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Immunopharmacology 41 (1999) 89–107

Immunopharmacology

PGG-Glucan, a soluble β-(1,3)-glucan, enhances the oxidative burst response, microbicidal activity, and activates an NF-κB-like factor in human PMN: Evidence for a glycosphingolipid β-(1,3)-glucan receptor

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Received 26 August 1998; revised 11 November 1998; accepted 12 November 1998

Abstract

PGG-Glucan, a soluble β-(1,6)-branched β-(1,3)-linked glucose homopolymer derived from the cell wall of the yeast *Saccharomyces cerevisiae*, is an immunomodulator which enhances leukocyte anti-infective activity and enhances myeloid and megakaryocyte progenitor proliferation. Incubation of human whole blood with PGG-Glucan significantly enhanced the oxidative burst response of subsequently isolated blood leukocytes to both soluble and particulate activators in a dose-dependent manner, and increased leukocyte microbicidal activity. No evidence for inflammatory cytokine production was obtained under these conditions. Electrophoretic mobility shift assays demonstrated that PGG-Glucan induced the activation of an NF-κB-like nuclear transcription factor in purified human neutrophils. The binding of ^{3}H-PGG-Glucan to human leukocyte membranes was specific, concentration-dependent, saturable, and high affinity ($K_d \sim 6$ nM). A monoclonal antibody specific to the glycosphingolipid lactosylceramide was able to inhibit activation of the NF-κB-like factor by PGG-Glucan, and ligand binding data, including polysaccharide specificity, suggested that the PGG-Glucan binding moiety was lactosylceramide. These results indicate that PGG-Glucan enhances neutrophil anti-microbial functions and that

Abbreviations: LMW-glucan, low molecular weight glucan; HLM, human leukocyte membranes; EMSA, electrophoretic mobility shift assay; fNLP, formyl–norleucine–leucine–phenyalanine; LPS, lipopolysaccharide; fcs, fetal calf serum; CR3, complement receptor 3; PMN, polymorphonuclear leukocyte; PE, phycoerythrin; TNP, trinitrophenol; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethylsulfonylfluoride; BSA, bovine serum albumin; HBSS, Hanks balanced salt solution; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; MCF, mean channel fluorescence; GPC, gel permeation chromatography; RPMI, Roswell Park Memorial Institute; RBCs, red blood cells; EMSA, electrophoretic mobility shift assay; mAb, monoclonal antibody; C/EBP-β, CCAAT enhancer binding protein-beta

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PII: S0162-3109(98)00059-9

interaction between this β-glucan and human neutrophils is mediated by the glycosphingolipid lactosylceramide present at the cell surface.

Keywords: Leukocyte priming; Immunomodulators; β-glucan; Receptors; Lactosylceramide; Signal transduction

1. Introduction

Recognition of microbial pathogens by phagocytic cells of the innate immune system is an essential step in the immune response to infection. Lectins and opsonin receptors on phagocyte plasma membranes bind to their cognate ligands on microbial cell walls to facilitate attachment and phagocytosis. In the absence of serum opsonins, carbohydrate recognition proteins on macrophages have been shown to be involved in the phagocytic uptake of fungi and mycobacterium (Ezekowitz et al., 1990; Roecklein et al., 1992; Giamis et al., 1993; Stokes et al., 1993; Castro et al., 1994). One of the most intensely studied microbial cell wall carbohydrate polymer is β-glucan. Both particulate and soluble β-glucans derived from a variety of plant, fungal, and bacterial sources have been shown to activate the innate immune system (reviewed in Stone and Clarke, 1992; Bohn and BeMiller, 1995). The reports of soluble β-glucans in patients with fungemia (Obayashi et al., 1995) imply that, like bacterial breakdown products such as LPS (Chen et al., 1992; Ulevitch and Tobias, 1995), soluble β-glucans are available during an infectious episode to potentially mobilize host defense responses.

Previously characterized β-glucans appear to produce a state of activation in leukocytes. Numerous laboratories have reported direct induction of arachidonic acid metabolite production (Czop and Austen, 1985; Czop et al., 1988; Pretus et al., 1989; Rasmussen et al., 1991; Doita et al., 1991), cytokines (Sherwood et al., 1987; Rasmussen et al., 1991; Doita et al., 1991; Sakurai et al., 1991; Abel and Czop, 1992; Gallin et al., 1992), and oxygen radical production (Cain et al., 1987; Sakurai et al., 1991; Gallin et al., 1992) by both particulate and soluble forms of β-glucans. PGG-Glucan (Betafectin®) is a novel soluble β-glucan derived from the cell wall of *Saccharomyces cerevisiae*. In contrast to other β-glucan preparations, experiments with PGG-Glucan have shown that it does not directly activate leukocyte functions such as oxidative burst activity (Mackin et al., 1994), cytokine secretion (Poutsiaka et al., 1993; Liang et al., 1998), or proliferation (Wakshull et al., 1994). Instead, PGG-Glucan primes cells for enhanced responses to secondary stimuli (Mackin et al., 1994; Wakshull et al., 1994; reviewed in Bleicher and Mackin, 1995), and enhances leukocyte phagocytosis and microbial killing (Bleicher and Mackin, 1995; Stashenko et al., 1995; Liang et al., 1998).

The biological activity of β-glucans is mediated through specific receptors located on target cells. Several groups of investigators have described receptors which bind to and mediate phagocytosis of particulate β-glucan preparations (e.g., zymosan-like particles; Czop, 1986; Goldman, 1988a; Engstad and Robertsen, 1994; Muller et al., 1994), and have partially characterized these receptors (Czop and Kay, 1991; Szabo et al., 1995). The leukocyte receptor CR3 has been reported to bind both particulate and soluble β-glucans, as well as other polysaccharides (Thornton et al., 1996). A soluble aminated β-glucan preparation has been shown to bind to murine peritoneal macrophages (Konopski et al., 1994), and a soluble phosphorylated β-glucan derivative has been reported to bind to monocyte cell lines (Müller et al., 1996).

In this report we show that PGG-Glucan can enhance PMN oxidative burst responses to secondary stimuli, increase microbial killing, and directly activate nuclear transcription factors, all in the absence of inflammatory cytokine production. In addition, we show that PGG-Glucan interacts with a unique binding site on PMN membranes, recently identified as the glycosphingolipid lactosylceramide (Zimmerman et al., 1998). The interaction between PGG-Glucan and lactosylceramide is shown to be high affinity, specific, and saturable, and is a component of the

biological responses to PGG-Glucan in human PMN. Preliminary reports of these results have been published (Wakshull et al., 1996a,b).

2. Materials and methods

2.1. Materials

PGG-Glucan and low molecular weight (LMW)-glucan were manufactured by Alpha-Beta Technology, (Worcester, MA). The molecular weights of PGG-Glucan and LMW-glucan are 120,000–205,000 Da and 8000–15,000 Da, respectively, as determined by GPC. PGG-Glucan was enzymatically digested by incubation with a β-(1,3)-specific exoglucanase purified from *Penicillium pinophylum*. OKM1, an IgG_{2b} monoclonal antibody against human CD11b, was purchased from Ortho Diagnostic Systems (Raritan, NJ); IgG_{2b} isotype control was purchased from Sigma (St. Louis, MO); rabbit polyclonal anti-idiotype antibody was developed against an anti-laminarin monoclonal antibody by Dr. Joyce Czop (Harvard, Boston, MA). For flow cytometric analysis of CR3 expression on murine cell lines, the following reagents were used: Mouse IgG (Sigma), PE-anti-CD11b (clone M1/70; Pharmingen, San Diego, CA), PE-anti-TNP (clone 49.2; Pharmingen). Monoclonal anti-lactosylceramide antibody (clone 8D12) has been described (Zimmerman et al., 1998); an irrelevant monoclonal IgM (anti-trinitrophenol; Pharmingen, San Diego, CA) served as isotype control; IgM binding was detected by FITC-conjugated goat anti-mouse IgM (Cappel, Organon Technika, Durham, NC). Laminarin, dextran, mannan, glycogen, and LPS (serotype 0128:B12) were purchased from Sigma; Lentinan was obtained from Ajinomoto, (Yokohama, Japan); aminated curdlan was kindly provided by Dr. Rolf Seljelid (University of Tromsø, Tromsø, Norway). Scleroglucan (Actigum®) was obtained from Sanofi Bio-Industries (Paris, France); Sonifilan was purchased from Kaken Pharmaceutical, (Chiba, Japan). Other chemicals were purchased either from Sigma or Boehringer Mannheim (Indianapolis, IN). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) was prepared as a 500 mM stock in DMSO and stored at −70°C. A 10^{-2} M stock of fNLP (Sigma) was prepared in DMSO and stored at −20°C. *Staphylococcus aureus* (*S. aureus*, ATCC #29213) was opsonized in normal human serum (Sigma).

