APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1996, p. 3176–3182 Vol. 62, No. 9
0099-2240/96/$04.00+0

Measurement of β(1→3)-Glucans in Occupational and Home Environments with an Inhibition Enzyme Immunoassay

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Received 7 March 1996/Accepted 19 June 1996

β(1→3)-Glucans are known for their potent ability to induce nonspecific inflammatory reactions and are believed to play a role in bioaerosol-induced respiratory symptoms. An inhibition enzyme immunoassay (EIA) was developed for the quantitation of β(1→3)-glucans in dust samples from occupational and residential environments. Immunospecific rabbit antibodies were produced by immunization with bovine serum albumin-conjugated laminarin [β(1→3)-glucan] and affinity chromatography on epoxy-Sepharose-coupled β(1→3)-glucans. The laminarin-based calibration curve in the inhibition EIA ranged from ≈40 to 3,000 ng/ml (15 to 85% inhibition). Another β(1→3)-glucan (curdlan) showed a similar inhibition curve but was three to five times less reactive on a weight basis. Pustulan, presumed to be a β(1→6)-glucan, showed a parallel dose-response curve at concentrations 10 times higher than that of laminarin. Control experiments with $NaIO_4$ and β(1→3)-glucanase treatment to destroy β(1→6)- and β(1→3)-glucan structures, respectively, indicated that the immunoreactivity of pustulan in the assay was due to β(1→3)-glucan and not to β(1→6)-glucan structures. Other polysaccharides, such as mannan and α(1→6)-glucan, did not react in the inhibition EIA. β(1→3)-Glucan extraction of dust samples in water (with mild detergent) was performed by heat treatment (120°C) because aqueous extracts obtained at room temperature did not contain detectable β(1→3)-glucan levels. The assay was shown to detect heat-extractable β(1→3)-glucan in dust samples collected in a variety of occupational and environmental settings. On the basis of duplicate analyses of dust samples, a coefficient of variation of approximately 25% was calculated. It was concluded that the new inhibition EIA offers a useful method for indoor β(1→3)-glucan exposure assessment.

Exposure to bioaerosols in indoor environments, both residential and occupational, can induce allergic, toxic, and inflammatory reactions resulting in acute and chronic respiratory symptoms. It is not clear, however, which components primarily account for the observed effects. Endotoxin, a cell wall component of gram-negative bacteria and a potent proinflammatory agent, has been recognized as an important causal factor, particularly in occupational environments. Recently, a similar role has been suggested for β(1→3)-glucans in bioaerosol-induced inflammatory responses and resulting respiratory symptoms and complaints (6, 10, 27, 33). β(1→3)-Glucans are glucose polymers with various molecular weights and degrees of branching and may originate from a large variety of sources, including most fungi and yeasts, some bacteria, most higher plants, and many lower plants. They are water-insoluble structural cell wall components of these organisms but may also be found in extracellular secretions of microbial origin. β(1→3)-Glucans can initiate a variety of biological responses in vertebrates such as host-mediated antitumor activity (16); stimulation of the reticuloendothelial system (7); activation of neutrophils (35), macrophages (2, 4, 29), and complement (28); and possibly activation of eosinophils (23).

Knowledge about airborne β(1→3)-glucan exposure as a potential respiratory health hazard is limited because of a lack of generally available methods to measure environmental β(1→3)-glucan. One relatively small population study has suggested a relationship between levels of airborne β(1→3)-glucans in office buildings and the occurrence of eye and throat irritation, dry coughing, and itching skin (27). In this study, a glucan-reactive lysate from *Limulus polyphemus* was used to quantify β(1→3)-glucans in airborne dust samples. This assay, based on the ability of β(1→3)-glucans to activate the *Limulus* coagulation system (3, 28), has only recently become commercially available and has some serious drawbacks, including its reactivity with various other polysaccharides and the high costs of the reagents required for the analyses.

There is a clear need for an improved specific, sensitive, and cost-efficient method to quantify β(1→3)-glucans which can be used in large-scale environmental hygiene and epidemiological studies. In this report, we describe a new assay which meets these requirements. An inhibition immunoassay was developed with which β(1→3)-glucans can be quantified with the use of affinity-purified rabbit anti-β(1→3)-glucan antibodies. The new assay was applied to organic dust samples from various environments, and preliminary results indicated high β(1→3)-glucan levels in house dust samples and personal dust samples from swine confinement workers and waste composting workers. We also investigated the β(1→3)-glucan contents of various plant materials and culture fluid of in vitro yeast cultures.

MATERIALS AND METHODS

Materials and reagents. Flat-bottom 96-well polystyrene microtiter plates with high binding capacity (no. 655061; Greiner, Nuertingen, Germany); bovine serum albumin (BSA) and *o*-phenylenediamine (no. P 1526; Sigma Chemical Co., St. Louis, Mo.); epoxy-activated Sepharose 6B (no. 17-0480-01) and Sephadex G-25 (Pharmacia LKB, Uppsala, Sweden); Centricon-30 microconcentrators (Amicon Inc., Beverly, Mass.); Microsep-100 centrifugal microconcentrators

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TABLE 1. β(1→3)-Glucans and related saccharides used in this study

Saccharide	Linkage(s)	Organization[a]	Source	Extraction procedure used	Reference(s)
Laminarin	β(1→3)(1→6)-Glucan	LSB	Algae	H_2O, 120°C	30, 31
Carboxymethylcurdlan	β(1→3)-Glucan	L	Bacteria	0.05 M NaOH	30, 31
Pustulan	β(1→6)-Glucan, β(1→6)(1→3)-Glucan	L	Lichen	H_2O, 120°C	12, 30
Dextran	α(1→6)(1→3)(1→4)(1→2)-Glucan	B	Bacteria	H_2O, room temp	30
Mannan	α(1→2)(1→3)(1→6)-Mannan	B	Fungi	H_2O, room temp	31

[a] L, linear; B, branched; LSB, linear with side branches.

(Filtron Technology Corp., Northborough, Mass.); peroxidase-labelled horse anti-rabbit immunoglobulin (no. M1234; Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands); Tween 20 (polyoxyethylenesorbitan monolaurate), H_2O_2, and gelatin (Merck, Darmstadt, Germany); zymolyase 100T (lot no. 109303; Seikagaku Corp., Tokyo, Japan); and BCA protein assay reagent (no. 23235; Pierce, Rockford, Ill.) were obtained from the sources indicated. All other chemicals were of analytical grade.

The glucans and other polysaccharides used in this study are described in Table 1 and were purchased from the following sources: dextran (no. D 5251) and mannan (no. M 7504), Sigma Chemical Co.; laminarin, Fluka AG, Buchs SG, Switzerland; pustulan, Calbiochem, La Jolla, Calif.; carboxymethylcurdlan, Wako Pure Chemicals Ind., Ltd., Osaka, Japan.

