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Human Vascular Endothelial Cells Express Pattern Recognition Receptors for Fungal Glucans Which Stimulates Nuclear Factor κ B Activation and Interleukin 8 Production

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Fungal cell wall glucans nonspecifically stimulate various aspects of innate immunity via interaction with membrane receptors on macrophages, neutrophils, and natural killer cells. We investigated the binding of water-soluble glucans in primary cultures of normal human coronary or dermal vascular endothelial cells (VECs). Membranes from VECs exhibited saturable binding. Competition studies demonstrated the presence of at least two glucan binding sites on VECs. Glucan phosphate competed for all binding sites with a K_D of 3.7 μ M for coronary VECs and 11 μ M for dermal VECs, respectively. Laminarin, a low molecular weight glucan, competed for 47 to 51 per cent of binding ($K_D = 2.8$ – 2.9 μ M), indicating the presence of at least two binding sites. Glucan (1 μ g/mL) stimulated VEC nuclear factor κ B nuclear binding activity and Interleukin 8 expression—but not that of vascular endothelial growth factor—in a time-dependent manner. This is the first report of pattern recognition receptors for glucan on human VECs. It also provides the first evidence that glucans can directly modulate the functional activity of VECs by stimulating cytokine gene. These results provide new insights into the mechanisms by which the host recognizes and responds to fungal cell wall products and suggests that the response to glucans may not be confined to leukocytes.

THE INNATE IMMUNE system has evolved a comprehensive network of receptors that rapidly identify microorganisms on the basis of the carbohydrates, lipids, and proteins expressed by the organism.^{1–3} These macromolecular structures are ideal recognition molecules because they are structurally distinct from those expressed on the surface of mammalian cells.^{1–4} Glucans are (1→3)- β -D-linked polymers of glucose that are part of the outer cell wall of saprophytic and pathogenic fungi as well as certain bacteria.⁴ Glucans are also released from the cell wall as exopolymers and circulating glucans have been detected in the blood of patients with fungal infections.^{5–7} Numerous studies have demonstrated that (1→3)- β -D-glucans will acti-

vate a wide array of innate host defenses and proinflammatory responses.^{8,9} This is due in part to the ability of these carbohydrate ligands to activate proinflammatory and immunoregulatory signaling pathways [nuclear factor (NF) κ B and NF-interleukin (IL) 6] in immune-competent cells by interacting with specific receptors.^{10–14} Based on these data it has been postulated that cell wall glucans may serve as fungal pattern recognition molecules for the innate immune system.^{1, 13}

Many species have pattern recognition receptors/binding proteins that recognize (1→3)- β -D-glucans.^{13, 15–18} Ligation of the glucan receptor(s) modulates immune function and proinflammatory responses in humans and animals.^{9, 13, 16–19} In mammals glucans are thought to induce biological activity through interaction with receptors on macrophages,^{11, 13, 15} neutrophils,^{20, 21} natural killer (NK) cells,^{21, 22} Binding of (1→3)- β -D-glucan in human and murine monocytes and macrophages is specific, saturable, and susceptible to displacement by other (1→3)- β -D-glucans.^{11, 13, 15}

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We have reported the binding/uptake of a variety of water-soluble (1→3)-β-D-glucans and control polymers with different physicochemical properties to investigate the relationship between complex polymer structure and receptor binding.¹³ We observed that there are multiple glucan receptors on human monocytes, that these receptors can distinguish between (1→3)-β-D-glucan polymers, and that large-affinity differences exist (24 μM to 11 nM) between glucan polymers derived from various sources.¹³ We also observed that certain glucans appear to interact nonselectively with glucan binding sites, whereas other glucans preferentially interact with only one site.¹³

In this study we investigated whether normal human vascular endothelial cells (VECs) expressed receptors for glucan and if so whether interaction of a chemically pure water-soluble glucan with endothelial cells activate immunoregulatory and/or proinflammatory intracellular signaling pathways and stimulate cytokine expression. We focused on VECs for several reasons. Recent data indicate that glucans are released from fungal cell walls into the blood of patients with systemic or deep fungal infections.⁵⁻⁷ Consequently vascular endothelium would be exposed to the circulating glucan polymers. Endothelial cells have been reported to respond to bacterial recognition patterns such as lipopolysaccharide (LPS).²³ Recently Kiechl et al.²⁴ have presented epidemiologic evidence that chronic infections increase the risk of atherosclerosis, a disease of the vascular endothelium. These investigators reported that systemic inflammation associated with circulating levels of endotoxin and other inflammatory mediators had a strong correlation with atherogenic pathophysiology.²⁴ Taken together these data indicate that endothelial cells can recognize and, in some cases, directly respond to macromolecular structures from microorganisms.

Herein, we report the existence of specific glucan binding sites on normal human VECs. Interaction of fungal glucan with endothelial membrane receptors increases NFκB activity and IL-8 gene and protein.

Materials and Methods

Carbohydrate Polymers

We selected glucan phosphate²⁵ and laminarin¹³ as the glucan ligands for this study because glucan phosphate has been shown to interact with all available (1→3)-β-D-glucan receptors¹³ whereas laminarin has been reported to interact with a subset of the glucan receptors.¹³ Water-insoluble (1→3)-β-D-glucan was extracted from *Saccharomyces cerevisiae*.²⁵ Insoluble glucan was converted to a water-soluble glucan phosphate as described by Williams et al.²⁵ and chemically characterized as previously described.²⁵⁻²⁹ The final

product was stored (-80°C) as a lyophilized powder. It was dissolved in aqueous medium and filter sterilized (0.45 μm) before use. Laminarin is a low molecular weight (7700 g/mol) (1→3)-β-D-glucan polymer which was obtained from Sigma Chemical Co. (St. Louis, MO). Laminarin was chemically characterized as previously described.^{13, 26, 27} Endotoxin contamination in the (1→3)-β-D-glucans was <1 Endotoxin Unit/mg as determined by the Endospecy assay (Seigakaku, Japan), which is specific for endotoxin but does not respond to (1→3)-β-D-glucans.^{30, 31} For *in vitro* studies glucan phosphate was suspended in tissue culture medium before addition to VECs.