2.2. Whole blood chemiluminescence assay

Blood was collected from healthy human volunteers in 10% v/v acid citrate dextrose. The blood was incubated with PGG-Glucan or saline for 3 h at 37°C. Red blood cells were removed by dextran sedimentation and leukocytes resuspended in autologous plasma at 8.4×10^6 cells/ml. Luminol (50 μM final concentration) was added to the cells and 180 μl aliquots were added in triplicate to the wells of a 96 well Microlite 1 plate (Dynatech Laboratories, Chantilly, VA). The cells were stimulated with 20 μl of 10^{-6} M solution of fNLP or serum opsonized *S. aureus* (5:1 target:effector ratio), and chemiluminescence monitored with a 96-well plate luminometer (ML3000, Dynatech Laboratories, Chantilly, VA).

2.3. Microbicidal assay

Whole blood was incubated with saline or PGG-Glucan and the leukocytes isolated as described above. The leukocytes were resuspended in RPMI +10% fcs at 8.0×10^6 cells/ml and opsonized *S. aureus* were added to the cells in a 96-well plate at a target:effector ratio of 5:1 and incubated for 60 min. At the end of the incubation period, surviving bacteria were enumerated by colony growth on agar plates. Percent killing was calculated from the colony counts obtained from leukocyte-containing wells relative to colony counts from control wells without leukocytes.

2.4. Measurement of cytokine secretion from whole blood

Whole blood was incubated with saline, LPS (100 ng/ml), or PGG-Glucan (1 μg/ml) as described above. Cells were removed by centrifugation and the plasma was assayed for the presence of cytokines by commercial ELISA kits (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

2.5. Preparation of ^{3}H-PGG-Glucan

PGG-Glucan was incubated with 20 fold molar excess of $NaIO_4$ for 72 h in the dark, followed by

addition of 10–20 fold molar excess of glycerol or ethylene glycol to quench the oxidation reaction. The PGG-Glucan was dialyzed against water, and then reductively labeled with 100 mCi of NaB^3H_4 (New England Nuclear, Boston, MA). Low molecular weight contaminants were removed by dialysis and ultrafiltration. Purity of the labeled product was confirmed by GPC. Hexose concentration was determined by the anthrone/sulfuric acid method (Somogyi, 1952; Dische, 1962). Specific activity varied between $1–2.4 \times 10^7$ dpm/μg hexose. No difference in molecular weight was detectable by GPC compared to native material. Periodate oxidation of β-(1,3)-glucans produces open chain polyaldehydes at the reducing and non-reducing ends. Subsequent reduction with tritiated sodium borohydride yields a polyalcohol at both ends, with the ^3H isotope incorporated into the hydroxyl groups thus generated (Stone and Clarke, 1992). The internal residues of the glucan will not be affected by the oxidation/reduction reaction. In control experiments, oxidation and reduction of PGG-Glucan did not alter its ability to compete with ^3H-PGG-Glucan for binding to HLM (data not shown).

2.6. Preparation of HLM and purified PMNs

Isolated leukocytes from human blood donors (Red Cross, Dedham, MA) were prepared by dextran (1.5% w/v in saline) sedimentation and hypotonic lysis to remove RBCs. The final cell pellet was resuspended in ~3–4 volumes PBS, and protease inhibitors added (5 mM EDTA, 40 μg/ml aprotinin, 1 μM pepstatin A, 1 μg/ml leupeptin, 500 μM PMSF). The cells were disrupted by probe sonication (50 W, 30 × 1 second pulses). Nuclei and remaining intact cells were removed by low speed centrifugation ($700 \times g$, 7 min), and membranes in the supernatants collected by high speed ultracentrifugation ($180{,}000 \times g$, 60 min, 4°C). Membrane pellets were resuspended in HBSS containing Ca^{++} and Mg^{++} (Life Technologies, Grand Island, NY). Membrane protein was determined using the BCA or Coomassie method (Pierce, Rockland, IL) using BSA as standard. BSA (Sigma; final concentration, 1 mg/ml) was added and the membranes stored in liquid nitrogen. The presence or absence of either divalent cations (see Results) or BSA did not effect binding.

Further purification of PMNs from mononuclear cells was achieved by layering the leukoycte-rich fraction from the dextran sedimentation procedure onto Lymphocyte Separation Medium (LSM; Organon Teknika, Durham, NC) followed by low speed centrifugation ($400 \times g$, 30 min). Cells recovered from the pellet (PMN and RBC) were hypotonically lysed to remove residual RBCs. The remaining cells were > 95% PMN as judged by morphological criteria, while mononuclear cells recovered from the LSM/plasma interface (monocytes and lymphocytes) had < 5% PMN contamination. Membranes were then prepared from these more highly purified cells as described above.

2.7. Preparation of membranes from cell lines

The human monocytic cell lines U937, HL-60, THP-1 and the murine monocytic cell lines J774.1, RAW264.7, P388D(1), and murine B cell line LB27.4 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Primary human fibroblasts, keratinocytes, and broncheal epithelial cells were purchased from Clonetics (San Diego, CA). The murine monocyte line BMC2.3 and T cell line DO11 were kindly provided by K.L. Rock from the Dana Farber Cancer Institute (Boston, MA). Cells were removed from culture, rinsed in ice cold PBS, and membranes prepared as described above.

2.8. ^3H-PGG-Glucan binding assay

Membranes were used fresh or from frozen stocks, and diluted with HBSS containing 1 mg/ml BSA to a concentration of 2–4 mg membrane protein/ml. The binding assay reaction mixture consisted of 280 μl membranes, 35 μl saline or test sample, and 35 μl ^3H-PGG-Glucan. Binding was allowed to proceed for various lengths of time at 37°C. At the end of the incubation, receptor-bound ligand was separated from free ligand by centrifugation through a double density gradient consisting of dibutyl phthalate and 8% (w/v) sucrose in PBS (Levesque et al., 1985). The membrane pellets were dissolved (Solvable™, New England Nuclear) and radioactivity determined by liquid scintillation counting.

The binding of ^3H-PGG-Glucan to the glycosphingolipid lactosylceramide was measured in a

solid phase microtiter plate assay format as previously described (Zimmerman et al., 1998). Briefly, lactosylceramide (Sigma) was dissolved in ethanol at 1 mg/ml. Aliquots (20 μl) were applied in triplicate to the wells of a 96-well polystyrene plate (Costar) and air dried. Plates were blocked by incubation with 300 μl of 1% gelatin (w/v) in PBS at 37°C for 1–2 h. Plates were then rinsed with PBS (2 × 200 μl). Fifty microliters of either PBS or unlabeled polysaccharide was added to each well, plates were equilibrated at either 37°C or 4°C, then ^{3}H-PGG-Glucan added (1 μg/ml, 100 μl final). Plates were incubated for 1–2 h at either 37°C or 4°C, then rinsed twice with 200 μl PBS. Radioactivity was solubilized with Solvable™ (150 μl) and counted.

2.9. Flow cytometric determination of CR3 expression and lactosylceramide

Cells were harvested and washed in PBS/0.1% BSA at 4°C. Cells (2×10^6 cells/100 μl) were pre-incubated for 30 min with 1 mg/ml mouse IgG in PBS/0.1% BSA at 4°C, followed by either 10 μg/ml PE-anti-CD11b or 5 μg/ml PE-anti-TNP as a negative control for an additional 30 min. Cells were washed twice in PBS/0.1% BSA, then fixed in 1% paraformaldehyde in PBS. Fluorescence was analyzed on a FACSCAN flow cytometer using CELLQuest software (Becton Dickinson, Palo Alto, CA). Background fluorescence (PE-anti-TNP stained cells) was subtracted from the fluorescence due to PE-anti-CD11b binding to obtain specific MCF.

For assessment of mAb 8D12 binding to the PMN cell surface, PMN were purified as described above and washed in PBS/0.1% BSA at 4°C. PMN (1×10^6/ml) were incubated with 8D12 (50 μg/ml) or control mouse IgM mAb for 30 min at 4°C, washed twice with PBS/0.1% BSA, then incubated with goat anti-mouse IgM-FITC (1:10) for an additional 30 min at 4°C. Cells were then washed twice in PBS/0.1%BSA and analyzed by flow cytometry as described above.

