Activation of rat macrophages by Betafectin® PGG-glucan requires cross-linking of membrane receptors distinct from complement receptor three (CR3)

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Abstract: PGG-glucan (Betafectin®) is a soluble, highly purified yeast (1,3)-β-glucan with broad anti-infective and immunomodulatory activities. These studies evaluated the ability of PGG-glucan to directly elicit O_2^- and tumor necrosis factor α (TNF-α) production by rat leukocytes in vitro. Particulate β-glucan stimulated O_2^- production by the rat NR8383 alveolar macrophage cell line and resident rat peritoneal macrophages, but soluble PGG-glucan did not. In contrast, presentation of PGG-glucan to cells after covalent immobilization to a plastic surface caused a direct stimulation of O_2^- and TNF-α production. The O_2^- response of rat leukocytes to immobilized PGG-glucan was inhibited by soluble PGG-glucan, indicating that cellular responses to both immobilized and soluble PGG-glucan occur via common cell surface receptors. Because complement receptor type three (CR3) has been proposed as a β-glucan receptor on human leukocytes, NR8383 cells were evaluated for the presence of CR3. Indirect immunofluorescence and flow cytometric analysis showed that despite being responsive to both particulate and immobilized β-glucans, NR8383 cells expressed no detectable CR3. These results indicate that the β-glucan receptors on NR8383 cells are not CR3 and suggest that physical presentation plays an important role in inducing pro-inflammatory leukocyte responses to PGG-glucan. *J. Leukoc. Biol.* 64: 337–344; 1998.

Key Words: *β-glucan · NR8383 cell line · respiratory burst · tumor necrosis factor α · CR3 receptor*

INTRODUCTION

(1,3)-β-Glucans are major structural components of fungal cell walls, consisting chemically of (1,3)-linked β-D-glucopyranosyl residues with (1,6)-linked β-D-glucopyranosyl side chains of varying length and distribution frequency [1, 2]. β-Glucans nonspecifically enhance the innate immune system and are pharmacologically classified as biological response modifiers [3–6]. In numerous animal models, (1,3)-β-glucans were shown to have broad anti-infective and anti-tumor activities, with the predominant immunopharmacological effects being the activation of neutrophils and macrophages (i.e., leukocytes) and the stimulation of pro-inflammatory mediators (i.e., cytokines, eicosanoids, and enzymes) [4–20].

PGG-glucan (Betafectin®) is a highly purified, aqueous soluble, pharmaceutical grade (1,3)-β-glucan manufactured from *Saccharomyces cerevisiae*. PGG-glucan has broad anti-infective activities in a variety of animal models [21–25] and has been shown to reduce the incidence of post-surgical infections in upper gastrointestinal surgery patients [26, 27, and E. P. Dellinger et al., unpublished results]. In contrast to most other β-glucan preparations, PGG-glucan does not directly stimulate leukocyte microbicidal functions in vitro, but rather primes or enhances their responsiveness to bacteria and other stimuli [3, 29]. PGG-glucan also does not directly stimulate or enhance the production of pro-inflammatory cytokines either in vitro or in vivo [3, 24, 29, 30, 31].

The biological activity profile of PGG-glucan clearly distinguishes it from other β-glucans, specifically particulate β-glucans, which directly activate leukocytes and stimulate pro-inflammatory cytokine production. One possible explanation for the marked differences in leukocyte activation elicited by particulate β-glucans relative to PGG-glucan is that, because of the soluble nature of PGG-glucan, it may be less efficient at cross-linking membrane β-glucan receptors and therefore may not fully activate leukocyte functions. To investigate this possibility, we constructed an experimental system that facilitated a direct comparison of leukocyte responses to a particulate β-glucan, soluble PGG-glucan, and PGG-glucan covalently attached to wells of a plastic microtiter plate. In these experiments, both the particulate β-glucan and PGG-glucan were purified from a single *Saccharomyces cerevisiae* strain to eliminate potential variation due to β-glucan sources. Our results demonstrate that immobilized PGG-glucan elicits macrophage functional responses identical to those seen with particulate β-glucan, whereas soluble PGG-glucan had no effect. These results suggest that the inability of soluble

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Abbreviations: TNF-α, tumor necrosis factor α; DPBS, Dulbecco's phosphate-buffered saline; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin; HSA, human serum albumin; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; PMA, phorbol myristate acetate.

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PGG-glucan to directly activate leukocyte microbicidal and cytokine responses is due to an inability to cross-link membrane β-glucan receptors.

