IgG to Various Beta-Glucans in a Human Adult Population

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Key Words

β-Glucan • IgG • Normal human sera • Fungi • Pustulan •
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Abstract

Background: Fungal β-(1,3)-glucans are pro-inflammatory agents, and exposures to β-(1,3)-glucans are associated with respiratory tract symptoms. IgG anti-(1,3)-glucan titers are measured in diagnosis of fungal infections. Although other β-glucan structures exist, like β-(1,6)-glucans, little is known about their antigenic or pro-inflammatory properties. We aimed to investigate IgG titers and specificities in human sera against different β-glucans with varying structures. **Methods:** IgG anti-β-glucan was measured by enzyme immunoassay in a random sample of 40 sera from healthy adults, with a panel of 8 differently structured glucans. In a subsequent larger series, IgG anti-β-(1,6)-glucan was measured in a random sample of 667 sera from three occupational populations with different organic dust exposures. Possible determinants of IgG anti-β-(1,6)-glucan titers were explored with linear-regression analysis. **Results:** We found wide variation in anti-β-glucan IgG levels. The highest titers were found for pure β-(1,6)-glucan pustulan. Moderate to strong reactions with other β-(1,6)-containing structures appeared to be due to cross-reacting anti-β-(1,6)-glucan anti-

bodies. Surprisingly, the mean IgG anti-β-(1,6)-glucan titer was significantly lower in agricultural workers – with highest organic dust exposure – than in spray painters and bakery workers. Smoking status was associated with lower IgG anti-β-(1,6)-glucan titers in all populations. **Conclusions:** IgG to β-(1,3)- and β-(1,6)-glucans can be found in normal human sera. β-(1,6)-glucans appear to be much more potent antigens. The health impact of high anti-β-(1,6)-glucan antibody levels remains unclear and further investigations are needed.

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Introduction

Fungal exposures have been related to diverse health effects, ranging from mycosis, like aspergillosis or candidiasis [1, 2] to IgE- [3, 4] and IgG-mediated [5, 6] allergies and nonspecific symptoms caused by innate immune responses to pro-inflammatory fungal cell wall components like β-glucans [7, 8].

β-Glucans are glucose polymers from plants, bacteria and fungi with large variations in proportion and arrangement of their 1,3-, 1,4- and 1,6-β-glycosidic linkages [9]. They constitute approximately 50–60% of the dry weight of fungal cell walls [10] and thereof approxi-

mately 65–90% are β -(1,3)-glucans and approximately 10–15% are β -(1,6)-glucans [11, 12]. The β -(1,3)-glucans are well-known pro-inflammatory agents [13, 14] and innate immune responses can be induced through binding to monocytes or macrophages via the receptor dectin-1 [15] or complement receptor 3 (CR3) [16]. Adaptive immune responses to β -glucan have received much less attention, but anti- β -(1,3)-glucan antibodies have previously been reported [17, 18] and their measurement has been suggested useful for the diagnosis of candidiasis [19]. Recently Chiani et al. [20] reported high anti- β -glucan IgG levels in human sera, in a study on the use of β -glucans in vaccines. In their study the levels of IgG against β -(1,6)-glucan (pustulan) were up to 10 times higher than the IgG anti- β -(1,3)-glucan (laminarin) titers.

We recently found that rabbit and mouse antibodies raised against nonbranched β -(1,3)-glucan showed large variation in their reactivity with glucans containing variable amounts of other linkage types additional to β -(1,3)-linkages [21]. This highly variable reactivity of rabbit and mouse antibodies to differently structured β -glucans, and the results of Chiani et al. [20], suggest that different antibody reactivity patterns to the various β -glucans may also be encountered in humans.

The aim of our study was therefore to investigate levels and specificities of anti- β -glucan IgG antibodies in sera from the general adult population. We measured IgG binding towards several different β -glucans and checked for cross-reactivities between structures. A subsequent aim was to investigate IgG anti- β -(1,6)-glucan titers and their main determinants in a larger set of sera from three occupational health surveys.

