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Evaluation of Fungal Extracts to Determine Immunomodulatory Properties

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Abstract

Background: Immunoglobulin E–mediated allergies have doubled in prevalence during recent decades in developed countries. This increase has been attributed, in part, to high hygiene standards, which have reduced exposure to microbes. The capacity of microbes to induce type 1 helper T cell (T_H1) responses may imply suppression of T_H2 responses. However, little research has been performed with fungal extracts.
Objectives: To evaluate the T_H1-inducing properties of fungal extracts.
Methods: A total of 24 fungal extracts, including Cetavlon-precipitated polysaccharides from different yeasts, molds, and mushrooms were prepared. The extracts were screened for production of interferon (IFN) γ in human peripheral blood mononuclear cells. The active compounds were further purified by mild acid hydrolysis and by column chromatography and studied in human peripheral blood mononuclear cells.
Results: Expression of IFN-γ was induced by several extracts. The strongest expression of IFN-γ was induced by *Candida albicans*. The Cetavlon-precipitated mannans of fungi induced cytokine responses that were similar or superior to those induced by whole extracts, *C albicans* being the most potent inducer of IFN-γ. Column chromatography–fractionated mild acid hydrolysis of *C albicans* mannan was performed. Fractions containing oligosaccharides of 12-16 mannoses induced production of tumor necrosis factor.
Conclusions: Several fungal extracts induce IFN-γ. The most promising preparations were yeast-derived oligosaccharides. Further research should be focused on purification and eventual synthesis of the extracts.

Key words: *Candida albicans*. Mannan. Mold. Mushroom. T_H1 cell. T_H2 cell. Yeast.

Resumen

Antecedentes: En los países desarrollados, la prevalencia de las enfermedades alérgicas mediadas por la inmunoglobulina E se han duplicado en las últimas décadas. Este aumento ha sido, en parte, atribuido a pautas de higiene excesivas que han reducido la exposición a microbios. La capacidad de los microbios para inducir la respuesta Th1 puede dar lugar a la supresión de la respuesta Th2. En este sentido, la investigación que se ha realizado con extractos fúngicos es escasa.
Objetivos: Evaluar las propiedades inmunomoduladoras Th1 que inducen los extractos de hongos.
Métodos: Se evaluaron un total de 24 extractos de hongos, incluyendo polisacáridos de diferentes levaduras, mohos y hongos. Se estudió la capacidad de estos extractos de inducir la producción de interferón-γ (IFN-γ) en células mononucleares de sangre periférica (PBMC) humanas. Los extractos fueron posteriormente sometidos a una hidrólisis ácida suave y a cromatografía en columnas.
Resultados: Los extractos procedentes de diferentes levaduras, mohos y hongos indujeron un incremento en la expresión de la producción de IFN-γ. La expresión más enérgica fue la provocada por *Candida albicans* (*C. albicans*). Los mananos fueron también capaces de conseguir un incremento de la expresión de IFN-γ similar o superior a la inducida por los extractos enteros, siendo el manano de *C. albicans* el más potente de todos ellos. Mediante los estudios de estimulación celular, con fracciones obtenidas por cromatografía del manano *C. albicans*, se observó que aquellas que contenían oligosacáridos de 12-16 manosas indujeron una mayor producción de TNF.
Conclusiones: Son varios los extractos fúngicos capaces de inducir la producción de IFN-γ. Los productos más potentes fueron los oligosacáridos derivados de las levaduras. Las investigaciones futuras deberían centrarse en la purificación y síntesis final de los mismos.

Palabras clave: *Candida albicans*. Manano. Moho. Hongos. Células Th1. Células Th2. Levadura.