MATERIALS AND METHODS

Reagents

Endotoxin-free whole glucan particles (WGPs) and PGG-glucan (Betafectin®; Alpha-Beta Technology Inc., Worcester, MA) were prepared from the cell walls of a proprietary, non-recombinant strain of *Saccharomyces cerevisiae* by a series of alkaline, acid, and organic extractions [32, 33]. The major component of WGPs are glucans with a (1,3)-linked β-D-glucopyranosyl backbone. WGPs lack the mannan, nucleic acid, and protein components found in zymosan [32]. PGG-glucan is a soluble β-glucan prepared from WGPs by a proprietary solubilization process and consists of a (1,3)-linked β-D-glucopyranosyl backbone with periodic (1,6)-linked β-D-glucopyranosyl side chains that are also composed of (1,3)-linked β-D-glucopyranosyl residues [32, 33]. PGG-glucan is highly soluble at physiological pH and has an apparent molecular mass of 150,000 ± 20,000 daltons as determined by multi-angle laser light scattering (MALLS) [34 and J. T. Park, Y. A. Guo, A. S. Magee, unpublished results]. The concentration of PGG-glucan was determined by the phenol/sulfuric acid assay [36]. The PGG-glucan preparation used herein contained <0.02% protein, <0.01% mannan, and 1% glucosamine.

Radiolabeled PGG-glucan was prepared as follows. Unlabeled PGG-glucan was oxidized with a 20-fold molar excess of sodium periodate in sterile, pyrogen-free (SPF) water (pH 7.4; McGaw Inc., Irvine, CA) for 72 h in the dark at room temperature. The periodate was then quenched with a 10–20 molar excess of glycerol. The oxidized PGG-glucan was dialyzed against SPF water and reductively radiolabeled with 100 mCi of [^{3}H]$NaBH_4$ at New England Nuclear (Boston, MA). The reaction mixture was then exhaustively reduced with a 20-fold excess of unlabeled $NaBH_4$ for 24 h. Radiolabeled low-molecular-weight PGG-glucan degradation products were removed by dialysis (10 k cutoff) and ultrafiltration with a Centriprep-30 Concentrator (Amicon Inc., Beverly, MA). ^{3}H-labeled and unlabeled PGG-glucan were indistinguishable by gel filtration chromatography using Toyopearl® HW-55F resin. The concentration of several radiolabeled PGG-glucan preparations was determined by the anthrone/sulfuric acid method [37] and the specific activity ranged from 1.0 to 2.4×10^7 dpm/μg PGG-glucan. In control experiments, unlabeled oxidized and reduced PGG-glucan and native PGG-glucan were identical in their ability to stimulate an oxidative burst in the NR8383 cell line when immobilized onto Costar Universal Binding plates (data not shown).

Dulbecco's phosphate-buffered saline (DPBS; without $CaCl_2$ and $MgCl_2$), Hanks' balanced salt solution (HBSS; with and without $CaCl_2$ and $MgSO_4$), human serum albumin (HSA), bovine serum albumin (BSA), bovine heart cytochrome *c*, and purified rat IgG were purchased from Sigma (St. Louis, MO). DPBS and HBSS were dissolved in sterile pathogen-free water and stored at 4°C. HSA and BSA were stored at 4°C as 5% (w/v) stock solutions in HBSS (with Ca^{2+} and Mg^{2+}). Cytochrome *c* was stored at −20°C as a 500 μM stock solution in HBSS (with Ca^{2+} and Mg^{2+}). Dextran was purchased from American Polymer Standards (Mentor, OH) and resuspended in saline at 1 mg/mL. Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (La Jolla, CA) and stored at −20°C as a 1 mg/mL stock solution in dimethyl sulfoxide. MRC OX-42 [38], ED7 [39], and ED8 [39] anti-CR3 mouse monoclonal antibodies were purchased from Serotec USA (Raleigh, NC). Fluorescein-conjugated goat anti-mouse IgG (no cross reaction with rat IgG) was purchased from Organon Teknika Corp. (Durham, NC).

Cells

Resident peritoneal macrophages were isolated from 350- to 500-g male Wistar rats (Charles River Labs, Wilmington, MA) by lavaging the peritoneal cavity with 60 mL of cold HBSS without Ca^{2+} and Mg^{2+}. Contaminating red blood cells were hypotonically lysed by a 10-s exposure to deionized water and then the peritoneal macrophages were resuspended in HBSS with Ca^{2+} and Mg^{2+} and HSA.

The NR8383 rat alveolar macrophage cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). This cell line was initiated from a normal adult male Sprague-Dawley rat and NR8383 cells exhibit the phenotype of mature macrophages, including phagocytic capabilities, nonspecific esterase activity, Fc receptor expression, oxidative burst potential, capacity to secrete interleukin-1 (IL-1), TNF-α, and IL-6, and a replicative response to exogenous growth factors [40–42]. NR8383 cells were passaged in vitro in F12 Nutrient Mixture (GIBCO-BRL, Grand Island, NY) supplemented with 15% heat-inactivated fetal calf serum (Summit, Ft. Collins, CO), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Fungizone® at 37°C in a humidified incubator with 5% (v/v) CO_2.

