Oral Delivery and Gastrointestinal Absorption of Soluble Glucans Stimulate Increased Resistance to Infectious Challenge


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ABSTRACT

Glucans are immunomodulatory carbohydrates found in the cell walls of fungi and certain bacteria. We examined the pharmacokinetics of three water-soluble glucans (glucan phosphate, laminarin, and scleroglucan) after oral administration of 1 mg/kg doses in rats. Maximum plasma concentrations for glucan phosphate occurred at 4 h. In contrast, laminarin and scleroglucan showed two plasma peaks between 0.5 and 12 h. At 24 h, 27% of the glucan phosphate and 20% of the laminarin remained in the serum. Scleroglucan was rapidly absorbed and eliminated. The liver did not significantly contribute to the clearance of plasma glucan. Biological effects were further studied in mice. Following oral administration of 1 mg, glucans were bound and internalized by intestinal epithelial cells and gut-associated lymphoid tissue (GALT) cells. Internalization of glucan by intestinal epithelial cells was not Dectin-dependent. GALT expression of Dectin-1 and toll-like receptor (TLR) 2, but not TLR4, increased following oral administration of glucan. Oral glucan increased systemic levels of interleukin (IL)-12 (151% in mice. Oral glucan administration also increased survival in mice challenged with Staphylococcus aureus or Candida albicans. These data demonstrate that orally administered water-soluble glucans translocate from the gastrointestinal (GI) tract into the systemic circulation. The glucans are bound by GI epithelial and GALT cells, and they modulate the expression of pattern recognition receptors in the GALT, increase IL-12 expression, and induce protection against infectious challenge.

Glucans are glucose polymers that are major constituents of the cell wall of fungi and certain bacteria. These carbohydrate polymers are evolutionarily conserved in the cell wall of fungi and a limited number of bacteria (Williams, 1997). Glucans are FDA-approved for cholesterol lowering, in purified form, have been shown to stimulate innate immunity (Williams, 1997), stimulate antitumor responses (Cheung et al., 2002; Hong et al., 2004), increase resistance to infections (Williams et al., 1996), and promote wound repair (Wei et al., 2002). Glucans are bound and internalized by pattern recognition receptors on neutrophils, macrophages, dendritic cells, NK cells, and a subset of T cells (Brown and Gordon, 2003; Williams et al., 2003). Recent evidence points to the existence of multiple glucan pattern recognition receptors, including Dectin-1, class A scavenger receptors, and the Type 3 complement receptor (Brown and Gordon, 2003; Williams et al., 2003). Receptors for glucans have also been identified on cells outside the immune system including epithelial cells (Ahren et al., 2001), vascular endothelial cells (Lowe et al., 2002), fibroblasts (Kougias et al., 2001), and anterior pi-

ABBREVIATIONS: FDA, Food and Drug Administration; IL, interleukin; GI, gastrointestinal; DAP, diaminopropane; PBS, phosphate-buffered saline; GALT, gut-associated lymphoid tissue; FITC, fluorescein isothiocyanate; TLR, toll-like receptor.
tuitary cells (Breuel et al., 2004). Thus, glucan receptors seem to be widely distributed throughout the body.

Although there have been numerous reports concerning the biological activity of systemically (i.v. or i.p.) administered soluble or insoluble glucans (Williams et al., 1996) over the past 25 years, there have been very few studies on the pharmacokinetics of glucans (Yoshida et al., 1997; Rice et al., 2004). We recently reported that the pharmacokinetics of systemically administered (i.v.) soluble glucan favored higher blood levels of glucans with certain physicochemical properties (Rice et al., 2004).

There have been no reports on the absorption and pharmacokinetics of orally administered soluble glucans, even though there are several reports indicating that orally administered glucans exert biological effects. Hong et al. (2004), Cheung et al. (2002), and colleagues have reported that orally administered glucans function as potent antitumor adjuvants when combined with antibodies that recognize tumor epitopes (Hong et al., 2004). Suzuki et al. (1989) reported that a branched water-soluble glucan increased splenocyte mitogenic response and NK cell activity following five daily oral administrations. In addition, these investigators reported that oral supplementation with glucan inhibited tumor growth of syngeneic Meth A fibrosarcoma, IMC carcinoma, and Lewis lung carcinoma (Suzuki et al., 1989). In another study, Suzuki et al. (1990) reported that oral administration of glucan to CDF1 mice increased peritoneal macrophage phagocytic activity as denoted by increased acid phosphatase activity, increased phagocytic activity, increased killing of Candida, and increased interleukin (IL)-1 production (Suzuki et al., 1990). These investigators also reported the oral efficacy of glucan in C3H/HeJ mice that are endotxin hyporesponsive, indicating the effect is not due to increased uptake of endotoxin from the gut following glucan dietary supplementation (Suzuki et al., 1989, 1990). Nicoletti et al. (1992) have reported that a branched glucan derived from Candida albicans exerted immunoadjuvant activity following oral administration. Oral glucans are FDA-approved for lowering serum cholesterol. They also decrease postprandial glucose surge in type II diabetes (Braaten et al., 1994; Ensley et al., 1994; Wu et al., 1998), and scleroglucan because the chemistry and biology of these water-soluble oligomers have been extensively studied (Mueller et al., 2000). These glucans vary according to molecular weight distribution, polydispersity, side chain branching, root mean square radius, and solution conformation (Mueller et al., 2000). By employing these three glucan ligands, we were able to establish that soluble glucans with very different physicochemical properties are absorbed from the GI tract into the systemic circulation where they exert biological effects.