1,3-Diaminopropane (DAP) Derivatization of Glucans

Glucan phosphate (85 mg) was dissolved in 6 mL of dimethylsulfoxide by stirring for one hour under a nitrogen atmosphere. When the glucan phosphate was dissolved 400 mL of DAP (355 mg, 4.8 mmol) was added. The solution was stirred under nitrogen and 35 mg of sodium cyanoborohydride (0.56 mmol) was added. After an additional 12 hours at room temperature 6 mL of water was added and the reaction mixture was dialyzed against ultrapure water using a 1000-molecular weight cutoff membrane. The lyophilized material was stored at -20°C for later use.

Normal Human Coronary and Dermal (VECs)

We studied VECs from two different anatomic locations. The VECs were obtained by Clonetics (Ogden, UT) and maintained in endothelial cell basal medium containing 10 ng/mL epidermal growth factor, one μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 μg/mL amphotericin B, 0.4 per cent bovine brain extract, and 10 per cent fetal bovine serum (Clonetics). The cells were grown as an adherent culture at 37°C and 5 per cent CO₂ tension in a humidified environment, and the membranes were harvested according to the sonication protocol described below. VECs were used in the third through sixth passage.

Isolation of VEC Membranes

Cells were harvested during the logarithmic phase of growth, centrifuged at 2000 rpm for 10 minutes, counted, centrifuged again at 2000 rpm (10 minutes), and frozen at -80°C. Cells were thawed in phosphate-buffered saline in the presence of 10 μL of protease inhibitor cocktail (Sigma P-8340) per 10⁶ cells. The solution was maintained at 4°C and sonicated at 35 per cent power for 30 seconds (three times) (Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA). The samples were centrifuged at 650g for 10 minutes at 4°C to spin out nuclei. The pellet was resuspended,

sonicated, and centrifuged. The combined supernatants were centrifuged at 68,000 rpm for 30 minutes at 4°C. The pellet containing VEC membranes was suspended in Hanks' Balanced Salt Solution at a concentration of 1 mg/mL. Aliquots of the membranes were stored in liquid nitrogen for later use.

Binding Assays

Binding assays were performed using a Biacore 2000 surface plasmon resonance instrument (Biacore, Piscataway, NJ). Samples were maintained at 4°C using an ISOTEMP circulating bath (Fisher Scientific). Experiments were performed at 37°C using a running buffer containing 150 mM NaCl, 10 mM HEPES, 3 mM EDTA, and 0.005 per cent surfactant P20 (Biacore).

Attachment of DAP-Glucan to the CM-5 Sensor Chip

DAP-glucan phosphate was freshly prepared in 10 mM sodium acetate and adjusted to a pH between 8.6 and 8.9 with one M NaOH. DAP-glucan was immobilized to a CM-5 (carboxymethyl dextran) sensor chip on the Biacore 2000 instrument at a flow rate of 5 μ L per minute. The sensor surface was first activated by exposure for 6 minutes to a freshly prepared solution of 100 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 25 mM *N*-hydroxysuccinimide (NHS). The surface was then exposed to DAP-glucan (3 mg/mL) for 7 minutes. This cycle of activation with EDC/NHS and exposure to DAP-glucan phosphate was repeated five times. Immobilization of DAP-glucan phosphate was typically about 2000 resonance units (RUs) or 2 ng/mm². After immobilization the biosensor surface was exposed to one M ethanolamine (pH 8.5) for 6 minutes to inactivate any remaining carboxyl groups. The Biacore biosensor contains four flowcells; DAP-glucan was immobilized on three flowcells, while the fourth flowcell served as a control for nonspecific binding to the dextran surface.

Saturation Experiment Using the CM-5 Sensor Chip

The saturation experiments were performed as described by Kougiaris et al.³² Specifically, membranes prepared from VECs were suspended at concentrations of 0.1 to 60 μ g/mL in HEPES buffer in siliconized vials. A continuous flow of 20 μ L/min buffer was used to establish a baseline measurement of RUs. Assays were performed at 37°C to approximate physiologic conditions. VEC membranes were injected for 5 minutes followed by continuous flow of HEPES buffer for 5 minutes to allow dissociation of membranes from the carbohydrate surface. At the end of each cycle the surface was regenerated by consecutive one-minute exposures to Triton X-100 (0.3%) and

guanidine hydrochloride (3 M) at 100 μ L/minute. Regeneration resulted in displacement of ≥ 90 per cent of the added RUs from the surface.

Glucan Phosphate and Laminarin Competition Studies

The competitive binding experiments were performed according to the method of Kougiaris et al.³² with modifications. A CM5 chip onto which DAP-glucan was attached was used for the competition experiments. For competition experiments samples containing a fixed concentration of VEC membranes (10 μ g/mL) in the absence and presence of competitor were alternately injected. VEC membranes were mixed with competing carbohydrates for at least one hour before injection on the Biacore instrument. After a 3-minute dissociation the surface of the chip was regenerated with consecutive one-minute exposures to 0.3 per cent Triton-X 100 and guanidine hydrochloride 3 mol/L at 100 μ L/minute.