BSA-laminarin conjugate for immunization. Since isolated carbohydrates are weak immunogens, conjugates of β(1→3)-glucan coupled to BSA were prepared for immunization. Laminarin was dissolved (20 mg/ml) in twice-distilled water by autoclaving for 20 min at 120°C. Immunoreactivity of β(1→6)-glucans was abolished by oxidation with 0.25 M $NaIO_4$ at 20°C for 60 min (18). The reaction was stopped by desalting the solution on a Sephadex G-25 column. Conjugation of oxidized laminarin to BSA was performed by reductive amination (25). Briefly, boric acid and borax were added to obtain a borate (0.2 M)-buffered glucan solution with a pH of 9.0. Cyanoborohydride (500 mg) and BSA (340 mg) were dissolved in 25 ml of this glucan solution. The mixture was shaken for 24 h at 50°C in a thermostat-equipped incubator. Precipitated material was removed and discarded by centrifugation at 3,000 × *g*.

Buffer exchange of the glyco-BSA conjugate in phosphate-buffered saline (PBS; pH 7.0)—removing cyanoborohydride and unreacted glucans—was performed with a Microsep-100 centrifugal microconcentrator (3,000 × *g*). Conjugation of glucan with BSA was confirmed by gel electrophoresis. The glucan-BSA conjugate contained approximately 8% carbohydrate on a weight basis.

Rabbit anti-β(1→3)-glucan antibodies. Male New Zealand White rabbits (Broekman Institute, Someren, The Netherlands) were immunized by subcutaneous injection of 1 ml of the glucan conjugate solution (0.1 mg of protein per ml) mixed 1:1 with Freund's complete adjuvant. Booster injections were administered at 4- to 8-week intervals with the same amount of antigen mixed 1:1 with incomplete Freund's adjuvant. Serum was collected at 1- and 2-week intervals after each booster and stored at −20°C. The antibody titer of the serum, which amounted to 10^6, was determined by using a direct enzyme immunoassay (EIA) in which laminarin (2 μg/ml of PBS, pH 7.0) was used to coat a microtiter plate directly.

Specific antibodies were isolated by affinity chromatography by using epoxy-activated Sepharose 6B as described by Hutchins and Bussey (15). Briefly, 360 mg of β(1→3)-glucan (laminarin) was dissolved in 21 ml of twice-distilled water by autoclaving for 20 min at 120°C. After heating, NaOH was added to yield a 0.1 M solution with a pH of 12 to 13. A 9-ml volume of preswollen gel was then added, and the mixture was shaken at 37°C for 20 h. Excess ligand was washed away, and any remaining active groups were blocked by incubation of the gel overnight in 1 M ethanolamine at 42°C. The gel was stored at 7°C in PBS (pH 7.0) containing 0.05% NaN_3 and washed extensively with PBS (pH 7.0) before use.

Immunoglobulins were precipitated from pooled antiserum (100 ml) with ammonium sulfate (0.2 g/ml of serum), redissolved in PBS (pH 7.0) in one-fifth of the original volume, filtered through a 0.45-μm-pore-size filter (Millipore), and applied at a flow rate of 0.25 ml/min on the packed affinity column (1.0 by 9.5 cm). The column was washed by using PBS (pH 7.0) with 0.5 M NaCl and eluted with 0.1 M glycine HCl (pH 2.5) at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and neutralized by addition of 0.2 ml of 0.5 M Na_2HPO_4. All fractions with an optical density at 280 nm of >0.1 were pooled, dialyzed against twice-distilled water for 16 h, and concentrated with Centricon-30 microconcentrators by centrifugation at 3,000 × *g*. The affinity-purified antibodies were stored at −20°C in aliquots in PBS (pH 7.0) at a concentration of 1.5 mg of protein per ml (total volume, 11 ml).

Rabbit anti-β(1→6)-glucan antibodies. Anti-β(1→6)-glucan antibodies were raised in rabbits as described by Montijn et al. (18).

Inhibition EIA. Laminarin (2 μg/ml) in PBS (pH 7.0) was used to coat each well (200 μl per well) of a microtiter plate, which was incubated overnight at 4°C. After extensive washing for three cycles with an automatic plate washer (LKB-Pharmacia) with PBS containing 0.05% (vol/vol) Tween 20 (PBT), 300 μl of 0.5% gelatin in PBT (PBTG) was applied, the mixture was incubated at 37°C for 30 min, and the gelatin solution was discarded. A test sample or a laminarin standard (100 μl diluted in PBTG) was then added to a microwell and subsequently mixed with an equal volume of affinity-purified anti-β(1→3)-glucan antibodies diluted 1/75,000 in PBTG. The microtiter plate was then shaken at 37°C for 1.5 h. After extensive washing, 200 μl of peroxidase-labelled horse anti-rabbit immunoglobulin antibodies diluted 1/5,000 in PBTG was added and the mixture was shaken for 1 h at 37°C. After extensive washing, 200 μl of *o*-phenylenediamine (2 mg/ml) in 0.05 M citrate-phosphate buffer, pH 5.5, containing 0.015% H_2O_2 was added and the mixture was incubated for 30 min at 20°C. The enzyme reaction was terminated by addition of 50 μl of 2 N HCl, and the optical density at 492 nm was read.

On each microtiter plate, 12 dilutions of the reference laminarin preparation (9.8 ng/ml to 20 μg/ml) were included and four control wells, which contained only antibodies mixed with PBTG, were also incubated. Samples were tested in 4 different dilutions, and β(1→3)-glucan concentrations were determined by interpolating the optical density at 492 nm of each test sample dilution on a semilog calibration curve obtained with the 12 dilutions of the reference preparation and calculated with the four-parameter curve-fitting program of the SOFTmax software package (Molecular Devices Corporation, Menlo Park, Calif.). Laminarin stock solutions of 1 mg/ml of twice-distilled water were prepared by autoclaving for 15 min at 120°C (1 bar). Stock solutions were stored at 7°C and autoclaved again prior to analysis.

β(1→3)-glucanase and $NaIO_4$ treatment. Specific destruction of β(1→3)- or β(1→6)-glucan conformational structures was accomplished by treatment with specific β(1→3)-glucanase (zymolyase 100T) (31) or $NaIO_4$, respectively (18). Solutions of pustulan and laminarin (0.5 mg/ml in PBS, pH 6.5) were preincubated overnight at 45°C with zymolyase (5,000 U/ml), which was dissolved in 50% glycerol in PBS (pH 6.5). The reaction was stopped by heating at 100°C for 5 min. Periodate oxidation of pustulan and laminarin was performed by incubation for 2 h with 0.25 M $NaIO_4$ in twice-distilled water at 20°C (18). The reaction was stopped by adding 1 M ethanolamine, which also blocked the newly formed aldehyde groups. Specific rabbit anti-β(1→6)-glucan antibodies were used to confirm the efficacy of $NaIO_4$ treatment and the specificity of the β(1→3)-glucanase activity of zymolyase.