### Materials and Methods

**Experimental Animals.** Age- and weight-matched male virus-free Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Age and weight-matched male virus-free ICR/Hsd mice were obtained from Harlan. The experimental animals were maintained on standard lab chow and water ad libitum but were fasted 12 h prior to experimentation. All animal procedures were reviewed and approved by the Animal Care and Use Committee at the James H. Quillen College of Medicine, East Tennessee State University.

**Glucans.** (1→3)-β-D-Glucan phosphate was prepared and chemically characterized as previously described by Williams et al. (1991), Ensley et al. (1994), and Kim et al. (2000). Scleroglucan, a β-(1→3),(1→6)-glucan, was prepared and chemically characterized as previously described (Pretus et al., 1991). Laminarin, a β-(1→3),(1→6)-glucan, was purchased from Sigma-Aldrich (St. Louis, MO) and was chemically characterized as previously described (Mueller et al., 2000). A comparison of the physicochemical characteristics of the glucan polymers is given in Table 1. The polymers were screened for endotoxin contamination with the Endospecy assay (Seikagaku/Cape Cod Associates, Falmouth, MA) according to manufacturer’s instructions. Endotoxin levels were ≤0.3 IU/mg.

**Preparation of Fluorescently Labeled Glucans.** A diaminopropane (DAP) moiety was added to the reducing terminus of each glucan by the method of Kougias et al. (2001). Briefly, the DAP was attached to the reducing terminus of the carbohydrate polymer by sodium borohydride reduction. The reaction mixture was diazoyed

### Table 1

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Molecular Mass (M_w)</th>
<th>Polydispersity I (M_p/M_w)</th>
<th>Radius of the Center of Gravity (r_g)</th>
<th>Intrinsic Viscosity (η)</th>
<th>V</th>
<th>α^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan phosphate</td>
<td>0.122 g/mol</td>
<td>1.57 × 10^3</td>
<td>1.67</td>
<td>20.3</td>
<td>0.33</td>
<td>0.302</td>
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<tr>
<td>Laminarin</td>
<td>0.164 g/mol</td>
<td>7.70 × 10^1</td>
<td>0.07</td>
<td>0.07</td>
<td>0.62</td>
<td>1.820</td>
</tr>
<tr>
<td>Scleroglucan</td>
<td>0.140 g/mol</td>
<td>1.02 × 10^9</td>
<td>3.21</td>
<td>35.4</td>
<td>1.08</td>
<td>1.820</td>
</tr>
</tbody>
</table>

dn/dc, refractive index increment; V, slope of the linear relationship between the log of the root-mean-square radius and log of the molecular mass moment (R_g = K_v × M^V) as described by Mueller et al. (2000); α, slope of the linear relationship between log intrinsic viscosity and log molecular mass ([η] = K_a × M^α) is known as the Mark-Houwink or α value for a polymer system (Braaten et al., 1984; Mueller et al., 2000); N.D., not detectable.

^2^H NMR analysis confirmed the primary structure.

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Wu et al. (1998) reported that dietary supplementation with protein-bound glucan did not enhance immune function, suggesting that the complex of glucan and protein may not be a suitable supplement. Unfortunately, details about the chemical nature and/or purity of the glucans employed in many of these studies were not provided. In addition, no data were presented that addressed the oral absorption and pharmacokinetics of the carbohydrates.

Clearly, there is a need to better understand the oral absorption and clearance of glucans. Therefore, we examined the absorption, pharmacokinetics, and biological effects of three water-soluble glucans that were administered by oral gavage to rodents. We employed glucan phosphate, laminarin, and scleroglucan because the chemistry and biology of these water-soluble oligomers have been extensively studied (Mueller et al., 2000). These glucans vary according to molecular weight distribution, polydispersity, side chain branching, root mean square radius, and solution conformation (Mueller et al., 2000). By employing these three glucan ligands, we were able to establish that soluble glucans with very different physicochemical properties are absorbed from the GI tract into the systemic circulation where they exert biological effects.
against 18-MF ultrapure pyrogen-free water and lyophilized to dryness. The glucan was stored at −20°C. Aliquots of the DAP-glucan derivatives were analyzed by gel permeation chromatography/multiangle laser light scattering, 1H NMR, and 13C NMR to confirm that the molecular weight, polydispersity, primary structure, and solution conformation were not altered by the derivatization.

