INFECTION AND IMMUNITY, June 2001, p. 3933–3938 Vol. 69, No. 6
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.6.3933–3938.2001

Normal Human Fibroblasts Express Pattern Recognition Receptors for Fungal (1→3)-β-D-Glucans

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Received 11 January 2001/Returned for modification 7 February 2001/Accepted 27 February 2001

Fungal cell wall glucans nonspecifically stimulate various aspects of innate immunity. Glucans are thought to mediate their effects via interaction with membrane receptors on macrophages, neutrophils, and NK cells. There have been no reports of glucan receptors on nonimmune cells. We investigated the binding of a water-soluble glucan in primary cultures of normal human dermal fibroblasts (NHDF). Membranes from NHDF exhibited saturable binding with an apparent dissociation constant (K_D) of 8.9 ± 1.9 μg of protein per ml and a maximum binding of 100 ± 8 resonance units. Competition studies demonstrated the presence of at least two glucan binding sites on NHDF. Glucan phosphate competed for all binding sites, with a K_D of 5.6 μM (95% confidence interval [CI], 3.0 to 11 μM), while laminarin competed for 69% ± 6% of binding sites, with a K_D of 3.7 μM (95% CI, 1.9 to 7.3 μM). Glucan (1 μg/ml) stimulated fibroblast NF-κB nuclear binding activity and interleukin 6 (IL-6) gene expression in a time-dependent manner. NF-κB was activated at 4, 8, and 12 h, while IL-6 mRNA levels were increased by 48% at 8 h. This is the first report of pattern recognition receptors for glucan on human fibroblasts and the first demonstration of glucan binding sites on cells other than leukocytes. It also provides the first evidence that glucans can directly modulate the functional activity of NHDF. These results provide new insights into the mechanisms by which the host recognizes and responds to fungal (1→3)-β-D-glucans and suggests that the response to glucans may not be confined to cells of the immune system.

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 (37). Glucans are also released from the microbial cell wall as exopolymers (14, 23, 28, 37). Numerous studies have demonstrated that (1→3)-β-D-glucans will activate a wide array of innate host defenses (4, 50). This is due, in part, to the ability of these carbohydrate ligands to activate proinflammatory and immunoregulatory signaling pathways (NF-κB and NF–interleukin 6 [NF–IL-6]) in immune competent cells by interacting with specific receptors (1–3, 26, 48). Recent data indicate that glucans are released from fungal cell walls into the systemic circulation of patients with systemic or deep fungal infections (14, 23, 28). It is not clear whether these circulating fungal polymers induce any of the sequelae associated with fungal infections. However, the innate immune system has evolved a complex network of receptors which rapidly identify microorganisms based on the carbohydrates, lipids, and proteins expressed by the organism (15, 20, 21). These macromolecular structures are ideal recognition molecules because they are structurally distinct from those expressed on the surface of mammalian cells (15, 20, 21, 37). It has been postulated that cell wall glucans may serve as pattern recognition molecules for the innate immune system (15, 26).

Many species have pattern recognition receptors or binding proteins which recognize (1→3)-β-D-glucans (10, 17, 26, 27, 30). Ligation of the glucan receptor(s) modulates immune function and proinflammatory responses in humans and animals (1–3, 47, 50, 51). In mammals, glucans are thought to induce biological activity through interaction with receptors on macrophages (3, 26, 27), neutrophils (34, 43), and NK cells (7, 43, 44). 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 (3, 26, 27). We have reported the binding and uptake of a variety of water-soluble (1→3)-β-D-glucans and control polymers with different physicochemical properties in order to investigate the relationship between complex polymer structure and receptor binding (26). 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 (24 μM to 11 nM) exist between glucan polymers derived from various sources (26). We also observed that certain glucans appear to interact nonselectively with glucan binding sites, while other glucans preferentially interact with only one site (26). The physical and/or functional basis for this selectively among glucan receptors is not clear.

To the best of our knowledge, there have been no reports of glucan-specific binding sites on cells other than leukocytes, nor is there evidence that glucans can directly activate cells other than immunocytes. In this study we investigated whether normal human dermal fibroblasts (NHDF) expressed receptors for glucan and, if so, whether interaction of a highly purified, water-soluble glucan with fibroblasts activated immunoregula-

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tory and/or proinflammatory intracellular signaling pathways. We focused on fibroblasts for several reasons. First, glucans have been reported to accelerate early wound repair (5, 6, 33). Of greater significance, glucans have been shown to increase collagen deposition in rodent skin wounds and intestinal anastomoses (6, 33). We speculated that the effect of glucan on collagen biosynthesis was indirect, i.e., that glucan stimulated macrophage release of wound growth factors which modulated fibroblast collagen biosynthesis (5, 6, 33). While this was a reasonable hypothesis the data did not preclude a direct interaction of the ligand with fibroblasts. Second, fibroblasts are known to respond to microbial recognition patterns, such as lipopolysaccharide (LPS) (29, 38, 39, 46) and lipoteichoic acid (38). Recently, Killcullen et al. (16) have reported that local application of *Staphylococcus aureus* peptidoglycan increases wound collagen accumulation. van Tol et al. have reported that bacterial peptidoglycan will directly stimulate in vitro expression of collagen α1 (I) and cytokine mRNA from rodent intestinal myofibroblasts (41). Finally, fibroblasts have been reported to express Toll-like receptors, which are crucial signal transducing molecules for LPS, lipoteichoic acid, and other microbial recognition patterns (39, 46). Taken together these data indicate that fibroblasts can recognize and, in some cases, directly respond to macromolecular structures from microbes.

Here, we report the existence of specific glucan binding sites on NHDF. Interaction of fungal glucan with fibroblast membrane receptors increases NF-κB activity and proinflammatory cytokine gene expression. This is the first report of pattern recognition receptors for glucans on cells other than leukocytes.

