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Evaluation of Immunostimulatory Activities of Synthetic Mannose-Containing Structures Mimicking the β-(1→2)-Linked Cell Wall Mannans of *Candida albicans*

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Immunostimulatory properties of synthetic structures mimicking the β-(1→2)-linked mannans of *Candida albicans* were evaluated *in vitro*. Contrary to earlier observations, tumor necrosis factor (TNF) production was not detected after stimulation with mannotetraose in mouse macrophages. Divalent disaccharide 1,4-bis(α-D-mannopyranosyloxy)butane induced TNF and some molecules induced low levels of gamma interferon (IFN-γ) in human peripheral blood mononuclear cells (PBMC).

Immunostimulatory molecules intensify and modify the lymphocyte-mediated immune response and its duration. Such molecules can, therefore, be potentially applied as adjuvants in vaccines and allergy preparations. Generally, allergen vaccines function by balancing the T helper 2 (Th2)-type responses by inducing Th1- and T regulatory-type responses (2, 3, 20, 21, 30, 31, 33).

β-(1→2)-Oligomannoside constituents of the *Candida albicans* cell wall have been shown to possess immunostimulatory properties, as evidenced by induction of cytokine production, including tumor necrosis factor (TNF) production, in humans and mice (6, 14, 16, 18, 19, 29, 34, 35, 36, 37). In particular, the oligosaccharide fractions consisting of four or more mannose units, isolated and fractionated from the *C. albicans* cell wall, have been shown to induce TNF production in mouse macrophages (19). However, being isolated from cell walls, such fractions may, at least in part, be contaminated with other biomolecules, including mannoproteins consisting of both oligosaccharides and proteins, which all may contribute to the biological activities observed. Therefore, biological studies employing well-defined synthetic β-(1→2)-linked oligomannoside compounds are of interest for verifying and studying in detail the proposed immunostimulatory properties of such constructs.

For the present biological study, 15 mannose-containing structures, a majority of these with β-(1→2)-linkages (Fig. 1A, 2A, 3A, and 4A), were prepared by applying and further modifying the recently developed methodologies for construction of β-(1→2)-mannosidic linkages by Crich and others (7). The synthesis procedures have been published previously by us (9, 10, 28). The compounds prepared were designed as simple mimics and analogues of the hydrolyzed oligosaccharide fractions from the *C. albicans* cell wall, with the β-(1→2) linkage serving as a basis for all structural modifications. Mild-acid-hydrolyzed *C. albicans* mannan was used as a positive control in all cell culture experiments. Initially, *C. albicans* mannan was prepared with the Cetavlon method as previously described (27); thereafter, it was hydrolyzed in mild acidic conditions with 0.1 N HCl for up to 60 min at 100°C. Neutralization of hydrolysis products was performed by adding NaOH. The outcome of the hydrolysis was analyzed by thin-layer chromatography (TLC) using silica gel-coated aluminum sheets (Merck, Darmstadt, Germany) and *n*-butanol-acetic acid-water (2:1:1 [vol/vol/vol]) as an eluent. *C. albicans* mannan and all synthetic compounds were screened for endotoxin contamination with the E-Toxate kit (Sigma-Aldrich, St. Louis, MO) by spot-checking during preparation and by double-checking all compounds showing any immunostimulatory activity (compounds 1, 2, and 10). Endotoxin levels in all tested samples (highest used stimulation concentration) were below 0.015 endotoxin units (EU)/ml.

Different concentrations of the synthetic β-(1→2)-linked mannotetraose (compound 3) were cultured for 24 h with cells from the mouse macrophage cell lineage J774.2 (lot 06/C/015; Sigma-Aldrich, Germany). TNF production was measured with the high-sensitivity mouse TNF cytokine Lincoplex kit (Linco Research, St. Charles, MO).