The carbohydrates were labeled with Alexa Fluor 488 succinimidyl ester (catalog no. A-20000; Molecular Probes, Eugene, OR). Briefly, the amine reactive AlexaFlour 488 succinimidyl ester (Pierce Chemical, Rockford, IL) was attached to the DAP moiety located at the reducing terminus. Specifically, DAP-glucan (3 mg) was dissolved into sodium borate buffer (0.1 M, pH 8.5) at a concentration of 10 μg/μl in a total volume of 300 μl. In the case of particulate glucan, the insoluble DAP glucan was suspended in borate buffer, and the suspension was maintained by gentle agitation. Alexa Fluor 488 dye (5 mg) was dissolved in 35 μl of dimethyl sulfoxide. The dye solution was added to the DAP-glucan and incubated for 1 h in the dark, followed by addition of 300 μl of sodium borate buffer. The mixture was incubated overnight at ambient temperature on a reciprocating shaker at slow speed. The samples were incubated in foil-covered microtuge tubes to prevent exposure to light. The excess dye was removed by dialyzing (1000 molecular weight cutoff) against PBS overnight at room temperature. The advantage to this approach is that a single DAP and a single fluorescent moiety are attached to the reducing terminus of the polymer without changing the basic physicochemical characteristics of the carbohydrate ligand. Addition of the DAP and fluorescent tag does not alter binding of the glucan polymer to recombinant Dectin (data not shown). The labeling efficiency (90%) was determined by flow cytometric analysis using recombinant Dectin-1.

**Experimental Protocol for Pharmacokinetic Studies.** In the pharmacokinetic studies, Sprague-Dawley rats were sedated using carbon dioxide, while approximately 2 mm of the tail was removed to obtain blood samples. The fluorescently labeled carbohydrates were orally administered using a 1 mg/ml solution in water at a dose 1 mg/kg. Blood samples were collected in 30-μl Fisherbrand Micro-Hematocrit Capillary Tubes (catalog no. 22-362-566; Fisher Scientific Co. (Pittsburgh, PA)), centrifuged using a Readacrit centrifuge (Clay Adams, Parsippany, NJ), then stored in deproteinized tubes. Samples were stored at 0°C (control), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after oral administration. Samples were maintained at 4°C prior to analysis. For studies on hepatic uptake of glucans, rats received 1 mg/kg of glucan orally, and paired sterile blood samples were drawn from the vena cava and hepatic portal vein at 4 h.

**Detection of Fluorescent Glucan in Serum Samples.** Fluorescent glucan in serum samples was detected and quantified with a FLUOstar® Galaxy Plate Reader (Biotechnology and Life Sciences, Maarssen, Netherlands) at ambient temperature. Samples, standards (0.1–10000 μg/ml) with plasma from control animals, and diluent controls were pipetted into COSTAR 96-well plates with black side walls and analyzed on a FLUOstar® Galaxy Plate Reader using an excitation filter of 485 nm and an emission filter of 520 nm.

**Preparation of Gastrointestinal Epithelial Cells and Gut-Associated Lymphoid Tissue Cells.** Murine gastrointestinal epithelial cells were isolated from the mucosa of rinsed intestinal segments with a cell scraper and suspended in PBS. Gut-associated lymphoid tissue (GALT) samples were harvested from the intestine by excision of Peyer’s patch nodules and dispersed into a single-cell suspension in PBS.

**Experimental Protocol for Detection of Dectin Expression and Glucan Binding to Intestinal Epithelial Cells and GALT.** Mice were gavaged with 1 mg/0.2 ml glucan phosphate, scleroglucan, or laminarin in PBS. PBS served as control. The mice were sacrificed 24 h after treatment, and GALT and small intestinal epithelial cells were harvested. The cells were screened for the presence of Dectin-1 by flow cytometry with anti-Dectin (2a.11) antibody (Brown et al., 2003).

In the glucan binding studies, epithelial and GALT cells isolated from mice that were not gavaged with glucan were washed in PBS and incubated with Alexa-fluor-labeled soluble glucan (1 μg/ml) at 4°C for 30 min. Cells were washed three times with PBS to removed unlabeled glucan and analyzed by flow cytometry.

**Flow Cytometric Analysis of Dectin Expression on Gut Epithelial Cells.** Intestinal epithelial cells (1 × 10^6), isolated as described above, were suspended in 100 μl of fluorescein isothiocyanate (FITC)-labeled 2a.11 antibody or isotype control antibody (BD Biosciences Pharmingen, San Diego, CA) diluted in BD Biosciences Pharmingen Stain Buffer. The cells were incubated on ice for 30 min, washed 2× with cold PBS, and suspended in stain buffer for analysis. Cells were analyzed on a FACScalibur flow cytometer with CellQuest software (BD Biosciences, San Jose, CA).

**Antibodies Employed for Phenotyping of GALT Cells.** Biotin-conjugated anti-Dectin antibody (2a11) was provided by Dr. Gordon Brown (Oxford University, Oxford, UK). FITC-conjugated anti-neutrophil antibody and its isotype control antibody were purchased from Serotec (Oxford, UK). Monocyte/macrophages were detected with allopurinol cyanin anti-F4/80 and its isotype control antibody (Caltag Laboratories, Burlingame, CA). Dendritic cells were identified with FITC-conjugated anti-CD11c (BD Biosciences Pharmingen), and T cells were identified with peridinin chlorophyll-a protein-conjugated anti-CD3 (BD Biosciences Pharmingen). NK cells were identified allopurinol cyanin-conjugated anti-NK1.1 (BD Biosciences Pharmingen). Isotype control antibodies were also purchased from BD Biosciences Pharmingen.