Immobilization of PGG-glucan

Varying concentrations of unlabeled PGG-glucan (0.1 mL) diluted in DPBS (pH 7.2) were incubated in Costar® Universal Binding plates (Corning Inc., Corning, NY) for 1 h at 37°C in a humidified incubator with 5% (v/v) CO_2. The liquid contents of the wells were removed by inversion and blotting onto paper towels. The plates were irradiated for 5 min in a UV Stratalinker 2400 (Stratagene, La Jolla, CA) equipped with a 254-nm light source and then used immediately. UV irradiation activates the surface of the plates facilitating the formation of a covalent linkage with passively adsorbed proteins or carbohydrates [43, 44]. In preliminary experiments, ^{3}H-PGG-glucan was used to assess the extent of PGG-glucan binding to the plates. ^{3}H-PGG-glucan was incubated in the plates as described above. The binding of the ^{3}H-PGG-glucan in the presence or absence of UV cross-linking was determined as the radioactivity remaining after washing with PBS containing 0.05% Tween 20 and 0.01% NaN_3. The amount of ^{3}H-PGG-glucan cross-linked to the plates was determined by releasing the covalently bound ^{3}H-PGG-glucan by hydrolysis (2 h incubation at 40–41°C) with 1 N H_2SO_4. The released radioactivity was measured using a liquid scintillation counter; no quenching by the H_2SO_4 was detected.

Measurement of respiratory burst activity

Macrophage production of superoxide anion (O_2^-) was measured by the reduction of cytochrome *c* in a microplate format [45]. Rat peritoneal macrophages (3.2×10^5 cells/well) or NR8383 cells (2.0×10^5 cells/well) were incubated for 90 min at 37°C with various stimuli in HBSS (0.2 mL) containing Ca^{2+}, Mg^{2+}, 100 μM cytochrome *c*, and 0.25% HSA. The absorbance at 550 nm was measured every 30 s in a Thermomax microplate reader (Molecular Devices, Palo Alto, CA) equipped with a 10-nm bandwidth filter. The change in absorbance during the 90-min incubation was determined and this value was converted into nanomoles of O_2^- by comparison with a standard curve of dithionite-reduced cytochrome *c* [45].

Measurement of cytokine secretion

Rat peritoneal macrophages (3.1×10^5 cells/well) or NR8383 cells (2.0×10^5 cells/well) were incubated in duplicate microcultures with the indicated stimuli for 24 h at 37°C in a humidified incubator with 10% (v/v) CO_2. The culture medium (0.2 mL/well) was Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 5% heat-inactivated fetal calf serum (Sigma), 4 mM L-glutamine (GIBCO-BRL), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Fungizone. Culture supernatants were tested for TNF-α by enzyme-linked immunosorbent assay (ELISA; Genzyme Corp., Cambridge, MA).

Immunofluorescence

Cells were prepared for indirect immunofluorescence using unlabeled murine mAbs specific for rat CR3 (MRC OX-42, ED7, or ED8) [38, 39] in combination with fluorescein-conjugated goat anti-mouse IgG (non-cross-reactive with rat IgG). Specifically, rat peritoneal macrophages (2.0×10^6) or NR8383 cells (1.0×10^6) were first incubated on ice for 15 min with rat IgG (30 μg) in PBS containing 0.1% BSA (BSA/PBS; 50 μL) to block surface Fc receptors. After the 15-min incubation, the cells were incubated for an additional 30 min on ice either with or without MRC OX-42, ED7, or ED8 (5 μg) and then washed twice with 0.1% BSA/PBS. The cells were then incubated for 30 min on ice in 0.1% BSA/PBS (250 μL) containing fluorescein-conjugated goat anti-mouse IgG (30 μg). After the 30-min incubation, the cells were washed three times with ice-cold 0.1% BSA/PBS and then fixed with 1% paraformaldehyde. Fluorescence of 5000 cells was quantitated on a FACSCalibur (Becton Dickinson, Mountain View, CA) flow cytometer.

TABLE 1. Whole Glucan Particles Induce A Respiratory Burst In Rat Macrophages

Stimulus	Superoxide produced[a] (nmol/10^6 cells) Rat peritoneal macrophages	NR8383 cells
Whole Glucan Particle Concentration		
625 µg/mL	8.3 ± 2.0[b]	NT[c]
200 µg/mL	NT	43.4 ± 0.8
100 µg/mL	NT	36.9 ± 0.8
50 µg/mL	NT	27.0 ± 0.3
PMA[d]	35.1 ± 0.8	20.5 ± 1.0
Buffer	0 ± 0	0.2 ± 0.1

[a] Cells were incubated with the indicated stimulus and the production of superoxide anion measured by the reduction of cytochrome *c* as described in Materials and Methods.