Introduction

Early exposure to microbes has been postulated as an explanation for the lower prevalence of atopy among children from farming environments [1,2]. Such exposure is thought to induce immune deviation from type 2 helper T cells (T_H2) to T_H1 and regulatory T-cell responses, thereby preventing development of allergic inflammation [3]. Consequently, microbial structures are promising adjuvants in allergy vaccines owing to their ability to suppress allergic inflammation [4-6]. In addition to microbes, fungi induce T_H1-type responses [4,7,8]. Most of the T_H1-inducing qualities of intact fungal cells have been attributed to their cell wall components. The components previously reported to be important in inducing a T_H1 response include mannan and the mannoproteins of yeasts such as *Candida albicans* and *Cryptococcus neoformans* and the β-glucans of mushrooms such as *Grifola frondosa*, *Lentinus edodes*, and *Sclerotinia sclerotiorum* [9-14]. In addition, some fungal proteins appear to activate monocytes [15]. Therefore, fungal antigens could be used as adjuvants in specific immunotherapy to favor the T_H1 pathway and inhibit the T_H2 response. The aim of this study was to evaluate various fungal extracts and the mannans of yeasts, molds, and mushrooms in order to determine their immunostimulatory properties.

Material and Methods

Fungal Antigens

The fungal extracts used in this study were isolated from 24 different yeasts, molds, and mushrooms. All fungal extracts were free of endotoxin contamination as determined using the E-TOXATE Kit (Sigma-Aldrich).

The yeast extracts used in the study are listed in Table 1. They included 2 commensal species (*C albicans, Pityrosporum ovale*), 3 environmental species (*Rhodotorula rubra, Cryptococcus albidus*, and *Candida utilis*), and 1 species used in commercial household products (*Saccharomyces cerevisiae* [baker's yeast]). The yeast cells were cultured, and their water-soluble antigens were extracted as previously described [16-18].

The mold extracts used in the study are listed in Table 1. The *Aspergillus umbrosus* strain was originally isolated from moldy hay and cultured to prepare whole antigen extract as previously described [19]. The whole extracts of *Aureobasidium pullulans, Acremonium furcatum, Aspergillus versicolor, Chaetomium globosum, Phialophora repens, Stachybotrys chartarum, Ulocladium botrytis*, and *Alternaria alternata* (kindly provided by Dr P Raunio, Department of Environmental Sciences, University of Kuopio, Finland) were originally isolated from water-damaged building materials and cultivated to prepare whole extracts as described elsewhere [20,21].

The mushroom extracts used in the study were prepared from 1 cultivated mushroom species (*Agaricus bisporus*, champignon) and 7 wild mushroom species (*Cantharellus tubaeformis*, trumpet-shaped chanterelle; *Paxillus involutus*, brown roll-rim; *Kuehneromyces mutabilis*, sheathed woodtuft; *Cortinarius triumphans*, birch webcap; *Lactarius rufus*, rufous milkcap; *Hydnum repandum*, wood hedgehog; and *Boletus edulis*, cep). *Agaricus bisporus* was acquired from a mushroom farm (Mykora). The wild mushrooms were collected from the Turku region in Finland by an experienced mushroom consultant. Special interest was paid to the purity and freshness of the material, and mushrooms with the slightest hint of larvae were excluded. Each species was collected in separate paper bags and deep-frozen within 3 hours. Prior to extraction, the mushrooms were washed with 10% ethanol and distilled water, and roughly homogenized in 0.125 M NH_4HCO_3 with a hand blender. After centrifugation at 4°C and 14 000*g* for 30 minutes, the pelleted fractions were resuspended in 0.125 M NH_4HCO_3, sonicated, and shaken for 24 hours at 4°C. In contrast with the other preparations, the A bisporus suspension was disrupted by freezing at –25°C and passing 4 times through X-press (AB Biox). After centrifugation (14 000*g*, 30 minutes at 4°C), the supernatants were sterile-filtered (0.2 µm Acrodisc PF filter, Pall Gelman Laboratory) and stored lyophilized at 4°C.