Electrophoretic Mobility Shift Assays

Double-stranded consensus binding site oligonucleotides for NF κ B were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The oligonucleotides were end-labeled with [γ -³²P]ATP (Amersham, Arlington Heights, IL) using T4 polynucleotide kinase (ProMega, Madison, WI). Binding assays were performed in 10 μ L of binding reaction mixture containing 10 μ g of nuclear proteins and ³²P-labeled NF κ B oligonucleotides. The binding reaction mixture was incubated at room temperature for 20 minutes and then electrophoresed on 4 per cent nondenaturing polyacrylamide gel electrophoresis (PAGE) gels. The specificity of binding was confirmed using three approaches. First, we performed competition assays in which a tenfold excess of cold oligonucleotide was added to separate reaction mixtures. Second, we performed a supershift assay by adding antibody to p50, antibody to p65, or antibodies to p50 and p65 to separate reaction mixtures. Finally a tenfold excess of cold oligonucleotide bearing the AP-II binding site was added to separate reaction mixtures. After PAGE the gels were analyzed by phosphor imaging (BioRad Laboratories, Hercules, CA) followed by drying and exposure to Kodak X-Omat film at -70°C.

RNA Isolation

Total cellular RNA was isolated from control and glucan-treated human dermal endothelial cells using the Ultraspect-II RNA isolating kit (Biotecx, Houston, TX).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

One microgram of total RNA was used for cDNA synthesis with Marine leukemia virus (MLV) reverse transcriptase (Perkin Elmer Inc, Branburg, NJ) in a 20- μ L final volume. The cDNA synthesis reaction was 15 minutes at 42°C and 5 minutes at 99°C. The reaction mixture (2 μ L) was subjected to PCR amplification (25 μ L) that contained one μ mol/L of each of two primers, 1.5 mM MgCl₂, 0.2 mM each of four deoxynucleotides, and 1.25 units Taq polymerase (Perkin Elmer Inc). PCR amplification of IL-8, VEGF, or toll-like receptor 4 (TLR4) cDNA was performed under the following conditions: 35 cycles, 45 seconds at 94°C, 35 seconds at 54°C, and 45 seconds at 72°C. GAPDH was used as the gene transcript control. The PCR data were imaged and quantified by computer-assisted densitometry and referenced to the gene transcript control. The IL-8 primer sequences were synthesized at the Johns Hopkins nucleotide facility (Baltimore, MD): 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' and 5'-TCTCAGCCCTCTTCAAAA-CTTCTC-3'.³³ The vascular endothelial growth factor (VEGF) primers were a gift from Dr. Balvin Chua (Quillen Center for Geriatric Research, East Tennessee State University, Johnson City, TN): 5'-CCATGA-ACCTTCTGCTCTCTTG-3' and 5'-GGTGAGAG-GTCTAGTTCCCGA-3'.

IL-8 and VEGF Enzyme-Linked Immunosorbent Assay (ELISA)

VEC culture supernatants were assayed for human IL-8 or VEGF by ELISA (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Experimental Protocols

The glucan binding characteristics of coronary and dermal VEC were strikingly similar. Therefore we arbitrarily chose to use normal human dermal vascular endothelial cells for the functional assays. For the NF κ B study VECs were incubated with glucan phosphate (1 μ g/mL) for varying periods of time. VECs incubated in medium alone served as the control. At each time point nuclear protein was harvested from control or glucan-treated VECs.

In IL-8 and VEGF experiments dermal VECs were incubated with glucan phosphate (1 μ g/mL), pyrrolidine dithiocarbamate (PDTC) (20 μ M) or glucan and PDTC for 8 hours. PDTC is a water-soluble antioxidant that inhibits NF κ B activation.³⁴ Cells incubated in medium alone served as the control. Total RNA was harvested at 8 hours and IL-8 and VEGF mRNA levels were determined by RT-PCR. PCR gels were quantified by computer-assisted scanning densitometry. In

parallel studies dermal VECs were incubated with glucan phosphate (1 μ g/mL). Supernatants were harvested at 24, 36, and 48 hours and were assayed for IL-8 and VEGF by ELISA.

Data Analysis

In saturation (Fig. 1) and competition (Figs. 2–5) studies data were normalized to the baseline established at the start of the experiment and analyzed by unweighted nonlinear regression using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). Because the bulk shift occurs over approximately 15 seconds we estimated the amount of membrane protein bound to the surface by measuring the increase in RUs at 30 seconds after changing from membrane to buffer exposure. For saturation experiments in which the DAP-glucan phosphate surface was exposed to varying concentrations of membrane protein RU values for each concentration of analyte were analyzed using the following model:

$$RU = \frac{RU_{MAX} * [analyte]}{K_D + [analyte]} + K_{NONSPECIFIC} * [analyte]$$

where RU_{MAX} is the maximum binding, K_D is the apparent K_D value, $K_{NONSPECIFIC}$ (K_{NS}) is the constant of linear nonspecific binding, and [analyte] is the protein concentration. K_D values are also accompanied by 95 per cent confidence intervals for the apparent K_D . In our experiments $K_{NS} = 0$.

For competition experiments in which the DAP-glucan phosphate surface was exposed to a fixed concentration of membrane protein in the absence or presence of competitor the RU values for each competitor concentration were further normalized to binding in the absence of competitor (100%) and analyzed using models for competitive displacement at a single binding site, a single binding site plus nonspecific binding, and/or a two-binding site model. The best model was chosen statistically using the sequential F-test.