Plant samples. Extracts were made of cereals (wheat, barley, and corn flour), soy (soy beans and soy flour), tapioca, potato, and potato starch. Potato starch was obtained from a potato processing plant in The Netherlands; the other products were purchased from a local grocery shop. Because of the insolubility of most β(1→3)-glucans under neutral conditions, alternative extraction procedures, i.e., heat and alkaline treatments, were explored. Each product was suspended (1% [wt/vol]) and homogenized in twice-distilled water with addition of 0.05% Tween 20 and in 0.05 M NaOH by using an Ultrathurax (Polytron). Samples suspended in twice-distilled water with 0.05% Tween 20 were rocked vigorously for 15 min, subsequently autoclaved at 120°C (1 bar) for 1 h, and then rocked once more for 15 min. Samples suspended in 0.05 M NaOH were rocked vigorously for 2 h. Sample suspensions from both extraction procedures were centrifuged at 1,000 × *g* for 15 min, and the supernatant was collected and stored at −20°C.

Yeast samples. Cell-free yeast culture media were tested in which wild-type and various mutants of *Saccharomyces cerevisiae* FY834 (*MATα his3Δ300 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63*) (34) had been cultured. Yeasts were grown at 28°C to the early exponential phase in standard minimal medium [0.17% (wt/vol) yeast nitrogen base without amino acids and $(NH_4)_2SO_4$] with addition of 0.5% (wt/vol) $(NH_4)_2SO_4$, buffered to pH 6.0 with 50 mM 2-(*N*-morpholino)ethanesulfonic acid, with the appropriate amino acids and uracil. Cell-free medium was obtained by centrifugation for 10 min at 2,000 × *g* and subsequent filtration through a 0.22-μm-pore-size filter (Millipore). Culture medium in which no yeast had been cultured was used as a control.

Environmental dust samples. Inhalable airborne dust sampling, both area and personal, was carried out in two waste composting facilities, for 2 days in the summer of 1994 (plant 1) and for 11 days in the winter of 1994 (plant 2). Personal inhalable dust samples were collected from 10 pig farmers during spring, autumn, and winter of 1991. Personal and area sampling of inhalable dust was performed on glass fiber filters (25-mm diameter; Whatman GF/A) by using PAS-6 sampling heads at a flow rate of 2 liters/min (32). Personal sampling was performed in the workers' breathing zone during full-shift periods of 6 to 8 h. Area samples were

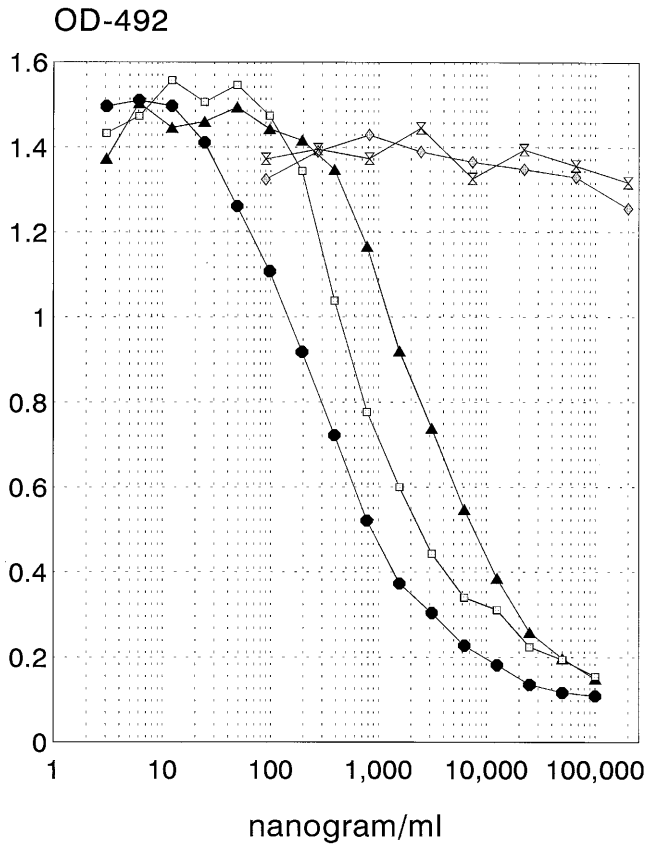

FIG. 1. Inhibition-dilution curves of four different glucans and mannan: laminarin, carboxymethylcurdlan [both β(1→3)-glucans], pustulan [β(1→6)-glucan, contaminated with β(1→3)-glucan], dextran [α(1→6)-glucan], and mannan (polymannose). Symbols: ●, laminarin □, curdlan ▲, pustulan; ⧖, mannan ◆, dextran. OD-492, optical density at 492 nm.

also collected during full-shift periods at ~1.5 m above the working floor. Filter samples were stored at −20°C until extraction. Settled-dust samples from floors of living rooms, bedrooms, and kitchens and from mattresses were collected in a series of 25 German homes on paper filters (70-mm diameter; 589 black ribbon; Schleicher & Schuell) in accordance with an internationally standardized protocol (22), as described earlier (8). As a negative control, nonorganic dust samples were also collected at a construction site. Both area and personal respirable dust sampling was performed by using Casella cyclones at a flow rate of 2 liters/min for 2- to 4-h periods.

Gravimetric measurements were performed in a preconditioned room at 18 to 22°C and 45 to 55% humidity with a Mettler AT261 analytical balance. The detection limit of this measurement was approximately 0.3 mg.

All dust samples were initially extracted for endotoxin analyses as described by Douwes et al. (8). Subsequent heat extraction to dissolve β(1→3)-glucans was performed on pellets after the first extraction resuspended in twice-distilled water with 0.05% Tween 20. Airborne dust samples were extracted in 5 ml, and settled dust samples were extracted in 5 to 20 ml, depending on the dust weight (<0.5 g, 5.0 ml; 0.5 to 1.0 g, 10.0 ml; >1.0 g, 20.0 ml). The extraction procedure was the same as that described for heat extraction of plant samples. After extraction, samples were centrifuged at 1,000 × *g* for 15 min and the supernatant was stored in several small portions (for duplicate analyses) at −20°C and analyzed within 3 months.