Table 1. Yeast and Mold Extracts Used in the Study

	Extract	Strain	Depositor[a]
Yeasts	*Candida albicans* IHR	CBS 5982, CBS 1894, CBS 6589	1
	Candida utilis	VTT-C-71015	2
	Cryptococcus albidus	71078	3
	Pityrosporum ovale ATCC	ATCC 42132	4
	Pityrosporum ovale CBS	CBS 7854	1
	Rhodotorula rubra	70765	3
	Saccharomyces cerevisiae	House-hold product	5
Molds	*Acremonium furcatum*	ATCC 208875	4
	Alternaria alternata	DSM 62006	6
	Aspergillus umbrosus	M118	7
	Aspergillus versicolor	UKU 3	8
	Aureobasidium pullulans	DSM 62074	6
	Chaetomium globosum	UKU 4	8
	Phialophora repens	KTL RE27	9
	Stachybotrys chartarum	ATCC 208877	4
	Ulocladium botrytis	UKU 11	8

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Mannans of *C albicans* (CBS 5982), *S chartarum* (ATCC 208877), and *P involutus* were isolated by hot citrate extraction and precipitated with cetyl trimethyl ammonium bromide (Cetavlon) according to Nakajima and Ballou [22].

Hydrolysis and Fractionation of C albicans Mannan

C albicans mannan (20 mg/mL) was hydrolyzed under mildly acidic conditions with 0.1 N HCl for up to 60 minutes at 100˚C. Hydrolysis products were neutralized with NaOH. The outcome of hydrolysis was analyzed by thin layer chromatography (TLC) using silica gel–coated aluminium sheets (Merck) and n-butanol-acetic acid-water (2:1:1 vol/vol/vol) as eluent. Hydrolysis products were detected using TLC with orcinol (2 g/L in 20% sulfuric acid) by heating the sheets for 10 minutes at 105˚C. Preliminary rough fractionation of hydrolysis products by size was performed with Amicon YM100, YM10, YM3, and YM1 ultrafiltration membranes (Millipore) according to the manufacturer's instructions. The resulting fractions of >100 kDa, 10-100 kDa, 1-10 kDa, and <3 kDa were sterile-filtered and lyophilized for storage at 4˚C.

Fractions of <3 kDa were further fractionated using liquid chromatography on Bio-Gel P-2 Fine (Bio-Rad) (1.7×131 cm) according to the manufacturer's instructions. Flow rates of 2 mL/h were used. An aliquot of each fraction (2 mL) was assayed for saccharide content with the anthrone method (Anthrone 8.014641, Merck-Schuchardt) and TLC, as described above. Thereafter, an adjacent 5 fractions were pooled and concentrated by lyophilization for immunological and chemical analyses.

Table 2. IFN-γ mRNA (Fold Increase to Unstimulated) After Stimulation With Fungal Extracts (n=10)

		μg/mL	Mean	SEM	*P*[a]
Yeasts	*Candida albicans* IHR	20 200	54.5 157.9	19.4 83.3	.022 .09
	Pityrosporum ovale CBS	20 200	6.8 16.9	2.4 8.9	.035 .11
	Pityrosporum ovale ATCC	20 200	7.7 15.9	1.6 4.3	.003 .007
	Saccharomyces cerevisiae	20 200	9.3 15.8	4.0 11.0	.07 .21
	Candida utilis	20 200	16.6 29.7	7.7 9.8	.07 .017
	Rhodotorula rubra	20 200	10.8 57.0	2.8 15.3	.010 .008
	Cryptococcus albidus	20 200	6.5 1.3	1.5 0.5	.008 .58
Mushrooms	*Agaricus bisporus*	20 200	3.2 1.1	1.6 0.5	.23 .82
	Boletus edulis	20 200	1.7 0.2	0.4	.15
	Cantharellus tubaeformis	20 200	3.6 0.7	2.2 0.2	.28 .16
	Cortinarius triumphans	20 200	2.6 0.1	0.9 0.1	.13 <.0001
	Hydnum repandum	20 200	1.6 4.0	0.3 2.5	.15 .29
	Kuehneromyces mutabilis	20 200	0.9 1.6	0.1 0.7	.45 .43
	Lactarius rufus	20 200	7.3 5.0	4.3 3.0	.23 .27
	Paxillus involutus	20 200	216.6 115.0	89.6 84.3	.040 .21

Abbreviation: IFN, interferon.
[a]Compared with unstimulated IFN-γ mRNA.