NF κ B integrated intensities were normalized to the nontreated control group (time 0) which was set at 1.0. IL-8 and VEGF mRNA integrated intensities were normalized to the gene transcript control. Figures 1 through 5 and Table 1 illustrate group mean \pm standard error of the mean responses for VECs treated as indicated at each study time point. Analysis of variance was used to compare group means and to assess the effects of treatment *versus* time. A *P* value of ≤ 0.05 was considered significant.

Results

VEC Membranes Bind to a Glucan-Coated Biosensor Surface in a Saturable Manner

The VEC membranes were injected over a concentration range of 0.1 to 60 mg/mL of protein. This was

followed by exposure of the surface to continuous flow of buffer (5 minutes) to allow dissociation of the analyte from the ligand. Bulk shift occurs over 15 seconds in the control flow cell, so we chose to measure responses 30 seconds after the end of the analyte injection. The binding response as a function of the VEC membrane protein concentration was proportional to the immobilized DAP-glucan attached to the flow channels (Fig. 1). As can be seen VEC membranes bound to immobilized ligand in a saturable manner (Fig. 1). Nonspecific binding was not significant at the protein concentrations that were used.

Glucan Phosphate Completely Inhibited Binding of VEC Membranes to a Glucan-Coated Sensor Surface

Glucan phosphate completely inhibited the binding of coronary and dermal VEC membranes to a glucan phosphate sensor surface with characteristics of a single binding site (Figs. 2 and 3). For coronary VEC membranes the K_D was $3.7 \mu\text{M}$ (95% confidence interval 2.3–6.0 μM) (Fig. 2). For dermal VEC membranes the K_D was $11 \mu\text{M}$ (95% confidence interval 5.6–23 μM) (Fig. 3).

Laminarin Partially Inhibited Binding of VEC Membranes to a Glucan-Coated Sensor Surface

Using a cell-based receptor ligand assay we have reported that laminarin will bind to a subset of (1→3)- β -D-glucan receptors on the human U937 monocyte cell line.¹³ Using surface plasmon resonance we have reported that laminarin also binds to a subset of (1→3)- β -D-glucan receptors on normal human dermal fibroblasts.³² In the present study laminarin partially inhibited the binding of coronary and dermal VEC membranes to the glucan phosphate biosensor surface

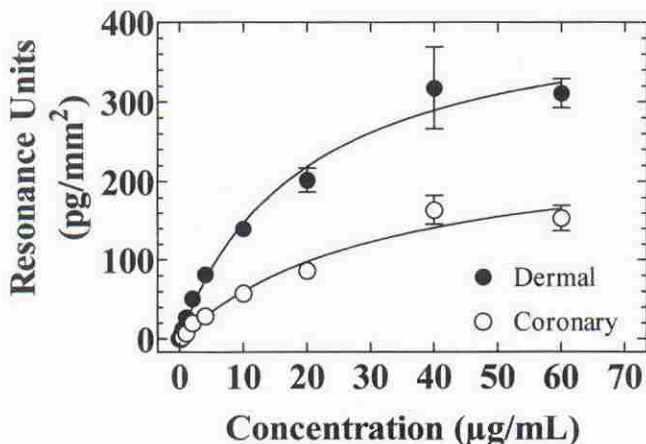


FIG. 1. Saturation curve for coronary and dermal VEC membrane binding to immobilized DAP-glucan. This figure shows results from a typical experiment in which averages were obtained from three flowcells and five cycles.

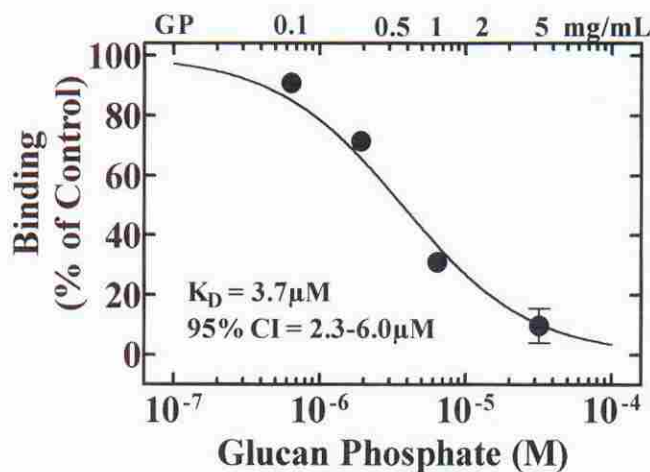


FIG. 2. Competition with glucan completely inhibits binding of coronary VEC membranes to immobilized glucan phosphate (GP). The K_D for coronary VECs was $3.7 \mu\text{M}$ (95% CI of 2.3–6.0 μM). The displacement relationship was characteristic of a single binding site.

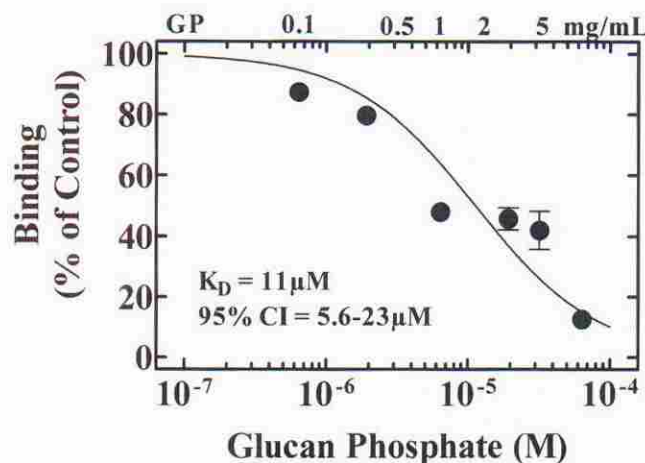


FIG. 3. Competition with glucan completely inhibits binding of dermal VEC membranes to immobilized glucan phosphate (GP). The K_D for dermal VECs was $11 \mu\text{M}$ (95% CI of 5.6–23 μM). The displacement relationship was characteristic of a single binding site.