Analytical methods. Protein concentrations were determined with BCA protein assay reagents from Pierce (no. 23223 and 23224) with BSA as the reference protein. Carbohydrate was measured with phenol-sulfuric acid with mannose as the reference (9).

RESULTS

Specificity of the inhibition EIA. In Fig. 1, dose-response curves are depicted for two β(1→3)-glucan preparations and various other polysaccharides described in Table 1. The inhibition curve for laminarin, the β(1→3)-glucan used for production and isolation of the antibodies, ranged from approximately 40 to 3,000 ng/ml (15 to 85% inhibition). The other β(1→3)-glucan (curdlan) showed a parallel inhibition curve but was approximately three to five times less reactive on a weight basis (200 to 10,000 ng/ml for 15 to 85% inhibition). Of the other tested polysaccharides, only pustulan, presumed to contain exclusively β(1→6)-glucan (30), showed a parallel dose-response curve, at concentrations approximately 10 times higher than that of laminarin. The other polysaccharides were all incapable of inhibiting the anti-β(1→3)-glucan antibodies, even at concentrations of 50 to 100 μg/ml.

The results shown in Fig. 1 suggested that the antibodies were not completely β(1→3)-glucan specific but also partially reactive with β(1→6)-glucans. Alternatively, the inhibitory activity of pustulan could be due to the presence of β(1→3)-glucosidic structures in that preparation. Therefore, control experiments were performed with laminarin and pustulan after pretreatment with either periodate or zymolyase, which specifically destroys β(1→6)- or β(1→3)-glucan structures, respectively (18, 31). Zymolyase almost completely abolished the inhibitory activity of both glucans (Fig. 2A), whereas periodate treatment did not abolish, and even slightly enhanced, the inhibitory capacity of the preparations (Fig. 2B). $NaIO_4$ and glycerol did not influence the reaction in the control wells with no inhibitor. Additional control experiments using an inhibition EIA with pustulan (inhibitor and coating) and anti-β(1→6)-glucan antibodies confirmed that the periodate pretreatment abolished the immunoreactivity of β(1→6)-glucan structures. The pustulan-based inhibition curve ranged from 100 to 9,000 ng/ml (15 to 85% inhibition), whereas $NaIO_4$-treated pustulan showed ≥15% inhibition only at concentrations higher than 10 μg/ml. After zymolyase treatment, 15 and 85% inhibition was observed at pustulan concentrations of 110 and 9,000 ng/ml, respectively, indicating that zymolyase had no effect on the immunoreactivity of β(1→6)-glucan structures.

Thus, the cross-reactivity observed with pustulan in the β(1→3)-glucan EIA was most probably due to the presence of β(1→3)-glucan structures in the preparation and not to lack of specificity of the anti β(1→3)-glucan antibodies. The specificity of the assay was also confirmed by the lack of reactivity of aqueous extracts of environmental dust samples obtained at room temperature, while subsequent heat extraction resulted in detectable immunoreactivity in the majority of samples. This indicates that the β(1→3)-glucan antibodies do not react with water-extractable (at room temperature) polysaccharides, which can be present in appreciable amounts in most organic dust samples (approximately 10% in dust from the pig farm environment [unpublished results]).

Sensitivity and reproducibility of the inhibition EIA. The detection limit of the inhibition EIA was determined by using the mean of 12 individually calculated detection limits based on the laminarin calibration curves of 12 assays, all performed with individual microtiter plates on different occasions. The detection limit for each individual microtiter plate was determined by calculating the minimum percentage of inhibition that significantly deviated (>3 times the standard deviation [SD]) from the four 0% inhibition controls included on each plate. The detection limit ranged from 4 to 20% with a mean of 10%. However, to minimize the chance of obtaining false-positive results, the detection limit was set at 15% inhibition, which corresponded to a mean detection limit of 42.6 (SD, 10.3) ng/ml. Since airborne inhalable dust was sampled at a flow rate of 2 liters/min for 8 h (≈1 m^3) and filters were extracted in 5 ml of extraction solution, the resulting detection limit for airborne inhalable dust samples was approximately 200 ng/m^3. For settled dust, 5 to 10% (wt/vol) suspensions were