Participants

Five atopic patients (mean [SD] age, 31.6 [7.2] years; 3 female, 2 male) and 5 healthy controls (34.4 [12.0] years; 5 female) underwent screening with fungal extracts (Tables 2 and 3). All atopic patients had skin prick test–verified pollen allergy and seasonal allergic rhinitis. Two of them had specific IgE to timothy grass pollen (*Phleum pratense*) and 3 to birch tree pollen (*Betula verrucosa*). Cell stimulation studies with acid–hydrolyzed *C albicans* mannan and its fractions were performed in 8 patients with atopic dermatitis and 8 healthy controls (Figure 1). The 8 patients with atopic dermatitis had specific IgE to the commensal yeasts *Malassezia furfur* and *C albicans*. Cell stimulation studies with the <3-kDa fraction of *C albicans* mannan were performed with 3 healthy controls (Figure 2). None of the healthy controls had a history of allergic diseases or specific IgE antibodies to common allergens. The participants gave peripheral blood samples for stimulations. The study was approved by the ethics committee of Turku University Central Hospital, Finland.

Peripheral Blood Mononuclear Cell Cultures

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples taken from atopic patients and healthy controls using density gradient centrifugation (Ficoll-Paque PLUS, Amersham Biosciences AB), washed twice with Hanks' balanced salt solution, and resuspended in RPMI-based medium (Invitrogen) supplemented with 5% autologous serum, 2 mM L-glutamine (Fluka Chemie AG), and 100 mg/mL gentamicin (Biological Industries Ltd). For detection of cytokines, cells (10^6 cells/mL) were stimulated individually for 3 days with different concentrations of fungal extracts, mannans, or hydrolyzed fractions with an unstimulated well as a control in a total culture volume of 300 μL/well. All experiments were performed in 48-well flat-bottomed Costar plates (Corning Inc) in duplicate at 37°C in a humidified atmosphere. After stimulation, PBMCs were harvested in 0.5 mL of TRIzol Reagent (Invitrogen) and stored at –70°C. Lymphoproliferation in PBMCs was determined with 3H-labeled thymidine (Amersham Biosciences AB).

Table 3. IFN-γ mRNA (Fold Increase to Unstimulated) After Stimulation With Fungal Extracts (n=10)

		μg/mL	Mean	SEM	*P*[a]
Molds	*Aspergillus umbrosus*	20 200	4.4 7.4	1.2 2.0	.020 .010
	Acremonium furcatum	20 200	18.0 0.2	12.9 0.1	.24 <.0001
	Alternaria alternata	20 200	19.5 0.2	11.6 0.1	.14 <.0001
	Aspergillus versicolor	20 200	2.2 0.6	0.9 0.1	.21 .07
	Aureobasidium pullulans	20 200	12.1 0.9	5.6 0.4	.09 .71
	Chaetomium globosum	20 200	9.9 0.2	5.8 0.1	.15 <.0001
	Phialophora repens	20 200	19.4 0.4	11.8 0.1	.15 .24
	Stachybotrys chartarum	20 200	72.6 0.6	15.9 0.1	<.0001 .17
	Ulocladium botrytis	20 200	28.6 0.4	18.2	.19
Mannans	*Candida albicans*	20 200	35.4 87.1	16.2 25.3	.06 .008
	Agaricus bisporus	20 200	6.3 16.4	3.6 6.9	.20 .07
	Aspergillus umbrosus	20 200	2.9 19.2	1.2 5.6	.14 .010

Abbreviation: IFN, interferon.
[a]Compared with unstimulated IFN-γ mRNA.