Materials and Methods

Glucans

We used the β -(1,3)-glucans curdian (Wako Chemicals, Neuss, Germany), laminarin and paramylon (both from Sigma-Aldrich, Zwijndrecht, The Netherlands), β -(1,3)-(1,4)-glucans barley glucan, lichenan and oat glucan (all from Megazyme, Wicklow, Ireland), the β -(1,3)-(1,6)-glucans baker's yeast glucan (Sigma-Aldrich) and scleroglucan (gift of Dr. U. Rau, Department of Biotechnology, Technische Universität Braunschweig, Germany), and pure β -(1,6)-glucan pustulan (EMD Chemicals Inc., Gibbstown, N.J., USA). Preparation of stock solutions has been described previously [21]. Structures were confirmed by proton NMR spectra produced and evaluated by Dr. D.W. Lowman (AppRidge International, LLC, Jonesborough, Tenn., USA) and Dr. J.P. Kamerling (Bio-Organic Chemistry of Carbohydrates, Utrecht University, Utrecht, The Netherlands). Structures are presented in table 1.

Serum Samples

Forty sera were randomly selected from a study among spray painters [22] and tested for IgG binding to β -glucans. Since this population had no specific organic dust exposures, glucan exposure and IgG anti-glucan levels in this group were supposed to be representative for the general adult population in the Netherlands. A larger serological screening of IgG binding to β -(1,6)-glucans was performed in 200 randomly selected sera from the same study, 220 sera from bakery workers [23] and 247 sera from a study in agricultural processing industries [24]. The agricultural population included several subcategories: 88 were organic farmers, 70 worked in onion-, 53 in flower bulb-, 17 in vegetable seed- and 19 in animal feed-producing industries. All sera had been collected in course of the respective studies between 2003 and 2006 and stored in aliquots at -20°C . The studies were all approved by the institutional review board and participants gave written consent [22, 25, 26].

IgG Anti- β -Glucan Measurement

The optimal coating concentration – at which the amount of anti-glucan IgG in the test sera is the limiting factor – was estimated for the various glucans by chessboard titrations. Optimal concentrations for human IgG binding to β -(1,3)-glucans were the same as for rabbit and mouse antibodies [27], but for pustulan, strong and maximal responses could already be found at coating concentrations of less than $0.3\text{ }\mu\text{g/ml}$. Thus, polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight at 4°C with glucans at $16\text{ }\mu\text{g/ml}$ in phosphate-buffered saline (PBS), except pustulan, for which maximal IgG binding was already reached at $0.3\text{ }\mu\text{g/ml}$ [21]. Wells were washed with PBS-Tween, blocked with PBS-Tween + 0.1% milk protein (PBTM; Nutricia, Zoetermeer, The Netherlands) and incubated for 1 h at 37°C with the sera diluted in PBTM. After another wash cycle, IgG binding was measured by incubation for 1 h at 37°C with 1/1,500 diluted peroxidase-labeled mouse monoclonal anti-human-IgG antibody (Sanquin Reagents, Amsterdam, The Netherlands), and finally o-phenylenediamine [21].

The 40 serum samples used for the initial screening of IgG binding to various glucans were tested at 1/1,000. Since most sera tested in 1/1,000 dilution showed maximal OD values (>3.0) in the IgG anti-pustulan inhibition enzyme immunoassay (EIA; fig. 1), measurement for a random selection of 23 sera was repeated at 1/40,000 and used for the correlation calculations.

Six strongly and broadly reactive sera were pooled and used as standard serum pool in the larger series testing for anti- β -(1,6)-glucan IgG binding with an assigned arbitrary value of 20,000 units/ml. The pool was serially diluted in each test plate from 1/10,000 to 1/320,000 and titers of sera were calculated by interpolation of the measured ODs on the standard curve. In the screening for anti- β -(1,6)-glucan IgG binding, sera were tested at 1/5,000 and 1/10,000 dilution. Sera were retested at lower (1/1,000 and 1/4,000) or higher (1/10,000 and 1/40,000) dilutions when necessary.

