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OPINION/HYPOTHESIS
Host-Microbe Biology

Potential of Chemically Synthesized Oligosaccharides To Define the Carbohydrate Moieties of the Fungal Cell Wall Responsible for the Human Immune Response, Using *Aspergillus fumigatus* Galactomannan as a Model

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ABSTRACT Methodologies to identify epitopes or ligands of the fungal cell wall polysaccharides influencing the immune response of human pathogens have to date been imperfect. Using the galactomannan (GM) of *Aspergillus fumigatus* as a model, we have shown that synthetic oligosaccharides of distinct structures representing key fragments of cell wall polysaccharides are the most precise tools to study the serological and immunomodulatory properties of a fungal polysaccharide.

KEYWORDS *Aspergillus fumigatus*, aspergillosis, antibodies, cytokines, chemokines, immunology, *Aspergillus*, galactomannan, glycoarray

Fungi are the only eukaryotes protected by a polysaccharide shell with an ambivalent function among pathogens, having a protective role against environmental stress and a negative role in the induction of an antifungal immune response (1). The carbohydrate fragments of the cell wall polysaccharide responsible for the induction of the immune response have often been poorly defined due to a lack of efficient tools. Currently, mutants lacking one polysaccharide due to the deletion of genes regulating its biosynthesis or polysaccharides purified from the cell wall are used. The first approach is indirect and does not take into account the putative compensatory reactions resulting from the gene deletion. The second approach results from purification of the cell wall polysaccharides. However, if it is possible to identify the target oligosaccharides in the case of long homogenous polysaccharides [and has been very appropriate in the identification of dectin-1, a receptor recognizing specifically the β-(1→3)-glucan chains (2)], this approach is more difficult when the composition of the repeating units of the polysaccharide is complex. This is the case for the galactomannan of *Aspergillus fumigatus*, which is composed of tetraose repeats with mannose units with α-(1→2) and α-(1→6) linkages bound to short side chains comprising β-(1→5)- and β-(1→6)-linked galactofuranose units of various lengths (3–5). It is even more difficult in the case of long polysaccharides without repeating units, such as the galactosaminogalactan of this species (6). The isolation of absolutely pure water-insoluble polysaccharide from the cell wall and the solubilization of immunoreactive oligosaccharides are difficult without structural modifications resulting from the harsh chemical extraction procedure required to solubilize the cell wall oligosaccharides.

In this report, we present an analysis of the oligosaccharides responsible for the immune response of the human host against the galactomannan (GM) of *A. fumigatus*. Even though galactofuran has been recognized as a potent *Aspergillus* immunogen (1, 7), the GM fragments modulating the host immune response have not been fully

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Cell wall glycoarray
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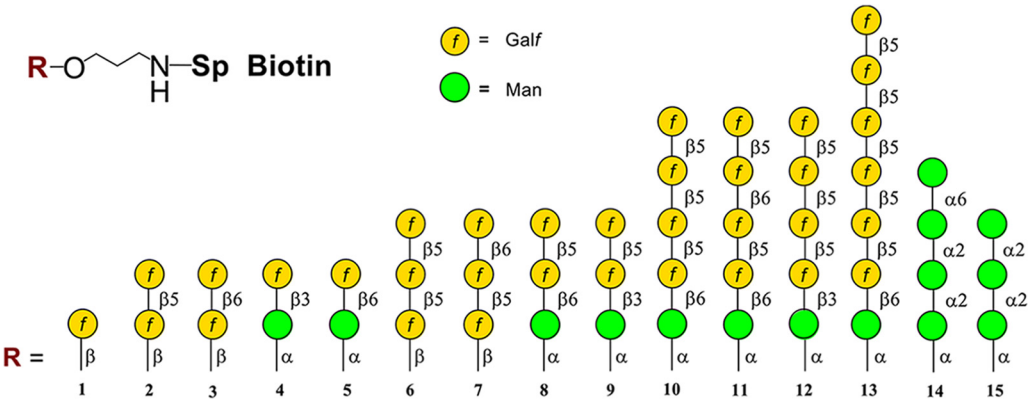

FIG 1 The structure of oligosaccharide ligands representing key structural elements of the galactofuranosylated side chains (ligands 1 to 13) and mannan backbone (ligands 14 and 15) of *A. fumigatus* galactomannan.

characterized. This study presents a new approach based on the use of synthetic oligosaccharides which allows a precise and unbiased identification of the carbohydrates responsible for the immune response.