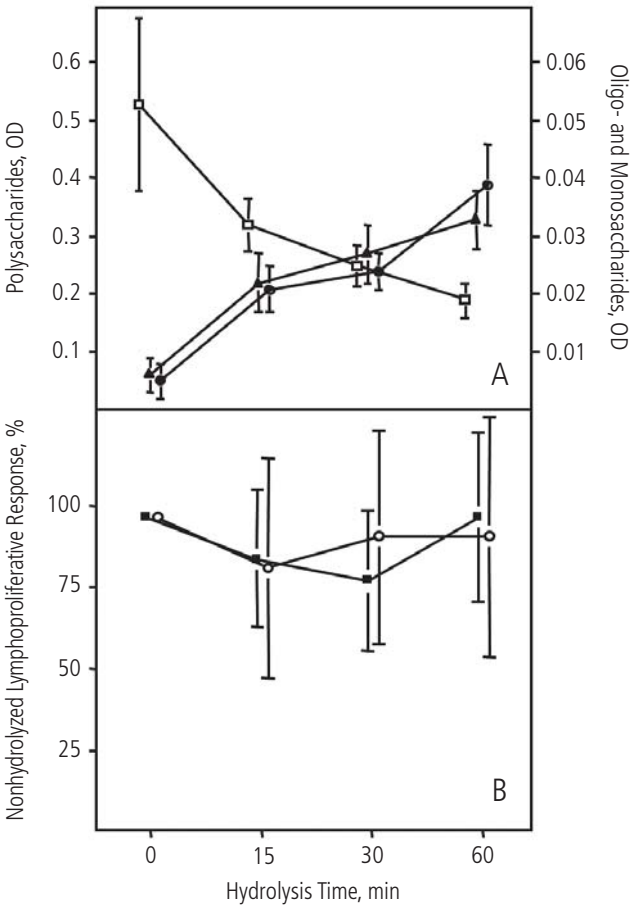

Figure 1. A, Effects of mild acid hydrolysis of *Candida albicans* mannan on lymphoproliferation in PBMCs. The Cetavlon-purified mannan of *C albicans* (20 mg) underwent mildly acidic hydrolysis with 0.1 N HCl for up to 60 minutes at 100˚C. The amounts of poly- (□), oligo- (●), and monosaccharides (▲) during hydrolysis were detected by TLC (mean [SEM]). B, 1:100 dilutions of the hydrolyzed fractions, or 200 µg/mL of nonhydrolyzed *C albicans* mannan, were used for stimulation of the PBMCs of 8 patients with atopic dermatitis (■) and 8 healthy controls (○). Lymphoproliferation was measured after 6 days of stimulation by incorporation of 3H-labeled thymidine. The results are represented as percentages of nonhydrolyzed *C albicans* mannan–induced responses (mean [SEM]). PBMC indicates peripheral blood mononuclear cell; TLC, thin layer chromatography.

In 3 replicate cultures (10^6 cells/mL), cells were incubated for 6 days in 96-well flat-bottomed Costar plates (Corning Inc). Lymphoproliferation, expressed as an index in relation to unstimulated cultures, was calculated from the mean of 3 replicate cultures.

Cytokine Detection

Isolation of RNA and TaqMan reverse-transcriptase polymerase chain reaction were performed as previously described [23]. Briefly, RNA was isolated according to TRIzol instructions, and the extracted RNA was stored in 75% ethanol at –20˚C. The reverse transcriptase reaction was performed with the First-Strand cDNA Synthesis Kit (Amersham Biosciences AB) using oligo (dT) primers. cDNA was stored at –70˚C. Relative quantitation of cytokine mRNA expression was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the housekeeping gene β-actin as an endogenous reference transcript. The cDNA-specific primer and dual-labeled probe sequences for β-actin and intergeron (IFN) γ, as well as the PCR reactions and data analysis, were as described elsewhere [23]. Tumor necrosis factor (TNF) and IFN-γ production by PBMCs in culture supernatants was measured using high-sensitivity human cytokine Lincoplex kits (LINCO Research). The assays were performed according to the manufacturer's protocol.

