



Anti- β -glucan antibodies in healthy human subjects

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ABSTRACT

Previous data by our group demonstrated the antifungal efficacy of a vaccine consisting of laminarin (β -(1,3)-glucan), conjugated with diphtheria toxoid, which generated protective anti-laminarin antibodies in mice. In this paper, we sought for the presence, isotype and subclass composition of natural anti-laminarin antibodies in an unselected population of human healthy subjects, in a comparison with antibodies directed against β -(1,6)-glucan (pustulan) and branched β -(1,3/1,6)-glucan (Pool 1) and mannan from *Candida albicans*. Almost all subjects showed detectable levels of anti- β -glucan antibodies, with IgG largely prevailing on IgM, little, if any, IgA and no IgE. However, the titer of anti- β -glucan antibodies was overall about 1 log lower than that of anti-mannan antibodies of the corresponding isotype. In particular, the level of anti-laminarin IgG was the lowest one, its geometrical mean titer (95% confidence interval, CI) being 1838 (1245–2714) as compared to 8157 (6067–10,931) and 3940 (2911–5332) for pustulan and Pool 1 fungal glucan, respectively. Analysis of IgG subclass composition showed that IgG2 was the prevalent subclass against any antigen, and again the concentration of anti-laminarin IgG2 was the lowest one, its geometrical mean concentration being 0.13 (0.07–0.24) μ g/ml as compared to anti-pustulan and anti-Pool 1 glucan and mannan IgG2 levels, which were 0.33 (0.2–0.5), 1.35 (0.9–2.0), and 36.1 (25.2–51.3) μ g/ml, respectively. These data show that anti-laminarin antibodies are present at low levels in humans as compared to other anti- β -glucan and, mostly, anti-mannan antibodies, and suggest that a protective antifungal vaccination in humans should attempt to tip the balance of antifungal antibodies in favour of the anti-laminarin ones.

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1. Introduction

After decades of neglect, antifungal vaccines have now gained general attention and consideration as critical novel tools to prevent and/or cure fungal infections. This has led to remarkable advances in identification of protective antigens, mechanisms of immuno-protection and vaccination targets [1,2]. Besides preventive vaccination approaches, use of antibodies for passive vaccination, with or without antimycotic therapy, is also being intensely pursued [3,4]. Within the various active and passive vaccination strategies, investigations with experimental models have suggested that some fungal antigens are endowed with the potential for inducing cross-immunity to even phylogenetically distant fungal organisms or even bacteria [5–7]. One of the most promising among these so-called “transphylectic” or “cross-kingdom” vaccines is a glycoconjugate between laminarin, an algal β -(1,3)-glucan polymer, and the diphtheria toxoid CRM197 (Lam-CRM vaccine [7]). This has been shown to induce protective anti- β -glucan antibodies

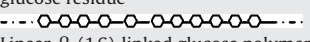
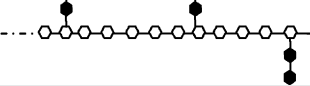
against the three major agents of opportunistic fungal infections in man, i.e. *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* [7,8].

β -Glucan is a major constituent of the fungal cell, conferring to the cell wall its typical strength opposing intracellular osmotic pressure. Of interest, β -glucan is also a constituent of a number of cell wall proteins which, besides their structural role in cell wall integrity, exert critical functions in fungus-host relationship [9,10]. Finally, β -glucan is also a natural ligand of various receptors present on cells of innate immunity, such as the Fc receptor, the complement receptor-3 and Dectin-1 [11].

Fungal β -glucans display two main isomeric configurations, namely β -(1,3) and β -(1,6), which are variously represented and intermixed in different fungi [9]. Laminarin, the antigen constituent of the protective Lam-CRM vaccine, is a linear, β -(1,3)-linked glucan, with only occasional, β -(1,6)-linked branches of a single glucose residue [12]. This suggests that the protective antibodies raised by the Lam-CRM vaccine recognize a β -(1,3)-configured epitope. Nonetheless, the nature of the specific molecule(s) bearing the protective epitope has not yet been clarified, and some antibodies recognizing pustulan, a linear, β -(1,6)-glucan were also produced in mice recipients of the Lam-CRM vaccine [7].

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Table 1
β-Glucan antigens used in this study.

Antigen	Structure	Source	Molecular weight
Laminarin	 Linear, β-(1,3)-linked glucose polymer with occasional β-(1,6)-linked side chains of a single glucose residue	<i>Laminaria digitata</i> (brown alga)	Approximately 5400 Da
Pustulan	 Linear, β-(1,6)-linked glucose polymer	<i>Umbilicaria papullosa</i> (lichen)	20,000 Da
Pool 1	 β-(1,6)-Linked glucan with β-(1,3)-linked side chains of 1–2 residues (1 every seven β-1,3)-linked glucosyl units	<i>Candida albicans</i>	Approximately 20,000 Da

● = β-(1,3)-linked glucose residue; ○ = β-(1,6)-linked glucose residue.