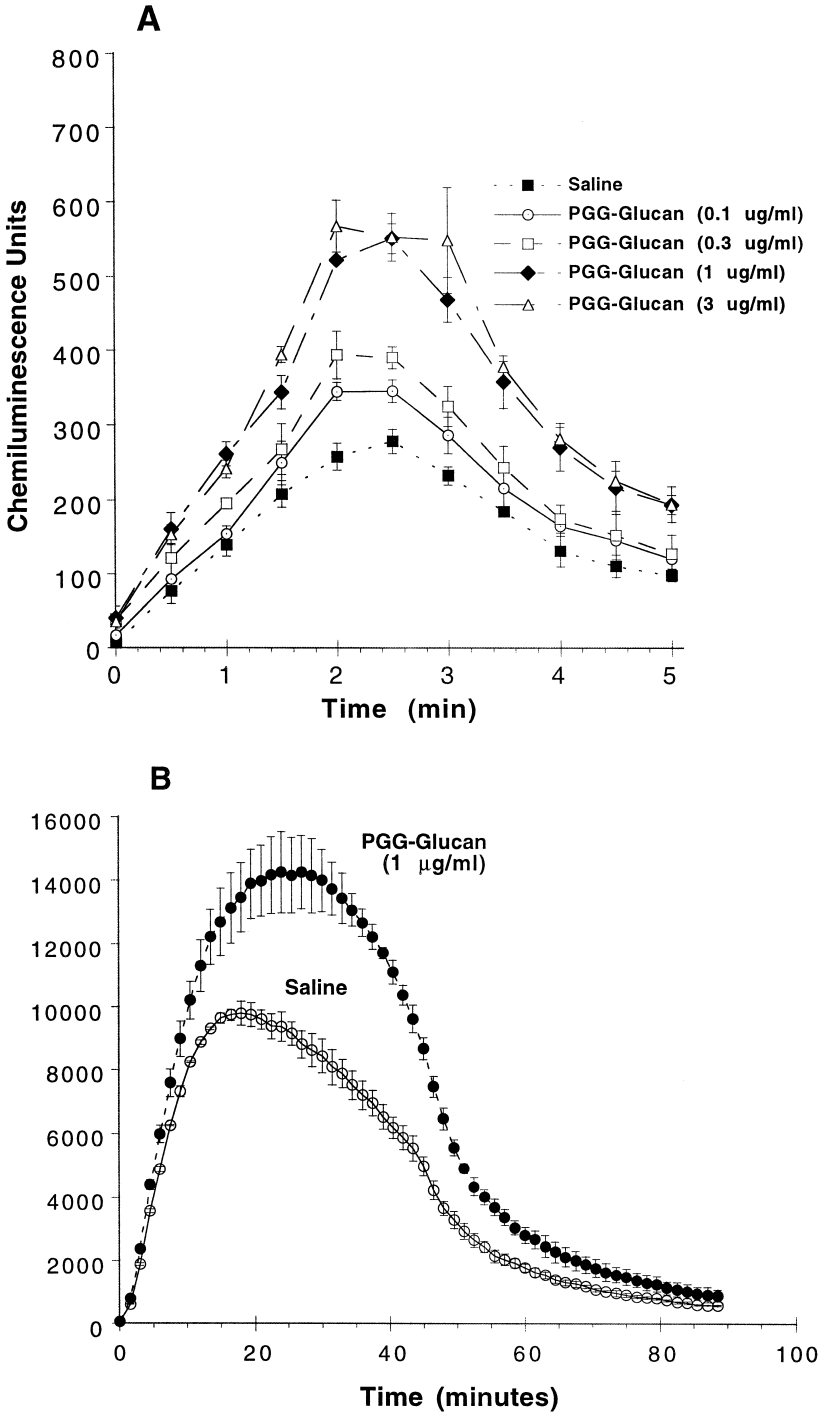
2.10. Electrophoretic mobility shift assay

Human PMNs (4.5×10^6 cells/ml; 10 ml total) purified as described above were incubated in RPMI + 10% fcs for 30 min at 37°C. PGG-Glucan, LPS, or dextran were then added at the indicated concentrations and incubation continued a further 60 min at 37°C. Untreated control cells were incubated in parallel. At the end of the incubation, cells were washed in ice cold PBS + 20 mM EDTA and nuclear extracts prepared and EMSAs performed as described in Adams et al. (1997). Wild type NF-κB probe contained the sequence 5′AGTTGAGGGGACTTTCCCAGGC (Leonardo and Baltimore, 1989). For specificity experiments, nuclear extracts were incubated with ^{32}P-labeled probe and either wild-type or mutant unlabeled probe (5′AGTTGAGGCGACTTTCCCAGGC, mutation underlined; Adams et al., 1997). In order to identify the components of the DNA binding protein, antibodies to specific Rel family members (see Results) were also included in EMSAs. Under the conditions used for these EMSA experiments, anti-Rel antibodies blocked formation of the protein/DNA complex rather than inducing a supershift, a result reported by others (see Adams et al., 1997, and references therein). In another series of experiments, the effect of an anti-lactosylceramide monoclonal antibody (ascites IgM, designated clone 8D12; Zimmerman et al., 1998) on either LPS or PGG-Glucan activation of NF-κB-like factors was assessed. Purified PMN (5×10^6/ml) were incubated with approximately 0.5 mg/ml 8D12 or a control monoclonal IgM (anti-*Cryptosporidium parvum*; Biodesign International, Kennebunk, ME) at the same concentration for 10 min at 37°C, followed by addition of LPS (1 μg/ml) or PGG-Glucan (3 μg/ml) and then incubation for 1 h. Nuclear extracts and EMSA were then performed as described above.

2.11. Calculations

The chemiluminescent PGG-Glucan dose–response data were analyzed by ANOVA, using the area under the curve (AUC); the chemiluminescent response to *S. aureus* and microbicidal data were analyzed by Student's *t*-test, also using the AUC (StatView, Abacus Concepts, Berkeley, CA). Binding data are reported either as the percent control binding (i.e., without competitor) ± percent coefficient of variation (%CV), or as the mean ± standard

deviation of triplicate samples in units as indicated in the figure legends. I.C.$_{50}$ values were obtained from the dose–response competition curves using Kaleidagraph software (Synergy, Reading, PA). Since not all



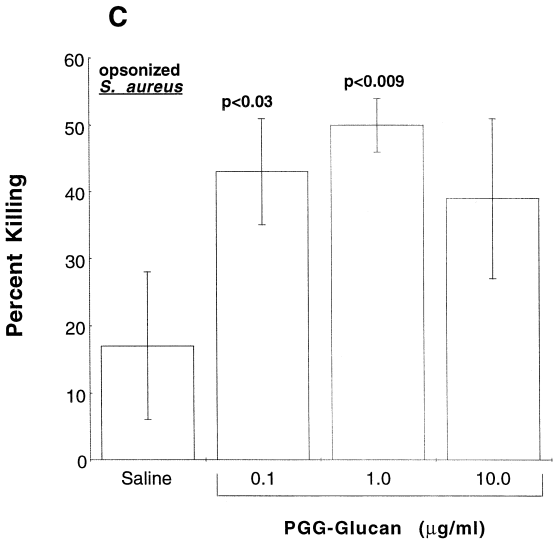


Fig. 1. PGG-Glucan enhances the oxidative burst response to both soluble and particulate activators, and enhances leukocyte microbicidal responses. A,B. Whole blood was incubated with the indicated concentrations of PGG-Glucan for 3 h at 37°C. RBCs were removed by dextran sedimentation, the leukocytes stimulated with 10^{-7} M fNLP (A) or opsonized *S. aureus* at a target:effector ratio of 5:1 (B), and chemiluminescence measured as described in Section 2. Data represent the mean ± standard deviation of triplicate wells. C. Leukocytes pretreated with the indicated concentration of PGG-Glucan as described above were resuspended in RPMI + 10% fcs then challenged with *S. aureus* for 60 min at 37°C. The percent of *S. aureus* killed relative to a leukocyte-free control is presented as mean ± standard deviation of triplicate wells. $p < 0.05$ versus saline for all PGG-Glucan doses in A, and for the 1 μg/ml dose in B and C.

competitors achieved an asymptotic minimum (i.e., non-specific binding), the $I.C._{50}$ values were operationally defined as the concentration of competitor at which 50% of ^{3}H-PGG-Glucan binding in the absence of competitor was achieved, without regard to non-specific binding. These values are therefore relative $I.C._{50}$ values. The saturation binding isotherm was evaluated by a one and two site model using non-linear regression analysis (Kaleidagraph) according to the equation $B = B_{\max}[L/(L + K_d)]$ (one site model) or $B_t = \sum B_{\max 1}[L/(L + K_{d_1})] + B_{\max 2}[L/(L + K_{d_2})]$ (two site model) where B_t represents the total amount of labeled ligand bound, L represents the concentration of labeled ligand, $B_{\max n}$ represents maximal binding capacity of site n, and K_{d_n} is the apparent dissociation constant of site n.