**Flow Cytometric Analysis of GALT Cells.** Red blood cells were lysed in all samples using PharmLyse buffer (BD Biosciences Pharmingen) according to the manufacturer’s directions. Cells were blocked with 5% rabbit serum, 0.5% bovine serum albumin, and 5 mM EDTA with anti-murine CD16/32 (BD Biosciences Pharmingen) prior to staining. Staining was performed according to conventional protocols at 4°C. Biotinylated antibodies were detected by streptavidin-PE (BD Biosciences Pharmingen). Cells were stained in BD Biosciences Pharmingen Stain buffer and analyzed using a FACScalibur flow cytometer with CellQuest software (BD Biosciences).

**Luminex Assay.** Blood was collected into serum separator microtainer tubes (BD Biosciences), centrifuged at 2000 rpm, and the serum harvested and stored at −80°C until analysis. Serum samples were diluted 1:2 with PBS prior to assay. The samples were assayed using the Biosource Mouse Multiplex 10 cytokine kit (BioSource International, Camarillo, CA) that included granulocyte monocyte colony-stimulating factor, interferon α, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, and tumor necrosis factor-α according to the manufacturer’s instructions. The samples were analyzed on a Luminex 100 (Luminex Corporation, Austin, TX). Cytokine concentrations were established with MasterPlex QT software version 1.2.8.58 (MiraBio, Inc., Alameda, CA).

**Confocal Imaging of Murine Intestinal Epithelial Cells Treated with Fluorescently Labeled Glucans.** The small intestine was harvested from mice, and ingesta was removed by flushing with PBS. The bowel was opened, and the epithelial cells were recovered by scraping into RPMI 1640 media supplemented with antibiotics. The cells were pelleted and then suspended in PBS. Remaining ingesta was allowed to settle out, and the cell-rich supernatant was collected. The cells were pelleted, suspended in media, counted, and plated in a six-well plate at 500,000 cells per well. After 24 h in culture, cells were treated with 100 μg/ml glucan phosphate labeled with Alexa Fluor 488 (Molecular Probes) for 30 min at 37°C and 5% CO₂. Cells were then washed 3× by scraping, fixed with 4% paraformaldehyde, counterstained with propidium iodide, and mounted onto a slide with Prolong Anti-Fade mounting medium (Molecular Probes). The cells were visualized using a Leica DM IRBE inverted microscope with the Leica TC2 SP2 confocal microscopy system (Leica, Wetzlar, Germany). The images were converted using Leica confocal software.

**Infection Studies.** Male ICR/HS (10/group) mice were gavaged with glucan phosphate (1 mg/mouse) in PBS 1 day prior to i.v.
challenge with $1 \times 10^6$ *Staphylococcus aureus* or $1 \times 10^5$ *C. albicans*. *S. aureus* and *C. albicans* were cultured and quantitated by the Clinical Microbiology Laboratory at East Tennessee State University. Mice were followed for survival. Mice that were moribund were euthanized, and the time of euthanasia was recorded as the survival time.

**Data Analysis.** In the glucan assays, standards were analyzed by linear regression, and sample concentrations were interpolated using Prism 3.0 (GraphPad Software Inc., San Diego, CA). Pharmacokinetic data were plotted as concentration versus time and analyzed using established noncompartmental methods. Bioavailability was determined as the ratio of the area under the glucan serum concentration versus time curve for oral versus i.v. administration. The i.v. pharmacokinetics of soluble glucan have been published (Rice et al., 2004). Survival trends were compared with the log-rank Wilcoxon nonparametric procedure. Flow cytometric binding data were summarized by the mean and S.E.M. Group mean responses were compared by analysis of variance and pair-wise multiple comparison testing (the least significant difference procedure or Tukey’s procedure for cases where analysis of variance was not significant). Probability levels of 0.05 or smaller were considered significant.

**Results**

**Pharmacokinetics and Bioavailability of Orally Administered Glucans.** Oral administration of the three soluble glucans studied produced measurable plasma levels. Plasma levels were normalized based on the maximum carbohydrate plasma level ($C_{\text{max}}$) during the experiment (Fig. 1; Table 2). Peak plasma levels for glucan phosphate occurred at 4 h after oral administration and gradually declined to 27 $\pm$ 3% of the maximum plasma level after 24 h. Given the short half-life for glucan phosphate (3.8 $\pm$ 0.8 h) following i.v. administration (Rice et al., 2004), these results suggest sustained absorption of the carbohydrate following oral glucan administration. Laminarin showed a biphasic response with two peak plasma concentrations occurring at 3 h and again at 12 h (Fig. 1). Plasma laminarin was 20 $\pm$ 7% of maximum at 24 h. Since the laminarin peak at 12 h followed both further food intake and nocturnal activity, the increased absorption of laminarin may be associated with either additional gastrointestinal activity or additional physical activity or both. Scleroglucan plasma levels increased rapidly following oral administration and showed peak concentrations at 15 min and 3 h. In contrast to glucan phosphate and laminarin, scleroglucan was essentially cleared from the systemic circulation after 12 h. There were undetectable levels of glucan following oral administration of a water-insoluble, particulate glucan preparation (data not shown), indicating that only soluble glucans are present in plasma. Hong et al. (2004) have reported that particulate glucans are subject to phagocytosis and transport in macrophages and so may be absent from plasma.