Assuming that the Lam-CRM vaccine owes its protection to the generation of anti-β-glucan antibodies in experimental models, it is important to assess presence, specificity and isotype of anti-β-glucan antibodies in human serum, in view of the possible use of this vaccine in humans. There are abundant sources of this polysaccharide in the environment, food and commensal microorganisms to which humans are currently exposed. While β-glucan is in itself a very poor immunogen, its presence as a moiety in complex, microbial cell wall molecules, particularly proteins [10], makes it a natural glycoconjugate, eventually enhancing its immunogenicity. While there is plenty of data on the presence in human serum of high levels of antibodies against fungal mannans or mannoproteins, very few data have been so far generated about the presence of anti-β-glucan antibodies and in no cases these antibodies have been characterized with respect to their subclass and specificity for the β-(1,3)- or β-(1,6)-glucan configuration [13–15].

In this paper we examined the anti-β-glucan antibody repertoire in normal, healthy Italian adults, in a comparison with antibodies against a mannoprotein preparation (>90% mannose) from *C. albicans*. Three different β-glucan antigens have been selected for the screening, representative of either linear β-(1,3)-glucan (laminarin) or linear β-(1,6)-glucan (pustulan) or branched, β-(1,3/1,6)-glucan of fungal origin (Pool 1 glucan from *C. albicans* [16]). We demonstrate here that anti-β-glucan antibodies of various isotypes and subclasses, mostly IgG2, are indeed present to noticeable levels in normal human serum. Their amount, however, is about 1 log lower than that of anti-*C. albicans* mannan antibodies, and they are prevalently directed against glucans with β-(1,6) rather than β-(1,3) configuration, these latter being practically absent or present at very low titers (<100) in some subjects.

2. Materials and methods

2.1. Antigens and sera

Laminarin and pustulan were purchased by Sigma Chem. Co., St. Louis, MO and Calbiochem, La Jolla, CA, respectively.

The Pool 1, β-(1,3/1,6)-glucan was separated by gel filtration chromatography from a purified, β-(1,3)-glucanase-solubilized preparation of *C. albicans* cell wall β-glucan, as described previously [16]. Molecular weight of the Pool 1 fraction was estimated by gel permeation chromatography and its molecular structure was determined by one- and two-dimensional nuclear magnetic reso-

nance [16]. Main characteristics and molecular structures of the β-glucan antigens used in this study are summarized in Table 1.

A mannan-rich, mannoprotein extract (polysaccharide >90%, w/w; protein 3–5%, w/w) containing major, immunodominant fungal antigens (MPF2) was prepared and purified from *C. albicans* cells as described in previous reports [17].

Human sera were obtained from 60 normal healthy adult volunteers. Blood donors were 34 females, aged 45.1 ± 9.2 years (mean $\pm$ S.D.), and 26 males, aged 47.4 ± 9.6 years, with no known history of present or prior infection by *C. albicans* or other fungi. Sera were heated at 56°C for 30 min and stored individually at -80°C .

2.2. Evaluation of anti-β-glucan and anti-mannan immunoglobulin levels in human sera

Comparison of IgG, IgM, IgA or IgE titers against the various β-glucan and mannan antigens was performed by ELISA, essentially as already described [7]. Briefly, polystyrene microtiter plates (MaxiSorp; NUNC, Roskilde, Denmark) coated with the antigens (50 μg/ml polysaccharide in 0.05 M carbonate buffer, pH 9.6, 100 μl/well, o.n. at $+4^\circ\text{C}$) and blocked 2 h at 37°C with 3% bovine serum albumin (Fraction V, Sigma) in PBS, were reacted with twofold dilutions of the human sera, followed by the appropriate, secondary antibody reagent (alkaline phosphatase-conjugated goat anti-human IgG, IgM, IgA or IgE antibodies, all purchased from Sigma). Plates were finally developed 30 min with p-nitrophenyl phosphate disodium (Sigma) as the enzyme substrate and read for adsorbance at 405 nm. Readings from negative controls (wells without human sera) were subtracted from all adsorbance values. Antibody titers were defined as the highest dilution of sera that gave an optical density at least twice that of negative controls. Ability by this ELISA format to reveal and discriminate antibody reactivity with β-glucans of different structure has been previously validated by the use of anti-β-glucan mAbs with selective specificity for β-(1,3), β-(1,6) and β-(1,3/1,6)-glucan antigens (Torosantucci et al., manuscript in preparation; see also Ref. [7]).