MATERIALS AND METHODS

Carbohydrate polymers. Water-insoluble (1→3)-β-D-glucan was extracted from *Saccharomyces cerevisiae* (49). Insoluble glucan was converted to a water-soluble form as described by Williams et al. (49) and chemically characterized as previously described (11, 19, 24, 25, 49). The final product was stored (−80°C) as a lyophilized powder. It was dissolved in aqueous medium and filter sterilized (0.45-μm-pore-size filter) prior to use. Laminarin is a low-molecular-weight (7,700-g/mol) (1→3)-β-D-glucan polymer which was obtained from Sigma Chemical Co. (St. Louis, Mo). Laminarin was chemically characterized as described by Mueller et al. (26). Endotoxin contamination was <0.5 endotoxin unit/mg of carbohydrate as determined by the endotoxin-specific Endospecy assay (Seigagaku, Tokyo, Japan). We used 1 μg of carbohydrate per ml in the NF-κB and IL-6 mRNA studies. Therefore, the maximum amount of endotoxin was <0.0005 endotoxin unit/μg of carbohydrate. This is well below the limit of detectability and well below the amount that would elicit a response in our assays.

DAP derivatization of glucans. Glucan phosphate (85 mg) was dissolved in 6 ml of dimethyl sulfoxide by stirring for 1 h under a nitrogen atmosphere. When the (1→3)-β-D-glucan phosphate was dissolved, 400 ml (355 mg, 4.8 mmol) of 1,3-diaminopropane (DAP) was added. The solution was stirred under nitrogen, and 35 mg of sodium cyanoborohydride (0.56 mmol) was added. After an additional 12 h at room temperature, 6 ml of water was added and the reaction mixture was dialyzed against ultrapure water using a 1,000-molecular-weight-cutoff membrane. The lyophilized material was stored at −20°C for later use.

NHDF cell line. NHDF were obtained by Clonetics (Ogden, Utah) and maintained in tissue culture with Dulbecco modified Eagle medium (Mediatech, Washington, D.C.) containing 9% heat-inactivated calf serum, 1% heat-inactivated fetal bovine serum (HyClone Corp., Logan, Utah), and 0.2% (vol/vol) penicillin-streptomycin (Sigma Chemical Co.). The cells were grown as an adherent culture at 37°C and 5% CO_2 tension in a humidified environment and were harvested according to the sonication protocol described below.

Isolation of NHDF membranes. Cells were harvested during the logarithmic phase of growth, centrifuged at 500 × *g* for 10 min, counted, centrifuged again at 500 × *g* for 10 min, 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 10^6 cells. The solution was maintained at 4°C and sonicated three times at 35% power for 30 s (Sonic Dismembrator; Fisher Scientific, Pittsburgh, Pa.). The samples were centrifuged at 650 × *g* for 10 min at 4°C to spin out nuclei. The pellet was resuspended, sonicated, and centrifuged. The combined supernatants were centrifuged at 435,000 × *g* for 30 min at 4°C. The pellet containing NHDF membranes was suspended in Hanks balanced salt solution and assayed using the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin standards. Aliquots of the membranes containing 1 mg of protein/ml were stored in liquid nitrogen for later use.

Binding assays. Binding assays were performed using a BIAcore 2000 surface plasmon resonance instrument (Biacore, Piscataway, N.J.). This technology measures changes in mass as resonance units (picograms per square millimeter) at a biosensor surface. We immobilized glucan phosphate on the biosensor and examined its interactions with membrane proteins and inhibition of the protein-glucan interaction by the carbohydrates glucan and laminarin. 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% 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 of between 8.6 and 8.9 with 1 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 μl per min. The sensor surface was first activated by exposure for 6 min to a freshly prepared solution of 100 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 25 mM *N*-hydroxysuccinimide. The surface was then exposed to DAP-glucan (3 mg/ml) for 7 min. This cycle of activation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide–25 mM *N*-hydroxysuccinimide and exposure to DAP-glucan phosphate was repeated five times. Immobilization of DAP-glucan phosphate was typically about 2,000 resonance units (RU), or 2 ng/mm^2. After immobilization, the biosensor surface was exposed to 1 M ethanolamine (pH 8.5) for 6 min to inactivate any remaining succinoylated carboxyl groups. The BIAcore biosensor contains four flow cells; DAP-glucan was immobilized on three flow cells, while the fourth flow cell served as a control for nonspecific binding to the dextran surface.

Saturation experiment using the CM-5 sensor chip. Membranes prepared from NHDF were suspended at concentrations of 0.012 to 12 μg/ml in HEPES buffer in siliconized vials. A continuous buffer flow of 25 μl/min was used to establish a baseline measurement of RU. Assays were performed at 37°C to approximate physiologic conditions. NHDF membranes were injected for 3 min, followed by continuous flow of HEPES buffer for 3 min to allow dissociation of membranes from the carbohydrate surface. At the end of each cycle, the surface was regenerated by consecutive 1-min exposures to Triton X-100 (0.3%) and guanidine hydrochloride (3 M) at 100 μl/min. Regeneration resulted in displacement of ≥90% of the added RU from the surface.

The BIAcore sensorgram displays the interactions between immobilized DAP-glucan and membrane receptors in real time. During the initial flow of buffer, a baseline is established. When the surface is exposed to NHDF membranes, there is an initial rapid change in the sensorgram due to the change in refractive index of the solution. This occurs in each flow cell, including the control which lacks DAP-glucan. This bulk shift is followed by a concentration-dependent interaction between DAP-glucan and membrane proteins. When the membrane preparation is replaced with buffer flowing over the DAP-glucan surface, there is an equal but opposite bulk shift, followed by dissociation of membrane from the DAP-glucan surface.