with characteristics of a single binding site (Figs. 4 and 5). In coronary VEC membranes laminarin inhibited 47 ± 9 per cent of the binding of VEC membranes to glucan phosphate. The K_D for the inhibition by laminarin was $2.9 \mu\text{M}$ [95% confidence interval (CI) 0.09–95 μM], which corresponded to a concentration of $0.029 \mu\text{g/mL}$. In dermal VECs laminarin inhibited 51 ± 4 per cent of the binding of VEC membranes to glucan phosphate. The K_D for the inhibition by laminarin was $2.8 \mu\text{M}$ (95% CI 0.42–19 μM), which corresponded to a concentration of $0.028 \mu\text{g/mL}$. The ability of laminarin to inhibit only a fraction of the binding interactions suggests that there are at least two types of interactions between VEC membranes and glucan phosphate. Laminarin is able to distinguish and

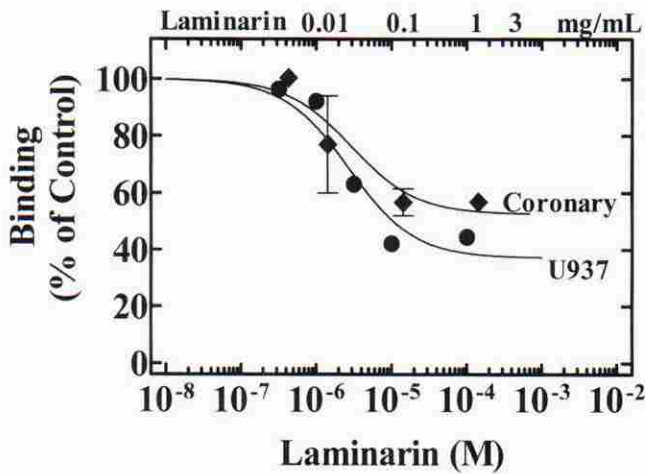


FIG. 4. Laminarin competition for coronary VEC binding to immobilized DAP-glucan phosphate. Laminarin did not completely inhibit the interaction of VECs and immobilized DAP-glucan phosphate suggesting the presence of two types of binding interactions, one of which is not inhibitable by laminarin. The displacement relationship was characteristic of a single binding site. The K_D for coronary VECs (diamonds) was $2.9 \mu\text{M}$ (95% CI of $0.09\text{--}95 \mu\text{M}$) with a maximum displacement of 47 ± 9 per cent. The K_D for U937 (circles) was $2.7 \mu\text{M}$ (95% CI of $0.58\text{--}13 \mu\text{M}$) with a maximum displacement of 63 ± 8 per cent.

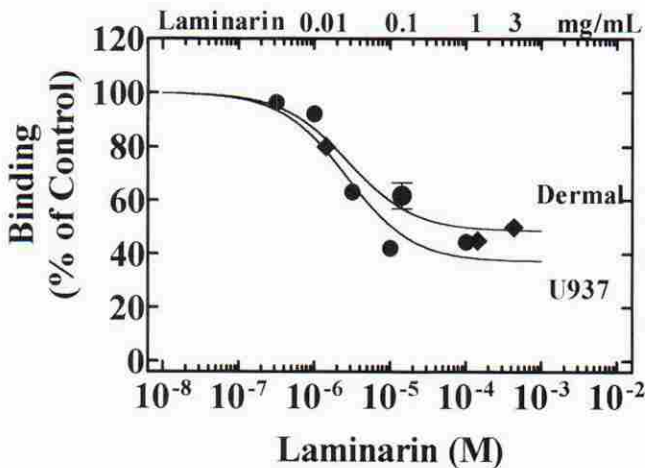


FIG. 5. Laminarin competition for dermal VEC binding to immobilized DAP-glucan phosphate. Laminarin did not completely inhibit the interaction of VECs and immobilized DAP-glucan phosphate suggesting the presence of two types of binding interactions, one of which is not inhibitable by laminarin. The displacement relationship was characteristic of a single binding site. The K_D for dermal VECs (diamonds) was $2.8 \mu\text{M}$ (95% CI of $0.42\text{--}18 \mu\text{M}$) with a maximum displacement of 51 ± 4 per cent. The K_D for U937 (circles) was $2.7 \mu\text{M}$ (95% CI of $0.58\text{--}13 \mu\text{M}$) with a maximum displacement of 63 ± 8 per cent.

inhibit the interaction at one of the sites, whereas glucan phosphate interacts with all sites.

As a control we examined the effect of laminarin on competitive binding using human U937 promonocytic cell membranes and compared it with the binding of VEC membranes. Figures 4 and 5 show that laminarin displaced 63 ± 8 per cent of binding in the U937 membranes with a K_D of $2.7 \mu\text{M}$ (95% CI $0.58\text{--}13 \mu\text{M}$)

and characteristics of a single binding site. This is strikingly similar to the binding characteristics of laminarin in VECs (Figs. 4 and 5).

Coincubation with Glucan Phosphate Increased VEC NFκB Activity

Glucan rapidly stimulated NFκB nuclear binding activity in dermal VECs (Fig. 6). NFκB was increased by 110 per cent one hour after incubation with glucan phosphate. NFκB levels decreased by 3 hours but remained ~20 per cent above control values for the duration of the experiment (Fig. 6).