Levels of the various IgG sub-classes were determined as described above, using biotin-conjugated anti-human IgG subclass-specific monoclonal antibodies (BD Pharmingen, USA) as secondary antibody reagents, followed by further reaction with streptavidin-alkaline phosphatase conjugate (BD Pharmingen). A human standard serum with known concentrations of the various IgG subclasses (Nordic Immunologic Laboratories, The Netherlands) was used as a calibrator for quantitative comparison of

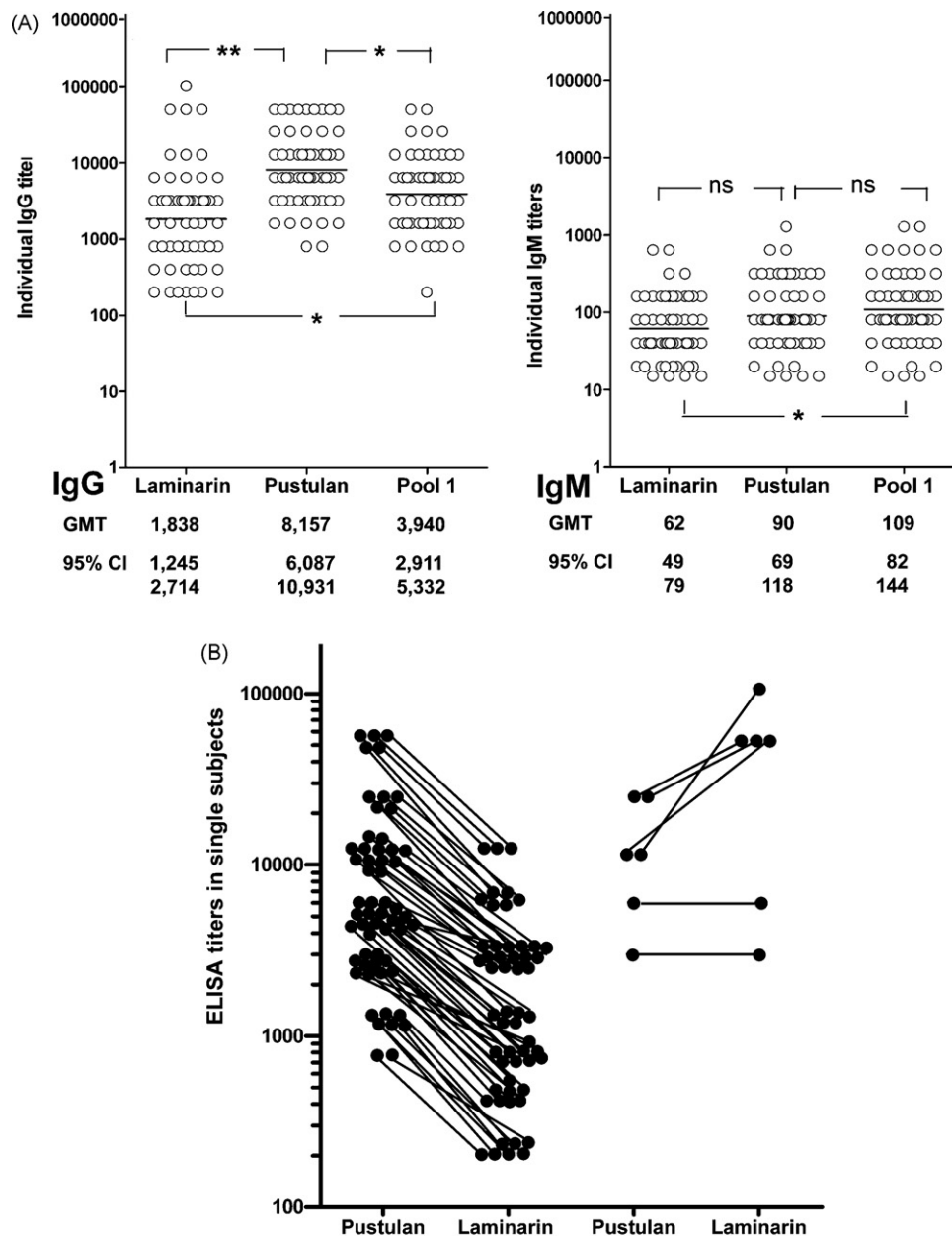


Fig. 1. Distribution of IgG and IgM, anti- β -glucan antibody titers in healthy human individuals. Panel A reports individual IgG and IgM titers against the different β -glucan antigens, as assessed by ELISA in 60 subjects. GMTs and 95%CI calculated for each antigen are reported at the bottom of the graphs and indicated (GMT) in the figure by lines. Asterisks point out statistically significant differences in antibody levels against the indicated antigens (* $P < 0.05$; ** $P < 0.001$). Panel B compares anti-laminarin and anti-pustulan IgG titers in each single subjects as above; subjects with anti-pustulan > anti-laminarin titers and those with anti-laminarin $\geq$ anti-pustulan titers are represented in the left or right part of the graph, respectively.