Glucan phosphate competition studies. A CM-5 chip onto which DAP-glucan was attached was utilized for the competition experiments. For competition experiments, samples containing a fixed concentration of NHDF membranes (10 μg/ml) in the absence and presence of competitor were alternately injected. NHDF membranes were mixed with competing carbohydrates for at least 1 h prior to injection on the BIAcore instrument. After a 3-min dissociation, the surface of the chip was regenerated with 0.3% Triton X-100 and 3 M guanidine hydrochloride at 100 μl/min for 1 min (two times). The control sample was then injected (20 μl/min, 3 min), followed by a new regeneration cycle.

Electrophoretic mobility shift assays. We employed the gel shift and supershift assays to assess the activation of NF-κB as well as the specificity of binding and the contribution of NF-κB components (p50 and p65) to the activity (2, 3, 45, 47, 48). Briefly, double-stranded consensus binding site oligonucleotides for NF-κB were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). The oligonucleotides were end labeled with [α-^{32}P]ATP (Amersham, Arlington Heights, Ill.) using T4 polynucleotide kinase (Promega, Madison, Wis.). Binding assays were performed in 10 μl of binding reaction mixture containing 10 μg of nuclear proteins and ^{32}P-labeled NF-κB oligonucleotides. The binding reaction mixture was incubated at room temperature for 20 min and then electrophoresed

on 4% nondenaturing polyacrylamide gels. The specificity of binding was confirmed by supershift and competition assays, which establish the specificity of the binding reaction as well as the relative contribution of p50-p65 heterodimer. To assess specificity of binding, a 10-fold excess of cold oligonucleotide was added. As an additional control, a 10-fold excess of cold oligonucleotide bearing the AP-II binding site was added to separate reaction mixtures. To assess the contribution of the NF-κB components (p50 and p65) to the activity observed, we performed a supershift assay in which antibody to p50, antibody to p65, or antibodies to both p50 and p65 were added to separate reaction mixtures. After polyacrylamide gel electrophoresis, the gels were analyzed by phosphorimaging (Bio-Rad Laboratories, Hercules, Calif.) followed by drying and exposure to Kodak X-Omat film at −70°C.

RNA isolation and reverse transcriptase PCR. Total cellular RNA was isolated from control and glucan-treated human dermal fibroblasts using the Ultraspect-II RNA isolation kit (Biotecx, Houston, Tex.). One microgram of total RNA was used for cDNA synthesis with murine leukemia virus reverse transcriptase (Perkin-Elmer Inc., Branburg, N.J.) in a 20-μl final volume. The cDNA synthesis reaction was for 15 min at 42°C and 5 min at 99°C. The reaction mixture (2 μl) was subjected to PCR amplification in a mixture (25 μl) that contained a 1 μM concentration of each of two primers, 1.5 mM $MgCl_2$, a 0.2 mM concentration of each of four deoxynucleotides, and 1.25 U of *Taq* polymerase (Perkin-Elmer Inc.). The upstream primer for human IL-6 was AACTCCTTCTCCAC AAGCG. The downstream primer for human IL-6 was TGGACTGCAGGAA CTCCTT. PCR amplification of IL-6 cDNA was performed under the following conditions: 35 cycles of 45 s at 94°C, 35 s at 54°C, and 45 s at 72°C. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was employed as the gene transcript control. The PCR data were imaged and quantified by computer-assisted densitometry and referenced to the gene transcript control.

NF-κB and IL-6 protocol. NHDF were incubated with glucan phosphate (1 μg/ml) for various periods of time. NHDF incubated in medium alone served as the control. At each time point, nuclear protein was harvested from control or glucan-treated NHDF. Parallel cultures of NHDF were harvested for total RNA.

Data analysis. In saturation (see Fig. 1) and competition (see Fig. 2 and 3) 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, Calif.). Since the bulk shift occurs over approximately 15 s, we estimated the amount of membrane protein bound to the surface by measuring the increase in RU at 30 s after changing from membrane to buffer exposure. For saturation experiments in which the DAP-glucan phosphate surface was exposed to various concentrations of membrane protein, RU values for each concentration of analyte were analyzed using the model $RU = \{(RU_{max} \times [analyte])/(K_D + [analyte])\} + K_{nonspecific} \times [analyte]$, where RU_{max} is the maximum binding, K_D is the apparent dissociation constant, $K_{nonspecific}$ is the constant of linear nonspecific binding, and [analyte] is the protein concentration. K_D values are also accompanied by 95% confidence intervals (CI) for the apparent K_D.

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, 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 two binding sites. The best model was chosen statistically using the sequential *F* test.

Mean values for NF-κB binding activity at time points up to 48 h (see Fig. 4) were compared by one-way analysis of variance and the least-significant-difference procedure. Probability levels of 0.05 or smaller were considered statistically significant.

RESULTS

NHDF membranes bind to a glucan-coated biosensor surface. The NHDF membranes were injected over a concentration range of 0.012 to 12 mg of protein per ml. This was followed by exposure of the surface to a continuous flow of buffer (3 min) to allow dissociation of the analyte from the ligand. The bulk shift occurs over 15 s in the control flow cell, so we chose to measure responses 30 s after the end of the analyte injection (Fig. 1). The binding response as a function of the NHDF membrane protein concentration was proportional to the immobilized DAP-glucan attached to the flow channels. The apparent K_D was 8.9 ± 1.9 μg of protein/ml, and the maximum binding was 100 ± 8 RU. Nonspecific binding was not significant at the protein concentrations which were used.