Glucan Increased IL-8 Gene and Protein Levels but Did Not Increase VEGF Levels in VECs

RT-PCR data indicate that IL-8 mRNA levels were increased by 78 per cent at 8 hours (Fig. 7). Addition of the NFκB inhibitor PDTC ($200 \mu\text{M}$) to dermal VECs blunted glucan induced IL-8 mRNA expression by 74 per cent suggesting that glucan stimulates endothelial cell IL-8 mRNA expression in part through an NFκB-dependent pathway (Fig. 7). There was a modest (16.4–39.7%) but significant increase in IL-8 levels in the supernatant of glucan-treated VECs (Table 1). Addition of PDTC to the VEC cultures inhibited both basal and glucan-induced IL-8 release. VEGF gene and protein expression were not increased by treatment with glucan up to 48 hours (data not shown).

Discussion

A number of significant observations have emerged from this study. First and foremost we found that nor-

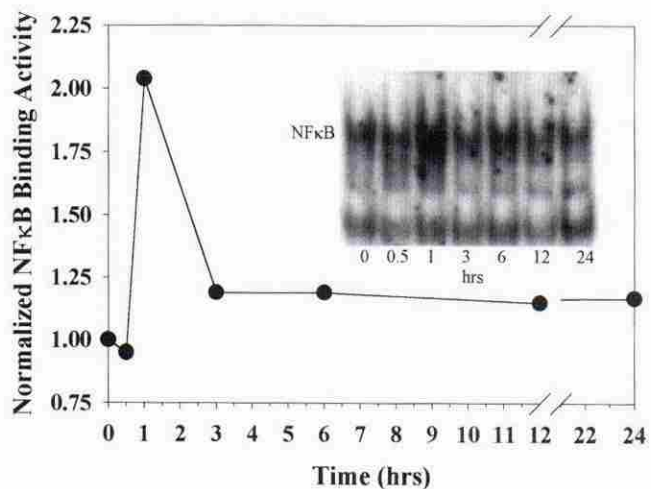


FIG. 6. Time-dependent increase in NFκB nuclear binding activity in human dermal VECs treated with glucan phosphate ($1 \mu\text{g/mL}$). A representative gel-shift assay is shown in the inset. The time in hours is given below each lane in the gel. The data were normalized to the 0 time point value which was set at 1.0. The experiment was repeated three times.

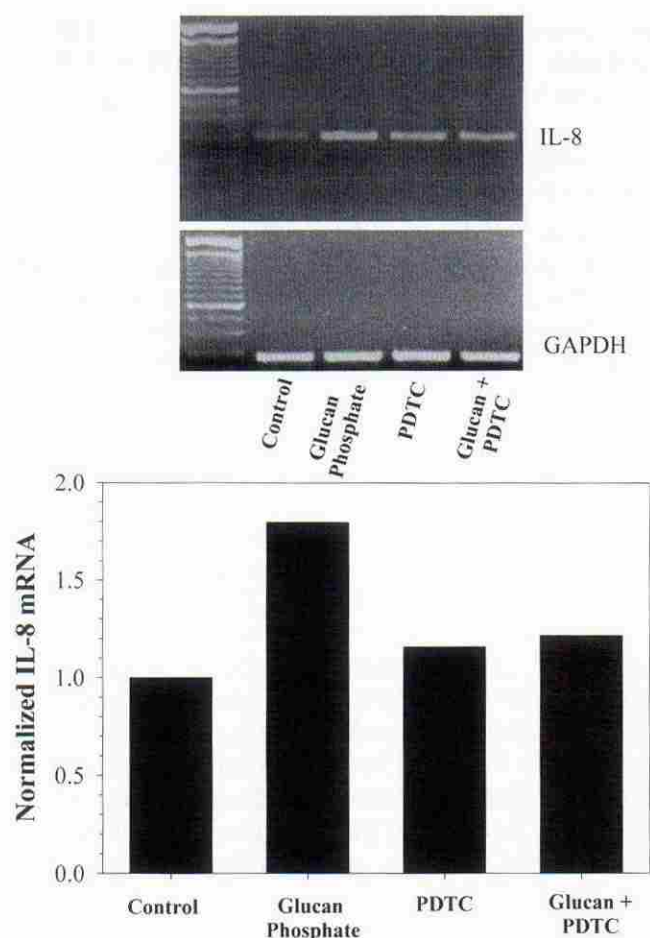


FIG. 7. Increased IL-8 mRNA levels in normal human dermal VECs treated with glucan phosphate (1 $\mu\text{g}/\text{mL}$). The VECs were coincubated with glucan, PDTC (200 μM), or glucan and PDTC for 8 hours. A representative IL-8 PCR product gel is shown beside the graph. The gels were imaged and the integrated intensity was determined. The IL-8 data were normalized to the GAPDH transcript control. The data are presented as normalized integrated intensity. The experiment was repeated three times.

mal human vascular endothelial cells express membrane receptors for fungal (1 \rightarrow 3)- β -D-glucans. To the best of our knowledge this is the first report of glucan-specific pattern recognition receptors on VECs. We studied normal human VECs derived from two different anatomic sites, *i.e.*, coronary and dermal endothelium. Both cell types expressed at least two specific receptors for fungal glucans. Interaction of glucan with membrane receptors on VEC activated NF κ B, an intracellular signaling pathway that is associated with regulation of cytokine/chemokine gene expression.³⁵ This is consistent with previous studies that have shown that glucans stimulate transcription factor activation in macrophages and neutrophils.¹⁰⁻¹² Glucan receptor interactions in VECs resulted in increased IL-8 gene and protein expression. However, VEGF gene and protein levels were not altered by glucan. The glucan-induced endothelial cell IL-8 mRNA and