β -glucan specific human IgG1, IgG2, IgG3 and IgG4. To this purpose, additional wells coated with doubling dilution of the serum standard (corresponding to 280 to 8.8, 60 to 0.47, 70 to 2.2 and 8.5 to 0.26 ng/ml of IgG1, IgG2, IgG3 or IgG4, respectively) were assayed alongside the test sera. Resulting O.D. 405 nm values, after subtraction of negative controls, were plotted against Ig concentration, generating a linear, standard curve which was used to interpolate O.D. units from test sera.

2.3. Statistical assessment

All calculations and statistical analysis were performed by the Prism 4.0 software (GraphPad Software Inc., San Diego, CA). Linear

regression and best-fit curve analysis were used for elaboration of IgG subclass standard curves and for interpolation of assay results. Statistical probability values (P) upon comparison of the various sets of serological data were assessed by analysis of variance (non-parametric Newman-Keuls ANOVA) followed by Dunn's post-test.

3. Results

3.1. Isotypes and antigen specificity of serum anti- β -glucan antibodies in humans

Because of the complexity of β -glucan constituents in fungi and the lack of data regarding the antigenicity of their different

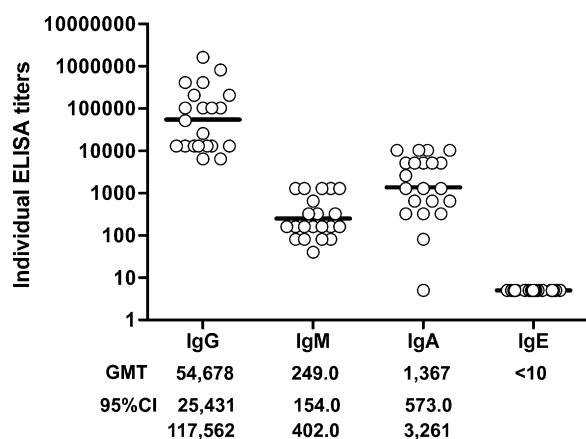


Fig. 2. Levels of anti-*C. albicans* mannan antibodies in healthy humans. ELISA titers of the various Ig isotypes against a purified, antigenic mannan preparation from *C. albicans* were measured in 21 subjects. Numerical values of GMTs (also indicated with lines in the graph) and 95%CI are reported at the bottom of the figure.

molecular configurations, we compared antibody titers of human sera against a panel of well-characterized β -glucan compounds of different molecular structure, which were tested as plastic-bound antigens in ELISA. They comprised a native β -glucan molecule from *C. albicans* with intermixed β -(1,3) and β -(1,6) configurations, and linear β -(1,3)-(laminarin) or linear β -(1,6)-(pustulan) glucans from non-fungal source. Some details about these antigens are reported in Table 1.

Almost all of the 60 subjects considered in the study showed detectable levels of antibodies recognizing laminarin, pustulan and *Candida* β -glucans, although with rather large individual variations (Fig. 1A). IgG largely prevailed on IgM which, in most subjects, were below an appreciable titer (GMT against the three antigens around or lower than 100). Remarkable differences were found in average IgG reactivity against the three different antigenic preparations: the highest reactivity of the human sera was against pustulan (GMT 8157; 95%CI 6087–10,931) and the lowest against laminarin (GMT 1838; 1245–2714), ($P < 0.001$), this latter showing an extremely broad titer range (from around 200 to around 100,000). The reactivity against *Candida* β -glucan was intermediate between the two (GMT 3940; 2911–5332) ($P < 0.05$ versus both pustulan and laminarin). Preferential specificity of human anti- β -glucan antibodies for pustulan-like (β -(1,6)-linked) rather than for laminarin-like (β -(1,3)-linked) molecules was also confirmed by direct comparison of anti-pustulan and anti-laminarin IgG titers in single individuals (Fig. 1B). In fact, among the 60 subjects considered, almost all showed 4–10 fold higher anti-pustulan than anti-laminarin IgG titers, while the reverse was only found in four subjects (Fig. 1B).

With rare individual exceptions, no IgA or IgE titers > 100 against any glucan preparation was found (data not shown). In addition, no qualitative or quantitative differences in anti- β -glucan antibody titers were noticed between male and female individuals (not shown).