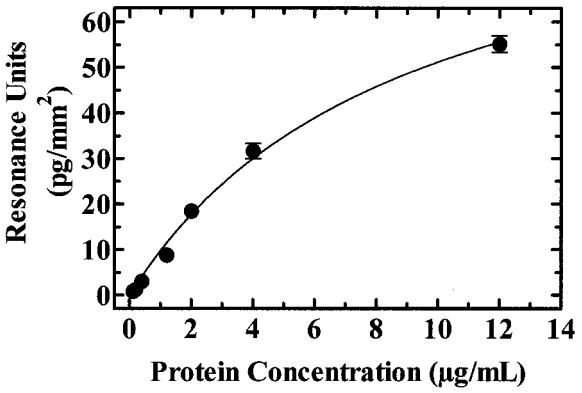

FIG. 1. Saturation curve for NHDF membrane binding to immobilized DAP-glucan, showing results from a typical experiment where averages were obtained from three flow cells and five cycles.

Glucan phosphate completely inhibits binding of NHDF to a glucan-coated sensor surface. Glucan phosphate completely inhibited the binding of NHDF membranes to a glucan phosphate biosensor surface with characteristics of a single binding site (Fig. 2). The K_D for the inhibition was 5.6 μM (95% CI, 3.0 to 11.0 μM), corresponding to a concentration of 0.88 μg of glucan phosphate per ml.

Laminarin partially inhibits binding of NHDF membranes to a glucan-coated sensor surface. Using a cell-based receptor ligand assay, we have observed that laminarin will bind to a subset of (1→3)-β-D-glucan receptors on the human U937 monocyte cell line (26). We have confirmed and extended this observation using surface plasmon resonance and U937 membranes (E. Lowe et al., submitted for publication). In the present study, laminarin partially inhibited the binding of NHDF membranes to the glucan phosphate biosensor surface with characteristics of a single binding site (Fig. 3). Laminarin could inhibit only 69% ± 6% of the binding of NHDF membranes to glucan phosphate. The K_D for the inhibition by lami-

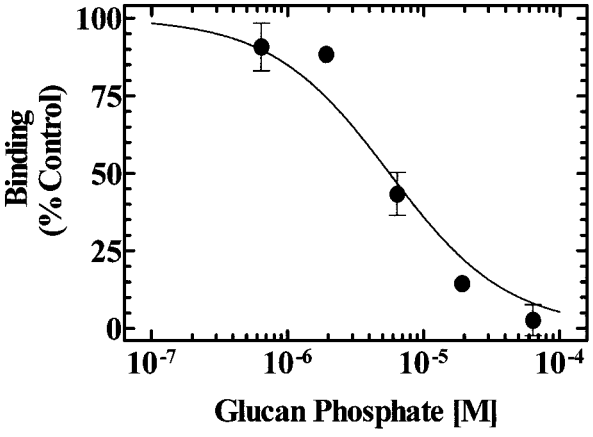

FIG. 2. Glucan phosphate competition for NHDF binding to immobilized DAP-glucan phosphate. The displacement relationship was characteristic of a single binding site with a K_D of 5.6 μM (95% CI, 3 to 11 μM).

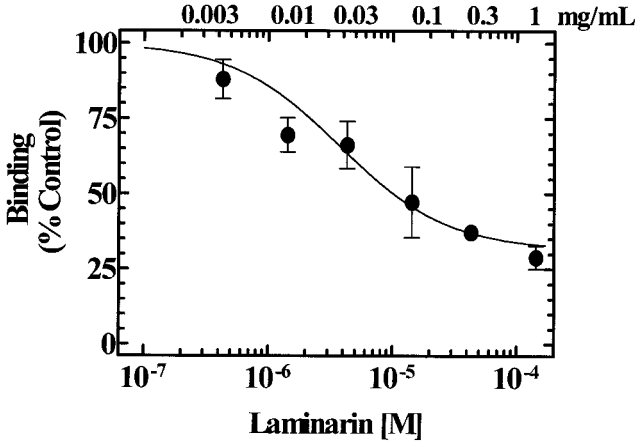

FIG. 3. Laminarin competition for NHDF binding to immobilized DAP-glucan phosphate. The displacement relationship was characteristic of a single binding site with a K_D of 3.7 μM (95% CI, 1.9 to 7.3 μM) and a maximum displacement of 69% ± 6%. The failure of laminarin to completely inhibit the interaction of NHDF and immobilized DAP-glucan phosphate suggests the presence of two types of binding interactions, one of which is not inhibitable by laminarin.

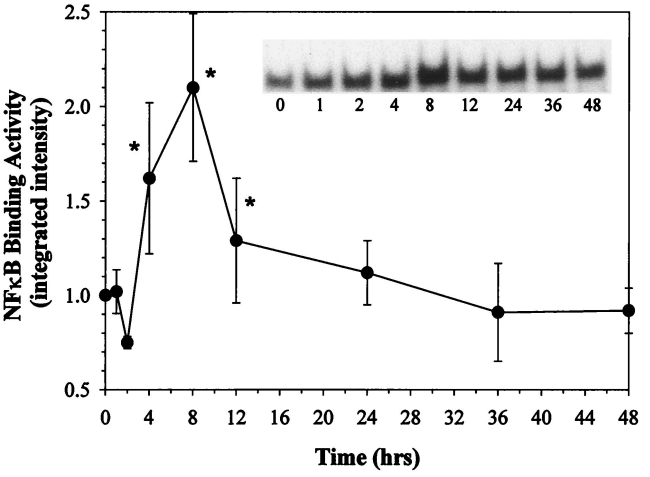

FIG. 4. Treatment of human fibroblasts with glucan phosphate (1 μg/ml) results in a time-dependent increase in NF-κB nuclear binding activity. The data are shown as a graph of normalized integrated intensity. A representative gel shift assay is shown in the inset. The density (integrated intensity) of the band is directly proportional to the degree of NF-κB nuclear binding and activity. The time is given below each lane in the gel. The gels are quantified by computer-assisted scanning density, and the data are presented as means ± standard errors, with an n value of 3 per time point. The data are normalized to the zero time point value, which was set at 1.0. *, $P < 0.05$.

narin was 3.7 μM (95% CI, 1.9 to 7.3 μM), corresponding to a concentration of 0.028 μg/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 NHDF membranes and glucan phosphate. Laminarin is able to distinguish and inhibit the interaction at one of the sites, while glucan phosphate interacts with both sites.