TABLE 1. Increased IL-8 Production in Glucan-Treated Human Dermal VECs*

Group	24 Hours	36 Hours	48 Hours
Control	174.6 \pm 4.7	157.0 \pm 12.8	239.8 \pm 6.6
Glucan	215.1 \pm 12.6 [†]	219.4 \pm 4.3 [†]	279.1 \pm 5.7 [†]
PDTC	104.4 \pm 2.3 [†]	95.4 \pm 5.5 [†]	157.0 \pm 4.5 [†]
Glucan + PDTC	90.46 \pm 7.0 ^{†‡}	110.6 \pm 8.8 ^{†‡}	149.3 \pm 3.7 ^{†‡}

* Human dermal VECs were incubated with glucan phosphate (1 $\mu\text{g}/\text{mL}$), PDTC (200 μM), or glucan phosphate + PDTC for 24, 36, or 48 hours. The supernatants were harvested and assayed for IL-8 using a commercial ELISA. Data are expressed as pg/mL \pm standard error of the mean.

N = eight per group.

[†] $P < 0.05$ vs control.

[‡] $P < 0.05$ vs glucan.

protein expression is mediated in part through an NF κ B-dependent signaling pathway.

We observed that binding of glucan by VEC membranes was saturable, dose dependent, and specific and competition for this interaction by glucan phosphate was complete. Thus the interaction of fungal glucan with VEC membrane receptors meets the criterion for specific binding. The binding characteristics for glucan phosphate in VECs were very similar to the K_D of 5.2 μM (95% CI .8-7.1 μM) for the human U937 promonocytic cell line.³⁶ Interestingly laminarin competition for this interaction was able to displace between 47 and 51 per cent of the interaction, respectively. Laminarin is equipotent with glucan phosphate based on molar concentration, but its lower molecular weight makes it more potent. The fact that laminarin cannot completely inhibit the interaction of VEC membranes with immobilized glucan phosphate suggests the presence of at least two different binding sites for glucan on VEC membranes. Laminarin selectively interacts with one site, whereas glucan phosphate interacts with all sites. We compared the effect of laminarin on competitive binding of the human U937 promonocytic cell line using surface plasmon resonance. Laminarin displaced 63 \pm 8 per cent of U937 binding with a K_D of 2.7 μM (95% CI 0.58-13 μM). Using a cell-based radioligand assay we have reported similar results for the human U937 cell line, where laminarin displaced 61 \pm 4 per cent of binding with a K_D of 2.6 μM (95% CI 1.7-4.2 μM) and glucan phosphate completely inhibited binding in a dose-dependent manner.¹³ This is excellent agreement between the cell-based radioligand assay and the surface plasmon resonance approach. The similarity in the affinities of these interactions in human promonocytes and endothelial cells suggests that similar receptors are present in both cell types. The data also strengthen our contention that there are multiple binding sites for glucans.

Although there are numerous reports that mono-

cytes, macrophages, neutrophils, and NK cells express membrane pattern recognition receptors for (1→3)- β -D-glucan the precise nature of the glucan receptor(s) is the subject of controversy. Di Renzo,³⁷ Thornton,³⁸ Vetvicka,²¹ and colleagues have reported that the type 3 complement receptor [CR3 (also known as CD11b/CD18)] is a glucan binding site on macrophages, neutrophils, and NK cells. The glucan binding is reported to be through one or more lectin sites located outside the CD11b I domain.^{21, 22, 38} Duan,³⁹ Di Renzo,³⁷ Vetvicka,^{21, 22} and colleagues have reported a β -glucan binding lectin on NK cells that contributes to NK cell-mediated cytotoxicity. Zimmerman et al.⁴⁰ reported that lactosylceramide binds PGG-glucan and that this glycosphingolipid may be a leukocyte glucan binding moiety. Dushkin,⁴¹ Vereschagin,⁴² and colleagues have reported that a carboxymethylated glucan binds to the macrophage scavenger receptor. We have reported the presence of two glucan binding sites on U937 cells that stimulate intracellular signaling pathways culminating in the activation, translocation, and nuclear binding of immunoregulatory and proinflammatory transcriptional activator proteins.¹¹ Our data suggest that neither of these sites is CR3.¹³ Michalek et al.⁴³ have extended this observation by reporting that glucans bind to a site distinct from CR3. CR3 is a β_2 integrin that is leukocyte restricted⁴⁴ and is involved in the recognition of microbial molecular patterns such as LPS.⁴⁵ However, endothelial cells have not been reported to express CR3; thus the binding and functional activation of endothelial cells by glucan cannot be attributed to a CR3-dependent mechanism. Brown and Gordon⁴⁶ have recently reported that Dectin-1 is a glucan binding site. These data do not diminish the potential importance of CR3 as a leukocyte binding moiety for glucans; rather they reinforce the notion that there are multiple glucan binding sites and they indicate that glucan receptors are not sequestered solely in leukocytes—suggesting that these receptors may be more widespread than previously thought. We have also recently identified two glucan-specific binding sites on normal human dermal fibroblasts.³² Thus there is now evidence for glucan receptors on macrophages, neutrophils, NK cells, VECs, and fibroblasts. It is not clear whether there are glucan binding sites on other cells. Furthermore we do not know whether the two binding sites we have identified on VECs activate the same or different signaling pathways within the cell. Why there are multiple glucan receptors on endothelial cells and other cells is not clear. However, Lowe et al.³⁶ have recently published data that support a cross-linking hypothesis for glucan activation of cells. In that study we demonstrated that a glucan polymer composed of seven glucose subunits (heptasaccharide) was the minimum binding subunit for hu-

man monocyte glucan receptors. However, the heptasaccharide was not able to stimulate NF κ B activation suggesting that the glucan polymer must be of sufficient size to cross-link spatially separated receptors to induce an activating signal.