Randomly selected sera from 21 subjects were also tested for anti-*C. albicans* mannan antibody isotypes by the same ELISA format. As shown in Fig. 2, very high IgG titers and appreciable IgA titers and, in some subjects, IgM were found. Overall, anti-mannan titers averaged nearly 1 log higher than the corresponding isotype titers of anti- β -glucan antibodies. For instance, as compared to anti- β -glucan GMTs (see Fig. 1A) the anti-mannan IgG GMT was 54,678 (25,431–117,562) and the IgM GMT was 258.7 (154.0–401.7) ($P < 0.01$ in all comparison with anti- β -glucan antibodies).

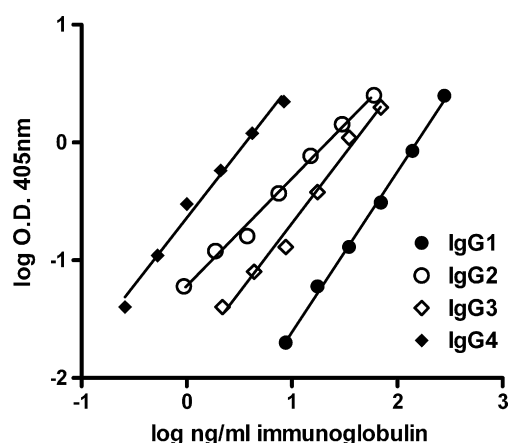


Fig. 3. Standard calibration curves for IgG subclass quantitation. The figure shows an example of ELISA dose-response curves by human IgG1, IgG2, IgG3 and IgG4. Lines represent best-fit curves, as inferred by linear regression analysis, whereas symbols refer to actual, experimentally measured values.

3.2. Anti- β -glucan and anti-mannan IgG subclass composition

Thirty human sera were further assayed by ELISA for their IgG subclass composition and reactivity with the different β -glucan and mannan antigens. In these experiments, IgG subclasses were quantified by comparison to standard curves employing defined concentrations of human IgG1, IgG2, IgG3 or IgG4 antibodies. An example of standard curves determined for the various subclasses is shown in Fig. 3. Fig. 4A–D reports, for all subjects examined, ranges and geometric mean (GM) concentration (ng/ml) of IgG subclass antibodies against the three β -glucan antigens and mannan. As shown in panels A–C, IgG1 and IgG2 against any glucan antigen were largely prevalent over IgG3 and IgG4 antibodies ($P < 0.001$ considering IgG1 or IgG2 versus IgG3 or IgG4 levels for all antigens). In turn, for each β -glucan antigen, the amount of IgG2 was significantly higher ($P < 0.01$) than the amount of IgG1. Namely, GM concentrations (95%CI) of IgG1 against *Candida* glucan, pustulan and laminarin were 1.35 (0.9–2.0), 0.33 (0.2–0.5) and 0.13 (0.07–0.24) μ g/ml, respectively, while the GM IgG2 concentrations against the same antigens were 24.5 (16.2–37), 6.3 (4.6–8.3) and 0.62 (0.34–1.1) μ g/ml, respectively. Levels of IgG3 and IgG4 were, instead, much lower (GM < 0.09 μ g/ml for all antigens).

In analogy to what found for total IgG titers, serum concentrations of any IgG subclass against laminarin resulted constantly lower as compared to those measured for the other β -glucan antigens, a difference which was particularly pronounced and highly significant ($P < 0.001$) in the case of IgG2 and IgG3 antibodies.

Concerning the anti-mannan IgG subclasses (shown in Fig. 4D), IgG1 and IgG2 were mostly represented, as found for the β -glucan antigens, but most of the sera had also appreciable amounts of IgG3 and IgG4 (GM 1.41; 0.7–2.8 95% and 0.4; 0.2–0.8 μ g/ml, respectively). As expected from comparison of total IgG levels, anti-mannan IgG1 and IgG2 were definitely higher than the corresponding subclass against any β -glucan antigens. Of interest, and in contrast with the anti- β -glucan antibodies, the IgG1 and the IgG2 were equally represented among anti-mannan antibodies since their mean serum concentrations did not significantly differ (GM 23.4 (12.8–42.8) and 36.1 (25.2–51.3) μ g/ml for IgG1 and IgG2, respectively).