Coincubation with glucan phosphate increases NHDF NF-κB activity and IL-6 mRNA expression. Glucan stimulated NF-κB nuclear binding activity and IL-6 mRNA expression in a time-dependent manner. NF-κB was activated at 4 (102%), 8 (131%), and 12 (85%) h (Fig. 4). NDHF were treated for 8 h with glucan phosphate (1 μg/ml). Reverse transcriptase PCR data indicate that IL-6 mRNA levels were increased by 48% at 8 h (Fig. 5).

DISCUSSION

A number of significant observations have emerged from this study. First and foremost, we found that NDHF express membrane receptors for (1→3)-β-D-glucans. To the best of our knowledge, this is the first report of glucan-specific receptors on cells other than leukocytes. Second, interaction of glucan with membrane receptors on NDHF activates NF-κB, an important intracellular signaling pathway which is associated with regulation of cytokine and chemokine gene expression. This is consistent with previous studies which have shown that glucans stimulate transcription factor activation in macrophages and neutrophils (1–3). Third, glucan receptor interactions in NHDF resulted in increased IL-6 mRNA expression. Previous studies have reported increased cytokine gene upregulation in macrophages treated with glucans (13, 18, 31). However, other studies have reported activation of immunocyte NF-κB by glucans without cytokine gene upregulation (45), while still other investigators have reported cytokine downregulation in response to glucan and proinflammatory stimuli (36, 47, 48). It is not clear whether this relates to the glucan used, the cytokines examined, the cells or tissues studied, or other factors.

We observed that binding of glucan by NHDF membranes was saturable, dose dependent, and specific. Competition for this interaction by glucan phosphate was complete and had a K_D of 5.6 μM (95% CI, 3.0 to 11.0 μM). This is very similar to the K_D of 5.2 μM (95% CI, 0.8 to 7.1 μM) for the human U937 promonocytic cell line (unpublished observation). Interestingly, laminarin competition for this interaction was able to

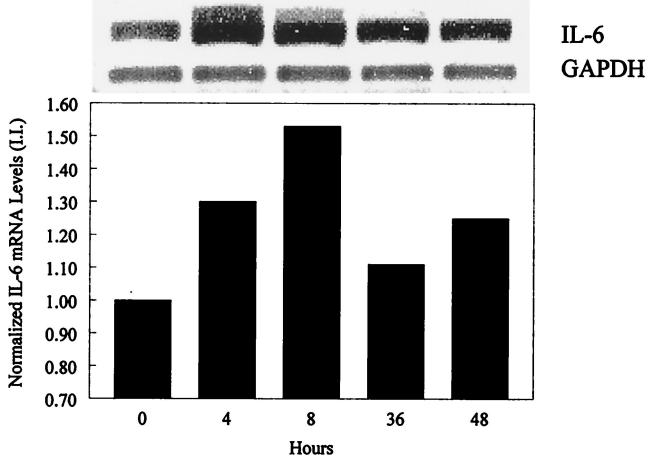

FIG. 5. Time-dependent increase in IL-6 mRNA levels in NHDF treated with glucan phosphate (1 mg/ml). A representative IL-6 PCR product gel is shown above the graph. The gels were imaged, and the integrated intensity (I.I.) was determined. The IL-6 data were normalized to the GAPDH transcript control. The data are presented as normalized integrated intensity.

displace a maximum of 69% ± 6% of the interaction, with a K_D of 3.7 μM (95% CI, 1.9 to 7.3 μM). Laminarin is equipotent with glucan phosphate based on molar concentration, but its lower molecular weight makes it more potent (28 versus 880 ng/ml). The fact that laminarin cannot completely inhibit the interaction of NHDF membranes with immobilized glucan phosphate suggests the presence of at least two different binding sites for glucan phosphate on NHDF membranes. Laminarin selectively interacts with one site, while glucan phosphate interacts with both sites. We have reported similar results using the human U937 promonocytic cell line, where laminarin displaced 61% ± 4% of binding, with a K_D of 2.6 μM (95% CI, 1.7 to 4.2 μM), and glucan phosphate completely inhibited binding in a dose-dependent manner (Lowe et al., submitted). The similarity in the affinities of these interactions in human promonocytes and fibroblasts suggests that similar receptors are present in both cell types. The data also strengthen the contention that there are multiple binding sites for glucans.

While there are numerous reports that monocytes, 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 et al. (7), Thornton et al. (40), and Vetvicka et al. (43) have reported that the type 3 complement receptor (CR3 [also known as CD11b or 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 (40, 43, 44). Duan et al. (8), Di Renzo et al. (7), and Vetvicka et al. (43, 44) have reported a β-glucan binding lectin on NK cells which contributes to NK cell-mediated cytotoxicity. Zimmerman et al. reported that lactosylceramide binds PGG-glucan (a proprietary glucan) and that this glycosphingolipid may be a leukocyte glucan binding moiety (52). Dushkin et al. (9) and Vereschagin et al. (42) 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 which stimulate intracellular signaling pathways culminating in the activation, translocation, and nuclear binding of immunoregulatory and proinflammatory transcriptional activator proteins (3, 26). Our data suggest that neither of these sites is CR3 (26). Michalek et al. have extended this observation by reporting that PGG-glucan also binds to a site distinct from CR3 (22). Whether PGG-glucan and the glucans described in this study bind to the same site(s) is not known. CR3 is a β2 integrin which is leukocyte restricted (35), and it is involved in the recognition of microbial molecular patterns, such as LPS (32). However, fibroblasts have not been reported to express CR3, and thus the binding and functional activation of fibroblasts by glucan cannot be attributed to a CR3-dependent mechanism. This does not diminish the potential importance of CR3 as a leukocyte binding moiety for glucans; rather, it reinforces the notion that there are multiple glucan binding sites and it indicates that glucan receptors are not sequestered solely in leukocytes, suggesting that these receptors may be more widespread than previously thought. Whether there are glucan receptors on cells other than fibroblasts, macrophages, neutrophils, and NK cells remains to be established. In addition, it is not clear whether the two binding sites which we have identified on human monocytes and fibroblasts activate the same or different signaling pathways within the cell. We are currently investigating both of these questions.