3. Results

3.1. PGG-Glucan enhances the leukocyte oxidative burst response and microbial killing

Human whole blood was incubated with increasing concentrations of PGG-Glucan for 3 h at 37°C. Leukocytes were isolated and then stimulated with 10^{-7} M fNLP and the production of reactive oxygen intermediates monitored by luminol-dependent chemiluminescence. In the absence of other activators, PGG-Glucan alone did not stimulate an oxidative burst (data not shown). However, pre-incubation with PGG-Glucan showed a significant dose-dependent increase in the oxidative burst response to 10^{-7} M fNLP (Fig. 1A). Enhanced chemiluminescence was detected at 0.1 μg/ml PGG-Glucan and reached a plateau between 1–3 μg/ml. A significant PGG-Glucan-induced increase in chemiluminescence was also obtained when opsonized *S. aureus* were used to stimulate the leukocytes (Fig. 1B). Experiments using purified PMN yielded similar results (data not shown). However, the PGG-Glucan-enhanced chemiluminescent response was more variable compared to the whole blood experiments, probably due to partial activation of the PMN during the purification procedure. In parallel flow cytometric experiments using whole blood incubated with PGG-Glucan, no change in the expression of receptors for fNLP, iC3b, or Fcγ was detected on leukocytes following incubation with PGG-Glucan (data not shown).

The involvement of the oxidative burst in leukocyte microbial killing is well established. Therefore, the ability of PGG-Glucan to enhance leukocyte microbicidal activity was tested. Isolated leukocytes pretreated with PGG-Glucan as described above were incubated with opsonized *S. aureus* for 60 min, and the number of surviving microorganisms enumerated. The results showed a dose-dependent increase in microbicidal activity following PGG-Glucan treatment, which was statistically significant at concentrations of 0.1 and 1 μg/ml (Fig. 1C).

3.2. PGG-Glucan does not stimulate cytokine production

Previous reports indicated that both particulate and soluble β-glucans induced the production of

pro-inflammatory cytokines. We therefore investigated whether incubation of human whole blood with PGG-Glucan induced the release of cytokines associated with leukocyte activation. Following incubation of whole blood with 1 μg/ml PGG-Glucan for 3 h, plasma cytokine levels were measured by ELISA. While LPS was shown to stimulate release of IL-1α, IL-1β, IL-6, IL-8, and TNF-α, no evidence for PGG-Glucan-induced release of these cytokines was found (Table 1). The same results were obtained when the PGG-Glucan concentration was increased to 100 μg/ml and the incubation period extended to 24 h (data not shown).

3.3. PGG-Glucan activates an NF-κB-like DNA binding protein

The results with the whole blood assays demonstrated that PGG-Glucan enhanced leukocyte oxidative burst and microbial killing activities, but without the production of pro-inflammatory cytokines. Although we felt it likely that the PMNs were the cells primarily responsible for the observed responses to PGG-Glucan, the whole blood assays did not allow us to rigorously demonstrate this. We therefore investigated signal transduction events and ^{3}H-PGG-Glucan binding activity in more purified cell populations in order to determine which cell type(s) was directly responding to PGG-Glucan.

The transcription factor NF-κB is involved in the immune response elicited by a variety of mediators. We therefore measured the ability of PGG-Glucan to activate NF-κB-like factors in human PMNs. Nuclear extracts from purified PMNs treated with either saline, dextran, or PGG-Glucan were measured for activation of an NF-κB-like factor by EMSA. PGG-Glucan, but not dextran or saline, stimulated the activation of an NF-κB-like factor in PMN nuclei (Fig. 2A). The specificity of the DNA-protein interaction was verified by competition experiments with wild type and mutant unlabeled oligonucleotides (Fig. 2B). Antibody-competition EMSA experiments were performed in order to specifically identify the components of the NF-κB-like factor. Antibodies to Rel family members p50 and p65 inhibited formation of LPS-stimulated DNA-protein complexes, indicating that LPS activated the classic p50/p65 heterodimer (Fig. 2C, left panel). PGG-Glucan-stimulated complex formation was inhibited by anti-p65, but not by anti-p50, or anti-p52 (Fig. 2C, right panel), indicating that this complex contained p65 but probably not p50, as previously shown by our laboratory in a murine monocytic cell line (Adams et al., 1997). This result suggests that PGG-Glucan utilizes a signal transduction pathway different from LPS, and shows that activation of an NF-κB-like DNA-binding protein by PGG-Glucan was not due to endotoxin contamination. Stimulation of the NF-κB-like DNA-binding protein by PGG-Glucan was found to be dose-dependent (Fig. 2D). Activation above control levels was observed at a PGG-Glucan concentration of 0.37 μg/ml and reached plateau levels (approximately 11-fold over control, assessed by densitometry) between 3–10 μg/ml.

Purification of the ^{3}H-PGG-Glucan binding site from PMNs demonstrated that it was the glycosphingolipid lactosylceramide (Zimmerman et al., 1998). We therefore investigated whether an anti-lactosylceramide antibody would inhibit PGG-Glucan activation of the NF-κB-like transcription factor. Incubation of purified PMNs with mAb 8D12 inhibited PGG-Glucan activation of the NF-κB-like factor as shown by EMSA (Fig. 3A). In contrast, activation of NF-κB by LPS was not altered by mAb 8D12. Incubation of PMN with an irrelevant IgM mAb had no effect on activation of NF-κB-like factors by either LPS or PGG-Glucan (Fig. 3B). Flow cytometric experiments were done to demonstrate that mAb 8D12 was binding to PMN. MCF for PMN incubated

Table 1
PGG-Glucan does not induce cytokine production from human blood cells

Cytokine	Plasma levels (pg/ml)[a,b]		
	Saline	LPS	PGG-Glucan
IL-1α	< 1.0	10 ± 6	< 1.0
IL-1β	< 4.0	285 ± 144	< 4.0
IL-6	15 ± 11	1179 ± 190	35 ± 30
IL-8	135 ± 73	2402 ± 858	179 ± 127
TNF-α	92 ± 33	1158 ± 358	13 ± 79

[a] Whole blood was pretreated with either saline, 100 ng/ml LPS, or 1 μg/ml PGG-Glucan for 3 h at 37°C. Similar results were obtained with 100 μg/ml PGG-Glucan incubated for 24 h.
[b] Data are mean values from 6–8 different plasma samples and are expressed as mean ± standard error of the mean.