Glycoarray of oligosaccharides encompassing the complete structure of the galactomannan of *Aspergillus fumigatus*. All fragments of the galactomannan molecule used in this study were chemically synthesized (Fig. 1). Oligosaccharides 1 to 13 related to galactofuranosylated side chains of galactomannan were obtained as previously described (4, 8, 9). Manno-oligosaccharides 14 and 15 (for preparation, see Text S1 in the supplemental material) represented a repeating unit of the mannan backbone of galactomannan. All oligosaccharides were biotinylated (10) and adsorbed to a streptavidin-coated plate to quantify the immune response.

The glycoarray with Gal*f*(1→5)Gal*f* blocks can be used to trace specific antibodies in sera from ABPA and CPA patients. No antibodies recognizing oligomannosides 14 and 15 were detected in the chronic pulmonary aspergillosis (CPA) or allergic bronchopulmonary aspergillosis (ABPA) patient sera (Fig. 2 and Text S2). Similarly, no antibodies recognizing ligands 1, 4, and 5 containing only one galactofuranose (Gal*f*) unit were detected in sera from controls and patients (Fig. 2). In contrast, the ligands with two Gal*f* units linked through a (1→5) linkage (ligands 2, 8, and 9), but not through a (1→6) linkage (ligand 3), gave antibody titers which were significantly higher in patients with ABPA or CPA than in the controls ($P < 0.0001$) (Fig. 2). Ligand 13 had the highest area under curve (AUC) value for both ABPA and CPA patient sera (Tables S1 to S4). However, the differences with oligosaccharides 6 to 13 were not statistically significant for patient discrimination (Tables S1 to S4). Interestingly, the presence of one to two Gal*f*(1→6)Gal*f* blocks in oligonucleotide-Gal*f* sequences with Gal*f*(1→5)Gal*f* blocks (ligands 7 and 11) did not affect their ability to distinguish between control and patient sera (Tables S1 to S4). The nature of the linkage between the oligonucleotide-Gal*f* chain and mannan (Man) unit [either a β-(1→3) linkage for ligands 9 and 12 or a β-(1→6) linkage for ligands 8 and 10] did not affect the level of antibody recognition (Fig. 2 and Tables S1 to S4).

Early studies with immunocompetent patients with CPA or ABPA have shown that high antibody titers against GM were detected in the sera of these patients (7). Even though the immunodominance of the oligonucleotide-Gal*f* sequences in GM has been repeatedly shown in the past, the chemical nature of the epitope recognized in GM was not precisely identified (1). The use of a set of chemically synthesized oligosaccharides representing different parts of side oligonucleotide-Gal*f* sequences in GM has permitted the identification of the epitope recognized by the anti-*A. fumigatus* antibodies. Interestingly, no antibodies bound to the oligomannosides which are fragments of the repeating units of the mannan backbone of *Aspergillus* cell wall GM. This situation is entirely different from the mannan of *Candida* species. The *Candida* cell wall mannans