In conclusion, we have identified at least two specific glucan binding sites on NHDF. The interaction of the glucan ligand with NHDF 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 cells other than leukocytes. The potential ramifications of these data are significant because they force us to reexamine the current hypotheses regarding the mechanisms by which the host recognizes and responds to these fungal cell wall carbohydrates. By way of example, glucans have been reported to exert a plethora of nonspecific effects on immune function (4, 50). The presumed mechanism was that glucans interact with leukocytes and other elements of innate immunity, resulting in either a primed or activated state (1, 12, 36). The systemic effects were attributed to release of proinflammatory and/or immunoregulatory mediators which serve as second messengers; i.e., the systemic effects were indirect. While this is a reasonable explanation for the observed effects, the present data suggest that glucans may also directly interact with and modify the functional state of cells such as fibroblasts. Since fibroblasts are present in many organ systems, it is reasonable to speculate that some of the nonspecific effects which have been ascribed to glucans may be more direct than previously thought.

ACKNOWLEDGMENTS

This material is based on work supported by an Office of Research and Development, Department of Veterans Affairs, Merit Review grant to I.W.B. This work was also supported, in part, by Public Health Service grant GM53522 from the National Institute of General Medical Sciences to D.L.W.

REFERENCES

1. **Adams, D. S., R. Nathans, S. C. Pero, A. Sen, and E. Wakshull.** 2000. Activation of a Rel-A/CEBP-beta-related transcription factor heteromer by PGG-glucan in a murine monocytic cell line. J. Cell. Biochem. **77:**221–233.
2. **Adams, D. S., S. C. Pero, J. B. Petro, R. Nathans, W. M. Mackin, and E. Wakshull.** 1997. PGG-glucan activates NF-κB-like and NF-IL-6-like transcription factor complexes in a murine monocytic cell line. J. Leukoc. Biol. **62:**865–873.
3. **Battle, J., T. Ha, C. Li, V. Della Beffa, P. Rice, J. Kalbfleisch, W. Browder, and D. Williams.** 1998. Ligand binding to the (1→3)-beta-D-glucan receptor stimulates NFκB activation, but not apoptosis in U937 cells. Biochem. Biophys. Res. Commun. **249:**499–504.
4. **Bohn, J. A., and J. N. BeMiller.** 1995. (1→3)-Beta-D-glucans as biological response modifiers: a review of structure-functional activity relationships. Carbohydr. Polym. **28:**3–14.
5. **Browder, W., D. Williams, P. Lucore, H. Pretus, E. Jones, and R. McNamee.** 1988. Effect of enhanced macrophage function on early wound healing. Surgery **104:**224–230.
6. **Compton, R., D. Williams, and W. Browder.** 1996. The beneficial effect of enhanced macrophage function on the healing of bowel anastomoses. Am. Surg. **62:**14–18.
7. **Di Renzo, L., E. Yefenof, and E. Klein.** 1991. The function of human NK cells is enhanced by β-glucan, a ligand of CR3 (CD11b/CD18). Eur. J. Immunol. **21:**1755–1758.
8. **Duan, X., M. Ackerly, E. Vivier, and P. Anderson.** 1994. Evidence for involvement of beta-glucan-binding cell surface lectins in human natural killer cell function. Cell. Immunol. **157:**393–402.
9. **Dushkin, I. M., A. F. Safina, E. I. Vereschagin, and Y. S. Schwartz.** 1996. Carboxymethylated beta-1,3-glucan inhibits the binding and degradation of acetylated low density lipoproteins in macrophages in vitro and modulates their plasma clearance in vivo. Cell Biochem. Funct. **14:**209–217.
10. **Engstad, R. E., and B. Robertsen.** 1994. Specificity of a beta-glucan receptor on macrophages from Atlantic salmon (Salmo salar L.). Dev. Comp. Immunol. **18:**397–408.
11. **Ensley, H. E., B. Tobias, H. A. Pretus, R. B. McNamee, E. L. Jones, I. W. Browder, and D. L. Williams.** 1994. NMR spectral analysis of a water-

