

Candida albicans mannan- and protein-induced humoral, cellular and cytokine responses in atopic dermatitis patients

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Summary

Background The cytokine observed most often in atopic dermatitis (AD) is IL-4, but a role for IL-5 and IFN- γ in the late and delayed phase reactions has been suggested. In AD with head, neck and shoulder distribution, hypersensitivity to saprophytic yeasts is an important pathogenetic factor. The yeast allergens include both the mannan polysaccharides and the proteins. Mannans are major cross-reacting allergens likely to be involved in the pathogenesis of AD.

Objective To characterize the humoral, lymphoproliferative and cytokine (IL-2, 4, 5 and IFN- γ) responses of peripheral blood mononuclear cells (PBMCs) induced by *Candida albicans* mannan and protein antigens in AD.

Methods Fifteen AD patients and seven healthy controls were included. Ficoll-isolated PBMCs were stimulated by PHA and laboratory-generated mannan and protein extracts of *C. albicans*. Lymphocyte proliferation was measured and cytokine production was studied by ELISA. The antigen-specific IgG and IgE antibodies were analysed by ELISA and nitrocellulose RAST.

Results In AD mannan ($P < 0.005$) and protein ($P < 0.002$), specific IgE levels were higher than in healthy controls. Both mannan and protein-specific lymphoproliferations (both: $P < 0.02$) were higher in AD than in healthy controls. Mannan, but not protein, induced long lasting IL-2 and IL-4 productions from 24 h lasting up to 66–96 h and IL-5 and IFN- γ productions with elevated levels at 66 and 96 h. The mannan-induced IL-2 ($P = 0.015$) and IFN- γ ($P < 0.005$) were increased in AD as compared with healthy controls. Significant correlations were seen between the protein-induced proliferation responses and both serum total IgE ($r = 0.59$, $P < 0.01$) and protein-specific IgE ($r = 0.65$, $P < 0.005$). The mannan-induced IL-2 responses correlated with the specific IgE ($r = 0.62$, $P < 0.01$) and proliferation ($r = 0.51$, $P < 0.02$) and S-IgE level ($r = 0.71$, $P < 0.002$). Mannan-induced IL-4 and IFN- γ productions also correlated ($r = 0.43$, $P < 0.05$).

Conclusions *C. albicans* mannan induced elevated IL-2 and IFN- γ responses in AD patients. The correlations of the cytokine responses with mannan-induced IgE and proliferation responses suggest that *C. albicans* mannan induced TH1 type cytokine responses are involved in AD.

Keywords: atopic dermatitis, *Candida*, cytokines, mannan, TH1, TH2

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Introduction

Atopic dermatitis (AD) is an inflammatory skin disease, in which the cytokines modulate the tissue inflammation of the

disease [1]. The role of environmental allergens in AD is disputed although allergy to foods and house dust mite, especially in children, have been accepted as important pathogenic factors. In AD involving the seborrheic regions of skin, head, neck and shoulder region, saprophytic growth and hypersensitivity to *Pityrosporum ovale* have been

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Table 1. The study patients and controls

Group	n	Gender	Age		
		M/F	Mean	SD	Limits
Atopic dermatitis	15	5/10	33.6	7.7	19–50
Health controls	7	3/4	32.4	11.2	25–56

shown to be important pathogenetic factors, with recovery of eczema by antifungal treatment [2–5]. Our previous studies suggested that this type of dermatitis implies a general hypersensitivity to yeasts and that the saprophytic growth of *Candida albicans* also has a clinical significance. We showed that *P. ovale* and *C. albicans* cross-react at IgE level, the finding having later been confirmed by other studies [6–8]. The major cross-reacting allergen of the yeasts is mannan polysaccharide, suggesting it to be involved in the pathogenesis of AD [6] [7].

The human CD4⁺ T cells, T-helper lymphocytes (TH), have been divided to subtypes TH0, TH1 and TH2, based on their cytokine production. The production of IgE antibodies and the immediate hypersensitivity are generated via the TH2 cells, producing IL-4, IL-5 and IL-10, whereas the delayed-type hypersensitivity and cellular immune responses are generated via the TH1 cells, producing IFN- γ and IL-2. The cytokine profiles of TH clones in AD have revealed mostly TH2-type cells and, in contact dermatitis, mostly TH1-type cells [9] [10], [11]. Recently, however, direct mRNA measurements from atopic skin and peripheral blood mononuclear cells (PBMCs), ELISA-analyses from peripheral blood, in antigen-specific and polyclonal models have given a nonuniform perspective of a more complex network of cytokine production, suggesting a role for IFN- γ in the late phase reactions [12–17].

C. albicans is a classical recall antigen inducing TH1-type immune responses with cell-mediated immune responses, which are crucial in retaining the saprophytic

yeast growth instead of invasive growth and infection [18]. A mannoprotein of *C. albicans* has been shown to induce lymphoproliferation and production of several cytokines, except IL-4 [19]. It also induces anti-*Candida* cell-mediated immunity in healthy humans [20]. On the other hand mannan is the main target for cross-reacting IgE antibodies in AD, suggesting it may play a role in the TH2-type immune response [6,7,21].

Even though mannan is the main cross-reacting allergen of yeasts and the main antigen for humoral immune responses, nothing is known about the cytokine pattern induced by it in AD. The present study was performed to characterize the cellular and humoral immune responses and the cytokine responses of PBMCs induced by *C. albicans* mannan and protein antigens in AD.