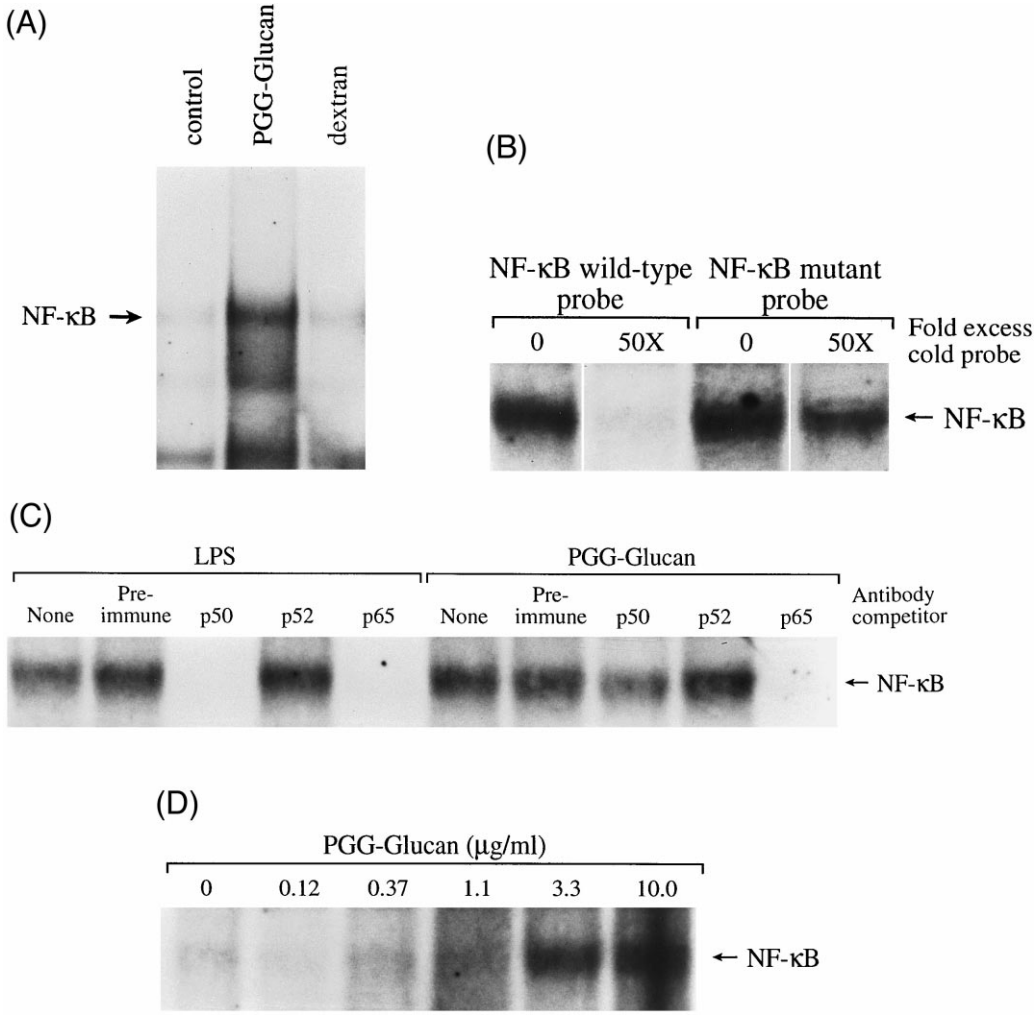


Fig. 2. PGG-Glucan stimulates the DNA-binding activity of an NF-κB-like nuclear protein. Isolated human PMN in RPMI + 10% fcs were incubated with saline, 3 μg/ml dextran, 1 μg/ml LPS, or 3 μg/ml PGG-Glucan for 1 h at 37°C. Nuclear extracts and EMSA reactions were done as described in Section 2. A. NF-κB-like DNA-binding activity was enhanced by PGG-Glucan but not dextran. B. Specificity of the DNA-protein interaction was confirmed by inhibition of complex formation by unlabeled wild type oligonucleotide, but not by mutant oligonucleotide. C. LPS or PGG-Glucan stimulated nuclear extracts were incubated with the indicated anti-Rel protein antibody during the EMSA reaction. D. Isolated human PMN were incubated with the indicated concentrations of PGG-Glucan. Increased DNA-binding activity was evident at 0.37 μg/ml and peaked between 3–10 μg/ml PGG-Glucan.

with mAb 8D12 was 98 versus 11 for the isotype control.

3.4. Characterization of ^{3}H-PGG-Glucan binding to HLM

We next sought to characterize the interaction between PGG-Glucan and its cellular recognition site(s). As stated previously, control experiments indicated that the labeling procedure did not effect binding activity (data not shown). The time course of ^{3}H-PGG-Glucan binding to HLM is shown in Fig. 4. At 37°C and with 1.0 μg/ml ^{3}H-PGG-Glucan, binding reached plateau levels after approximately 60 min. The binding was concentration-dependent and reached saturation at 25 nM ^{3}H-PGG-Glucan (Fig. 5). Non-linear regression analysis of the data was

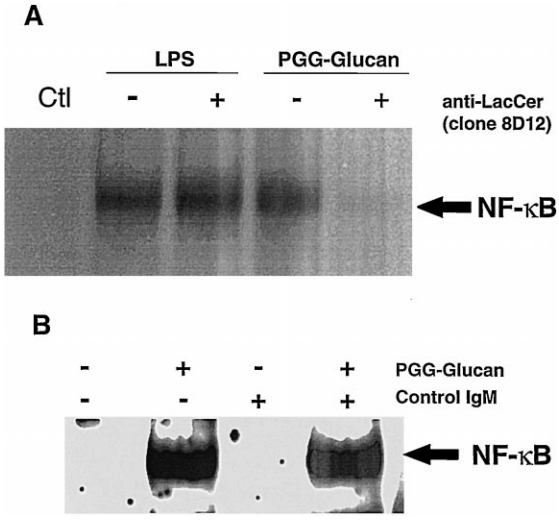


Fig. 3. Anti-lactosylceramide monoclonal antibody 8D12 inhibits PGG-Glucan activation of an NF-κB-like nuclear protein. Purified human PMN were incubated with or without 0.5 mg/ml (A) 8D12 or (B) control IgM for 10 min at 37°C, followed by addition of LPS (1 μg/ml) or PGG-Glucan (3 μg/ml), or nothing (control). Following a 1 h incubation, nuclear extracts were prepared and EMSA run as described in Section 2. (A) The presence of monoclonal antibody 8Dn12 inhibited PGG-Glucan activation of an NF-κB-like nuclear protein, whereas there was no effect on LPS-stimulated NF-κB activation. (B) Control IgM had no effect on PGG-Glucan-induced activation of an NF-κB-like nuclear protein.

consistent with a single homogeneous binding site having an apparent dissociation constant (K_d) of approximately 6 nM and a binding maximum (B_{max}) of 33 fm/mg protein. The binding of ^{3}H-PGG-Glucan to HLM was found to be temperature sensitive. Incubation of membranes with ^{3}H-PGG-Glucan (1 μg/ml) at either 4°C or 25°C showed almost no specific binding (1.0 and 1.4 ng/mg protein, respectively), while significant specific binding was observed at 37°C (15.5 ng/mg protein).

Attempts were made to measure the binding of ^{3}H-PGG-Glucan to intact purified PMNs. Reliable and reproducible specific binding of ^{3}H-PGG-Glucan was difficult to demonstrate. We believe that this was, in part, due to a high degree of pinocytotic activity contributing to apparent non-specific binding. Efforts were made to reduce this non-specific binding through the use of low temperatures (see above) and metabolic poisons, but without success.

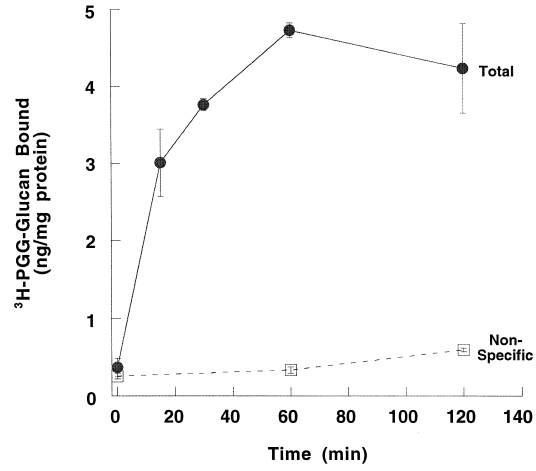


Fig. 4. Time Course of ^{3}H-PGG-Glucan binding to human leukocyte membranes. ^{3}H-PGG-Glucan (1 μg/ml) was incubated for the indicated times with human leukocyte membranes in the presence or absence of unlabeled PGG-Glucan (70 μg/ml). Aliquots were removed and membrane-associated radioactivity determined as described in Section 2. Data represent the mean ± standard deviation of triplicate samples in units of ng ^{3}H-PGG-Glucan bound/mg membrane protein.