[b] Mean ± standard deviation of triplicate determinations.

[c] Not tested.

[d] The PMA concentrations for peritoneal macrophages and NR8383 cells were 5 ng/mL and 25 ng/mL, respectively.

RESULTS

Particulate (1,3)-β-glucan stimulates a respiratory burst in rat macrophages but soluble PGG-glucan does not

Numerous previous studies have demonstrated that particulate and/or poorly soluble (1,3)-β-glucans directly stimulate leukocyte O_2^- production in vitro [4–6, 17, 18, 46–48]. We first determined whether WGPs purified from *S. cerevisiae* stimulated O_2^- production by NR8383 cells and rat peritoneal macrophages. In a dose-dependent manner, WGPs stimulated NR8383 macrophages to produce O_2^- (**Table 1**). Resident rat peritoneal macrophages also produced O_2^- in response to WGP stimulation, but at a much higher concentration (Table 1). In studies similar to those performed with WGPs, NR8383 cells and rat peritoneal macrophages were co-incubated with various concentrations of soluble PGG-glucan and O_2^- production measured. As shown in **Table 2**, soluble PGG-glucan did not induce the production of O_2^- by NR8383 cells. Even with as much as 7 mg/mL of PGG-glucan in solution (data not shown), no stimulation of O_2^- production was observed in these cells.

TABLE 2. PGG-Glucan In Solution Does Not Stimulate A Respiratory Burst In Rat Macrophages

Stimulus	Concentration	Superoxide produced[a] (nmoles/10^6 cells) NR8383 cells
PGG-Glucan	20 µg/mL	17.8 ± 2.2
	5 µg/mL	15.3 ± 1.3
	1.25 µg/mL	18.0 ± 0.5
	0.31 µg/mL	14.8 ± 2.2
	0.078 µg/mL	17.2 ± 0.8
PMA	50 ng/mL	52.6 ± 0.7
Buffer		17.9 ± 3.7

[a] Cells were incubated with the indicated stimulus and the production of superoxide anion measured by the reduction of cytochrome *c* as described in Materials and Methods.

[b] Mean ± standard deviation of triplicate determinations.

Rat peritoneal macrophages likewise failed to produce O_2^- in response to soluble PGG-glucan (data not shown).

Immobilization of PGG-glucan to a plastic surface

Cross-linking membrane surface receptors by a specific ligand is a common paradigm for cell activation and is thought to mimic the receptor/ligand interaction that occurs in vivo to trigger signal transduction [43, 49, 50]. Particulate β-glucans are likely to be more efficient at receptor cross-linking due to an increased density of ligands on the particle surface. To evaluate potential involvement of ligand presentation in mediating responses induced by β-glucans, we constructed a system whereby we covalently linked soluble PGG-glucan to a plastic surface so that the biological activity elicited by immobilized PGG-glucan could be directly compared with that elicited by soluble PGG-glucan or WGPs. In preliminary experiments, the ability to covalently link PGG-glucan to the surface of Costar Universal Binding plates was determined by UV cross-linking radiolabeled PGG-glucan to the plates and determining binding efficiency by measuring the amount of radioactivity released by acid hydrolysis. A concentration-dependent increase in the amount of bound ^{3}H-PGG-glucan was obtained when the plates were UV-irradiated after incubation with increasing concentrations of ^{3}H-PGG-glucan (**Fig. 1**). Without UV-irradiation, washing the plate wells with 0.05% Tween 20 decreased the amount of immobilized ^{3}H-PGG-glucan by 85–90% (Fig. 1). These results demonstrate that PGG-glucan can be immobilized onto the surface of Costar Universal Binding plates via a covalent association.

Immobilized PGG-glucan stimulates O_2^- and TNF-α production in rat macrophages

To examine the importance of β-glucan presentation in eliciting O_2^- production by rat peritoneal macrophages, cells were incubated with immobilized PGG-glucan in the presence of excess cytochrome *c*. Immobilized PGG-glucan induced a dose-dependent production of O_2^- by the peritoneal macrophages (**Fig. 2**). It is interesting to note that a slight increase in O_2^- production was also obtained when peritoneal macrophages were incubated in wells that had not been UV-irradiated after incubation with PGG-glucan, suggesting that some PGG-glucan was non-covalently associated with the plastic surface and could also stimulate slight respiratory burst activity (Fig. 2). The above results demonstrate that PGG-glucan presentation has a dramatic effect on its ability to directly stimulate a respiratory burst in rat peritoneal macrophages.