Statistical Analysis

The Wilcoxon signed rank test was used to assess statistically significant differences in cytokine expression and lymphoproliferation between stimulated and unstimulated cell cultures. Statistical significance was set at $P<.05$ in all tests.

Results

The induction of IFN-γ by different fungal extracts was studied in PBMC cultures from 10 study participants (Tables 2 and 3). For the statistical analysis, data for atopic patients and healthy controls were merged, as no significant differences were observed between them. The whole yeast extracts generally induced a strong, dose-dependent increase in IFN-γ mRNA expression. The strongest induction of IFN-γ mRNA expression was obtained after stimulation with *C albicans* extract. In general, the mold and mushroom extracts were weaker inducers of IFN-γ production than the yeast extracts. The strongest inducers of IFN-γ were *S chartarum* mold extracts and *P involutus* mushroom extracts. Most mold and some mushroom extracts induced downregulation of IFN-γ mRNA expression. Downregulation with the 200-µg/mL dose was associated with diminished β-actin expression, indicating cytotoxicity of these extracts at high concentrations.

As the fungal cell wall is rich in saccharides and mannan is the major component of the yeast cell wall and its water-soluble extracts, different fungal mannans were prepared to investigate how they induced IFN-γ in PBMCs. The mannans of *C albicans* (yeast), *A bisporus* (mushroom), and *A umbrosus* (mold) induced similar or increased IFN-γ expression as compared to the whole extracts (Table 3). In a subset analysis, *S chartarum* and *P involutus* mannans enhanced IFN-γ expression in PBMCs at a similar level to that of the whole extracts (data not shown).

To further characterize the immunostimulatory components of fungal mannans, the most potent *C albicans* mannan was selected. Mannan was hydrolyzed under mildly acidic conditions (0.1 N HCl) for up to 60 minutes, and hydrolysis products were roughly fractionated by Amicon ultrafiltration to fractions of >100 kDa, 10-100 kDa, 1-10 kDa, and <3 kDa fractions. Prolonged hydrolysis of *C albicans* mannan increased the oligosaccharide and monosaccharide content of the hydrolysates (Figure 1A), although no reduction