Comparison of the area under the plasma concentration versus time curve allowed us to estimate the oral absorption of each glucan (Table 2). Bioavailability was higher for the neutral polysaccharides, laminarin and scleroglucan, than for soluble glucan phosphate, a polyelectrolyte. Bioavailability for laminarin and scleroglucan were 4.9 and 4.0%, respectively. However, bioavailability for glucan phosphate was only 0.5% (Table 2).

We also determined whether glucans were removed from the blood by the liver in a first pass effect using the portal-systemic concentration difference. Blood was harvested from the portal vein and the inferior vena cava after oral administration of labeled glucan. Hepatic uptake of glucans was studied in rats receiving soluble glucan (1 mg/kg) administered orally. Glucan concentrations in central venous blood at 4 h were only 15% lower (not significant) than concentrations in the portal circulation, suggesting that at this dosage hepatic uptake does not significantly contribute to removal of glucan from the systemic circulation.

**Binding of Labeled Glucans by Cells Isolated from Peyer’s Patches.** Flow cytometric analysis of GALT cells, isolated from Peyer's patches, confirmed that oral administration of fluorescent carbohydrates resulted in the presence of fluorescence in the GALT cells isolated from Peyer's patches after 24 h (Fig. 2). This demonstrates that GALT cells are capable of recognizing and binding glucans. Cells

![Fig. 1. Pharmacokinetics of glucan phosphate, laminarin, and scleroglucan following a single oral administration in rats. The pharmacokinetic profiles for laminarin and scleroglucan revealed two plasma peaks, whereas glucan phosphate showed a single peak. At 24 h, both glucan phosphate and laminarin were present in the systemic circulation, albeit at low concentrations. Scleroglucan was rapidly cleared from the circulation. n = 4/group.](image-url)
administration of soluble glucan phosphate (1,289 pg/ml; p < 0.016). Interestingly, IL-6 was elevated at 8 h following oral administration of water-insoluble, particulate glucans. Interleukin-12 was elevated 24 h following oral administration of water-insoluble particulate glucans, but modest increases in systemic levels of IL-6 and IL-10 were also observed. Systemic administration of glucans has been reported to modulate systemic cytokine levels (Engstad et al., 2002). We screened sera from mice that had been treated with oral glucan for 10 immunoregulatory and/or pro-inflammatory cytokines. Orally administered glucans resulted in significant, but modest, increases in systemic levels of IL-6 and IL-10. Interleukin-12 was elevated 24 h following oral administration of soluble glucan phosphate (299 ± 30 versus 198 ± 37 pg/ml; p < 0.016). Interestingly, IL-6 was elevated at 8 h following oral administration of water-insoluble, particulate glucan (110 ± 20 versus 72 ± 5 pg/ml; p < 0.012) even though there was no evidence for oral absorption of the insoluble preparation. No changes were observed in serum levels of IL-1β, IL-2, IL-4, IL-5, IL-10, tumor necrosis factor-α, IFNγ, or granulocyte monocyte-colony-stimulating factor.

Oral Administration of Glucans Increased Dectin-1 and TLR2 Expression in GALT Cells. Dectin-1 expression was increased in GALT cells, isolated from Peyer’s patches, by oral administration of laminarin, glucan phosphate, and scleroglucan (Table 3). Phenotypic analysis of GALT cells from oral glucan-treated mice revealed that the increase in Dectin-1 expression was solely due to increased macrophage Dectin-1 expression. TLR2 expression was increased in dendritic cells isolated from Peyer’s patches following oral administration of laminarin, glucan phosphate, or scleroglucan. TLR4 levels did not change in response to glucans (data not shown). We also observed that oral glucan administration resulted in a nonsignificant increase in GALT-associated CD3+ T cells and a slight decrease in neutrophils (data not shown).

Oral Administration of Soluble Glucan Increased Long-Term Survival in Mice Challenged with S. aureus or C. albicans. Oral gavage with glucan phosphate 1 day prior to i.v. challenge with S. aureus resulted in increased long-term survival (Fig. 4). Oral administration of a soluble glucan resulted in a 50% long-term survival (p < 0.05). In contrast, control mice showed 100% mortality by day 6.

Oral gavage with glucan phosphate 1 day prior to i.v. challenge with C. albicans resulted in increased long-term survival (Fig. 5). Oral administration of a soluble glucan

**Fig. 2.** Oral administration of glucans increases percentage of Dectin-1-positive cells in the GALT. GALT cells were harvested 24 h after gavage, stained with anti-Dectin-1 antibody, and analyzed by flow cytometry. This representative histogram shows Dectin fluorescence for an isotype control, control, and the three glucans studied. Control cells (unstained) were stained with isotype control antibody and cells from glucan-treated mice stained with anti-dectin 2a11 antibody. Cells from control animals that did not receive glucan did not exhibit fluorescence.