Quality control of the IgG measurements included assessing intra- and interassay coefficients of variation (CV). The average intra-assay CV – based on values obtained at different dilutions of each serum in the same test plate – was $<7\%$ and the interassay CV – based on repeated measurements of more than 20% of all sera from each of the three study populations – was $<24\%$.

Table 1. Glucan structures and linkages liable to β -(1,3)-glucanase or periodate treatment

Type of glucan (minor linkage type)	Structure
<i>β</i> -(1,3)-glucan	
Curdlan (<10% β-(1,6)-glycosidic linkages [40])
Laminarin (a β-(1,6)-side chain branch approx. every 3rd–15th glucose subunit along the backbone [38, 39])	
Paramylon [9]	
<i>β</i> -(1,6)-glucan	
Pustulan [35]
<i>β</i> -(1,3)-(1,6)-glucan	
Baker's yeast glycosidic linkages (approx. 10% β-(1,6)-glucan [11])
Scleroglucan (a β-(1,6)-side chain branch approx. every 3rd glucose subunit along the backbone [38])	
<i>β</i> -(1,3)-(1,4)-glucan	
Lichenan (three β-(1,3)- in relation to seven β-(1,4)-linked glucose subunits per molecule [41])
Barley β-glucan (30% β-(1,3)- and 70% β-(1,4)-glycosidic linkages [42])	
Oat β-glucan (30% β-(1,3)- and 70% β-(1,4)-glycosidic linkages [42])	
↑β-(1,3)-glucanase; ↑periodate.

Fig. 1. Screening of 40 human sera for IgG binding to different β -glucans. IgG reactivity was defined as the OD measured at a serum dilution of 1/1,000.

EIA Experiments

Lichenan [α β -(1,3)-(1,4)-glucan] and baker's yeast glucan [α β -(1,3)-(1,6)-glucan] were coated overnight to microtiter plates in PBS at 16 μ g/ml, and the pure β -(1,6)-glucan pustulan at 0.3 μ g/ml. After a blocking step, the various glucans used as inhibitors were added in serial dilutions in PBTM at concentrations from 0.003 to 100 μ g/ml, directly followed by the standard human serum pool, diluted 1/500 in the lichenan- and baker's yeast glucan-coated wells, and 1/2,000 in pustulan-coated wells. IgG binding to microwells was measured as described for the direct EIA.

β -(1,3)-Glucanase Treatment

Lichenan, baker's yeast glucan and pustulan were treated with β -(1,3)-glucanase (Sigma-Aldrich), which would specifically destroy any antigenic structure depending on intact β -(1,3)-linkages. The linkages liable to β -(1,3)-glucanase are presented in table 1.

Lichenan and pustulan were solved in 50 mM Tris-HCl (pH 5.0) at 120°C. Baker's yeast glucan was dissolved in 0.05 M NaOH at 120°C after which the pH was adjusted to 5 with 50 mM Tris-HCl (pH 2.0). This procedure did not affect the glucan reactivity in the inhibition EIA [28] when compared to the original solving procedure [21] (data not shown). Four units of β -(1,3)-glucanase (3 mg) were added to 200 μ l of 1 mg/ml glucan. After 2 min vortexing, the mixtures were incubated at 55°C (pH 5) overnight, according to the manufacturer's protocol and the reaction was stopped by boiling for 20 min. A negative control consisted of similarly treated glucanase in buffer alone.

Periodate Treatment

Lichenan, baker's yeast glucan and pustulan were also treated with sodium-periodate (Sigma-Aldrich), which oxidizes the C²-

and C³-linked hydroxyl groups and opens the glucopyranose ring [29]. The resulting structural change is usually reflected in a significant change of antigenic properties. In β -(1,3)-glucans, however, the C²-C³ bond is protected by the glycosidic linkage at C³, and the antigenic properties of (pure) β -(1,3)-glucans are therefore not affected. The linkages liable to periodate are presented in table 1.