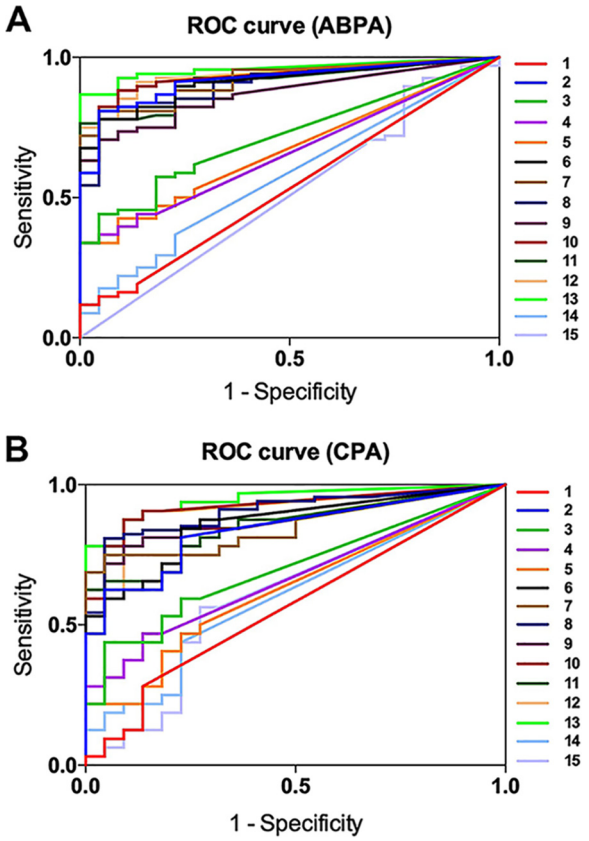

FIG 2 Results of enzyme-linked immunosorbent assay (ELISA) data with different oligosaccharide ligands related to *A. fumigatus* galactomannan and sera of aspergillosis patients. (A and B) The results are expressed as receiver operating characteristic (ROC) curves plotted for ABPA patient sera (A) and CPA patient sera (B) with regard to the control sera. Sensitivity represents the fraction of patient sera ranking as positive (true positive), and specificity represents the fraction of control sera ranking as negative (true negative). See Tables S1 and S2 for the statistical significance of the results.

are well-known antigens recognized in patient sera and have been used in the past for serotyping this species (11). The antibody response against *Candida* mannan is mainly associated with the linear α-(1→2)-linked side chains of the mannan core and the β-(1→2)-mannan oligosaccharides. Accordingly, the linkages between the mannose residues are essential for determining an immune response (11). Similarly, in *A. fumigatus*, the linkages between the galactofuranose residues and the size of the oligosaccharides are important since β-(1→6) linkages are not recognized unless they are intercalated with β-(1→5) linkages. These results showed that the recognition of the GM by the human antibodies is not dependent on a strict three-dimensional structure of the oligonucleotides. In addition, it is interesting to note that a Gal*f* disaccharide is a recognized antigenic epitope, while earlier studies have specified that the best oligosaccharide sequences usually recognized by antibodies have a degree of polymerization of 4 or 5.

Oligosaccharides with Gal*f*(1→5)Gal*f* blocks can be used to understand the secretion of cytokines and chemokines by immune cells. The production of the specific cytokines interleukin 1 beta (IL-1β), IL-1Ra, IL-6, and tumor necrosis factor alpha (TNF-α) and chemokines CCL2, CCL3, CCL4, CCL5, and CXCL1 (known to be associated with aspergillosis [12, 13]) produced by peripheral blood mononuclear cells (PBMCs) in the presence of the oligosaccharides was quantified (Fig. 3 and 4 and Text S3). The findings and conclusions based on the analysis were basically similar for the chemokines and cytokines. Like for the antibody study, the ligands with only one Gal*f* unit (ligands 1 and 3 to 5) and oligomannosides (ligands 14 and 15) did not induce the