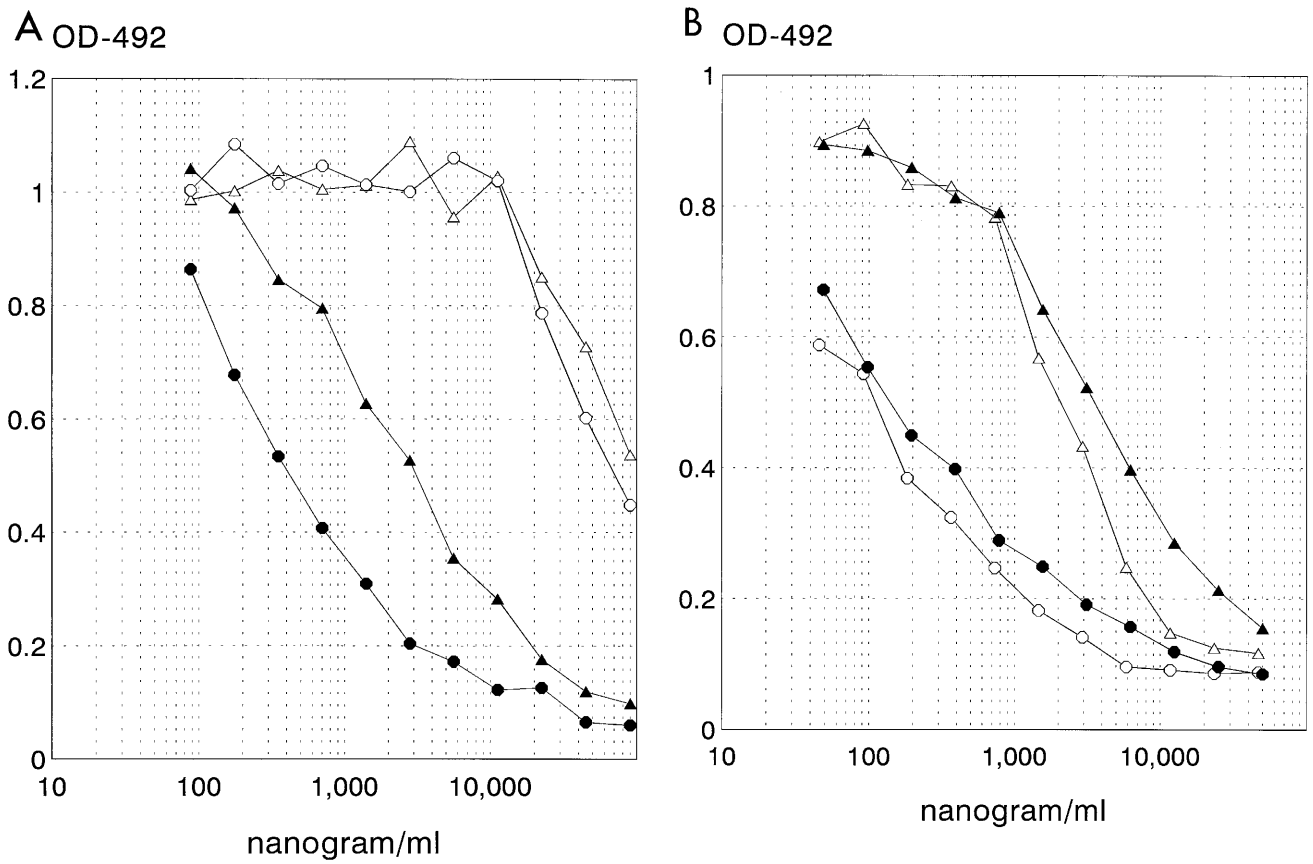

FIG. 2. Inhibition-dilution curves for untreated- and zymolyase-treated laminarin and pustulan (A) (symbols: ●, laminarin; ▲, pustulan; ○, laminarin and zymolyase; △, pustulan and zymolyase) and untreated and $NaIO_4$ treated laminarin and pustulan (B) (symbols: ●, laminarin; ▲, pustulan; ○, laminarin and $NaIO_4$; △, pustulan and $NaIO_4$. OD-492, optical density at 492 nm.

made for extraction; thus, the detection limit for settled-dust samples was approximately 0.5 μg/g of dust.

To evaluate the use of the new assay to measure β(1→3)-glucans in different environments, extracts of dust samples from various origins were tested. Dilution curves obtained with autoclaved (120°C) extracts of environmental samples (house dust, waste composting dust, and swine confinement building dust) were essentially parallel to the calibration curve, as shown in Fig. 3 for five dust samples from swine confinement workers. This indicates that the reference curve can be applied for different types of samples at various dilutions. The reproducibility of the inhibition EIA was determined on detectable (>40 ng/ml) duplicate analyses of the environmental dust extracts (duplicate extract aliquots from the same dust sample) and expressed as a coefficient of variation. Duplicate analyses were performed on different microtiter plates at different days. On the basis of waste composting facility samples (plant 2), samples from swine confinement workers, and house dust samples, mean coefficients of variation for the inhibition assay of, respectively, 20% (n = 26), 20% (n = 59), and 27% (n = 100) were calculated.

β(1→3)-Glucan levels in plant extracts. As shown in Table 2, all tested extracts of cereals, soy, tapioca, and potato contained measurable amounts of β(1→3)-glucan. Dilution curves of all extracts were essentially parallel to the calibration curve in the β(1→3)-glucan assay, and calculated concentrations ranged from approximately 0.01% (wt/wt) for potato to approximately 0.7% (wt/wt) for barley. Differences between the results obtained with two extraction methods (autoclaving at 120°C and 0.05 M NaOH extraction) were small and not systematic.

β(1→3)-Glucan in yeast culture media. The β(1→3)-glucan levels in yeast culture media were moderate, ranging from approximately 0.2 to 1 μg/ml, while in the control medium (without yeast) no immunoreactivity was detected (data not shown).

Exposure levels in various environments. In two small pilot studies, appreciable levels of β(1→3)-glucan were measured in compost plant and swine confinement building environments (Table 3). These environments—which are known for their high bioaerosol exposures (5, 21)—were also characterized by relatively high mean dust exposure (Table 3). House dust sampled from mattresses and from floors of living rooms, bedrooms, and kitchens also contained substantial amounts of β(1→3)-glucan (Table 4). The nonorganic dust samples collected at the construction site did not contain detectable β(1→3)-glucan levels (Table 3). In Tables 3 and 4, β(1→3)-glucan and dust levels are expressed as geometric means with geometric SDs, since exposure data in occupational and home environments are, in general, best described by a lognormal distribution. Concentrations below the detection limit were considered to have a value of two-thirds of this limit (11).

Blank glass fiber filters like those used for sampling of airborne dust did not contain detectable levels of β(1→3)-glucan. Heat extracts (5 ml, 122°C, 1 bar) of blank paper filters like those used for house dust sampling, however, contained sig-

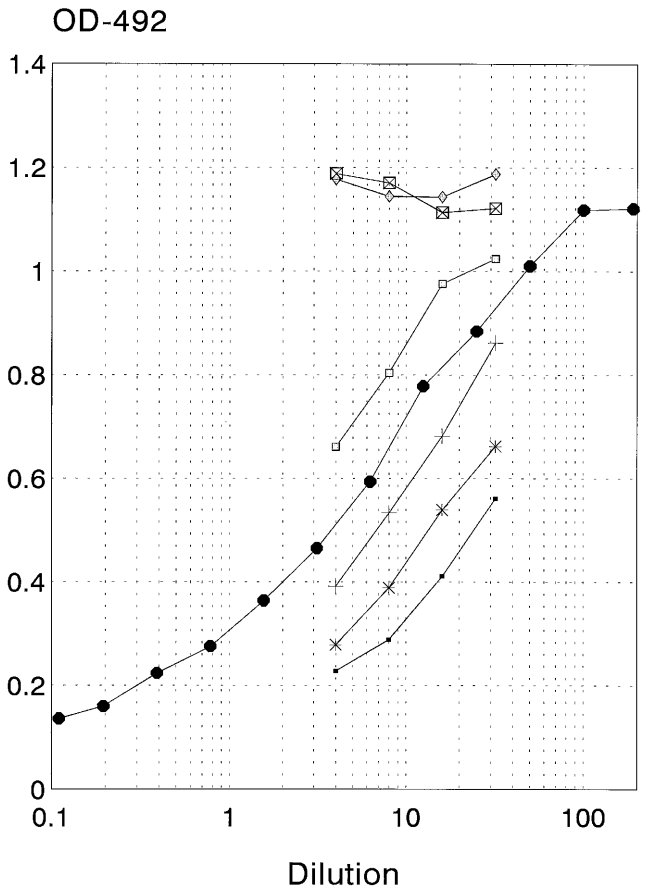

FIG. 3. Inhibition-dilution curves of heat extracts of personal dust samples from five swine confinement workers. Calibration was done with laminarin. Symbols: ●, laminarin; +, sample 1; ∗, sample 2, □, sample 3; ☒, sample 4; ■, sample 5; ◆, blank. OD-492, optical density at 492 nm.

nificant amounts of β(1→3)-glucans. In six blank filters, a mean concentration of 188 (SD, 39.3) μg of β(1→3)-glucan per filter was detected, which was, on average, 21% (minimum, 2%; maximum, 53%) of the total amount of β(1→3)-glucan detected in each dust sample. To calculate the β(1→3)-glucan level per gram of house dust with correction for the β(1→3)-glucan contribution of the filter, the filter background level was subtracted from the absolute β(1→3)-glucan level measured for the whole sample (including the dust and the filter). After this correction, β(1→3)-glucans were still detected in all house dust samples at concentrations ranging from 180 to 6,500 μg/g.