In a group of 18 randomly selected individuals, we also assessed, for each IgG subclass, percentage amounts of Abs directed against the various β -glucan antigens. As shown in Fig. 5, the results of this analysis confirmed that Abs directed against laminarin were constantly the least represented among anti- β -glucan IgG1, IgG2

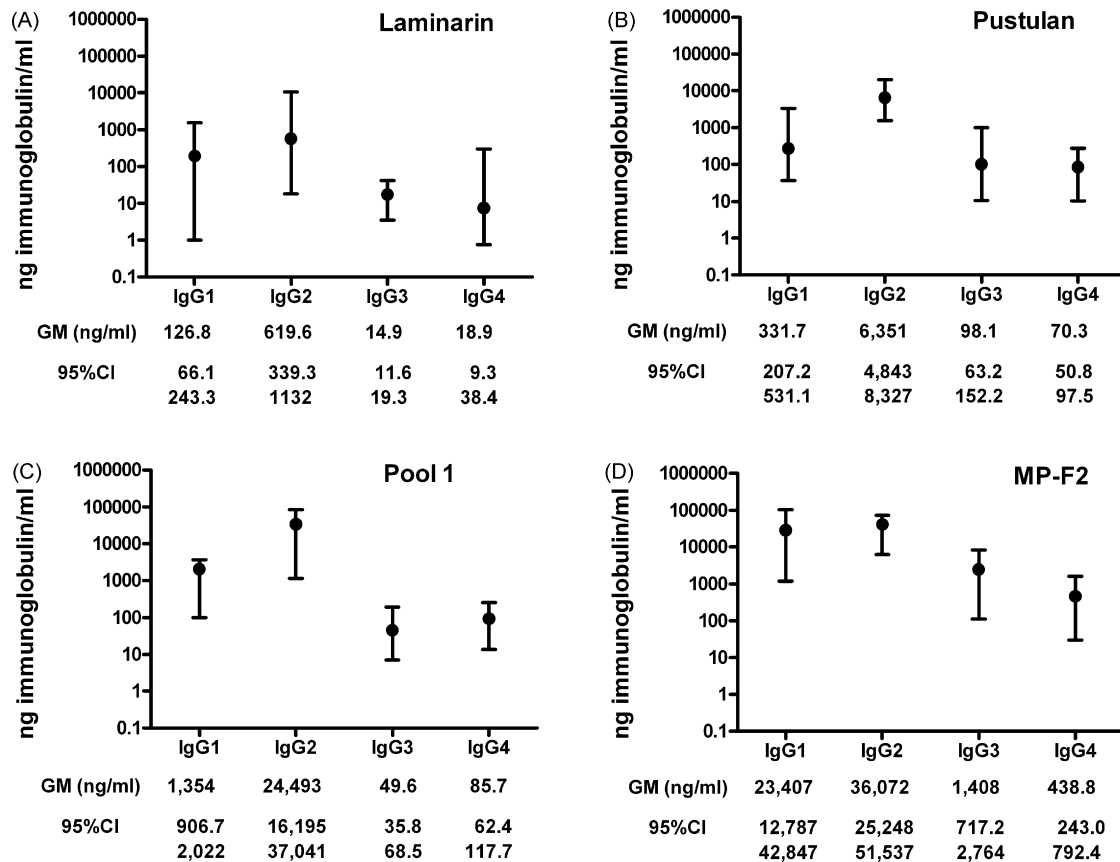


Fig. 4. Subclass composition of anti-β-glucan and anti-mannan, natural IgG antibodies. The graphs present median values (symbols) and ranges (lines) of IgG subclass, anti-β-glucan antibody concentration in serum from 30 individuals, expressed in ng/ml. Panels A–D show reactivity against laminarin, pustulan, *C. albicans* glucan (Pool 1) and *C. albicans* mannan (MP-F2), as indicated. For each antigen, geometric mean antibody concentrations (GM) and 95%CI are reported at the bottom of the panel.

and IgG3 subclasses, accounting for no more than 20% of the total amount of any of these IgGs in almost all tested subjects. In contrast, percent amount of anti-laminarin, anti-pustulan and anti-Pool 1 IgG4 was almost similar. The most striking difference in terms of cognate antigen specificity was seen among the IgG2, which, displayed a well-evident “hierarchy” of specificity, with net predominance of Abs binding to Pool 1 fungal β-glucan ($76.9 \pm 2.6\%$, mean $\pm$ S.E.M., of all anti-β-glucan IgG2), followed by

pustulan-binding Abs, which averaged $18.8 \pm 2.5\%$ and, lastly, by laminarin-binding Abs which represented only a $3.4 \pm 1.0\%$ of the anti-β-glucan IgG2 repertoire (all comparison $P < 0.01$).