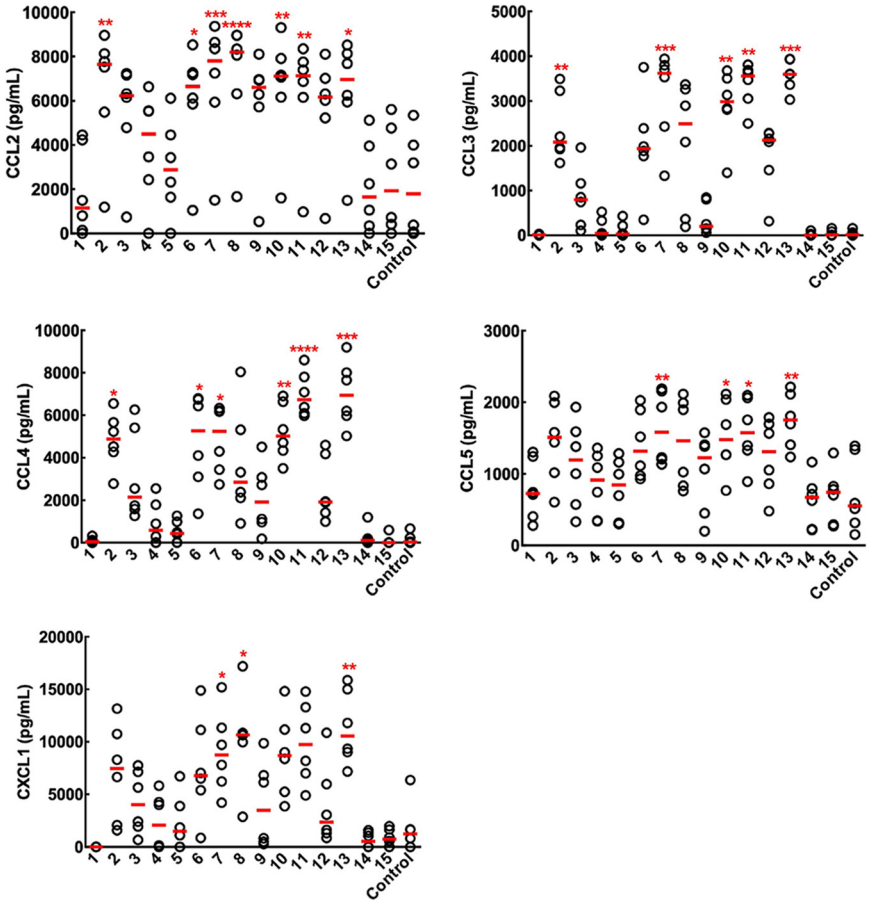

FIG 3 Chemokine induction by the oligosaccharides. The biotinylated oligosaccharides 1 to 15 were coated onto streptavidin microtiter plates, and PBMCs (six donors) were added to the microtiter plates in RPMI medium supplemented with 10% normal human serum. Controls are unstimulated PBMCs on streptavidin or nonstreptavidin microtiter plates. The chemokine concentrations obtained from each donor are plotted as dots, where the center red line indicates the median. The median chemokine concentrations were compared with those of the unstimulated PBMCs (control) by a Kruskal-Wallis test and Dunn's multiple-comparison test (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

production of cytokines and chemokines (Fig. 3). Similar amounts of cytokines and chemokines are produced in the presence of heat-inactivated serum or normal serum, indicating that the production of cytokines and chemokines is independent of the complement (data not shown). The analysis of the response of the trisaccharide and pentasaccharide 8 and 10, respectively, and their isomers, 9 and 12, respectively, showed that the nature of the chemical linkage of the galactofuran to the mannan chain significantly influenced the production of cytokines and chemokines (in contrast to the antibody data); the β-(1→3) linkage between tetra-Gal*f*- and Man was found to be detrimental to the production of cytokines. In contrast, the occurrence of a β-(1→6) linkage inside the galactofuran composed of (1→5) linkages did not influence cytokine and chemokine production (compare ligands 10 and 11). Like for the antibody epitope, galactofuranosides of two units with one β-(1→5) linkage between Gal*f* residues induced the production of cytokines and chemokines, although at a very limited amount, especially for the cytokines. Heptasaccharide 13 significantly induced the highest expression of all the chemokines and cytokines tested (Fig. 4).

The chemokines (CCL3, CCL2, and CCL5) followed in this study have been previously shown to be associated with aspergillosis since they are involved in the recruitment of neutrophils, platelets, and monocytes, but the molecules responsible for the chemokine production have not been identified (12, 13). GM has been previously recognized to induce the production of cytokines, but the conclusions on the pro- or anti-