Of each glucan, 200 μ l (1 mg/ml) was incubated overnight at 20°C with 200 μ l periodate (2 M) in PBS. The reaction was stopped with 25 μ l 16 M ethanolamine (Sigma-Aldrich). As negative controls we included buffers treated with periodate similar to the glucans.

For comparison with previous results [28], we included laminarin in the glucanase and periodate treatment experiments, while it was not included in the present serologic screening.

Statistical Analysis

Since serological data showed a log-normal distribution, all statistical analyses were based on ln-transformed OD values and titers. Differences between groups were assessed by Student's t test on ln-transformed values. Relations with determinants of IgG anti- β -(1,6)-glucan titers were investigated by multiple regression analysis. From the agricultural study we had information on dust and endotoxin exposure levels, smoking status, gender, age and childhood in farm environment [24]. For the bakery study we had similar information on dust exposure levels, gender, age and smoking [23], and for the population of spray painters age, gender and smoking [22]. Statistical analyses were performed with SAS statistical software (version 9.1; SAS Institute, Cary, N.C., USA).

Table 2. Pearson correlation of (ln-transformed) OD values for human serum IgG binding to various glucans (n = 40)

		β -(1,3)-(1,6)-glucan		β -(1,3)-glucan		β -(1,3)-(1,4)-glucan		
		baker's yeast glucan	sclero-glucan	curdlan	para-mylon	barley glucan	lichenan	oat glucan
β -(1,6)-glucan	pustulan ¹	0.68	0.75	0.31	-0.18	0.11	0.52	0.19
β -(1,3)-(1,6)-glucan	baker's yeast glucan		0.79	0.34	0.38	0.53	0.69	0.52
	scleroglucan			0.23	0.41	0.39	0.78	0.30
β -(1,3)-glucan	curdlan				0.15	0.22	0.24	0.22
	paramylon					0.29	0.34	0.30
β -(1,3)-(1,4)-glucan	barley glucan						0.63	0.86
	lichenan							0.52

¹ Based on 23 sera for which the analysis was repeated at 1/40,000, since most sera tested in 1/1,000 dilution showed maximal OD values (>3.0) in the IgG anti-pustulan EIA.

Results

IgG Reactions with Different β -Glucans

IgG binding of 40 sera to 8 different β -glucans is shown in figure 1. Clear differences were observed, with the highest reactivity for pustulan, a β -(1,6)-glucan; in fact, many sera showed at 1/1,000 dilution a maximal (OD = $\pm$ 3.8–3.9) or near maximal EIA reaction in pustulan-coated microwells. Much lower, but considerable IgG binding was measured for the β -(1,3)-(1,6)-glucans baker's yeast glucan and scleroglucan as well as for the β -(1,3)-(1,4)-glucan lichenan. Paramylon and curdlan – glucans containing only β -(1,3)-linkages – as well as barley and oat glucan, both β -(1,3)-(1,4)-glucans – showed low reactivities with only incidental OD values >0.5 at a 1/1,000 serum dilution.

In table 2, correlations between IgG reactions of each serum with different glucans are presented. A cluster of strong correlations can be identified in the upper left corner (marked grey) for glucans containing β -(1,6)-structures, pustulan, baker's yeast glucan and scleroglucan, and another cluster in the lower right corner of β -(1,3)-(1,4)-glucans, lichenan, barley and oat glucan. Remarkably strong correlations were also found between reactions towards lichenan [β -(1,3)-(1,4)-glucan] and the three glucans containing β -(1,6)-structures (table 2; upper right corner). IgG reactions to the linear β -(1,3)-glucans, paramylon and curdlan, showed no or only weak correlations with any of the other glucans.