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples donated by voluntary laboratory personnel after informed consent by Ficoll-Paque density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Cells were resuspended in RPMI-based culture medium and stimulated on 48-well flat-bottomed cell culture plates at a density of 10^6/ml as previously described (31). The cells were stimulated with the compounds (1 to 15) (0.2 to 100 μg/ml), and medium alone served as an unstimulated control. After 72 h, the cells were collected and centrifuged. RNA was extracted from the pellets, cDNA synthesis was performed, and relative quantitation of cytokine mRNA expression in the PBMC was performed by TaqMan PCR using the ABI Prism 7700 sequence detection system as described previously (26). The data analysis was performed according to the manufacturer's instructions (User Bulletin number 2, P/N 4303849; Applied Biosystems) using a comparative threshold cycle (C_T) ($2^{-\Delta\Delta CT}$) method, in

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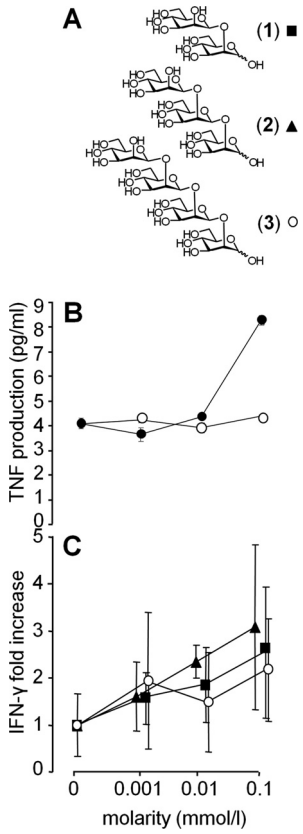

FIG 1 (A) Structures of the β-(1→2)-linked mannosides mannobiose (1), mannotriose (2), and mannotetraose (3). (B) TNF production (mean and standard error of the mean [SEM]) from mouse macrophage cell lineage J774.2 as measured with Luminex after stimulation with compound 3 (○) and mild-acid-hydrolyzed *Candida albicans* mannan (●). (C) Inductions of IFN-γ secretion in PBMC by compounds 1 (■), 2 (▲), and 3 (○). IFN-γ responses were measured either as mRNA expression in PBMC with TaqMan or as IFN-γ production by PBMC measured with Luminex after stimulation with at least two concentrations of the compounds. Fold changes in the IFN-γ responses (mean and SEM) compared to that in the unstimulated culture are shown on the *y* axis.

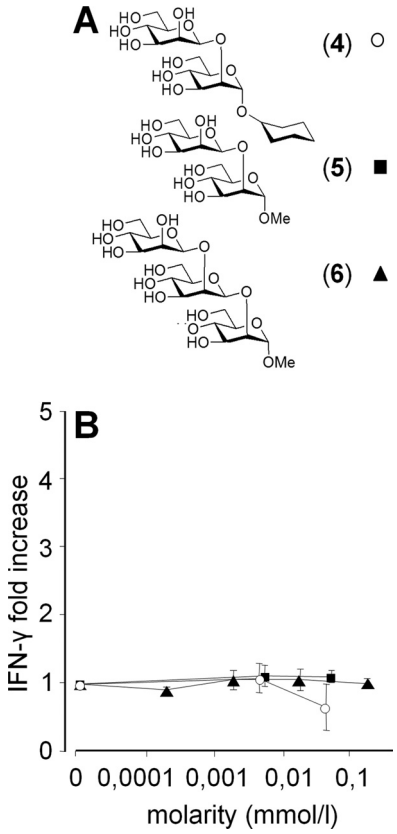

FIG 2 (A) Structures of the β-(1→2)-linked mannoglycosides cyclohexyl β-D-mannopyranosyl-(1→2)-α-D-mannopyranoside (4), methyl β-D-mannopyranosyl-(1→2)-α-D-mannopyranoside (5), and methyl β-D-mannopyranosyl-(1→2)-β-D-mannopyranosyl-(1→2)-α-D-mannopyranoside (6). (B) IFN-γ responses in PBMC after stimulation with compounds 4 (○), 5 (■), and 6 (▲). IFN-γ responses were measured either as mRNA expression in PBMC with TaqMan or as IFN-γ production by PBMC measured with Luminex. Fold changes in the IFN-γ responses compared to that in the unstimulated culture (mean and SEM) are shown on the *y* axis.