DISCUSSION

In this report, we describe a β(1→3)-glucan inhibition EIA that can be used to assess β(1→3)-glucan levels in airborne and settled dust. Application of the new inhibition EIA showed that β(1→3)-glucans could be measured with high specificity and reproducibility in dust samples from various environments.

We immunized rabbits with BSA-conjugated glucan and obtained polyclonal antibodies with high affinity for β(1→3)-glucans. Affinity chromatography with epoxy-Sepharose-bound β(1→3)-glucan was used to remove antibody activity against BSA, and, if relevant, newly formed (during conjugation) neoantigens of BSA. The resulting affinity-purified antibodies were, indeed, completely devoid of activity against BSA but showed strong reactions against the β(1→3)-glucan used for immunization, as well as another β(1→3)-glucan (curdlan). Thus, direct coupling of β(1→3)-glucan to epoxy-Sepharose was proven to be suitable for antibody isolation. Direct coupling of β(1→3)-glucan to the microtiter plate—which is not a common practice for polysaccharides—was also successfully applied in our inhibition assay. This also precludes false-positive results due to antibodies directed against BSA or BSA-associated neoantigens. Moreover, this procedure avoids the use of the often limited amount of the BSA conjugate for coating.

In our study, we immunized rabbits with linear β(1→3)-glucan in which β(1→6) branches were destroyed by $NaIO_4$ to avoid reactivity of the antibodies with β(1→6)-glucans. This was in contrast to other studies on the production of anti β(1→3)-glucan antibodies, in which branched β(1→3)(1→6)-glucans were used for immunization (1, 13, 14). Two different linear β(1→3)-glucans, curdlan and laminarin, were demonstrated to react in the inhibition assay. Control experiments showed that the antibodies also reacted with pustulan, which is presumed to contain only β(1→6)-glucan (Fig. 1) (30). The reactivity of pustulan, however, was, like that of laminarin, retained after $NaIO_4$ treatment, whereas it was completely lost after treatment with β(1→3)-glucanase (Fig. 2A and B). Similar experiments with a polyclonal anti-β(1→6)-glucan serum confirmed the specific β(1→3)-glucanase activity of zymolyase and the complete destruction of β(1→6)-glucan structures by $NaIO_4$ treatment. Consequently, we conclude that pustulan also contains β(1→3)-glucan structures. One other study has also suggested the presence of β(1→3)-glucan in pustulan (12). Since all of the other tested polysaccharides were not reactive in our assay, we conclude that the inhibition EIA specifically recognizes β(1→3)-glucan epitopes. Preliminary results indicating reactivity with yeast and plant glucans (see below), consisting mainly of β(1→3)(1→4) and β(1→3)(1→6) linkages, respectively, suggest that both linear and branched β(1→3)-glucans are recognized in the inhibition assay.

Our inhibition EIA was less sensitive (40 ng/ml) than a sandwich EIA for β(1→3)-glucans described by Hirata et al. (14) (1 ng/ml) but more sensitive than an inhibition EIA described by Adachi et al. (1) (>1 μg/ml). Comparison on a weight basis, however, using different reference glucans (schizophyllan and grifolan, respectively, versus laminarin) may not be justified, since the purity and, possibly, the chain length and conformation of the glucans may significantly influence the detection limit expressed on a weight basis (see below). An example might be the three- to fivefold difference between the detection limits and reactivities on a weight basis of the two β(1→3)-glucans shown in Fig. 1.

TABLE 2. β(1→3)-Glucans in cereal and other plant materials extracted by heat or alkaline treatment

Source	β(1→3)-Glucan concn (μg/mg)	
	Heat treatment[a]	Alkaline treatment[b]
Potato	0.13	0.19
Potato starch	0.16	0.90
Barley	6.17	7.21
Corn flour	1.38	1.63
Soy beans	1.75	2.41
Soy flour	3.51	1.49
Tapioca	4.87	3.78
Wheat	4.19	2.96

[a] Autoclaved at 120°C and 1 bar for 1 h.
[b] Shaken in 0.05 M NaOH for 2 h.

TABLE 3. β(1→3)-Glucan exposure measurements in three occupational environments

Sample source (no. with glucan/total)	Geometric mean[a] dust concn (mg/m^3)	β(1→3)-Glucan concn (μg/m^3)			
		Geometric mean	Geometric SD	Minimum	Maximum
Waste composting facilities					
Plant 1 (July 1994)					
Offices, control rooms (0/2)[b]	<0.3[c]	<0.2[c]			
Compost ripening area (4/4)[b]	0.43	1.02	2.1	0.53	2.95
Process hall (12/12)[b,d]	4.6	19.35	1.6	11.00	47.03
Plant 2 (December 1994)					
Offices, control rooms (0/5)[b]	<0.3[c]	<0.2[c]			
Compost ripening area (1/6)[b]	0.77	0.39	11.0	<0.2[c]	28.61
Composting hall (0/8)[b]	0.46	<0.2[c]			
Process hall (2/16)[b,e]	0.38	<0.2[c]	1.6	<0.2[c]	0.51
Inorganic waste unloading area (8/8)[b]	2.87	3.80	2.1	2.02	15.97
Personal (19/21)[f]	2.67	6.57	7.2	<0.2[c]	210.11
Swine confinement workers, personal (55/59)[f]	2.1	4.34	3.4	<0.2[c]	38.49
Construction workers, personal and stationary (0/15)[f]	2.7	<0.2[c]			

[a] Geometric means were calculated by including nondetectable results (nondetectable values were set at two-thirds of the detection limit [11]).
[b] Ambient air sampling.
[c] Below detection limit (dust concentration, 0.3 mg/m^3; airborne concentration, glucan 0.2 μg/m^3).
[d] Area for unloading and sieving of organic waste and transfer of compost.
[e] Area for unloading and sieving of organic waste.
[f] Personal air sampling.