These results suggest a clustering of different anti-glucan IgG antibody responses: a strong response to pure

β -(1,6)-glucans, a moderate response to glucans with combinations of β -(1,3)- with either β -(1,6)- or β -(1,4)-linkages, and a relatively poor response to pure β -(1,3)-glucans. The correlations within these clusters suggest that there might be considerable cross-reactivity due to shared epitopes among structurally related glucans.

Specificity and Cross-Reactivity of Anti-Glucan IgG

To explore whether the high correlations of antibody binding were due to cross-reactivities, we conducted inhibition experiments with the glucans with the strongest IgG-binding capacity – the β -(1,3)-(1,4)-glucan lichenan, the β -(1,3)-(1,6)-glucan baker's yeast glucan and the pure β -(1,6)-glucan pustulan. To minimize the risk of incidental findings due to exclusive properties of single sera, a pool of 6 strongly and broadly reactive sera was used in these experiments. Inhibitory potency was expressed as the concentration at which 50% inhibition was reached (c_{50}).

IgG binding to β -(1,3)-(1,4)-glucan (lichenan) was only moderately inhibited by lichenan itself (c_{50} = 10 μ g/ml), less avidly by β -(1,3)-(1,6)-glucan baker's yeast glucan (c_{50} = 60 μ g/ml) and only weakly by pure β -(1,6)-glucan pustulan (c_{50} = 400 μ g/ml; fig. 2a). IgG binding to the β -(1,3)-(1,6)-glucan from baker's yeast was also moderately inhibited by the coated antigen itself (c_{50} = 20 μ g/ml) and weakly by the β -(1,3)-(1,4)-glucan lichenan (c_{50} = 200 μ g/ml; fig. 2b); the reaction was, however, strongly inhibited by the β -(1,6)-glucan pustulan, even at remarkably low concentrations (c_{50} = 0.2 μ g/ml; fig. 2b). As expected, binding of IgG to the β -(1,6)-glucan pustulan was strongly inhibited by pustulan (c_{50} = 0.8 μ g/ml)

and weakly by the β -(1,3)-(1,6)-glucan from baker's yeast ($c_{50} = 200 \mu\text{g/ml}$), while β -(1,3)-(1,4)-glucan lichenan showed hardly any significant ($>10\%$) inhibition at these concentrations (fig. 2c). These results confirm that the β -(1,6)-glucan structure is a much more potent IgG-binding antigen than the β -(1,3)-containing glucans. The strong 'cross-inhibition' potency of pustulan shown in figure 2b suggests that IgG binding by the β -(1,3)-(1,6)-glucan from baker's yeast is largely due to cross-reactive β -(1,6)-linkage-associated epitopes.

Periodate and β -(1,3)-Glucanase Treatment

Since the β -(1,6)-glucan pustulan also showed some inhibition of IgG binding to the β -(1,3)-(1,4)-glucan lichenan (fig. 2a), we considered the possibility of contamination in either glucan preparation, and assessed the effects of periodate and β -(1,3)-glucanase treatment on IgG binding. Effect of each treatment was assessed by comparing IgG binding from the standard serum pool to microwells coated with treated and untreated glucans. The pure β -(1,3)-glucan laminarin was treated in parallel to confirm specificity of both treatments.

As expected, IgG-binding reactivity of the β -(1,3)-glucan laminarin (fig. 3d) was not affected by periodate treatment but sensitive to glucanase, while IgG binding to the β -(1,6)-glucan pustulan (fig. 3c) was practically completely abolished by periodate treatment but not or only slightly affected by glucanase. The IgG-binding capacity of the β -(1,3)-(1,6)-glucan from baker's yeast (fig. 3a) and the β -(1,3)-(1,4)-glucan lichenan (fig. 3b) appeared to be sensitive to both periodate and glucanase treatment.

