of the neutrophil bone marrow reserve pool), and very low expression in other lymphoid
tissues, that might have been due to contamination of these tissues with blood. This interpretation is consistent with the expression of Tag7, that could only be detected in freshly isolated spleen cells, but not in B and T lymphocytic cell lines (19). It is also possible, however, that some other cells than neutrophils express very low levels of PGRP mRNA and do not express, or express only very low levels of PGRP protein (undetectable by Western Blot).

Second, in contrast to Tag7 (19), our PGRP has no TNF-like cytotoxicity for L929 cells and other tumor cell lines, even at concentrations of PGRP 10^3 times higher than the cytotoxic concentrations of TNF-α. The reason for this discrepancy is unclear, although it may be related to the fact that we have used purified PGRP protein, whereas previously crude culture supernatants containing Tag7, rather than purified Tag7 protein, were tested (19). Therefore, our results firmly establish PGRP as a peptidoglycan binding protein that interacts with bacteria and has no TNF-like cytotoxicity. Also consistent with PGN-binding activity of PGRP and its expression in neutrophils is our finding that PGRP inhibits growth of Gram-positive bacteria in vitro.

PGRP also has no effect on the cytotoxicity of TNF-α, but it inhibits some of the bacterially-induced functions of neutrophils and macrophages, such as phagocytosis of Gram-positive bacteria by macrophages, induction of oxidative burst by Gram-positive bacteria in neutrophils, and induction of cytokine production by PGN in macrophages. This inhibitory effect does not seem to be due to inhibition of PGN binding to CD14, because PGRP and CD14 do not inhibit each others’ binding to PGN, but it could possibly be due to inhibition of activation through other PGN-recognition systems, such as TLR2 (14-16). However, the significance of these effects of PGRP in vivo still needs to be elucidated, because PGRP is mostly located intracellularly, and because some of these effects (inhibition of cytokine production) were only observed in serum-free, but not in serum-containing medium. These inhibitory effects of PGRP are reminiscent of the inhibitory effects of other antibacterial intracellular neutrophil proteins, such as BPI, whose main function is cytotoxicity to Gram-negative bacteria inside phagolysosomes, but which, when used as soluble extracellular proteins, also inhibit cell activation by Gram-negative bacteria and LPS (40, 41).
In summary, mammalian PGRP binds to PGN and Gram-positive bacteria (with unmodified PGN) with nanomolar affinity, is expressed in neutrophils, and inhibits growth of Gram-positive bacteria. Therefore, PGRP may function as an intracellular antibacterial protein in neutrophils.

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REFERENCES


FOOTNOTES

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1 Abbreviations: PGRP, peptidoglycan-recognition protein; BPI, bactericidal permeability-inducing protein; BSA, bovine serum albumin; fMLP, fromyl-methyl-leucyl-methionine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; LBP, LPS-binding protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MCP, monocyte chemoattractant protein; MDP, muramyl dipeptide (MurNAc-L-Ala-D-isoglutamine); NBT, nitroblue tetrazolium; PBMC, peripheral blood mononuclear cells; PDB, phorbol dibutyrate; PGN, peptidoglycan; PMNs, polymorphonuclear leukocytes; ReLPS, LPS from Salmonella minnesota Re 595; sPGN, soluble PGN; TA, teichoic acid; TLR, Toll-like receptor; TNF, tumor necrosis factor.

2 R. Dziarski, unpublished data.

3 C. Liu and R. Dziarski, unpublished data.

4 M. C. Nehls and R. Dziarski, unpublished data.
LEGENDS TO FIGURES

Fig. 1. $^{125}$I-PGRP binds to sPGN-agarose: dose-dependence (A), kinetics (B), and purity of PGRP (C). $^{125}$I-PGRP (200 ng/ml) was incubated with the indicated amounts of sPGN-agarose or control agarose for 10 min (A), or 1 µl of sPGN-agarose, ReLPS-agarose, or control agarose for 4 to 64 min (B) at 37°C, centrifuged through 0.8 M sucrose, and the amount of $^{125}$I bound to agarose was measured. The results are means of 4 samples from 2 experiments; the SE were less than 15% and are not shown. Unlabeled (3 µg/lane, C, lane 1) and $^{125}$I-labeled (100,000 cpm/lane, C, lane 2) PGRP yielded one 20 kDa band that co-migrated on a 12% SDS-PAGE gel, visualized by Coomassie blue staining (lane 1) or autoradiography (lane 2).

Fig. 2. $^{125}$I-PGRP specifically binds to sPGN, but not to other bacterial polymers. (A) Binding of $^{125}$I-PGRP (200 ng/ml) to 0.5 µl of sPGN-agarose or to the indicated agarose conjugates was measured as in Fig. 1A. The results are means ± SE of 3 experiments. (B) Competitive inhibition of binding of $^{125}$I-PGRP (200 ng/ml) to 0.5 µl of sPGN-agarose in the absence (control) or presence of various concentrations of the indicated competitors was measured, and the results (means from 3 experiments) were calculated as the percent of control binding without a competitor; the SE were less than 15% and are not shown.