Effects of PGG-glucan presentation on respiratory burst activity were also investigated using the NR8383 rat alveolar macrophage cell line. As with rat peritoneal macrophages, immobilized PGG-glucan also induced a dose-dependent production of O_2^- in NR8383 cells (**Fig. 3**). It is interesting to note that NR8383 cells appeared more sensitive, responding to lower concentrations of PGG-glucan and producing higher O_2^- levels compared to rat peritoneal macrophages (Fig. 3). The PGG-glucan coating concentration required for half-maximal stimulation of O_2^- production in NR8383 cells was approxi-

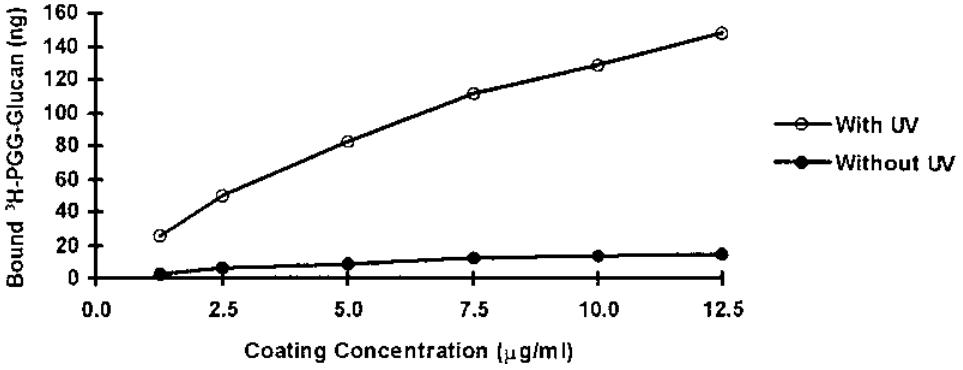

Fig. 1. Binding of ^{3}H-PGG-glucan to a Costar Universal Binding plate. ^{3}H-PGG-glucan at the indicated concentrations was incubated for 1 h at 37°C in a Costar Universal Binding plate. The ^{3}H-PGG-glucan was then removed and a portion of the plate was either UV-irradiated (open circles) or covered (filled circles) to prevent exposure as described in Materials and Methods. After irradiation, non-covalently associated ^{3}H-PGG-glucan was removed by washing with PBS containing 0.05% Tween 20 and 0.01% NaN_3. Covalently bound ^{3}H-PGG-glucan was released by hydrolysis with H_2SO_4 and quantitated as described in Materials and Methods. Data are expressed as mean values of duplicate determinations. Only 10–20% of the total amount of ^{3}H-PGG-glucan added to the wells was bound.

mately 50-fold less than that required to stimulate O_2^- production in peritoneal macrophages.

Because TNF-α is one of many cytokines and growth factors secreted by activated macrophages [51], we also determined whether immobilized PGG-glucan could stimulate the release of TNF-α by rat macrophages. Cells were incubated for 24 h with immobilized PGG-glucan or soluble PGG-glucan and the culture supernatants analyzed for TNF-α by ELISA. In a dose-dependent manner, immobilized PGG-glucan stimulated the secretion of TNF-α by both peritoneal macrophages and NR8383 cells (**Table 3**). In similar experiments, soluble PGG-glucan at concentrations up to 100 μg/mL had no effect on TNF-α production by either cell type (data not shown).

Soluble PGG-glucan inhibits macrophage responses to immobilized PGG-glucan

One possible explanation for the different responses to soluble versus immobilized PGG-glucan is that the leukocyte responses may be mediated via different cell surface receptor systems. To test this possibility, the ability of soluble PGG-glucan to compete or inhibit NR8383 responsiveness to immobilized PGG-glucan was determined. The O_2^- response of NR8383 cells to immobilized PGG-glucan was inhibited in a dose-dependent manner by soluble PGG-glucan, whereas dextran [a (1,6)-linked α-D-glucopyranosyl molecule] had no effect on the O_2^- response of NR8383 cells to immobilized PGG-glucan (**Table 4**). The ability to inhibit activation of NR8383 cells with soluble PGG-glucan and not dextran suggests that the inhibition was specific for a (1,3)-linked β-D-glucopyranosyl molecule. In addition, coincubation of NR8383 cells with PGG-glucan in solution did not inhibit the production of O_2^- when the cells were stimulated with phorbol 12-myristate 13-acetate (PMA), suggesting that PGG-glucan in solution does not nonspecifically down-regulate leukocyte respiratory burst activity (Table 4). The above competition results suggest that soluble PGG-glucan binds to the same β-glucan receptors as the immobilized PGG-glucan.