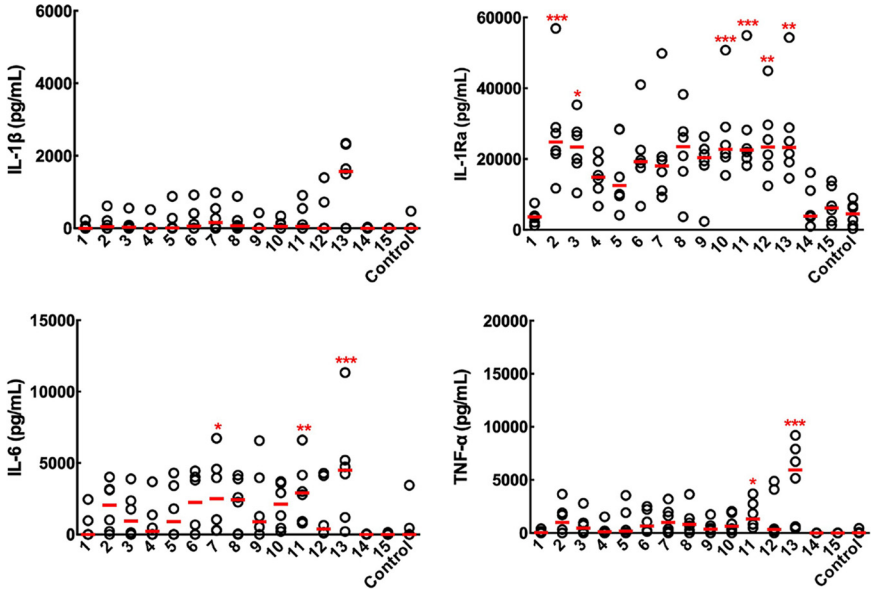

FIG 4 Cytokine induction by the oligosaccharides. The amounts of cytokines in the culture medium were examined by ELISA. The median chemokine concentrations were compared with those of the unstimulated PBMCs (control) by a Kruskal-Wallis test and Dunn's multiple-comparison test (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

inflammatory function of GM have been controversial (14–16). The results indicated that in contrast to antibodies, the production of cytokines and chemokines was facilitated by longer chains of β-(1→5)-linked Gal*f* units. It will be now possible to analyze the presentation of the carbohydrate antigen on the major histocompatibility complexes. Similarly to patient antibodies, the production of cytokine and chemokines from PBMCs was not induced by GM-related oligomannosides. Again, this situation is opposite from the immune response against *Candida* spp. *Candida* mannan induces a strong immune cellular response. However, these studies were mostly based on the use of mannosyltransferase mutants rather than on the use of pure oligosaccharides, and conclusions were indirectly based on the lack of or modified response seen with the mutants rather than on a direct positive response of the cell toward pure oligosaccharides (17). Compensatory reactions resulting from the gene deletion often modify the composition of the cell wall; consequently, the immune response against the mutant may not result from the lack of the target polysaccharides but may be hidden by the modifications of the cell wall resulting from these compensatory reactions (1). Moreover, the discrepancy between immunological studies run with polysaccharides might be due to the insufficient purification of the polysaccharide used, since the precise characterization of the immunostimulatory activities of polysaccharides requires the use of pure polysaccharides (1, 18).

The purity of chemically synthesized oligosaccharides can be easily controlled and will make synthetic oligosaccharides a perfect tool to understand the immune role of carbohydrate moieties. In addition, the biotinylation of the oligosaccharides allows their quantitative immobilization onto streptavidin-coated devices, which can allow amplification of the response between the oligosaccharides and the immune cell surface. Even though many receptors (mannose-binding lectin, mannose receptor, dectin-2, dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin [DC-SIGN], intelectin-1, and even dectin-1) have been claimed to bind to GM (1, 14, 15, 19, 20), the binding has not been demonstrated biochemically. Such chemical tools will be the best use for such a demonstration.

Conclusion. Even though glycoarrays have been developed in the past (21, 22), they have not been focused specifically on fungal polysaccharides. Recent studies demonstrated that glycoarrays cannot be universal and that the carbohydrate molecules

blotted on the array have to be specifically designed for the specific research project considered since not all oligosaccharides present in nature can be deposited on a single array. This study demonstrated that chemically synthesized oligosaccharides specifically designed to represent fragments of the fungal cell wall are appropriate tools to investigate precisely the immune response against this insoluble polysaccharide shell. We have in our hands oligosaccharides from all essential cell wall polysaccharides of *A. fumigatus* cell wall [α- and β-(1→3)-glucans, chitin, galactomannan, and galactosaminogalactan]. These oligosaccharides have now been bound to streptavidin beads to follow their internalization and their recognition at the phagosome level using a strategy previously developed by others to analyze the interactions between β-(1→3)-glucan and dectin-1 at the phagosomal level (23). Such approach will allow a precise definition of the mediators other than immunoglobulins, which can play a role in inducing an immune response against the cell wall components. In addition, such a strategy can also be applied to all cell wall polysaccharides from various human fungal pathogens.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.1 MB.

TEXT S2, DOCX file, 0.1 MB.

TEXT S3, DOCX file, 0.1 MB.

TABLE S1, DOCX file, 0.1 MB.

TABLE S2, DOCX file, 0.1 MB.

TABLE S3, DOCX file, 0.1 MB.

TABLE S4, DOCX file, 0.1 MB.

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