Fig. 3. PGRP binds to sPGN with nanomolar affinity. (A) Increasing concentrations of $^{125}$I-PGRP were incubated with 0.5 µl of sPGN-agarose or control agarose, and the amounts of $^{125}$I-PGRP bound to agarose and remaining unbound (free) were measured. Nonspecific binding was the amount of $^{125}$I associated with control agarose; specific binding was total amount of $^{125}$I associated with sPGN-agarose minus nonspecific binding. The results are means of 3 experiments. (B) The Scatchard plot was fitted using Cricket Graph software, and mean apparent dissociation constants ($K_d$) ± SE were calculated using curve fitting to a hyperbolic function (SigmaPlot software).

Fig. 4. PGRP cooperatively binds to sPGN and PGRP binding is inhibited by lysozyme. Competitive inhibition of binding of $^{125}$I-PGRP (200 ng/ml) to 0.5 µl of sPGN-agarose by various concentrations of the indicated PGN-binding proteins was measured, and the results (means from 3
experiments) were calculated as the percent of control binding without a competitor; the SE were less than 15% and are not shown.

**Fig. 5.** PGRP binds to bacteria, insoluble cell wall PGN, zymosan, and chitin. $^{125}$I-PGRP (200 ng/ml) and 25 µg of the indicated bacteria or cellulose (A) or the indicated amounts of insoluble *S. aureus* cell wall PGN, zymosan, chitin, or cellulose (B) were incubated for 10 min at 37°C, centrifuged through 0.8 M sucrose, and the amount of $^{125}$I bound to the pellet was measured. The results are means of 4 samples from 2 experiments; in (B), the SE were less than 15% and are not shown.

**Fig. 6.** Both recombinant and native PGRP bind to sPGN and bacteria. Recombinant PGRP or bone marrow cell lysates were incubated with sPGN-agarose, *Micrococcus*, control agarose, or latex particles, then the agarose, bacteria, or latex were spun down, and PGRP eluted from the sediments or remaining in the supernatants was detected by Western blots with anti-PGRP Abs. Recombinant PGRP standard (1 µg) is shown on the left.

**Fig. 7.** PGRP mRNA is predominantly expressed in PMNs. Expression of PGRP mRNA in mouse cells (A), mouse cell lines (B), and human cells and cell lines (C) was determined by RT-PCR; 20 cycles were used to amplify GAPDH (451 bp) and PGRP (357 bp) for mouse cells (A) and 48 cycles for mouse cell lines (B); 30 cycles were used to amplify human GAPDH (451 bp) and PGRP (342 bp) (C). The products were separated on 2% agarose gel and stained with ethidium bromide. The results are from one out of three similar experiments.

**Fig. 8.** PGRP protein is expressed in PMNs. Western blots of the lysates of the indicated mouse cells (1 x $10^6$/lane) or purified PGRP were probed with anti-PGRP Abs. (A) PGRP protein that co-migrated with the PGRP standard was detected in PMNs and PMN-rich bone marrow cells, but not in other cells. (B) The appearance of recombinant PGRP or native PGRP in cell lysates (but not of other immunoreactive non-specific bands) was inhibited by pre-incubation of anti-PGRP Abs with PGRP C-terminal and N-sub-terminal peptides. The results are from one out of two similar experiments.

**Fig. 9.** PGRP inhibits growth of Gram-positive, but not Gram-negative bacteria. Growth
of bacteria in the absence or presence of 45 μg/ml of mouse PGRP was measured by viable counts. The results are duplicate samples for each time point (A-C) or means from duplicate samples (D) from one out of two similar experiments.

**Fig. 10. PGRP inhibits phagocytosis and oxidative burst in leukocytes.** (A) Phagocytosis of *Micrococcus*, but not of latex particles, by mouse macrophage RAW264.7 cells was inhibited by 3.3 μg/ml PGRP. (B) Oxidative burst, measured by NBT test, induced in human neutrophils by *Micrococcus* and *Bacillus*, but not by *E. coli*, LPS, or PDB + ionomycin, was inhibited by 3.3 μg/ml PGRP. The results are means of 3 experiments.

**Fig. 11. PGRP inhibits sPGN-induced cytokine production in macrophages.** Production of TNF-α (A) and IL-6 (B) by mouse macrophage RAW264.7 cells grown in a serum-free medium and stimulated with sPGN or ReLPS in the absence or presence of 10 μg/ml PGRP was measured. The results are means of 3 experiments.

**Fig. 12. PGRP has no TNF-α-like cytotoxicity and has no effect on cytotoxicity of TNF-α.** L929 cells were incubated for 18 h with the indicated concentrations of PGRP, TNF-α, or PGRP plus TNF-α in the presence of actinomycin D, and the numbers of live cells were measured spectrophotometrically. The results are from one out of 3 similar experiments.
Fig. 1
Fig. 2
Specific binding
Nonspecific binding

Free PGRP (µg/ml)

Bound PGRP (ng/ml)

B/F

Kd1 = 12.8 ± 1.0 nM

Kd2 = 70 ± 3.0 nM

Specific binding (ng/ml)

0.0 0.2 0.4 0.6 0.8 1.0

Fig. 3
Fig. 4
Fig. 5

A

PGRP binding (cpm ± SE)

Cellulose
E. coli
S. aureus
Bacillus
Micrococcus

B

PGRP binding (cpm)

Concentration (µg/tube)

Insoluble S. aureus PGN
Zymosan
Chitin
Cellulose
Fig. 6
Fig. 8

**A**

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kDa

89  50  34

PGRP
Fig. 9
Fig. 10
Fig. 11