Another method used to measure β(1→3)-glucans with a glucan-reactive preparation of *Limulus* amebocyte lysate (LAL) has recently been described by Rylander et al. (27) and Obayashi et al. (20). The reactivity of several β(1→3)-glucans in this assay was reported earlier (3, 17, 19, 24, 31). The glucan-reactive LAL test is very sensitive [1 to 10 pg of β(1→3)-glucan per ml] but probably not highly specific, since it also reacts with other glucans, such as gyrophoran [β(1→6)-glucan], at nanogram-per-milliliter concentrations and with very high concentrations (1 to 100 μg/ml) of D-mannans and dextran (17, 31) but not with bacterial lipopolysaccharides (endotoxin). A possibly more serious problem may be that activation of LAL depends on the chain length and conformation of the glucans (3, 19, 31). β(1→3)-Glucan conformers with triple-helix structures are severely underestimated compared with single-helix and random-coil β(1→3)-glucan structures (3, 19), while all three conformers appear to be biologically active (19). A similar problem with our inhibition assay may be due to steric hindrance of β(1→3)-glucan epitopes, especially in large triple-helix conformations. Our data, however, do not support this, since alkaline and heat extracts of plant samples showed similar results in the β(1→3)-glucan EIA (Table 2). Heat treatment increases the triple-helix-to-single-helix ratio, while alkaline treatment transforms triple-helix formation to single-helix or random-coil formation (19).

TABLE 4. β(1→3)-Glucan exposure measurements in house dust

Sampling location (no. of samples with glucan/total)	β(1→3)-Glucan concn (μg/g)			
	Geometric mean	Geometric SD	Minimum	Maximum
Living room (25/25)	1,293	1.4	627	2,915
Bedroom (25/25)	1,286	1.7	408	3,507
Kitchen (25/25)	1,168	2.0	376	6,540
Mattress (25/25)	757	1.7	182	1,654

The use of a specific immunoassay that is also less expensive and possibly less dependent on the structural conformation of β(1→3)-glucans may be advantageous in hygiene and epidemiological-effect studies, compared with the modified LAL test that has recently become commercially available.

Thus far, discussions on the immunobiological effects of glucans have focused mainly on fungal β(1→3)(1→6)-glucans. However, it is not clear whether plant β(1→3)(1→4)-glucans have similar properties. Plant glucans like laminarin and barley β-D-glucan also interact with *Limulus* coagulation factor G (31). This suggests that plant glucans are, indeed, also biologically active and detection of both plant and fungal β(1→3)-glucans in a β(1→3)-glucan assay—as described in this report—may therefore be relevant.

Very high β(1→3)-glucan levels were found in the studied occupational and residential dust samples. Mean β(1→3)-glucan concentrations in the waste composting facilities ranged from <0.2 to 19 μg/m^3. The mean personal exposure for waste composting workers was 7 μg/m^3, and that for pig farmers was 4 μg/m^3. Comparable β(1→3)-glucan concentrations (1 μg/m^3) have been measured in an experimental cotton cardroom by Rylander et al. (26) with a glucan-reactive LAL assay. The detection limit of 40 ng/ml allows quantification of the exposure in most samples from these environments, but airborne β(1→3)-glucan measurements in low-exposure environments such as office buildings (0.00 to 0.55 ng/m^3 [27]) and dwellings probably require a more sensitive assay. The high β(1→3)-glucan content of organic dust from both occupational and residential origins (0.5 to 20‰) suggests a large contribution of plant and/or fungal material in that dust. We therefore reconsidered the possibility of nonspecific false-positive reactions and analyzed aqueous dust extracts obtained at room temperature and heat extracts of (nonorganic) dust samples from construction workers with comparable gravimetric dust exposure. None of these extracts contained detectable β(1→3)-glucan levels.

High β(1→3)-glucan content of organic dust—as measured in our study—seems plausible, considering that β(1→3)-glucans occur as major structural cell wall or storage components of many plants and microorganisms, which are known to contribute largely to the content of most organic dust. Moreover, β(1→3)-glucans (as well as other polysaccharides) are likely to be relatively degradation resistant in the environment, which may result in a high carbohydrate content of organic dust. Organic dust contains a substantial proportion of extractable carbohydrate of approximately 10% (wt/wt) when the carbohydrate is extracted at room temperature and 20% when it is heat extracted (determined for pig farm dust; unpublished results). Thus, β(1→3)-glucans would, in fact, contribute only approximately 1 to 2% to the total carbohydrate concentration.

In conclusion, our β(1→3)-glucan inhibition assay offers a specific and sensitive method for assessment of exposure to airborne glucans, at least in high-exposure environments, and for assessment of β(1→3)-glucan levels in settled dust from the home environment. The new assay is therefore expected to be useful for epidemiological studies investigating the relationship between β(1→3)-glucan exposure and respiratory health.

ACKNOWLEDGMENTS

This project was conducted with the assistance of P. Noomen and K. Verkuijlen. Samples from swine confinement workers were kindly provided by L. Preller. House dust samples were provided by H. Wichman and J. Heinrich from the GSF, Neuherberg, Germany. Yeast samples were given by A. Ram from the Institute of Molecular Cell Biology of the University of Amsterdam.