which β-actin served as an endogenous reference gene and unstimulated cell culture served as a calibrator. The resulting $2^{-\Delta\Delta CT}$ value was used to indicate the fold change in cytokine expression relative to that of unstimulated cultures.

The cytokines in supernatants (gamma interferon [IFN-γ], interleukin-4 [IL-4], IL-10, and TNF) were measured with high-sensitivity human cytokine Lincoplex kits (Linco Research, St. Charles, MO, USA). The Lincoplex assays were performed in accordance with the manufacturer's protocol by employing Luminex technology. The study was approved by the local ethics committee.

In the present study, the synthetic β-(1→2)-linked mannotetraose (compound 3) did not induce any detectable TNF production in a mouse macrophage cell line. Under the same experimental conditions, the mild-acid-hydrolyzed *C. albicans* mannan induced TNF production (Fig. 1B). In an earlier work by Poulain and coworkers, similar mannotetraose-containing fractions prepared by acidic hydrolysis and subsequent fractionation of the *C. albicans* cell wall oligosaccharides induced TNF production in peritoneal mouse macrophages (19). In addition, native fractions containing β-(1→2)-linked oligomannosides with a degree of polymerization (DP) of eight or more appeared to improve the induction of TNF secretion (19). It should be noted that in that study, the macrophages were purified from peritoneal exudate cells of 20- to 24-week-old BALB/c mice, whereas the present work was performed with immortalized macrophages from a mouse cell lineage, a difference which may partially explain the contradictory results obtained.

It is known that J774 mouse macrophage cells are heterogenous and dependent on several factors in their expression of the macrophage mannose receptor (MR) (11, 32). It is thus possible that the expression of the functional mannose receptor was low or absent in our experiments. MR and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) are well documented for their capacity of recognizing *N*-linked mannans (1, 5). However, macrophage activation by β-(1→2)-linked mannoside structures is not dependent solely on MR because the recognition of the different components of the *C. albicans* cell wall by immune cells is mediated by a diverse range of pattern recognition receptors (29). In addition, responses by β-(1→2)-linked oligomannoside stimulation are mediated through galectin 3, a 30-kDa receptor expressed on macrophages,