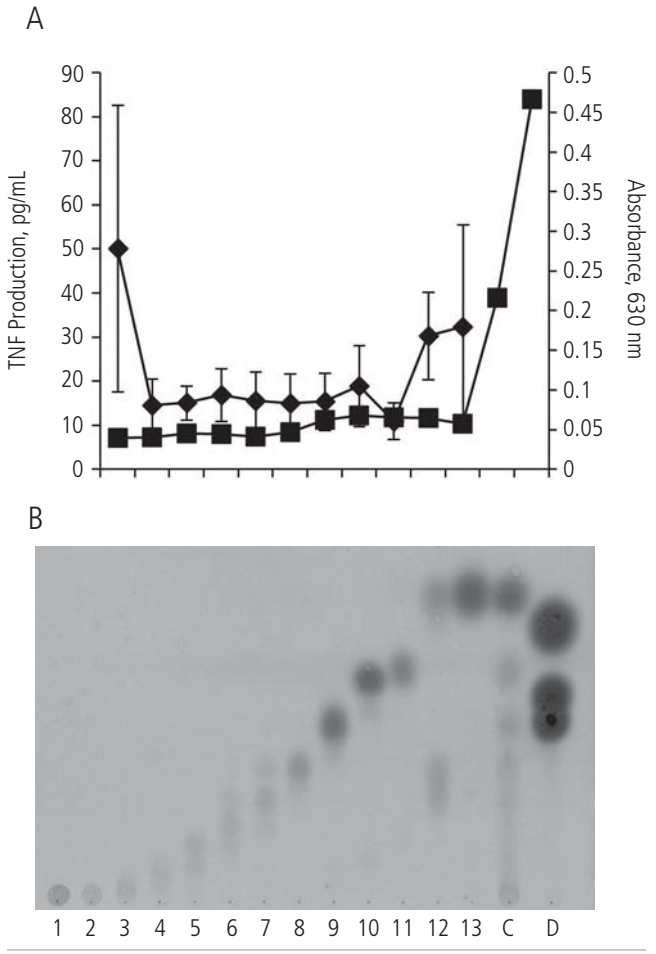

Figure 2. Biogel P2 column liquid chromatography of <3-kDa fractions of acid-hydrolyzed *Candida albicans* mannan. A, TNF production (♦) (mean [SEM]) in human PBMCs (n=3) stimulated by fractions is shown on the left y-axis and saccharide content (■) of each fraction measured with the anthrone method is shown on the right y-axis. B, TLC of pooled fractions. C represents the acid-hydrolyzed *C albicans* mannan that was used as a control; D represents the mixture of raffinose, lactose, and galactose that was used as a control. PBMC indicates peripheral blood mononuclear cell; TLC, thin layer chromatography; TNF, tumor necrosis factor.

in the lymphoproliferation of PBMCs was observed after prolonged hydrolysis (Figure 1B). A subset analysis revealed no suppression of IFN-γ secretion after prolonged hydrolysis (data not shown). All gained fractions induced relatively more IFN-γ secretion in the PBMCs of 3 participants (2.54±2.08 μg/mL; 1.06 [0.78] μg/mL; 1.22 [0.91] μg/mL; 3.76 [1.63] μg/mL) than the intact mannan (0.83 [0.54] μg/mL) at the corresponding concentrations.

To further analyze the immunostimulatory components in the <3-kDa fraction, additional fractionation was performed by size using Biogel P2 liquid chromatography (Bio-Rad). The highest concentration of saccharides was in the 11-13–kDa fraction, and the production of TNF was induced by pooled fraction 1 (Figure 2A). TLC revealed the saccharide-rich fractions to consist of disaccharides and monosaccharides (Figure 2B).

Discussion

In this study, 24 fungal extracts were evaluated to determine their immunostimulatory properties. The results indicated that <3-kDa *C albicans* mannan was the most promising T_H1-inducing structure; the extract was further fractionated by column chromatography. The capacity of the fractions to induce TNF production in PBMC stimulations was considered a measurement of antiallergic activity. However, regardless of the thoroughness of fractionation, the cell wall extracts evaluated may have contained other biomolecules, including glycoproteins, in addition to saccharides, with the result that the biological activity observed may not derive solely from the immunostimulatory properties of the saccharide structures. Therefore, to identify the T_H1-deviating, antiallergic factors of *C albicans* saccharide structures, synthesized saccharide molecules should be further studied.