REFERENCES

1. **Adachi, Y., N. Ohno, and T. Yadomae.** 1994. Preparation and antigen specificity of an anti-(1→3)-β-D-glucan antibody. Biol. Pharm. Bull. **17:**1508–1512.
2. **Adachi, Y., M. Okazaki, N. Ohno, and T. Yadomae.** 1994. Enhancement of cytokine production by macrophages stimulated with (1→3)-β-D-glucan, grifolan (GRN), isolated from *Grifola frondosa*. Biol. Pharm. Bull. **17:**1554–1560.
3. **Aketagawa, J., S. Tanaka, H. Tamura, Y. Shibata, and H. Saitô.** 1993. Activation of limulus coagulation factor G by several (1→3)-β-D-glucans: comparison of the potency of glucans with identical degree of polymerization but different conformations. J. Biochem. **113:**683–686.
4. **Brattgjerd, S., Ø. Evensen, and A. Lauve.** 1994. Effect of injected yeast glucan on the activity of macrophages in Atlantic salmon, *Salmo salar* L., as evaluated by in vitro hydrogen peroxide production and phagocytic capacity. Immunology **83:**288–294.
5. **Clark, C. S., R. Rylander, and L. Larsson.** 1983. Levels of gram-negative bacteria, *Aspergillus fumigatus*, dust, and endotoxin at compost plants. Appl. Environ. Microbiol. **5:**1501–1505.
6. **De Lucca, A. J., II, K. A. Brogden, and A. D. French.** 1992. Agglutination of lung surfactant with glucan. Br. J. Ind. Med. **49:**755–760.
7. **Di Luzio, N. R.** 1979. Lysozyme, glucan activated macrophages and neoplasia. J. Reticuloendothel. Soc. **26:**67–81.
8. **Douwes, J., P. Versloot, A. Hollander, D. Heederik, and G. Doekes.** 1995. Influence of various dust sampling and extraction methods on the measurement of airborne endotoxin. Appl. Environ. Microbiol. **61:**1763–1769.
9. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
10. **Fogelmark, B., M. Sjöstrand, and R. Rylander.** 1994. Pulmonary inflammation induced by repeated inhalations of β(1→3)-D-glucan and endotoxin. Int. J. Exp. Pathol. **75:**85–90.
11. **Helsel, D. R.** 1990. Less than obvious—statistical treatment of data below the detection limit. Environ. Sci. Technol. **24:**1766–1774.
12. **Hirata, A., Y. Adachi, W. Itoh, M. Komoda, K. Tabata, and I. Sugawara.** 1994. Monoclonal antibody to proteoglycan derived from *Grifola frondosa* (Maitake). Biol. Pharm. Bull. **17:**539–542.
13. **Hirata, A., W. Itoh, K. Tabata, T. Kojima, S. Itoyama, and I. Sugawara.** 1993. Preparation and characterization of murine anti-schizophyllan monoclonal antibody, SPG1-HS. Biosci. Biotechnol. Biochem. **57:**125–126.
14. **Hirata, A., M. Komoda, W. Itoh, K. Tabata, S. Itoyama, and I. Sugawara.** 1994. An improved sandwich ELISA method for the determination of immunoreactive schizophyllan. Biol. Pharm. Bull. **17:**1437–1440.
15. **Hutchins, K., and H. Bussey.** 1983. Cell wall receptor for yeast killer toxin: involvement of (1→6)-β-D-glucan. J. Bacteriol. **154:**161–169.
16. **Kraus, J., and G. Franz.** 1991. β(1→3)-Glucans: anti-tumor activity and immunostimulation. NATO Adv. Study Inst. Ser. H Cell Biol. **53:**431–444.
17. **Mikami, T., T. Nagase, T. Matsumoto, S. Suzuki, and M. Suzuki.** 1982. Gelation of *Limulus* amoebocyte lysate by simple polysaccharides. Microbiol. Immunol. **26:**403–409.
18. **Montijn, R. C., J. van Rinsum, A. van Schagen, and F. Klis.** 1994. Glucomannoproteins in the cell wall of *Saccharomyces cerevisiae* contain a novel type of carbohydrate side chain. J. Biol. Chem. **269:**19338–19342.
19. **Nagi, N., N. Ohno, Y. Adachi, J. Akettagawa, H. Tamura, Y. Shibata, S. Tanala, and T. Yadomae.** 1994. Application of *Limulus* test (G pathway) for the detection of different conformers of (1→3)-β-D-glucans. Biol. Pharm. Bull. **16:**822–828.
20. **Obayashi, T., M. Yoshida, T. Mori, H. Goto, A. Yasuoka, H. Iwasaki, H. Teshima, S. Kohno, A. Horiuchi, A. Ito, H. Yamaguchi, K. Shimada, and T. Kawai.** 1995. Plasma (1→3)-β-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. Lancet **345:**17–20.
21. **Preller, L., D. Heederik, H. Kromhout, J. S. M. Boleij, and J. M. T. Tielen.** 1995. Determinants of dust and endotoxin exposure of pig farmers: development of a control strategy using empirical modelling. Ann. Occup. Hyg. **39:**545–557.
22. **Platts-Mills, T. A. E., and A. L. De Weck.** 1989. Dust mite allergens and asthma: a world wide problem. International workshop report. J. Allergy Clin. Immunol. **11:**199–206.
23. **Ramani, M., C. J. Howell, B. W. Spur, L. J. F. Youlten, T. J. M. Clark, M. H. Lessof, and T. H. Lee.** 1988. The generation and cellular distribution of leukotriene C_4 in human eosinophils stimulated by unopsonized zymosan and glucan particles. Clin. Immunol. **81:**696–705.
24. **Roslansky, P., and T. J. Novitsky.** 1991. Sensitivity of *Limulus* amebocyte lysate (LAL) to LAL-reactive glucans. J. Clin. Microbiol. **29:**2477–2483.
25. **Roy, R., E. Katzenellenbogen, and H. J. Jennings.** 1984. Improved procedures for the conjugation of oligosaccharides to protein by reductive amination. Can. J. Biochem. Cell Biol. **62:**270–275.
26. **Rylander, R., R. Bergstrøm, H. Goto, K. Yuasa, and S. Tanaka.** 1989. Studies on endotoxin and β(1→3)-D-glucan in cotton dust, p. 46–47. *In* R. R. Jacobs and P. J. Wakelyn (ed.), Proceedings of the Thirteenth Special Session on Cotton Dust Research. National Cotton Council, Memphis, Tenn.
27. **Rylander, R., K. Persson, H. Goto, K. Yuasa, and S. Tanaka.** 1992. Airborne beta-1,3-glucan may be related to symptoms in sick buildings. Indoor Environ. **1:**263–267.
28. **Saito, K., M. Nishijma, N. Ohno, N. Nagi, T. Yadomae, and T. Miyazaki.** 1992. Activation of complement and *Limulus* coagulation system by an alkali-soluble glucan isolated from *Omphalia lapidescens* and its less branched derivates. Chem. Pharm. Bull. **40:**1227–1230.
29. **Sakurai, T., N. Ohno, and T. Yadomae.** 1994. Changes in immune mediators in mouse lung produced by administration of soluble (1→3)-β-D-glucan. Biol. Pharm. Bull. **17:**617–622.
30. **Stone, B. A., and A. E. Clarke.** 1992. Chemistry and biology of (1→3)-β-glucans. La Trobe University Press, Victoria, Australia.
31. **Tanaka, S., J. Aketagawa, S. Takahashi, Y. Shibata, Y. Tsumuraya, and Y. Hashimoto.** 1991. Activation of a limulus coagulation factor G by (1→3)-β-D-glucans. Carbohydr. Res. **218:**167–174.
32. **ter Kuile, W. M.** 1984. Vergleichungsmessungen mit verschiedenen Geräten zur Bestimmung der Gesamtstaubkonzentration am Arbeitsplatz: Teil II. Staub Reinh. Luft **44:**211–216.
33. **Williams, D. L.** 1994. (1→3)-β-D-glucans, p. 83–85. *In* R. Rylander and R. R. Jacobs (ed.), Organic dusts: exposure, effects, and prevention. Lewis Publishers, Chicago.
34. **Winston, L., C. Dollard, and S. L. Ricupero-Havasse.** 1995. Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast **11:**53–55.
35. **Zhang, K., and H. R. Petty.** 1994. Influence of polysaccharides on neutrophil function: specific antagonists suggest a model for cooperative saccharide-associated inhibition of immune complex-triggered superoxide production. J. Cell. Biochem. **56:**225–235.