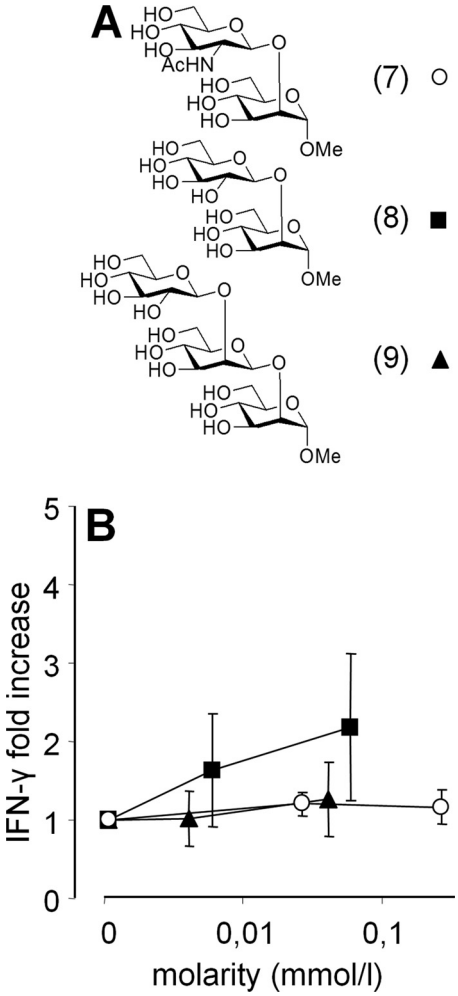

FIG 3 (A) Glucose-containing structures of methyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranoside (7), methyl β-D-glucopyranosyl-(1→2)-α-D-mannopyranoside (8), and methyl β-D-glucopyranosyl-(1→2)-β-D-mannopyranosyl-(1→2)-α-D-mannopyranoside (9). (B) Induction of IFN-γ secretion in PBMC after stimulation with compounds 7 (○), 8 (■), and 9 (▲). IFN-γ responses were measured as mRNA expression in PBMC with TaqMan. Fold changes in the IFN-γ expression (mean and SEM) compared to that in the unstimulated culture are shown on the *y* axis.

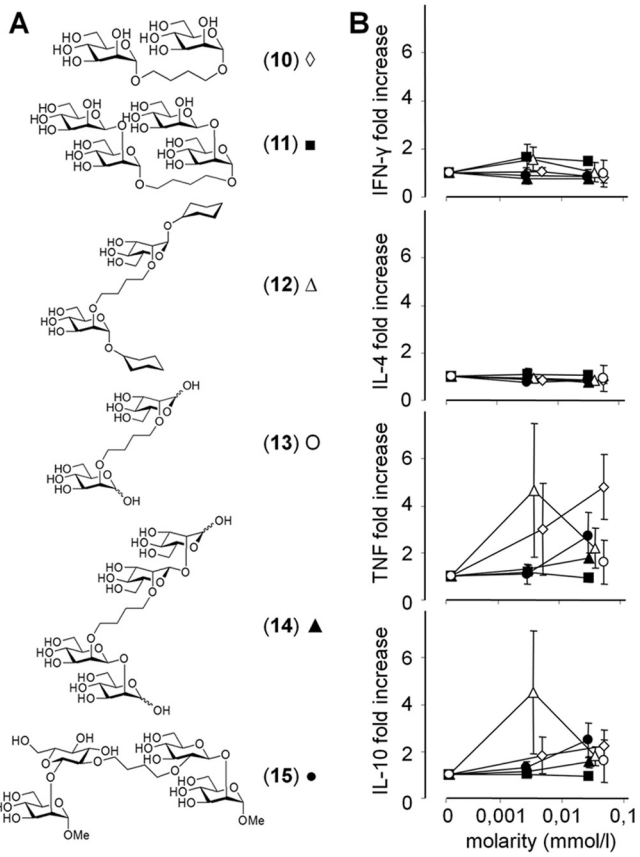

FIG 4 (A) Divalent structures of 1,4-bis(α-D-mannopyranosyloxy)butane (10), 1,4-bis(2-*O*-β-D-mannopyranosyl-(1→2)-α-D-mannopyranosyloxy)butane (11), 1,4-bis(cyclohexyl 2-*O*-α-D-mannopyranosyl)butane (12), 1,4-bis(2-*O*-D-mannopyranose)butane (13), 1,4-bis(2-*O*-β-D-mannopyranosyl-(1→2)-D-mannopyranose)butane (14), and 1,4-bis(methyl-α-D-mannopyranosyl-(2→1)-2-*O*-β-D-glucopyranosyl)butane (15). (B) Fold increases in PBMC cytokine production after divalent oligosaccharide stimulations. Production of IFN-γ, IL-4, TNF, and IL-10 was measured (mean and SEM) with Luminex at 72 h after stimulations with various concentrations of the divalent saccharide compounds 10 (◊), 11 (■), 12 (△), 13 (○), 14 (▲), and 15 (●).