The PGG-glucan receptor is not CR3

Ross et al. [46, 48, 52, 53] have recently provided evidence that the human CR3 (CD11b/CD18) receptor contains a binding site that interacts with both soluble and particulate (1,3)-β-glucans. We next examined whether PGG-glucan may be mediating its biological effects via the rat CR3 receptor. Using MRC OX-42

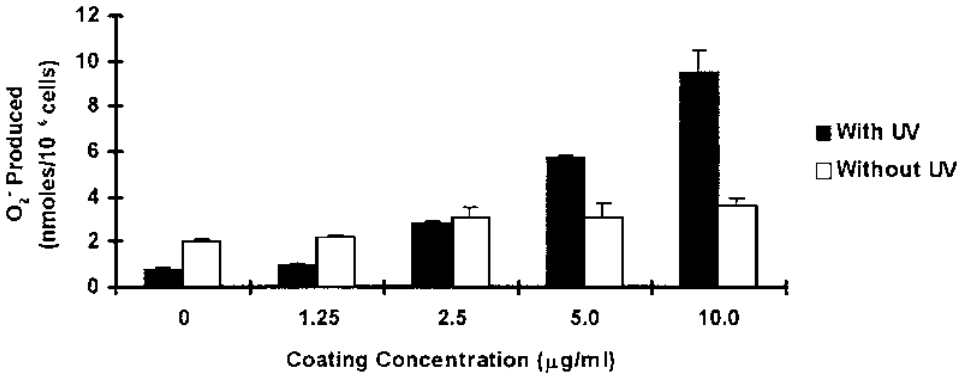

Fig. 2. Covalent binding of PGG-glucan is required for efficient stimulation of O_2^- by rat peritoneal macrophages. PGG-glucan at the indicated concentrations was incubated in a Costar Universal Binding plate for 1 h at 37°C and then the wells were either exposed (filled bars) to UV-irradiation or covered (open bars) during UV irradiation to prevent exposure as described in Materials and Methods. Rat peritoneal macrophages were then incubated in the plate for 90 min at 37°C. The production of superoxide anion was measured by the reduction of cytochrome *c* as described in Materials and Methods. All values are the mean ± standard deviation of triplicate determinations.

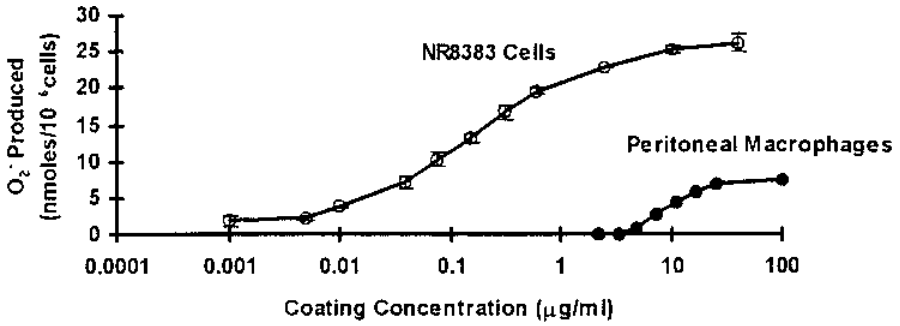

Fig. 3. Immobilized PGG-glucan stimulates a respiratory burst in NR8383 rat alveolar macrophages and rat peritoneal macrophages. PGG-glucan at the indicated concentrations was incubated in a Costar Universal Binding plate for 1 h at 37°C and immobilized to the plate as described in Materials and Methods. NR8383 cells (open circles) or peritoneal macrophages (filled circles) were added and then incubated in the plate for 90 min at 37°C. The production of O_2^- was measured by the reduction of cytochrome *c* as described in Materials and Methods. All values are the mean ± standard deviation of triplicate determinations. In many instances error bars are obscured by the symbols.

(a specific CR3 reactive monoclonal antibody) to detect rat CR3, virtually no CR3 was detected on NR8383 cells (**Fig. 4A**), whereas CR3 was observed on two distinct populations of rat peritoneal macrophages (Fig. 4B). Similar results were also obtained with the rat specific anti-CR_3 mAbs ED7 and ED8 (data not shown). Although these results cannot eliminate CR_3 as a potential PGG-glucan binding unit on rat peritoneal macrophages, they do demonstrate that a surface receptor distinct from CR3 must be responsible for mediating the functional responses induced by immobilized PGG-glucan in NR8383 cells.