These data may also be of significance with regard to understanding the mechanisms by which the host recognizes and responds to bacterial pathogens. Recent data from Ahren et al.⁴⁷ indicate that glucan receptors play a pivotal role in mediating the uptake of nontypeable *Hemophilus influenzae* in human monocytes and epithelial cells. Specifically these investigators reported that *H. influenzae* entered cells primarily through a glucan receptor-mediated endocytosis that was inhibitable by laminarin.⁴⁷ They did not identify the glucan-specific receptor(s) that were involved in *H. influenzae* binding.⁴⁷ However, the fact that they observed similar results in monocytes and epithelial cells supports our observations that glucan receptors are not sequestered solely in immunocytes.⁴⁷ Furthermore the data of Ahren et al.⁴⁷ indicate that glucan receptors may play an important role in host recognition and response to bacterial as well as fungal pathogens.

In conclusion we have identified at least two specific glucan binding sites on normal human dermal endothelial cells. The interaction of the glucan ligand with VECs results in the activation of proinflammatory intracellular signaling pathways and upregulation of cytokine gene expression. This is the first report of a glucan binding site on VECs. The potential ramifications of these data are significant because they force us to re-examine the current hypotheses regarding the mechanisms by which the host recognizes and responds to fungal cell wall carbohydrates. By way of example glucans have been reported to exert a plethora of systemic effects.^{8, 9} The presumed mechanism was that glucans interact with leukocytes and other elements of innate immunity resulting in either a primed or activated state.^{12, 48, 49} The systemic effects were attributed to release of proinflammatory and/or immunoregulatory mediators that serve as second messengers; *i.e.*, the systemic effects were indirect. Although this is a reasonable explanation for the observed effects the present data indicate that glucans may also directly interact with and modify the functional state of vascular endothelium. This observation is significant because we know that VECs line all of the vessels and are present in every organ system. We also know that glucans and other cell wall macromolecules are released into the systemic circulation during fungal infections.⁵⁻⁷ Thus it is reasonable to speculate that some of the effects that have been ascribed to glucans may be mediated through direct interaction with vascular endothelium.

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DISCUSSION

RAUL J. GUZMAN, M.D. (Nashville, TN): In this manuscript the authors continue to present their findings on this interesting class of molecules found in fungal and some bacterial cell wall membranes. Their findings may have broad implications for treating our patients with sepsis as we have heard today in the pathophysiology of vascular disorders. The authors have convincingly demonstrated that vascular endothelial cells express receptors for fungal wall glucans. I believe that they have presented ample data to support their hypothesis that glucan polymers can have significant effects when liberated into their circulation. The overall effect on endothelial cells appears to be harmful, whereas these authors and others have previously observed that glucan polymers can have beneficial effects by partially inhibiting some of the negative inflammatory responses seen during sepsis. For example, in septic mice the authors have shown that treatment with glucan phosphate increased long-term survival. Glucans have appeared to exert some of their beneficial effects by activating the immune response of

leukocytes and this is also potentially a NF κ B-mediated mechanism.

This brings to mind several questions. I would like to know if the authors feel that their new findings suggest that glucan-type molecules have a limited therapeutic role in the treatment of sepsis and perhaps contrary to some previous reports, these agents may be one of the culprits in the inflammatory response. In the discussion portion of their manuscript the authors describe several potential natural ligands for these receptors and immune cells. However, their normal function on endothelial cells is not known. Regards to these apparently ubiquitous cells surface receptors. What do the authors believe might be the native function of these cell surface receptors in endothelial cells and there are other known ligands for these receptors on the endothelium?

Finally Dr. Lowe suggests that this class of agents may affect endothelial cell function and several studies have implicated infectious agents in the etiology of arterial occlusive disease. For example, there are some intriguing data to suggest that chronic infection with chlamydia or viral agents

such as cytomegalovirus is associated with atherosclerosis. I would like to know if the authors are aware of any data linking chronic fungal infection with an increased risk of atherosclerosis.

ELIZABETH P. LOWE, M.D. (Closing Discussion): I would like to address the first question as to whether glucans have a limited therapeutic role in the treatment of sepsis and if they may be a culprit in the inflammatory response. The purpose of this paper was to better understand the interaction of glucans with endothelial cells and to see if there were even receptors present. Not only did we discover that there were receptors present, but those receptors activated intracellular signaling pathways and those pathways resulted in protein expression. This gives a better understanding of the host response to infection. This better un-

derstanding may give us the potential to manipulate the response in the future and possible for therapeutic implications. What is the native function of these cell surface receptors? We don't know. We just discovered these receptors are present. Not only are they on endothelial cells but they are also on leukocytes and fibroblasts. This may mean because they are so ubiquitous that they may be involved in native immunity and survival of the hosts. Are these chronic fungal infections linked to increased risk of atherosclerosis? This report was the first to show that glucans bond to endothelial cells and that this increases IL-8 production. IL-8 is a chemoattractant for neutrophils and neutrophils have been implicated in the pathogenesis of atherosclerosis. This could lead to further understanding of this disease, but as yet it is too early to say for sure.