4. Discussion

The Lam-CRM vaccine, which confers protection against infections by different fungal pathogens in experimental rodent models,

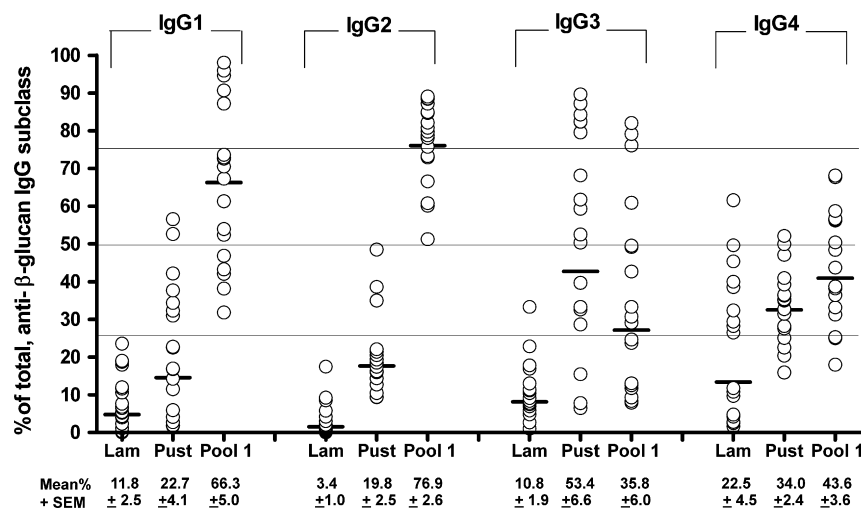


Fig. 5. Distribution by specificity of the human anti-β-glucan IgG subclass repertoire. Percent amounts of Abs directed against the different β-glucan antigens among each IgG subclass have been individually calculated for 18 subjects. Mean% values are indicated with lines in the graph and reported, together with the relative SEM, at the bottom of the figure.

has been shown to induce the production of IgG prevalently directed against linear β -glucans with a β -(1,3) configuration (such as the compositional vaccine antigen laminarin), and, to some extent, also against β -glucans in β -(1,6) configuration as, for instance, pustulan. Both laminarin- and pustulan-like molecular structures are commonly present in β -glucans of most pathogenic fungi [7]. The balance between these two specificities is strongly influenced by the nature of the adjuvant and route of administration (data not shown). Although the protective role of the two specificities has not been wholly clarified, previous data [7] and some still unpublished results with the use of monoclonal antibodies preferentially or exclusively recognizing laminarin or pustulan in passive vaccination experiments (Torosantucci et al., manuscript in preparation), strongly suggest that the protective β -glucan epitope has a linear, β -(1,3), laminarin-like configuration. Previous data have also indicated that anti-mannan antibodies may exert competing, inhibitory effects on the protective capacity of anti- β -glucan antibodies [18], and that the antibody isotype and subclass may strongly influence, if not reverse, the specificity and the antifungal, protective capacity of certain anti-saccharide antibody [19,20].

Since β -glucans with a similar molecular organization as laminarin or fungal β -glucans are variably but commonly present in food, environment and in human-commensal microorganisms, mostly complexed with other constituents, in particular with proteins, the detection of anti- β -glucan antibodies in human sera is quite a logical expectation. They have indeed been found [13–15] and assigned a presumptive role in defense against fungal pathogens [13]. It has been reported that anti- β -glucan antibodies possess a good specificity and positive predictive value for diagnosing invasive *Candida* infections in human patients [14]. Anti-laminaribioside antibodies have been also recently described as new serological markers of Crohn's disease in humans [21] and, interestingly, were also shown to be generated during *C. albicans* infection (Poulain D, personal communication).

To our knowledge, however, no detailed data about the ability of human antibodies to recognize β -glucans with different molecular organization, nor about their isotype and IgG subclass composition have been reported in previously published investigations. Overall, investigations on anti- β -glucan antibodies have been very few and limited to very few subjects, as compared to the plethora of those concerning other fungal antigens such as, for instance, the anti-*Candida* mannan antibodies. The search for the presence, fine specificity, isotype and subclass of anti- β -glucan antibodies in healthy, naive subjects is a critical aspect for identifying the actual correlates of Lam-CRM vaccine protection and to define the potential target of this vaccine in humans.

In this paper, we tried to fill this gap and show for the first time remarkable differences in natural antibody reactivity with different β -glucans, also tested through quantification of glucan-reactive IgG subclasses.

The first evidence provided by our investigation is that, altogether, anti- β -glucan antibodies are indeed commonly present in human serum, although with rather large individual subject variations. These antibodies are of the IgG isotype and more specifically of the IgG2 and IgG1 subclasses, as expected from a memory response to persistent exposure to β -glucan complexed with proteins or other immunogenicity-enhancing constituents, also suggesting a mixed Th1/Th2-type T-cell response on natural exposure to these components. Different exposure to the different sources of this antigen, coupled with the possible different immunogenicity of the β -glucans present in the different sources, could account for the wide differences in individual antibody response. For this aspect, our data are in substantial agreement with those reported by other authors, although we measured much higher levels of IgG2 than those measured by Kondori et

al. in healthy subjects [14] and also found well-detectable levels of IgG1, IgG3 and IgG4, in contrast to that reported by Keller et al. in an early study [15]. These discrepancies may be explained by methodological differences, particularly the use by the above authors of less refined and characterized antigens (fungal cells or complex β -glucan preparation from non-pathogenic yeasts) than those employed here. Particularly, no distinction could be previously made between antibodies directed against β -(1,3)- or β -(1,6)-linked, β -glucan moieties.