Thus, the strong IgG binding by pustulan is not due to a contamination with β -(1,3)-linkages, and most likely due to specific epitopes consisting of its main and typical chemical structure, chains of β -(1,6)-linked glucose residues. The β -(1,3)-(1,6)-glucan from baker's yeast and the β -(1,3)-(1,4)-glucan lichenan were sensitive to both treatments, suggesting that their IgG-binding epitopes comprise both intact β -(1,3)-structures, but also intact β -(1,6)- (for baker's yeast glucan) or intact β -(1,4)-linked (for lichenan) glucose residues.

Anti- β -(1,6)-Glucan Titers in Populations with Different Exposure Levels

Overall, the inhibition as well as the periodate and glucanase treatment experiments indicate that the strong human IgG anti- β -(1,6)-glucan (pustulan) reactivity (fig. 1) is due to a highly specific IgG response. We therefore measured IgG anti-pustulan reactivity in a much larger set of sera from three populations with different levels of work-

Fig. 2. Inhibition of IgG binding to coated lichenan, baker's yeast glucan or pustulan by either lichenan, baker's yeast glucan or pustulan.

Fig. 3. IgG binding of a pool of human sera to baker's yeast glucan, lichenan, pustulan or laminarin after treatment of the glucans with periodate (○), β -(1,3)-glucanase (+) and without treatment (x).

related organic dust exposures. In most sera we found high titers of anti- β -(1,6)-glucan IgG (fig. 4), but levels were significantly lower in the agricultural population (GM: 2,700, $p < 0.015$) as compared to bakery workers (GM: 5,700) and spray painters (GM: 5,400). Further stratification of the agricultural population according to the sectors showed no significant differences in IgG titers between these subpopulations (fig. 4), and all had significantly lower anti- β -(1,6)-glucan IgG titers (GM range 2,500–3,200) than bakery workers and spray painters.

Determinants of Anti- β -(1,6)-Glucan IgG Titers

In a first exploratory analysis we investigated which factors might determine anti- β -(1,6)-glucan IgG titers. Mean age, number of males and smokers were similar for

the three studies and are summarized in table 3. Mean dust exposures were similar, approximately 1.4 mg/m^3 , for the agricultural and the bakery population. Approximately 35% of the workers in the agricultural populations were currently farmers (the other 65% were agricultural industry workers), approximately 30% had grown up on a crop farm and approximately 40% had grown up on a livestock farm.

The only significant relation in the multiple regression analysis with (ln-transformed) anti- β -(1,6)-glucan IgG titers as dependent variable was found for current smoking status (table 4). Smokers had approximately 30% lower titers compared to nonsmokers. Among nonsmokers, no difference was noted between never smokers and former smokers.

Fig. 4. Serum IgG against pustulan in the study populations of bakery workers, spray painters and agricultural workers; organic farmers and workers in onion-, seed-, animal feed- and flower bulb-producing industries were all part of the agricultural population (n = 247).

Table 3. Demographic description of the study populations

	Agriculture	Bakery worker	Spray painters
N	247	220	200
Mean age, years	41.7 ± 11.0	39.8 ± 11.0	37.6 ± 10.6
Male, %	85.43	96.34	90.62
Current smokers, %	28.74	33.18	32.62
Dust exposure, mg/m ³ GM	1.56 (3.0)	1.27 (1.7)	
Endotoxin exposure, EU/m ³ GM	742 (4.2)		
Childhood on livestock farm, %	36.03		
Childhood on crop farm, %	29.15		
Currently a farmer, %	35.63		

Figures in parentheses are GSD.

Discussion

Most normal human sera were shown to contain anti- β -glucan IgG antibodies with specificities for different configurations of β -glucans. The strongest reactions were found to pustulan, a pure β -(1,6)-glucan, and in general the sera showed higher reactivities with glucans containing β -(1,6)-linkages. Chiani et al. [20] found similar results when screening healthy human donors for an-

tibodies against laminarin (β -(1,3)-glucan), pustulan (β -(1,6)-glucan) and a preparation of *Candida albicans* cell wall β -(1,3)-(1,6)-glucan. They reported twice as much IgG binding to the β -(1,3)-(1,6)-glucan as compared to laminarin, and 4 times as much to pustulan. We also found relatively high levels of IgG antibodies against lichenan, a β -(1,3)-(1,4)-glucan without β -(1,6)-structures.