DISCUSSION

In these studies, we have demonstrated that particulate (1,3)-β-glucan and immobilized PGG-glucan, but not soluble PGG-glucan, stimulate respiratory burst activity and TNF-α production in rat macrophages. These results demonstrate the importance of β-glucan presentation in inducing macrophage responses to these stimuli and are consistent with the interpretation that cross-linking of β-glucan receptors is needed to stimulate macrophage function. Soluble PGG-glucan may not induce a respiratory burst or cytokine secretion because of inefficient receptor cross-linking. Although the identity of the receptors responsible for these effects is not known at this time, CR3 does not appear to be involved.

TABLE 3. Immobilized PGG-Glucan Stimulates The Secretion of TNF-α by Rat Peritoneal Macrophages

		TNF-α (pg/mL)	
Stimulus	Concentration	Rat peritoneal macrophages[a]	NR8383 cells
Immobilized PGG-glucan	100.0 μg/mL	NT[b]	83[c]
	40.0 μg/mL	NT	85
	12.5 μg/mL	919	NT
	10.0 μg/mL	837	63
	7.5 μg/mL	462	NT
	5.0 μg/mL	214	NT
	2.5 μg/mL	85	61
	1.25 μg/mL	54	NT
	0.60 μg/mL	NT	<35
Immobilized Dextran	12.5 μg/mL	<35	NT
Media		<35	<35

[a] Rat peritoneal macrophages or NR8383 cells were incubated with the indicated stimulus for 24 h at 37°C and then the culture supernatants were tested for the presence of TNF-α with a commercial ELISA kit as described in Materials and Methods. The lower limit of TNF-α detection was 35 pg/mL. At the indicated concentrations each stimulus was incubated in Costar universal binding plates and then covalently linked to the surface as described in Materials and Methods.

[b] Not tested.

[c] All values are the mean of duplicate determinations.

A central feature of the innate immune system is the phagocytic response of macrophages and neutrophils to particulate antigens and insoluble immune complexes. Leukocyte responses to these type of stimuli have been experimentally characterized in vitro by immobilizing soluble antigens onto plastic. The phagocytic response to such immobilized ligands has been termed "frustrated phagocytosis" [54, 55]. Immobilized and cross-linked ligands have been shown to directly induce a respiratory burst in phagocytic cells [56, 57] and the cross-linking of receptors has been shown to stimulate the secretion of cytokines [49, 50, 58]. Furthermore, β-glucans conjugated to plastic or conjugated to biodegradable microbeads have been shown to induce the production of IL-1 and arachidonic acid metabolites by macrophages [20, 59, 60–62] and PGG-glucan passively adsorbed to a plastic surface has been shown to induce the secretion of IL-1Ra by human peripheral blood mononuclear cells (PBMCs) [30]. Our results with immobilized PGG-glucan provide an additional example of a non-stimulating soluble ligand that, when immobilized, triggers phagocytic cell activation. We have recently also observed that immobilized PGG-glucan can induce O_2^- production in human monocytes and peripheral blood neutrophils and nitric oxide production in NR8383 cells as well as the secretion of TNF-α and IL-1β by murine peritoneal macrophages and IL-1β by human PBMCs (data not shown).

Numerous (1,3)-β-glucan receptors have been reported in the literature [46, 48, 52, 53, 63–69]. Ross et al. [46, 48, 52, 53] have reported that human CR3 contains a (1,3)-β-glucan lectin binding site. However, there is controversy regarding the functional role of the human neutrophil CR3 β-glucan receptor [46, 48, 52, 53, 63, 64]. Our results demonstrate that NR8383 cells express virtually no surface CR3 and that this cell line is

TABLE 4. Stimulation of a Respiratory Burst in the NR8383 Rat Alveolar Macrophage Cell Line by Immobilized PGG-Glucan Can be Inhibited by Soluble PGG-Glucan

		Superoxide produced[a] (nmol/10^6 cells)		
		Soluble inhibitor (100 μg/mL)[b]		
Stimulus	Concentration	None	PGG-Glucan	Dextran
Immobilized PGG-glucan	0.62 μg/mL[c]	60.7 ± 2.0[d]	41.1 ± 0.1 (33%)	59.3 ± 2.1 (1%)
	0.31 μg/mL	55.2 ± 2.3	25.2 ± 1.0 (54%)	53.7 ± 0.6 (3%)
	0.15 μg/mL	48.2 ± 2.6	17.7 ± 0.3 (64%)	47.8 ± 0.3 (1%)
Buffer		11.3 ± 0.8	11.5 ± 0.9 (0%)	11.2 ± 1.2 (1%)
PMA	10 ng/mL	41.8 ± 1.6	46.2 ± 1.2 (0%)	44.5 ± 1.2 (0%)

[a] Cells were incubated with the indicated stimulus and the production of superoxide anion was measured by the reduction of cytochrome *c* as described in Materials and Methods.