It is hypothesized that early exposure to microorganisms can protect against allergic inflammation. In general, immune deviation from a T_H2 response to a T_H1 response is considered to be the key factor behind this protective capacity. Consequently, interest has grown in the search for microbial components that could be used as adjuvants to develop a local T_H1-inducing environment and reduce allergen-induced T_H2 responses, thereby suppressing allergic inflammation. To date, several microbial components, including bacterial lipopolysaccharide, unmethylated oligodeoxynucleotide sequences containing CpG motifs (CpG ODN), monophosphoryl lipid A (MPL), and tuberculin, have been found to induce T_H1-type immune responses and downregulate allergen-induced T_H2 responses [24-29]. Clinical studies have also been performed with immunostimulatory MPL and CpG ODNs. Our data emphasize that many fungal extracts and mannans are also potent inducers of IFN-γ expression and could therefore be used as T_H1-inducing adjuvants for downmodulation of allergic T_H2 immune responses. This clear induction of IFN-γ secretion may be due to earlier priming of the immune system by natural exposure to these molds in outdoor air, for example, in forests, and by natural saprophytic exposure to yeasts. Similar priming may also be involved in mushroom antigens, suggesting that the fungal extracts acted as recall antigens. This hypothesis was supported by Heintel et al [30], who found that a number of yeast genera were capable of eliciting extensive major histocompatibility complex (MHC) class I-restricted yeast-specific memory T-cell responses with IFN-γ production in cultures of whole blood from healthy individuals. Another possibility is that of immunological cross-reactivity between certain components of fungi from different environmental sources. Since mannose and mannan are common saccharide constituents of the fungal cell wall, the shared mannan epitopes could represent cross-reactivity between fungi. This type of cross-reactivity was also suggested to be involved in the induction of MHC class I-restricted $CD8^+$ memory T-cell responses against different yeast genera [30].

Although MHC-restricted antigen recognition mechanisms are likely to be involved in the induction of IFN-γ by fungal antigens, fungal components may also be capable of inducing IFN-γ in a non–MHC-dependent manner. For example, the involvement of Toll-like receptors (TLRs), particularly TLR2

and TLR4, in the recognition of fungal antigens has been demonstrated in a number of studies [31-33]. In addition, it has been shown that innate effector cells are capable of recognizing fungal cell wall molecules directly through a variety of other pattern-recognition receptors, including mannose, β-glucan, and complement receptors [34,35]. In our study, the most promising fungal adjuvant was *C albicans* mannan and its acid hydrolysis products, as they induced strong lymphoproliferative and IFN-γ responses in PBMCs. *C albicans* mannan derived from <3-kDa acid hydrolysis products were thus found to share the same immunostimulatory characteristics as the intact *C albicans* mannan. This supports the assumption that low-molecular-weight components of *C albicans* mannan may be the key immunostimulatory components of the parent molecule.

The structures accounting for the immunostimulatory capacity of <3-kDa hydrolysis products of *C albicans* mannan were further fractionated by size. As microbial immunostimulatory molecules are shown to induce especially good TNF responses, TNF was chosen as an indicator cytokine [5,36]. Other cytokines were also studied, although no significant production was detected. A pooled fraction containing oligosaccharides of 12-16 mannoses induced production of TNF. Our results are in accordance with those of previous studies showing that an oligosaccharide has to comprise at least 4 mannose units before production of TNF is stimulated [37]. Nuclear magnetic resonance spectroscopy has shown the presence of β-1,2 linkages in the fractions corresponding to our active fractions [38-41]. Our results are consistent with those of previous studies showing the role of β-(1,2)-linked oligomannosides in mediating the attachment of *C albican*s to murine macrophages and stimulating early and transient TNF production [41,42]. In addition, improved TNF secretion with β-(1,2)–linked oligomannosides and a degree of polymerization of ≥8 has been reported [42]. β-(1,2)–Linked oligomannosides may be, at least in part, behind the immunological activity observed in our study. However, it is impossible to rule out the possibility that other molecules can induce cytokine production. In these fractions, very low amounts of active molecules can induce cytokines and yet be undetectable by nuclear magnetic resonance spectroscopy or mass spectrometry.

The purpose of this study was to clarify the immunopotency of various fungal extracts and their potential use as T_H1-inducing adjuvants for downmodulation of T_H2-biased allergic immune responses. In addition, the methods for preparation of extracts and mannans from the wide variety of fungal species presented in this study may be used in further studies. The most promising T_H1-inducing extracts appear to be yeasts, especially *C albicans*, its cell wall mannan, and hydrolysis products. Further studies are needed to determine the composition and capacity of these fractions to modulate allergen-induced immune responses in human PBMCs in vitro and to investigate the effect of the extracts on the predominant T_H2 allergic response.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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