The strong correlations between IgG reactions to the three β -(1,6)-containing glucans, as well as between IgG

Table 4. Influence of potential determinants on anti- β -(1,6)-glucan IgG titers in three populations

	Agriculture e ^a	Bakery workers e ^a	Spray painters e ^a
Intercept e	1,955	6,700	7,280
Age ¹	0.99 (0.98–1.01)	1.00 (0.99–1.02)	1.00 (0.98–1.01)
Gender (male)	1.26 (0.83–1.90)	0.69 (0.31–1.55)	0.85 (0.54–1.34)
Smoking (current vs. non)	0.72 (0.52–0.99)	0.76 (0.56–1.05)	0.66 (0.50–0.87)
Dust exposure ²	0.95 (0.70–1.28)	0.99 (0.74–1.31)	
Endotoxin exposure ²	1.09 (0.85–1.28)		
Childhood on livestock farm	0.75 (0.52–1.10)		
Childhood on crop farm	1.16 (0.84–1.59)		
Currently a farmer	1.05 (0.66–1.66)		

e^a represents the relative increase in titer in the presence of the determinant (vs. its absence), or the increase associated with a 1-year increase in age (¹) or a 1-ln unit increase in exposure (²). Figures in parentheses are confidence intervals.

binding to the three β -(1,3)-(1,4)-glucans suggest at least two clusters of cross-reactive epitopes. Surprisingly, IgG binding to the β -(1,3)-(1,4)-glucan lichenan also showed moderate to high correlations with reactions to β -(1,6)-containing glucans, and less with the reactions to the other β -(1,3)-(1,4)-glucans. The inhibition EIA results (fig. 2a) also suggest that the IgG-binding epitope(s) of β -(1,3)-(1,4)-glucan lichenan may be structurally more related to that of the β -(1,3)-(1,6)-glucan from baker's yeast.

Results from the specificity experiments indicate that the strong antigenicity of pustulan must be due to its characteristic β -(1,6)-linked glucose chain structure. The loss of IgG-binding reactivity to the β -(1,6)-glucan pustulan after the periodate treatment in combination with NMR spectral analyses confirming the β -(1,6)-structure as the major component in the commercial pustulan preparation indicate that a major part of the human anti-glucan serum IgG specifically binds to the β -(1,6)-glucan structure. Although the NMR analysis does not exclude the presence of minor contamination at <5% it is highly unlikely that such contaminants might account for the strong IgG binding seen at the low coating concentration of pustulan (0.3 μ g/ml). The IgG binding to the baker's yeast β -(1,3)-(1,6)-glucan may be largely due to cross-reacting β -(1,6)-dependent epitopes, although it also depends on intact β -(1,3)-structures, as indicated by the partial loss of IgG-binding reactivity after both the glucanase and the periodate treatment. IgG binding to β -(1,3)-(1,4)-glucan lichenan similarly depends on the combination of glucanase-sensitive β -(1,3)-structures and periodate-sensitive β -(1,4)-structures. Thus, human sera

contain IgG antibodies specific for both β -(1,6)- and β -(1,3)-glucan structures.

Interestingly, the β -(1,3)-(1,4)-glucans from oat and barley bound human IgG only weakly, in spite of a similar primary structure as lichenan [9], which might be explained by variation in tertiary structures. Substantial contamination of lichenan by β -(1,6)-structures seemed highly unlikely. NMR spectral analysis (data not shown) confirmed the primary structures, with typical proton peaks characteristic for the β -(1,3)-(1,4)-structures in lichenan, while no typical β -(1,6)-linkage-associated peaks as in β -(1,6)-glucan pustulan and baker's yeast β -(1,3)-(1,6)-glucan were found. According to the manufacturers' information, all glucan preparations would be >90% pure, but since they all are extracted from natural sources, the possibility of minor impurities cannot be excluded. The observed differences between the binding of IgG towards the different glucans are, however, large, up to in some cases several orders of magnitude. It thus seems unlikely that such vast differences could be caused by minor impurities in the glucan preparations.