insoluble (1–3)-β-D-glucan isolated from *Saccharomyces cerevisiae*. Carbohydr. Res. **258:**307–311.
12. **Falch, B. H., T. Espevik, L. Ryan, and B. T. Stokke.** 2000. The cytokine stimulating activity of (1→3)-β-D-glucans is dependent on the triple helix conformation. Carbohydr. Res. **329:**587–596.
13. **Hirata, N., A. Tsuzkui, N. Ohno, M. Saita, Y. Adachi, and T. Yadomae.** 1998. Cytokine synthesis of human monocytes stimulated by triple or single helical conformer of an antitumour (1→3)-beta-D-glucan preparation, sonifilan. Zentbl. Bakteriol. **288:**403–413.
14. **Hiyoshi, M., S. Tagawa, S. Hashimoto, C. Sakamoto, and N. Tatsumi.** 1999. Evaluation of a new laboratory test measuring plasma (1→3)-beta-D-glucan in the diagnosis of Candida deep mycosis: comparison with a serologic test. Kansenshogaku Zasshi **73:**1–6.
15. **Janeway, C. A., Jr., and R. Medzhitov.** 1999. Lipoproteins take their Toll on the host. Curr. Biol. **9:**R879–R882.
16. **Killcullen, J. K., Q. P. Ly, T. H. Chang, S. M. Levenson, and J. J. Steinberg.** 1998. Nonviable *Staphylococcus aureus* and its peptidoglycan stimulate macrophage recruitment, angiogenesis, fibroplasia and collagen accumulation in wounded rats. Wound Repair Regen. **6:**149–156.
17. **Lee, S. Y., R. Wang, and K. Soderhall.** 2000. A lipopolysaccharide- and beta-1,3-glucan-binding protein from hemocytes of the freshwater crayfish Pacifastacus leniusculus. Purification, characterization, and cDNA cloning. J. Biol. Chem. **275:**1337–1343.
18. **Ljungman, A. G., P. Leanderson, and C. Tagesson.** 1998. (1→3)-Beta-D-glucan stimulates nitric oxide generation and cytokine mRNA expression in macrophages. Environ. Toxicol. Pharmacol. **5:**273–281.
19. **Lowman, D., H. Ensley, and D. Williams.** 1998. Identification of phosphate substitution sites by NMR spectroscopy in a water-soluble phosphorylated (1→3)-β-D-glucan. Carbohydr. Res. **306:**559–562.
20. **Medzhitov, R., and C. A. Janeway.** 1998. Self-defense: the fruit fly style. Proc. Natl. Acad. Sci. USA **95:**429–430.
21. **Medzhitov, R., and C. A. Janeway.** 2000. An ancient system of host defense. Curr. Opin. Immunol. **10:**12–15.
22. **Michalek, M., D. Melican, D. Brunke-Reese, M. Langevin, K. Lemerise, W. Galbraith, M. Patchen, and W. Mackin.** 1998. Activation of rat macrophages by betafectin PGG-glucan requires cross-linking of membrane receptors distinct from complement receptor three (CR3). J. Leukoc. Biol. **64:**337–344.
23. **Mori, T., H. Ikemoto, M. Matsumura, M. Yoshida, K. Inada, S. Endo, A. Ito, S. Watanabe, H. Yamaguchi, M. Mitsuya, M. Kodama, T. Tani, T. Yokota, T. Kobayashi, J. Kambayashi, T. Nakamura, T. Masaoka, H. Teshima, T. Yoshinaga, S. Kohno, K. Hara, and S. Miyazaki.** 1997. Evaluation of plasma (1→3)-beta-D-glucan measurement by the kinetic turbidimetric Limulus test, for the clinical diagnosis of mycotic infections. Eur. J. Clin. Chem. Biochem. **35:**553–560.
24. **Mueller, A., W. Mayberry, R. Acuff, S. Thedford, W. Browder, and D. Williams.** 1994. Lipid content of macroparticulate (1→3)-beta-D-glucan isolated from *Saccharomyces cerevisiae*. Microbios **79:**253–261.
25. **Mueller, A., H. Pretus, R. McNamee, E. Jones, I. Browder, and D. Williams.** 1995. Comparison of the carbohydrate biological response modifiers Krestin, schizophyllan and glucan phosphate by aqueous size exclusion chromatography with in-line argon-ion multi-angle laser light scattering photometry and differential viscometry detectors. J. Chromatogr. B **666:**283–290.
26. **Mueller, A., J. Raptis, P. J. Rice, J. H. Kalbfleisch, R. D. Stout, H. E. Ensley, W. Browder, and D. L. Williams.** 2000. The influence of glucan polymer structure and solution conformation on binding to (1→3)-β-D-glucan receptors in a human monocyte-like cell line. Glycobiology **10:**339–346.
27. **Mueller, A., P. J. Rice, H. E. Ensley, P. S. Coogan, J. H. Kalbfleisch, J. L. Kelley, E. J. Love, C. A. Portera, T. Ha, I. W. Browder, and D. L. Williams.** 1996. Receptor binding and internalization of water-soluble (1→3)-beta-D-glucan biologic response modifier in two monocyte/macrophage cell lines. J. Immunol. **156:**3418–3425.
28. **Nakamura, Y., S. Tazawa, and M. Tsutiya.** 1998. The clinical significance of plasma (1→3)-beta-D-glucan measurement by the kinetic turbidimetric Limulus test for fungal febrile episodes. Rinsho Biseibutshu Jinsoku Shindan Kenkyukai Shi **9:**33–39.
29. **Nemoto, E., S. Sugawara, H. Tada, H. Takada, H. Shimauchi, and H. Horiuchi.** 2000. Cleavage of CD14 on human gingival fibroblasts cocultured with activated neutrophils is mediated by human leukocyte elastase resulting in down-regulation of lipopolysaccharide-induced IL-8 production. J. Immunol. **165:**5807–5813.
30. **Ochiai, M., and M. Ashida.** 2000. A pattern-recognition protein for beta-1,3-glucan. The binding domain and the cDNA cloning of beta-1,3-glucan recognition protein from the silkworm, *Bombyx mori*. J. Biol. Chem. **275:**4995–5002.
31. **Okazaki, M., Y. Adachi, N. Ohno, and T. Yadomae.** 1995. Structure-activity relationship of (1→3)-β-D-glucans in the induction of cytokine production from macrophages in vitro. Biol. Pharm. Bull. **18:**1320–1327.