The second evidence presented here is that anti- β -glucan antibody titers are as average 1 log lower than anti-*C. albicans* mannan antibodies of the same subclass. Fungal mannans are also complexed with proteins and are generally present in the same materials with β -glucans, for instance in yeasts and molds [9]. Although mannoproteins of some fungal species play important role in host–fungus relationship and in fungal virulence [22], it is not clear to what extent antibodies against the mannan moiety of mannoproteins are protective. In the best studied model, namely experimental disseminated candidosis, some antibodies against a β -mannoside cell wall moiety have been shown to transfer protection in passive vaccination, but antibodies against α -mannan, which is the largely prevalent configuration in *C. albicans*, may also be inhibitory [23]. In human candidosis, the value of anti-mannan and anti- β -glucan antibodies, and their interaction in terms of protection from fungal disease, are debated [24] but it is a common clinical observation that the natural anti-*Candida* antibody repertoire in human serum does not confer antifungal protection, suggesting that either the amount or the prevalent specificity or the isotype of natural anti- β -glucan antibodies is non-protective, or that the plethora of irrelevant anti-mannan antibodies (or others) negates the action of potentially protective, anti- β -glucan ones.

In the above context, the third evidence gathered here is that human IgGs and in particular the IgG2 subclass, which is the main IgG subclass elicited against microbial (and β -glucan) polysaccharides in humans [15,25], mostly recognize β -(1,6)-linked β -glucans, such as pustulan and, even more strongly, β -(1,3) branched- β -(1,6)-glucans such as the native *C. albicans* glucan. In contrast, they recognized to a much lesser extent the β -(1,3)-linked glucan of the laminarin type. This suggest that the sources of β -glucans to which humans are naturally exposed are prevalently enriched with β -(1,6)-linked, linear or branched glucans or that these types of β -glucan are more immunogenic under native conditions. Whatever the reason, the marked prevalence, in humans, of natural anti- β -glucan antibodies directed against β -(1,6) rather than against β -(1,3) antigenic determinants is of particular importance in view of our previous reports suggesting that the protective antibodies elicited by the Lam-CRM vaccine in mice were directed against a β -(1,3)-linked, linear epitope ([7]; Torosantucci et al., manuscript in preparation). Compared with the plethora of all other possibly irrelevant if not inhibitory antibodies present in human serum, these data point out the likely inadequacy of the low average quantity of anti- β -(1,3)-glucan antibodies present in human serum to play a significant role in natural protection against fungal infection.

Human IgG subclasses differ with respect to physical, chemical, and biological properties. The IgG2, prevalent anti- β -glucan IgG subclass, show poor binding to Fc receptors on macrophages and neutrophils and do not efficiently fix complement [26,27]. These characteristics may render these antibodies inefficient in promoting antifungal protective immunological responses such as phagocytosis. This may add to non-optimal β -glucan fine specificity in determining a low protective valence of the natural repertoire of anti- β -glucan antibodies in humans.

We are fully aware of some limitations inherent in the present study, as for instance the relatively low number of subjects

examined and the unavailability of data about the influence of diet, use of yeast as probiotics and possible mucosal colonization by *Candida* or other saprophytic microorganisms expressing β -glucans. As previously noted, differences in all the above factors may possibly account for the wide range of antibody levels found in our subjects. A caveat is also necessary regarding our ELISA test for quantification of anti- β -glucan IgG subclasses. This, in fact, has been mostly designed to negate any bias due to differences among the IgG subclass-specific, secondary Ab reagents, but it might be not fully reliable because of competition among IgGs for β -glucan binding, leading to possible underestimation of some less abundant or less affine IgGs. Nonetheless, our data, while expanding the very poor, previous knowledge on the presence of natural anti- β -glucan antibodies, definitely show their composition in terms of isotypes and IgG subclasses and document their fine antigenic specificities with regard to the different β -glucan molecular structures by the use of purified and well-characterized antigenic reagents. Particularly, if the protective epitope of the Lam-CRM vaccine is a β -glucan with a linear, β -(1,3)-linked molecular structure, as initial data suggest, the demonstration provided here of the low level of natural anti-laminarin antibodies as compared to all other anti-glucan antibodies, strongly suggest that the schedule of Lam-CRM vaccine administration with adjuvants in humans should be such as to tip the anti- β -glucan antibody balance in favour of those mostly recognizing the β -(1,3)-glucan configuration.

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