**Intestinal Epithelial Cells Bind and Internalize Soluble Glucans through a Dectin-Independent Mechanism.** Flow cytometric analysis of murine intestinal epithelial cells failed to show the presence of Dectin-1 receptors (data not shown). However, intestinal epithelial cells were able to actively uptake and internalize a fluorescently labeled soluble glucan (Fig. 3). Approximately 10% of the epithelial cells incorporated glucan, even though cell viability was quite high. This suggests that only a subpopulation of intestinal cells binds glucans, but these cells do not express Dectin-1. We concluded that the glucan is intracellular rather than bound to the cell membrane because glucans were present in the same plane as the nuclei in cells imaged by confocal microscopy, and the glucan was distributed throughout the cytoplasm rather than forming a ring around the cells.

**Cytokine Expression after Oral Glucan Administration.** Systemic administration of glucans has been reported to modulate systemic cytokine levels (Engstad et al., 2002). We screened sera from mice that had been treated with oral glucan for 10 immunoregulatory and/or pro-inflammatory cytokines. Orally administered glucans resulted in significant, but modest, increases in systemic levels of IL-6 and IL-12. Interleukin-12 was elevated 24 h following oral administration of soluble glucan phosphate (299 ± 30 versus 198 ± 37 pg/ml; p < 0.016). Interestingly, IL-6 was elevated at 8 h following oral administration of water-insoluble, particulate glucan (110 ± 20 versus 72 ± 5 pg/ml; p < 0.012) even though there was no evidence for oral absorption of the insoluble preparation. No changes were observed in serum levels of IL-1β, IL-2, IL-4, IL-5, IL-10, tumor necrosis factor-α, IFNγ, or granulocyte monocyte-colony-stimulating factor.

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**Fig. 3.** Uptake and internalization of fluorescently labeled glucan phosphate by murine gastrointestinal epithelial cells. Epithelial cells were harvested from murine small intestine and incubated with labeled glucan phosphate (1 µg/ml) for 24 h. The cells were harvested and examined on a confocal microscope. Approximately 10% of the total cell population actively internalized the labeled glucan. The glucan is seen as green. The nucleus is stained red.

**TABLE 2**

<table>
<thead>
<tr>
<th>Glucan</th>
<th>AUC i.v.*</th>
<th>AUC p.o.</th>
<th>Bioavailability</th>
<th>C_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg × h/ml</td>
<td></td>
<td>%</td>
<td>ng/ml</td>
</tr>
<tr>
<td>Glucan phosphate</td>
<td>26.0 ± 3.4</td>
<td>0.120 ± 0.038</td>
<td>0.5</td>
<td>41.5 ± 3.4</td>
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<tr>
<td>Laminarin</td>
<td>10.8 ± 1.2</td>
<td>0.521 ± 0.147</td>
<td>4.9</td>
<td>115 ± 33</td>
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<tr>
<td>Scleroglucan</td>
<td>9.5 ± 0.8</td>
<td>0.381 ± 0.229</td>
<td>4.0</td>
<td>355 ± 281</td>
</tr>
</tbody>
</table>

* Area under the curve (AUC) i.v. values from Rice et al. (2004). n = 5 to 6, mean ± S.E.M.

* AUC p.o., oral administration of glucans. n = 9 (glucan phosphate), 5 (laminarin), and 5 (scleroglucan); mean ± S.E.M.
Mice were gavaged with glucan phosphate (1 mg/mouse) 24 h prior to i.v. challenge with 10^5 S. aureus (p < 0.01). Mice were gavaged with glucan phosphate (1 mg/mouse) at time 0. The mice were sacrificed 24 h after treatment, and GALT cells were harvested. 

### TABLE 3

Increased Dectin-1 and TLR2 expression in murine GALT cells following oral administration of water-soluble glucans

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Laminarin</th>
<th>Glucan Phosphate</th>
<th>Scleroglucan</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percentage of Dectin-positive</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Total</td>
<td>38.2 ± 1.8</td>
<td>71.8 ± 9.1a</td>
<td>76.9 ± 3.1a</td>
<td>66.0 ± 1.6a</td>
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<td>Dendritic cells (CD3+)</td>
<td>91.7 ± 2.2</td>
<td>94.9 ± 2.9</td>
<td>98.3 ± 0.4</td>
<td>96.2 ± 2.0</td>
</tr>
<tr>
<td>Lymphocytes (CD11+)</td>
<td>70.2 ± 4.8</td>
<td>59.9 ± 20.4</td>
<td>70.5 ± 15.0</td>
<td>52.6 ± 21.1</td>
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<tr>
<td>PMN</td>
<td>95.5 ± 0.7</td>
<td>80.9 ± 7.3</td>
<td>83.6 ± 7.5</td>
<td>95.2 ± 3.3</td>
</tr>
<tr>
<td>Macrophages (F4/80+)</td>
<td>67.6 ± 5.2</td>
<td>90.2 ± 5.0a</td>
<td>91.2 ± 4.5a</td>
<td>98.7 ± 0.7a</td>
</tr>
<tr>
<td><strong>Percentage of TLR2-positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>41.8 ± 3.0</td>
<td>47.2 ± 9.5</td>
<td>52.5 ± 1.1</td>
<td>51.6 ± 5.6</td>
</tr>
<tr>
<td>Dendritic cells (CD3+)</td>
<td>75.3 ± 3.7</td>
<td>98.6 ± 0.8a</td>
<td>98.8 ± 0.2a</td>
<td>98.8 ± 0.8a</td>
</tr>
<tr>
<td>Lymphocytes (CD11+)</td>
<td>13.7 ± 2.6</td>
<td>24.5 ± 3.8</td>
<td>36.1 ± 12.3</td>
<td>45.2 ± 24.2</td>
</tr>
<tr>
<td>PMN</td>
<td>89.7 ± 15.8</td>
<td>78.2 ± 1.0</td>
<td>70.6 ± 3.8</td>
<td>70.9 ± 8.8</td>
</tr>
<tr>
<td>Macrophages (F4/80+)</td>
<td>66.5 ± 10.3</td>
<td>82.3 ± 5.1</td>
<td>72.6 ± 12.7</td>
<td>81.7 ± 2.1</td>
</tr>
</tbody>
</table>