[b] Soluble inhibitors were coincubated with the cells and cytochrome *c*.

[c] PGG-glucan at the indicated concentrations was incubated in a Costar universal binding plate and then covalently linked to the surface as described in Materials and Methods.

[d] Mean ± standard deviation of triplicate determinations. Percent inhibition compared with the response with no inhibitor is shown in parentheses.

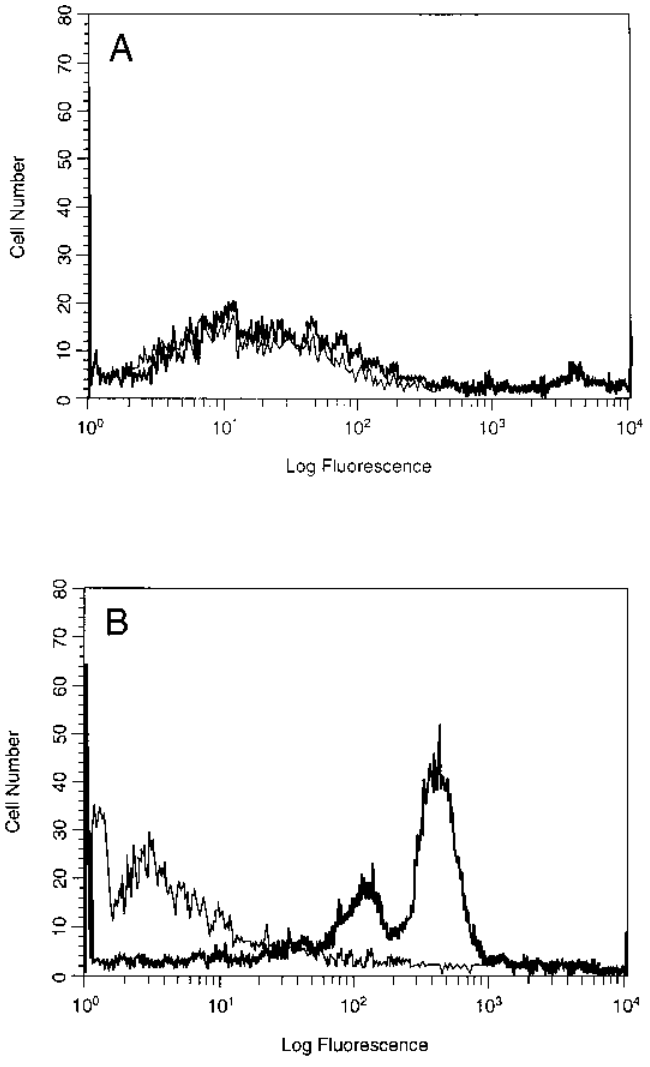

Fig. 4. Cell surface expression of CR3 by NR8383 cells and rat peritoneal macrophages. NR8383 cells (A) and normal rat peritoneal macrophages (B) were stained for surface CR3 using unlabeled mAb MRC OX-42 as the 1° antibody followed by fluorescein-conjugated goat anti-mouse IgG as the 2° antibody (bold line). The thin line represents the fluorescence of cells incubated with only the fluorescein-conjugated goat anti mouse IgG. Fluorescence was measured on a FACSCalibur flow cytometer, and the data are expressed as histograms of cell number (ordinate) vs. log fluorescence intensity (abscissa) for 5000 cells.

significantly more sensitive to immobilized PGG-glucan than rat peritoneal macrophages, which express large amounts of surface CR3. Thus, these data demonstrate that the responsiveness of leukocytes to β-glucans is unrelated to CR3 expression and raises further doubt as to whether CR3 is a functionally relevant β-glucan receptor.

Numerous (1,3)-β-glucan preparations from fungal sources are known to induce biological responses in mammalian leukocytes in vitro [3–6]. These observations suggest that β-glucan receptors likely play an important role in vivo in defense against fungi and other organisms that contain surface (1,3)-β-glucan molecules. In this communication, we have described an experimental system designed to directly compare the effects on rat leukocytes of a highly-purified (1,3)-β-glucan preparation when in solution or immobilized onto a plastic surface. These studies have demonstrated that immobilized PGG-glucan, but not soluble PGG-glucan, induces O_2^- and TNF-α secretion by rat macrophages. These results suggest that immobilized PGG-glucan activates rat macrophages by cross-linking or immobilization of a β-glucan receptor and that soluble PGG-glucan is ineffective or incapable of this process. Biochemical characterization of the response of rat macrophages to immobilized PGG-glucan and to soluble PGG-glucan is currently underway and should be helpful in understanding why soluble PGG-glucan enhances innate immunity against infections in vivo without overt cellular activation and the production of pro-inflammatory cytokines.

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