High IgG anti- β -(1,6)-glucan titers were found in three occupational populations. Surprisingly, the mean titer was significantly and approximately two-fold lower in the agricultural population, with the highest microbial and presumably the highest glucan exposures. Since spray painters have a specific work-related chemical, but no enhanced organic dust exposure, we assumed that their β -glucan exposure levels are comparable to that of the general population. Bakery workers are known to have increased airborne exposure to β -(1,3)-glucans [26], and wheat, oat and barley contain β -(1,3)-(1,4)-glucans

[9]. The agricultural workers are exposed to high levels of organic dust [30], of which bacteria and fungi form a large part [31], thus high microbial β -(1,3)-(1,6)-glucan exposures would be expected. The lower anti- β -(1,6)-glucan antibody titers in the agricultural population might indicate that the β -(1,6)-glucan exposure levels in the agricultural setting is much lower than assumed. However, there is thus far no method for the direct measurement of β -(1,6)-glucans in airborne or settled dust samples. Nevertheless, since dust levels and other characteristics of the environments were as expected, lower glucan exposure in the agricultural population than in the other populations is very unlikely. Knowledge of airborne β -(1,6)-glucan exposure levels depends on the development and validation of such specific techniques.

As a further complication, humoral anti- β -(1,6)-glucan immune responses may not only be driven by airborne exposures, but just as much by β -(1,6)-glucan exposure via the gastrointestinal tract, due to its presence in food specimens like edible mushrooms. Similarly, dermal contact might also contribute to the total glucan exposure. A recent study has identified β -(1,6)-glucan as the main structural cell wall component of the skin yeast *Malassezia sympodialis* [32]. These yeasts, commonly found on the skin of animals or humans, may cause skin diseases like eczema and dandruff, and could be another important factor in the body's total β -(1,6)-glucan exposure. Although there is little reason to expect huge differences between our study populations in exposure via the oral and dermal routes, our assumptions regarding airborne exposure may be irrelevant, if stimulation by other routes dominates the adaptive humoral anti- β -(1,6)-glucan immune response.

The only factor significantly associated with lower anti- β -(1,6)-IgG titers was smoking. Such a relation has been found for many other IgG responses [33], but it does not explain the difference in titers between the three populations, as the frequencies of smokers in the three popu-

lations were alike. Thus, there must be other yet unknown factors influencing the anti- β -(1,6)-glucan IgG production. In fact, the determinants in our regression models explained only a small proportion of the variance in anti- β -(1,6)-glucan IgG titers.

The health impact of these high levels of IgG anti- β -(1,6)-glucan is as yet unknown. They might play a role in immune modulation, for example, by inhibiting or even blocking in vivo binding of β -glucans to innate immune receptors [15, 16, 34] or by initiating a phagocytic immune response by complement activation and/or opsonization via the F_c receptor on immune cell surfaces. Such a mechanism may be supported by the results of Rubin-Bejerano et al. [35], who reported much higher neutrophil activities towards β -(1,6)-glucan- than β -(1,3)-glucan-coated particles and suggested a role of specific IgG- and complement-driven opsonization response. Thus, immune modulation as reported previously for β -(1,3)-glucans [36, 37] may also be investigated for β -(1,6)-glucans.

In conclusion, high anti- β -glucan antibody can be found in many normal human sera, and the predominantly reactive antigenic structures appeared to be associated with β -(1,6)-linkages. Anti- β -(1,6)-IgG titers in three different human populations were high. In the population with the presumably highest microbial exposures we found significantly lower levels of anti- β -(1,6)-IgG than in the other two less exposed populations. While the health impact of these antibodies remains unclear, our study clearly shows a need to include β -(1,6)-glucans in future exposure and immunological research.

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