*p < 0.05 versus control.

**Fig. 4.** Oral administration of water-soluble glucan phosphate increased long-term survival of ICR/HSD mice challenged with S. aureus (p < 0.01). Mice were gavaged with glucan phosphate (1 mg/mouse) 24 h prior to i.v. challenge with 10^8 S. aureus. n = 10/group.

**Fig. 5.** Oral administration of water-soluble glucan phosphate increased long-term survival of ICR/HSD mice challenged with C. albicans (p < 0.01). Mice were gavaged with glucan phosphate (1 mg/mouse) 24 h prior to i.v. challenge with 10^6 C. albicans. n = 10/group.

resulted in a 40% long-term survival (p < 0.05). In contrast, control mice showed 100% mortality by day 6.

**Discussion**

The present study demonstrates that soluble glucans can translocate from the GI to the systemic circulation. The three soluble glucans studied were each absorbed after oral administration despite substantial differences in molecular weights, branching frequencies, root-mean-square radii, and solution conformations. Although the liver contains a large number of macrophages (i.e., Kupffer cells) that have been reported to recognize glucans (Suda et al., 1996), we found an insignificant (15%) difference in plasma concentration in the central and portal circulations at the time of peak concentration.

There were significant differences in oral pharmacokinetics between the glucans. Glucan phosphate had 10-fold lower bioavailability than laminarin and scleroglucan. This may be influenced by carbohydrate charge because glucan phosphate is a polyelectrolyte, whereas the other carbohydrates are negatively charged. Absorption is clearly more complex, though. For laminarin and glucan phosphate, approximately 25% of the maximum plasma level was present in the plasma 24 h after oral administration. Since these glucans are eliminated more rapidly following i.v. administration (Rice et al., 2004), this result suggests that glucan phosphate and laminarin are slowly absorbed following oral administration. Scleroglucan, the largest of the polymers studied, was completely eliminated by 24 h. It is not clear why the largest molecular weight carbohydrate was absorbed most rapidly, but this result could reflect preferential absorption early in intestinal transit. Hashimoto et al. (1991) have suggested that large molecular weight glucans are taken up by the Microfold cells in areas of the intestine, where they interact with the GALT. Our data support an active GI uptake mechanism for glucans. We observed that a subpopulation (~10%) of murine intestinal epithelial cells were capable of actively uptaking and internalizing a fluorescently labeled soluble glucan (Fig. 2). We have not been able to establish the identity of these epithelial cells, but it is possible that they are Microfold cells. Hong et al. (2004) speculated that uptake and transport of glucans by GI macrophages was Dectin-dependent. Saegusa et al. (2004) have reported that zymosan and S. cerevisiae glucan increase Dectin-1 mRNA expression in the intestinal epithelial-like Caco-2 cells. Our data demonstrate the presence of Dectin-1-positive cells in the GALT, but we also observed that intestinal epithelial cells uptake and internalize glucan even though we could not detect Dectin-1 expression. Thus, the movement of glucan from the GI tract into the systemic circulation may not be solely dependent upon Dectin-1. We also observed that laminarin and scleroglucan had two peak plasma levels, whereas glucan phosphate showed a single peak plasma level. At present, we do not know the precise reason for this difference in plasma concentration. It is possible that neutral glucan polymers are preferentially absorbed at multiple sites along the GI tract, thus accounting for multiple peaks. It is also possible that...
Glucans produce an array of biological effects following oral administration. Although changes in cytokine expression were modest and selective for individual carbohydrates, it is noteworthy that oral administration of soluble glucan resulted in increased serum IL-12, an immunomodulatory and pro-inflammatory cytokine. Interestingly, particulate glucan increased serum levels of the pro-inflammatory cytokine IL-6, even though particulate glucan was not found in the plasma following oral administration. Hong et al. (2004) have reported that particulate glucans are internalized by macrophages, which transport glucan to various sites throughout the body and slowly degrade the particulate and release a bioactive soluble glucan product. The interaction of particulate glucan with macrophages, which are known to release IL-6, may account for the changes we observed in IL-6 levels. It was interesting to note that oral soluble glucan did not stimulate IL-6 production, whereas oral administration of water-insoluble glucan did not stimulate IL-12 production. The physical state of the carbohydrate has a significant effect on the biological activity observed.