dendritic cells, and epithelial cells (13, 17, 23). The contact between galectin 3 and the β-(1→2)-oligomannosides of the *Candida* cell wall is essential in *C. albicans* colonization and invasion (8) as well as recognition of *C. albicans* by murine macrophages (12). Since acid-hydrolyzed mannan, containing β-(1→2)-oligomannosides, induced TNF production by the J774 cells, the negative results with the synthetic mannotetraose cannot be explained solely by a lack of macrophage mannose receptor expression alone. A possible explanation for our results is that, in the earlier studies, the oligomannoside fractions evaluated may have been cell wall glycoproteins comprising both carbohydrate and protein. In the present study, well-defined, chemically synthesized mannosides without a protein component were used, and accordingly, the responses detected can only be due to interactions between the pure small carbohydrate residues and the macrophages. As such, these interactions appear inadequate to induce TNF production.

Structural details concerning the possible interactions between oligomannosides and human lymphoid cells have remained largely unknown. It is, nevertheless, reasonable to assume that the interactions between the sugar moiety and the cell depend on both the valency and the three-dimensional structure of the carbohydrate (26). These, in turn, are influenced by chain length, stereochemistry, and the nature of the glycosidic linkages and the individual sugar units. In the present work, variations of these parameters were aimed at when selecting and constructing the compounds for the biological screening. As an indicator of Th1 immunostimulation, the main Th1-type cytokine, IFN-γ, was selected.

Of the β-(1→2)-linked oligomannoside compounds, β-(1→2)-mannobiose (compound 1), -mannotriose (compound 2), and -mannotetraose (compound 3) all showed moderate inductions of IFN-γ expression in PBMC (Fig. 1C). With these small-size compounds having DPs of ≤4, previously reported correlations between the immunomodulatory activity and the chain length of the oligomannoside were not able to be demonstrated (4, 15, 19). The corresponding structures with locked anomeric configura-

tions, i.e., the mannobioside with cyclohexyl or methyl aglycon (compounds 4 and 5, respectively) and the mannotrioside with methyl aglycon (compound 6), did not induce any measurable IFN-γ production in the PBMC (Fig. 2B). In order to investigate the significance of the stereochemistry at C-2 of the individual sugar units, the glucose- and *N*-acetyl glucose amine-containing analogues (compounds 7 to 9) with modifications to the nonreducing end of the oligosaccharide compound were likewise screened. Structurally related oligosaccharide fragments have been identified in the native *C. albicans* cell wall. These modified analogues did not, however, induce any cytokine production (Fig. 3B).

In contrast to simple monovalent oligosaccharides, the oligo- and multivalent carbohydrate assemblies may simultaneously interact with multiple receptors, potentially enhancing the binding affinities of such constructs and, hence, affecting the biological activities (22, 24, 25). As the most simple compounds for oligovalent mannoside structures, a series of divalent mono- and disaccharide-based constructs, prepared earlier by us using olefin cross-metathesis (9), were here screened for their potential immunostimulatory responses with a wider array of cytokines. However, none of the divalent compounds (compounds 10 to 15) investigated in the present work induced any measurable IL-4, IL-10, or IFN-γ responses. For one single compound, 1,4-bis(α-D-mannopyranosyloxy)butane (compound 10), dose-dependent stimulation of TNF production was observed (Fig. 4B).

In summary, none of the synthetic oligomannosides investigated in the present work were shown to induce any significant cytokine production in the human white blood cell. One single divalent mannoside was shown to induce TNF production, whereas in contrast to earlier reports using native oligosaccharides from *C. albicans*, synthetic well-defined β-(1→2)-linked mannotetraose did not induce any TNF production in mouse macrophages. To conclude, the results obtained herein indicate that further studies are needed in order to verify that the biological responses that are assumed in earlier studies to stem from the β-(1→2)-linked oligomannosides are due solely to the presence of these molecules. It is possible that the heterogeneous native extracts also contain some other biologically active components partly contributing to the observed biological activities. Similarly, further synthetic efforts should be directed toward preparation of truly multivalent β-(1→2)-oligomannosides and their analogues for possible identification of synthetic immunostimulatory molecular candidates.

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