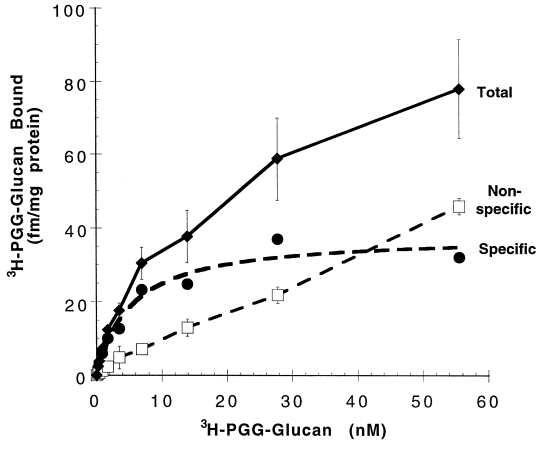


Fig. 5. Dose-dependence of ^{3}H-PGG-Glucan binding. Human leukocyte membranes were incubated with increasing concentrations of ^{3}H-PGG-Glucan in the presence (non-specific) or absence (total) of 300-fold excess unlabeled PGG-Glucan for 90 min at 37°C. Membrane-associated radioactivity was determined as described in Section 2. Specific binding was calculated by subtracting non-specific from total binding. The specific binding data were fit by linear regression analysis as described in Section 2. The dissociation constant determined by this method was Kd ~6 nM; maximal binding B_{max} was ~33 fmol/mg protein. Data points represent the mean ± standard deviation of triplicate samples in fm ^{3}H-PGG-Glucan bound/mg membrane protein.

3.5. Polysaccharide selectivity of the PGG-Glucan binding site

A variety of polysaccharides were tested for their ability to compete for ^{3}H-PGG-Glucan binding to the PGG-Glucan binding site on HLM (Table 2). Unlabeled PGG-Glucan (100 μg/ml) was able to inhibit approximately 80% of ^{3}H-PGG-Glucan binding. Full competition curves showed that 50% inhibition ($I.C._{50}$) of 1 μg/ml ^{3}H-PGG-Glucan binding to HLM was achieved at a concentration of 6.9 ± 3.5 μg/ml ($n = 30$). Examples of several competition curves are shown in Fig. 6. PGG-Glucan, Sonifilan, and scleroglucan were able to compete for ^{3}H-PGG-Glucan binding to HLM, whereas laminarin and aminated curdlan were not. Additional polysaccharides were used to assess the structural selectivity of the ^{3}H-PGG-Glucan binding activity (Table 2). Dextran and mannan, α-linked glucose and mannose polymers, respectively, failed to inhibit binding. Similarly, glycogen, an α-(1,4)-linked glucose polymer, did not inhibit binding. These results suggest that the receptor is selective for glucose polymers with β-(1,3)-linkages. This was confirmed when PGG-Glucan digested by a β-(1,3)-specific exoglucanase failed to inhibit ^{3}H-PGG-Glucan binding to HLM (Table 2). To further assess the selectivity of the ^{3}H-PGG-Glucan binding site, several additional β-(1,6)-branched, β-(1,3)-glucans were tested in competition assays. Neither the LMW-glucan nor laminarin (5000 Da; see also Fig. 7) competed for binding. Lentinan, a large (~500,000 Da) triple helical glucan (Hamuro and Chihara, 1985), also did not inhibit binding. Scleroglucan (~850,000 Da) and Sonifilan (~450,000 Da) were comparable to PGG-Glucan as competitors (see also Fig. 7). These β-(1,3)-glucans differ from PGG-Glucan not only in molecular size, but also in branch frequency and branch length. Thus, not only was the binding specific for β-(1-3)-glucans relative to other linkage

Table 2
Effect of various polysaccharides, divalent cations, and antibodies on ^{3}H-PGG-Glucan binding to HLM

Category	Addition	Concentration	% Control binding[a]	% CV
Control	PGG-Glucan	0.1 mg/ml	22	5
	digested PGG-Glucan[b]	0.2 mg/ml	93	5
Other polysaccharides	LMW glucan	0.1 mg/ml	117	12
	dextran	1 mg/ml	88	12
	mannan	1 mg/ml	88	5
	laminarin	1.0 mg/ml	83	5
	glycogen	0.1 mg/ml	134	20
	lipopolysaccharide	0.1 mg/ml	89	16
	Lentinan	1 mg/ml	91	10
	aminated glucan[c]	0.1 mg/ml	111	1
	scleroglucan	1 mg/ml	41	4
	Sonifilan	0.1 mg/ml	17	2
Chelators	EDTA	5 mM	82	13
	EGTA	5 mM	87	0.1
Antibodies	anti-idiotype[d]	10 μg/ml	88	7
	anti-CR3[e]	28 μg/ml	123	19
	anti-lactosylceramide[f]	1:2 dilution[h]	40	1
	control IgM[g]	1:2 dilution[h]	100	2

[a] Represents the percent of binding relative the amount bound in the absence of unlabeled competitor.
[b] Represents β-(1,3)-exoglucanse-digested PGG-Glucan.
[c] Animated curdlan was prepared from curdian by formic acid hydrolsis and reductive animation.
[d] Rabbit anti-(mouse anti-lamiranin mAb).
[e] IgG2b isotype controls did not inhibit binding (data not shown).
[f] Anti-lactosylceramide (clone 8D12).
[g] Anti-Crytospridium parvum mAb served as IgM isotypes control.
[h] Refers to dilution of ascites fluid used.

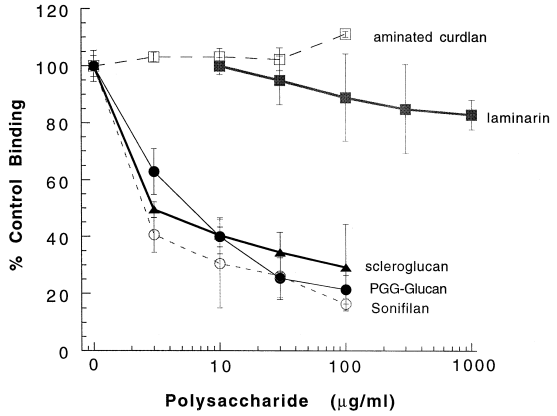


Fig. 6. Inhibition of ^{3}H-PGG-Glucan binding to human leukocyte membranes is β-glucan specific. Human leukocyte membranes were incubated with ^{3}H-PGG-Glucan (1 μg/ml) and increasing concentrations of PGG-Glucan, aminated curdlan, laminarin, scleroglucan, or Sonifilan for 1 h at 37°C. Membrane-associated radioactivity was determined as described in Section 2. Data are presented as the mean percent control binding ± percent coefficient of variation of triplicate determinations.

groups, but was also selective within the structural class of β-(1-3)-glucans. In addition, divalent cation chelators did not affect binding, indicating that the binding site is not a C-type lectin.

Finally, two antibodies which have been used by other investigators to characterize β-glucan receptors were tested for their ability to interfere with ^{3}H-PGG-Glucan binding to HLM. Neither an anti-idiotype antibody, which was shown to inhibit β-glucan particle phagocytosis (Czop et al., 1990), or anti-CR3, which was shown to inhibit both particulate and soluble β-glucan binding (Ross et al., 1987; Thornton et al., 1996), inhibited ^{3}H-PGG-Glucan binding to HLM (Table 2). These results indicate that PGG-Glucan is binding to different recognition sites, if not entirely different receptor molecule(s), from those described previously. The possibility that ^{3}H-PGG-Glucan was binding to an entirely separate molecular entity was strengthened by the observation that an anti-lactosylceramide monoclonal antibody (clone 8D12) inhibited binding to HLM (Table 2), consistent with the EMSA results (Fig. 3).

3.6. Characterization of ^{3}H-PGG-Glucan binding to purified lactosylceramide

The observation that ^{3}H-PGG-Glucan binding to human PMN membranes is mediated by lactosylceramide (Zimmerman et al., 1998; present results)

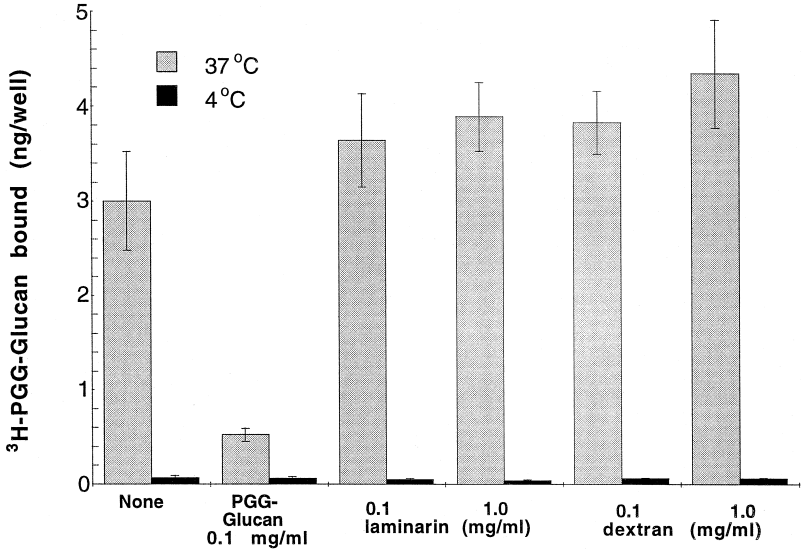


Fig. 7. ^{3}H-PGG-Glucan binding to purified lactosylceramide is temperature dependent. Lactosylceramide was dried onto 96-well polystyrene microtiter plates, blocked with gelatin, then incubated with ^{3}H-PGG-Glucan (1 μg/ml) with or without unlabeled PGG-Glucan, laminarin, or dextran at the either 37°C or 4°c as described in Section 2. After 1 h, wells were rinsed and radioactivity removed and counted as described. Data points represent the mean of triplicate samples ± standard deviation in units of ng ^{3}H-PGG-Glucan bound/well.