We also observed that oral glucan increased Dectin-1 and TLR2 expression but not TLR4 expression on GALT cells isolated from Peyer’s patches. This is surprising given the report by Herre et al. (2004) that indicates that Dectin-1 is rapidly internalized, thus removing Dectin-1 from the cell surface. Furthermore, the nature of the glucan determined whether Dectin-1 was recycled to the cell surface (Herre et al., 2004). In this study, we found that GALT macrophages showed increased Dectin-1, whereas dendritic cells showed increased TLR2, at 24 h following oral gavage. This may represent a glucan-induced increase in innate immune surveillance at the interface between the GI tract and the body.

Until this time, there has been little evidence that oral administration of glucan would increase resistance to infection in mammals (Dritz et al., 1995). Indeed, Dritz et al. (1995) have reported that dietary supplementation with particulate glucan actually increased susceptibility to infection. To address this issue, we evaluated the effect of oral soluble glucan in two murine models of infection. C. albicans is a fungal pathogen that expresses glucan in its cell wall. S. aureus is a bacterial pathogen that does not produce glucan. Previous studies have shown that i.v. administration of soluble glucans will increase resistance to S. aureus (Williams and Di Luzio, 1980) or C. albicans (Williams et al., 1978). Oral administration of soluble glucan but not insoluble glucan (D. L. Williams, unpublished data) increased long-term survival in fungal and bacterial sepsis, demonstrating that GI uptake of glucans is sufficient to protect from subsequent exposure to fungal and bacterial pathogens.

Our results may have important ramifications for several aspects of glucan biology. There are numerous studies that have proposed the use of glucan and related biopolymers as pharmaceuticals for the treatment of experimental infections, tumors, and other diseases (Williams and Browder, 1994). The vast majority of those studies have involved administration of glucans via the i.v., i.p., s.c., or i.m. routes (Williams and Browder, 1994). The uptake and biological effects of orally administered glucans have been highly controversial (Wu et al., 1998). There are some glucan preparations that have been purported to work via the oral route (Suzuki et al., 1989; Nicoletti et al., 1992), but until recently (Hong et al., 2004), the evidence for this was not compelling, and it was never clear whether the activity was due to interaction with cells in the GI tract or whether the carbohydrate entered the bloodstream to mediate its biological effects. Our data demonstrate that highly purified soluble glucans interact with a variety of GI cells, enter the systemic circulation, and persist in the plasma up to 24 h after a single oral administration. These data may be particularly significant in light of the recent studies by Cheung et al. (2002), Hong et al. (2004), and colleagues indicating that oral glucans are potent antitumor adjuvants, when combined with antitumor antibodies. Soluble glucans can now be produced in large quantity and pure form. When taken as a whole, these data suggest that it may be possible to develop an oral glucan formulation for disease prevention and/or treatment.

Another potential implication of this data concerns dietary supplements. Dietary glucans lower serum cholesterol, decrease postprandial glucose surge in type II diabetes (Braaten et al., 1994; Wuersch and Pi-Sunyer, 1997), and stimulate innate immune defenses (Suzuki et al., 1989, 1990). Our data demonstrate that highly purified, water-soluble glucans can enter the systemic circulation similar to other orally administered drugs. However, a highly purified water insoluble glucan was not present in plasma. Furthermore, the cytokine profiles elicited by soluble and insoluble oral glucan preparations were strikingly different, and the effect of soluble versus particulate glucan on infection seems to be different. This indicates that the physical state of the carbohydrate is important in absorption and eliciting specific biological effects. Our study did not address whether particulate glucans in the GI tract are internalized by cells, which then exit the GI tract.

Finally, numerous studies have demonstrated that glucans are found in the systemic circulation of patients with fungal infections (Digby et al., 2003; Gonzalez et al., 2004). Since glucans are evolutionarily conserved in microbes, it has been assumed that blood-borne glucans in the infected patient are derived entirely from pathogens (Digby et al., 2003). Consequently, serum glucan levels have been proposed as a diagnostic assay for fungal infections, and the FDA has approved a glucan-specific serum assay as an indicator of fungal sepsis. However, recent evidence indicates that serum glucan levels are elevated in patients infected by bacteria that do not produce glucans (Digby et al., 2003). Even more perplexing was the observation that normal or control patients had low (≤20 pg/ml), but detectable, levels of serum glucan (Digby et al., 2003; Gonzalez et al., 2004). Our data indicate that soluble glucans can translocate from the GI tract into the systemic circulation in normal rats. Critically ill patients frequently develop increased GI permeability, which results in movement of microbes and macromolecules from the GI tract into the systemic circulation (Brinkmann et al., 1996; Wang et al., 2004). Thus, plasma glucan in patients with bacterial infections, and the low levels of glucan found in normal individuals, may be attributable to movement of glucan from the GI tract into the blood and not necessarily to the presence of a pathogen.

In conclusion, we have demonstrated that fungal-derived soluble glucans translocate from the GI tract into the systemic circulation in normal animals. Whether this is an active or passive process remains to be fully elucidated, but it is...
clear that several cell types within the GI tract are interacting with glucans. Thus, glucans in the GI tract can serve as a reservoir for glucan absorption into the systemic circulation, and these GI-derived glucans can produce significant biological effects.

References


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