32. **Perera, P. Y., T. N. Mayadas, O. Takeuchi, S. Akira, M. Zaks-Zilberman, S. M. Goyert, and S. N. Vogel.** 2001. CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TOR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression. J. Immunol. **166:**574–581.
33. **Portera, C. A., E. J. Love, L. Memore, L. Zhang, A. Mueller, W. Browder, and D. L. Williams.** 1997. Effect of macrophage stimulation on collagen biosynthesis in the healing wound. Am. Surg. **63:**125–131.
34. **Ross, G. D., J. A. Cain, B. L. Myones, S. L. Newman, and P. J. Lachmann.** 1987. Specificity of membrane complement receptor type three (CR3) for β-glucans. Complement **4:**61–74.
35. **Ross, G. D., and V. Vetvicka.** 1993. CR3 (CD11b, CD18): a phagocyte and NK cell membrane receptor with multiple ligand specificities and functions. Clin. Exp. Immunol. **92:**181–184.
36. **Soltys, J., and M. T. Quinn.** 1999. Modulation of endotoxin- and enterotoxin-induced cytokine release by in vivo treatment with beta-(1,6)-branched beta-(1,3)-glucan. Infect. Immun. **67:**244–252.
37. **Stone, B. A., and A. E. Clarke.** 1992. Chemistry and biology of (1→3)-β-glucan, p. 283–363. La Trobe University Press, Melbourne, Australia.
38. **Sugawara, S., R. Arakaki, H. Rikiishi, and H. Takada.** 1999. Lipoteichoic acid acts as an antagonist and an agonist of lipopolysaccharide on human gingival fibroblasts and monocytes in a CD14-dependent manner. Infect. Immun. **67:**1623–1632.
39. **Tabeta, K., K. Yamazaki, S. Akashi, K. Miyake, H. Kumada, T. Umemoto, and H. Yoshie.** 2000. Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. Infect. Immun. **68:**3731–3735.
40. **Thornton, B. P., V. Vetvicka, M. Pitman, R. C. Goldman, and G. D. Ross.** 1996. Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). J. Immunol. **156:**1235–1246.
41. **van Tol, E. A. F., L. Holt, F. L. Li, F. M. Kong, T. Rippe, M. Yamauchi, J. Pucilowska, P. K. Lund, and R. B. Sartor.** 1999. Bacterial cell wall polymers promote intestinal fibrosis by direct stimulation of myofibroblasts. Am. J. Physiol. **277:**G245–G255.
42. **Vereschagin, E. I., A. A. Van Lambalgen, M. I. Dushkin, Y. S. Schwartz, L. Polyakov, A. Heemskerk, E. Huisman, L. G. Thijs, and G. C. Van den Bos.** 1998. Soluble glucan protects against endotoxin shock in the rat: the role of the scavenger receptor. Shock **9:**193–198.
43. **Vetvicka, V., B. P. Thornton, and G. D. Ross.** 1996. Soluble beta-glucan polysaccharide binding to the lectin site of neutrophil or natural killer cell complement receptor type 3 (CD11b/CD18) generates a primed state of the receptor capable of mediating cytotoxicity of iC3b-opsonized target cells. J. Clin. Invest. **98:**50–61.
44. **Vetvicka, V., B. P. Thornton, J. Wieman, and G. D. Ross.** 1997. Targeting of natural killer cells to mammary carcinoma via naturally occurring tumor cell-bound iC3b and beta-glucan-primed CR3. J. Immunol. **159:**599–605.
45. **Wakshull, E., D. Brunke-Reese, J. Lindermuth, L. Fisette, R. S. Nathans, J. J. Crowley, J. C. Tufts, J. Zimmerman, W. Mackin, and D. S. Adams.** 1999. PGG-glucan, a soluble beta-(1,3)-glucan, enhances the oxidative burst response, microbicidal activity, and activates an NF-kappaB-like factor in human PMN: evidence for a glycosphingolipid beta-(1,3)-glucan receptor. Immunopharmacology **41:**89–107.
46. **Wang, P. L., Y. Azuma, M. Shinohara, and K. Ohura.** 2000. Toll-like receptor 4-mediated signal pathway induced by *Porphyromonas gingivalis* lipopolysaccharide in human gingival fibroblasts. Biochem. Biophys. Res. Commun. **273:**1161–1167.
47. **Williams, D. L., T. Ha, C. Li, J. H. Kalbfleisch, J. J. Laffan, and D. A. Ferguson.** 1999. Inhibiting early activation of tissue nuclear factor-κB and nuclear factor interleukin 6 with (1→3)-β-D-glucan increases long-term survival in polymicrobial sepsis. Surgery **126:**54–65.
48. **Williams, D. L., T. Ha, C. Li, J. Laffan, J. Kalbfleisch, and W. Browder.** 2000. Inhibition of LPS induced NFκB activation by a glucan ligand involves down regulation of IKKβ kinase activity and altered phosphorylation and degradation of IκBα. Shock **13:**446–452.
49. **Williams, D. L., R. B. McNamee, E. L. Jones, H. A. Pretus, H. E. Ensley, I. W. Browder, and N. R. Di Luzio.** 1991. A method for the solubilization of a (1→3)-β-D-glucan isolated from *Saccharomyces cerevisiae*. Carbohydr. Res. **219:**203–213.
50. **Williams, D. L., A. Mueller, and W. Browder.** 1996. Glucan-based macrophage stimulators: a review of their anti-infective potential. Clin. Immunother. **5:**392–399.
51. **Williams, D. L., H. A. Pretus, R. B. McNamee, E. L. Jones, H. E. Ensley, I. W. Browder, and N. R. Di Luzio.** 1991. Development, physicochemical characterization and preclinical efficacy evaluation of a water soluble glucan sulfate derived from *Saccharomyces cerevisiae*. Immunopharmacology **22:**139–156.
52. **Zimmerman, J. W., J. Lindermuth, P. A. Fish, G. P. Palace, T. T. Stevenson, and D. E. DeMong.** 1998. A novel carbohydrate-glycosphingolipid interaction between a beta-(1–3)-glucan immunomodulator, PGG-glucan, and lactosylceramide of human leukocytes. J. Biol. Chem. **273:**22014–22020.

Editor: T. R. Kozel