was further explored by evaluating the temperature sensitivity and polysaccharide specificity of ^{3}H-PGG-Glucan binding to purified lactosylceramide. As previously reported (Zimmerman et al., 1998) ^{3}H-PGG-Glucan binding to purified lactosylceramide was found to be temperature dependent, and not inhibited by either laminarin or dextran (Fig. 7). ^{3}H-PGG-Glucan binding to HLM showed these same characteristics (see above). The ability of a variety of other polysaccharides to inhibit ^{3}H-PGG-Glucan binding to HLM and lactosylceramide was compared. I.C.$_{50}$ data were derived from competition curves (not shown) similar to those in Fig. 6. PGG-Glucan, Sonifilan, and scleroglucan inhibited ^{3}H-PGG-Glucan binding to HLM versus lactoscerylceramide with the same relative potency (I.C.$_{50} = 7$ versus 9, 3 versus 8, and 8 versus 5 μg/ml, respectively), while LMW-glucan, laminarin, Lentinan, glycogen, dextran, and mannan were ineffective as inhibitors (I.C.$_{50} > 100$ μg/ml). There is thus an almost exact correspondence in polysaccharide specificity between HLM binding and lactosylceramide binding. These data confirm and extend previous observations on the polysaccharide binding selectivity of lactosylceramide (Zimmerman et al., 1998). Taken together, these data support the observation that the ^{3}H-PGG-Glucan receptor on human PMN is lactosylceramide.

3.7. Distribution of ^{3}H-PGG-Glucan binding to membranes derived from various cell types

The expression of ^{3}H-PGG-Glucan (1 μg/ml) binding activity was determined in membranes derived from a number of different cell types, both primary and cell line, of murine and human origin (Table 3). Because HLM consists of membranes from several different cell types, we used density gradient purified PMN and mononuclear leukocytes to determine the distribution of binding activity in these cells. In human blood, membranes derived from purified human PMN had the highest density of binding sites (12.8 ng/mg protein). The mononuclear cell population, containing both monocytes and lymphocytes, exhibited much lower binding activity (1.2 ng/mg protein). The human myeloid cell lines U937, KG-1, HL-60, bound ^{3}H-PGG-Glucan minimally or not at all, while THP-1 membranes yielded small (0.6 ng/mg protein) but consistent binding activity. Four different murine monocytic cell lines

Table 3
^{3}H-PGG-Glucan binding to membranes from various cell types

Cell type	Description	^{3}H-PGG-Glucan bound[a]			CR3[d] (MCF)
		Total	Non-specific[b]	Specific (%)[c]	
PMN[e]	human polymorphonuclear leukocyte	16.0 ± 0.54	3.2 ± 0.30	12.8 (80)	–
mononuclear cells[f]	human monocytes and lymphocytes	2.8 ± 0.68	1.6 ± 0.52	1.2 (43)	–
U937	human monocytic cell line	0.3 ± 0.17	0.3 ± 0.02	0.0 (0)	–
HL-60	human monocytic cell line	1.8 ± 0.11	1.6 ± 0.02	0.2 (9)	–
KG-1	human myeloid cell line	1.1 ± 0.75	1.0 ± 0.08	0.1 (4)	–
THP-1	human monocytic cell line	2.6 ± 0.34	2.0 ± 0.01	0.6 (24)	–
J774.1	murine monocytic cell line	2.7 ± 0.18	0.7 ± 0.02	2.0 (74)	531
BMC2.3	murine monocytic cell line	2.4 ± 0.19	0.6 ± 0.07	1.8 (74)	542
RAW264.7	murine monocytic cell line	1.7 ± 0.14	0.4 ± 0.06	1.3 (75)	605
P338D (1)	murine monocytic cell line	4.6 ± 0.53	1.9 ± 0.12	2.7 (58)	26
DO11	murine T cell line	0.7 ± 0.30	0.7 ± 0.05	0.0 (0)	–
LB27.4	murine B cell line	1.1 ± 0.23	1.2 ± 0.01	0.0 (0)	–

[a] Expressed as mean ± standard deviation in ng/mg protein.
[b] Defined as ^{3}H-PGG-Glucan bound in the presence of > 100-fold excess unlabeled PGG-Glucan.
[c] Determined by (Total) − (Non-Specific), number in parenthesis refers to % Total binding.
[d] Quantitation of CR3 was done by flow cytometry using PE-labeled anti-CD11b. Data are expressed as MCF in arbitrary fluorescence units. Background fluorescence for isotype controls were subtracted. '–', indicates not tested.
[e] > 95% PMNs by morphology.
[f] 40–50% monocytes by morphology.

(BMC2.3, RAW264.7, P388D(1), and J774.1) were found to have binding activity roughly comparable to human mononuclear cells (1.3–2.7 ng/mg protein), whereas murine B-cell and T-cell lines showed no binding activity. Analysis of CR3 expression on the murine monocytic cell lines (J774.1, BMC2.3, RAW264.7, and P388D(1)) showed that CR3 expression did not correlate with ^{3}H-PGG-Glucan binding activity, particularly in the P388D(1) cell line (Table 4). This is consistent with the observation that ant-CR3 did not inhibit ^{3}H-PGG-Glucan binding (see above).

4. Discussion

We have investigated the immunomodulatory activity of PGG-Glucan in human leukocytes in vitro. In this report we have shown that PGG-Glucan enhanced human leukocyte anti-microbial defense responses and increased their ability to kill microorganisms. Pretreatment of leukocytes with PGG-Glucan, followed by either soluble or particulate activators, significantly increased the production of reactive oxygen intermediates (ROI). This was paralleled by a significant increase in the capacity of leukocytes to kill *S. aureus*. PGG-Glucan alone did not induce the production of ROI by human leukocytes. Thus, PGG-Glucan primed cells for an enhanced functional response to secondary stimuli, rather than directly activating them. Importantly, no evidence for PGG-Glucan-induced release of pro-inflammatory cytokines from human blood leukocytes was observed, nor was there increased cytokine production observed when PGG-Glucan was tested in an in vivo infection model (Liang et al., 1998). This lack of direct cytokine induction appears to be unusual among immunomodulators, and may in part be explained by the novel receptor and signaling pathway(s) used by PGG-Glucan.

We investigated whether the signal transduction pathway stimulated by PGG-Glucan activated NF-κB, a transcription factor known to be involved in immune responses (Baeuerle and Henkel, 1994; Siebenlist et al., 1994; Baldwin, 1996), including PMN responses to inflammatory stimuli (McDonald et al., 1997). PGG-Glucan activated an NF-κB-like DNA binding protein in human PMNs. The activation was specific and dose-dependent. Although the PGG-Glucan-induced DNA-protein complex had a similar mobility to that produced by LPS stimulation, the DNA binding protein was composed of p65 (Rel A) and an unknown subunit. A similar result has been obtained with PGG-Glucan stimulation of a murine monocytic cell line (Adams et al., 1997). Investigations are currently underway to identify the unknown subunit. Preliminary results indicate that, following PGG-Glucan stimulation, the NF-κB-like DNA binding protein extracted from PMN nuclei is composed of p65(Rel A) and a C/EBP-β-like molecule (unpublished observations), in concurrence with results obtained in a murine monocytic cell line (Adams et al., J. Leuk. Biol., submitted). Interaction of Rel family proteins with members of the C/EBP family of transcription factors has been previously reported (Stein et al., 1993; Ruocco et al., 1996; Kalkhoven et al., 1996). The exact make-up of NF-κB dimers has profound consequences on DNA binding specificity and transcriptional activity (Kunsch et al., 1992; Baeuerle and Henkel, 1994; Ziegler-Heitbrock et al., 1995; Baldwin, 1996), and may in part explain the lack of cytokine induction by PGG-Glucan (Table 1). However, not until a PGG-Glucan-regulated gene is identified and its promoter studied will the role of this transcription factor in the biological response to PGG-Glucan become clear.

The binding of ^{3}H-PGG-Glucan to HLM was specific, high affinity, and was time, temperature, and concentration dependent. Saturation was observed at approximately 25 nM ^{3}H-PGG-Glucan, and the apparent dissociation constant was 6 nM. Most of the binding detected in membranes from white blood cell preparations was due to PMNs, which had about 10-fold higher binding activity per mg protein than mononuclear cell membranes. Unfortunately, ^{3}H-PGG-Glucan binding to intact PMNs was problematic and could not be routinely measured. The reasons for this are currently unclear, but may in part be due to pinocytotic activity present at the temperature (37°C) required for binding activity. Similar difficulties with ligand binding to chemokine receptors on intact cells has been reported (Boring et al., 1996). However, results presented here clearly show that PGG-Glucan induced biological responses in intact cells. The coincidence of the dose–response curves

with the binding isotherm, as well as polysaccharide specificity (discussed below), argues in favor of the signal transduction events and subsequent biological responses described here being mediated by the ^{3}H-PGG-Glucan binding entity.

^{3}H-PGG-Glucan binding to HLM was specific for β-(1,3)-glucan. Neither dextran (α-1,6-linked glucan), glycogen (α-1,4-linked glucan), or mannan (predominately α-1,2, α-1,3, and α-1,6-linked mannose polymer) inhibited binding. Moreover, other more subtle structural characteristics also appear to be important since not all β-(1,3)-glucans tested were capable of inhibiting ^{3}H-PGG-Glucan binding. The contribution of structural differences between the various β-glucans (main chain length, branch frequency, branch length, and aggregation number) to binding selectivity is not yet clear. More rigorous structure-activity studies are needed to define the binding epitope(s) recognized by the PGG-Glucan receptor in order to understand this selectivity among seemingly similar β-(1,3)-glucans. The implication from this structural selectivity is that different biologically active β-(1,3)-glucans may interact with different receptors.

Several lines of evidence indicate that the binding site which recognizes PGG-Glucan is distinct from those previously described for other soluble or particulate β-glucans. We base this conclusion on a) differences in polysaccharide specificity (Goldman, 1988a; Janusz et al., 1989; Czop et al., 1990; Engstad and Robertsen, 1994; Konopski et al., 1994; Thornton et al., 1996) b) differences in cell type distribution (Goldman, 1988a,b; Czop et al., 1990; Müller et al., 1996), and c) no inhibition of ^{3}H-PGG-Glucan binding by antibodies directed against other β-glucan receptors (Ross et al., 1987; Czop et al., 1990; Thornton et al., 1996). While this latter observation could be interpreted to mean that PGG-Glucan interacts at a different epitope from the anti-receptor antibodies, additional evidence that PGG-Glucan does not bind to CR3 is the lack of correlation between ^{3}H-PGG-Glucan binding and CR3 expression on P388D(1) cells, as well as on human PMNs and monocytes, which show a 10-fold difference in ^{3}H-PGG-Glucan binding but express comparable amounts of CR3 (Repo et al., 1993; Repo et al., 1995; unpublished observations). Finally, Michalek et al. (1998) have recently shown in a rat alveolar macrophage cell line that immobilized PGG-Glucan can elicit functional responses in the absence of detectable CR3. Curiously, CR3 has been shown to interact with iC3b in a temperature-sensitive fashion (Van Strijp et al., 1993; Cai and Wight, 1995), much like that shown here for PGG-Glucan and lactosylceramide, except in the latter case binding activity is lost at a higher temperature. However, these authors concluded that CR3 does not bind β-glucan.

Our laboratory has recently identified the ^{3}H-PGG-Glucan binding moiety on human PMNs as the glycosphingolipid lactosylceramide, and elucidated some of the structural requirements for binding activity (Zimmerman et al., 1998). The data in the present report complement these observations, and show that a) a monoclonal antibody to lactosylceramide can inhibit signal transduction responses to PGG-Glucan in intact PMNs, and b) ^{3}H-PGG-Glucan binding to purified lactosylceramide shows the same temperature sensitivity, relative affinity, and polysaccharide specificity as it does to HLM. The case for lactosylceramide as a plasma membrane receptor involved in signal transduction events has support in the literature. Lactosylceramide is present in large quantities in PMN, a portion of which is located on the external leaflet of the plasma membrane, and has been given the designation CDw17 (Symington et al., 1984, 1987; Symington, 1989; Kniep and Skubitz, 1998). Glycosphingolipids as mediators of cell–cell interactions and signal transduction events through carbohydrate–carbohydrate interactions have numerous literature precedents (Song et al., 1998; Iwabuchi et al., 1998; for reviews see Hakomori, 1993; Riboni et al., 1997). Monoclonal antibodies to lactosylceramide have been shown to partially degranulate PMN and induce an oxidative burst (Lund-Johansen et al., 1992), and when incubated with smooth muscle cells in vitro lactosylceramide has been shown to induce signaling through MAP kinase pathways (Bhunia et al., 1996). Furthermore, lactosylceramide has been shown to be a cellular binding site for both fungal and bacterial microorganisms (Stromberg et al., 1988; Jimenez-Lucho et al., 1990; Sandberg et al., 1995; Angstrom et al., 1998). Our data, therefore, suggest that PMN lactosylceramide can interact with microorganisms through β-glucan structures located in their cell walls, inducing a primed state when the β-glucan is in a soluble form.

We conclude that PGG-Glucan augments the ability of human leukocytes to respond to secondary stimuli, resulting in enhanced microbicidal function. The concordance of the biological dose–responses with the ^{3}H-PGG-Glucan binding isotherm, the inhibition of cellular signaling by an anti-lactosylceramide monoclonal antibody, and the striking similarity of the ^{3}H-PGG-Glucan binding characteristics between HLM and lactosylceramide strongly suggest that the functional response to PGG-Glucan in human PMN is mediated through interaction with cell surface lactosylceramide. This conclusion therefore suggests the existence of a novel receptor capable of recognizing β-glucans.

The concept of multiple β-glucan receptors is supported not only by the data presented here and cited above, but by the range of biological responses elicited by various particulate and soluble β-glucan preparations (for reviews, see Stone and Clarke, 1992; Bohn and BeMiller, 1995; Bleicher and Mackin, 1995). The nature of the biological responses elicited by a given β-glucan preparation may depend upon which and how many receptor(s) it is able to engage. This idea is further supported by reports that single helical β-(1,3)-glucan chains induce different biological responses than triple helical β-(1,3)-glucan aggregates (Tagteishi et al., 1997; Hashimoto et al., 1997). Additionally, the differences in elicited responses to soluble versus particulate β-glucans may relate to their relative affinity/avidity for their receptor, as has been shown for Fcε and T cell receptors (Torigoe et al., 1998; Kersh et al., 1998). The strength of typically weak carbohydrate–glycosphingolipid interactions is critically dependent upon multivalency and/or high density recognition sites (Bovin, 1997); a polysaccharide with a repeat structure such as β-(1,3)-glucan and clusters or 'islands' of membrane glycosphingolipids (Hakomori, 1993) such as lactosylceramide provide an interacting carbohydrate–glycosphingolipid pair which fit this model, and suggests one possible mechanism for selectivity. PGG-Glucan has been shown to enhance host defense mechanisms in animal infection models (Onderdonk et al., 1992; Stashenko et al., 1995; Kernodle et al., 1998) as well as in humans (Babineau et al., 1994a,b) without directly eliciting an oxidative burst or inflammatory cytokines (Liang et al., 1998; Patchen et al., 1998; present results). These observations are consistent with the idea that PGG-Glucan does not directly activate, but primes leukocytes for response to secondary signals (Poutsiaka et al., 1993; Mackin et al., 1994). Similar conclusions on priming of human leukocyte phagocytic and tumoricidal activities by other β-glucan preparations have been reported (Vetvicka et al., 1996, 1997 and references therein).

Acknowledgements

The authors would like to thank Ms. P. Fish for providing the glucanase-treated PGG-Glucan, Ms. K. Lemerise and Y. Gu for the flow cytometric analysis of CR3 and lactosylceramide, respectively, Dr. J. Park for determination of polysaccharide molecular weight, and Drs. M. Patchen, S. Jamas, and J. Smart for review of the manuscript.

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