Materials and methods

Patients

The patients participating the study were outpatients from the Department of Dermatology of the University Central Hospital in Turku. Fifteen patients had AD, established by a specialist in dermatology according to the criteria of Hanifin and Rajka [22]. Seven controls with no history of allergic diseases or atopic dermatitis were included. The age and gender of the patients are shown in Table 1. A heparinized blood sample and a serum sample were taken.

Preparation of antigens

A *C. albicans* strain from the Centraalbureau voor Schimmelmcultures, Delft, The Netherlands (CBS 5982, serotype A) was cultured on agar plates on synthetic media, as previously described [23]. The chemically purified mannan, Cetavlon-mannan, was prepared by precipitation in neutral citrate buffer after 2 h heating of the yeast suspension in an autoclave and thereafter with repeated precipitations with methanol and finally after borate-alkali

Table 2. The medians (interquartile) of the humoral response of the study patients and controls

Group	S-IgE ^a	IgE ^b		IgG ^c	
		Mannan	Protein	Mannan	Protein
Atopic dermatitis	2080 (3698)	0.96 (7.14)	3.10 (14.0)	0.85 (0.56)	0.10 (0.05)
Health controls	9 (20)	0.01 (0)	0.05 (0.08)	0.88 (0.66)	0.13 (0.07)
P-value	<0.001	<0.005	<0.005	n.s.	n.s.

a) kU/L, b) RAST index, c) optical density. P-values calculated using the Mann–Whitney U-test.

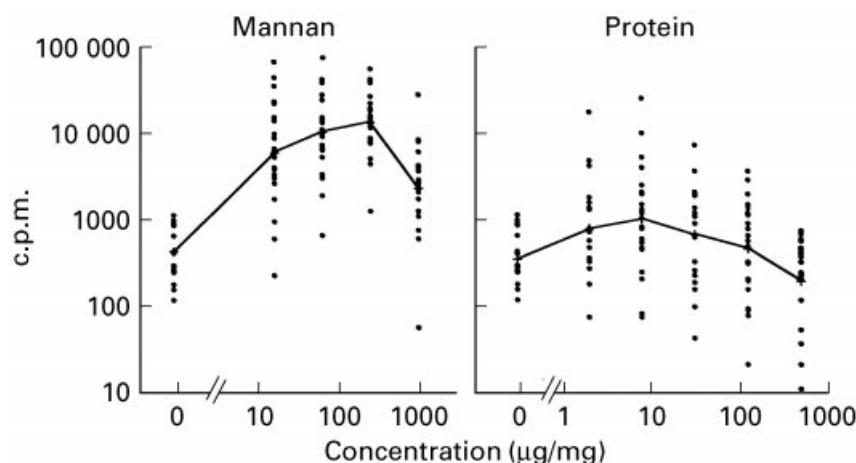


Fig. 1. Dose-response curves of different antigen concentrations of *C. albicans* mannan and protein in lymphocyte stimulation. The medians (c.p.m.) of each concentration are connected. All study subjects ($n = 22$) are included.

treatment with CTAB (cetyltrimethylammoniumbromide) (Sigma, St Louis MO, USA) as earlier described [24]. The crude cytoplasmic extract of the yeast cells was prepared after disruption in X-press as previously described [23]. The extract was then prepared in carbohydrate-free form by ConA affinity chromatography purification as previously described [23]. Based on parallel serial dilutions of purified protein and ConA in immunoblotting using rabbit anti-ConA antibodies, the amount of contaminant ConA in the purified protein preparation was found to be $<0.5\%$ (w/w). Both extracts were dialysed and stored in lyophilized form. The extracts were found to be free of endotoxin contamination by E-Toxate kit (Sigma).

IgE and IgG determinations

The antigen-specific IgE antibodies were analysed by nitrocellulose RAST and antigen-specific IgG antibodies with ELISA as previously described [25].

Lymphocyte stimulations in vitro

PBMCs were isolated from heparinized whole blood with Ficoll (Ficoll-Hypaque, Pharmacia Biotech Ab, Uppsala, Sweden) gradient centrifugation as previously described [26]. The mononuclear cells were suspended after washing in RPMI, supplemented with 10% human AB serum, 2 mmol/L L-glutamine, 100 U/mL penicilline and 100 µg/mL streptomycine. The cell suspension was diluted in the RPMI-based culture medium to 10^6 cells/mL and applied on culture plates (Nuncclon-Delta, Roskilde, Denmark). 96-well plates, 100 µL suspension/well in triplicate, were used for proliferation assays and 24-well plates, 400 µL suspension/well in duplicate, for collection of supernatants for cytokine ELISA. The cultures were incubated at 37°C, in humidified atmosphere with 5% CO₂ for 5–7 days. In the proliferation assay tritium-labelled thymidine was used in

the culture medium during the last 18 h and the incorporated radioactivity was measured from the cells. For cytokine ELISA the culture supernatants were collected at 24, 66 and 96 h of incubation.

Cytokine determinations by ELISA

Cytokines released by PBMCs were measured by cytokine-specific ELISA in 96-well, flat-bottomed microtitre plates (Nunc-Immuno Plate Maxisorp, Roskilde, Denmark) as described previously [26] with slight modifications. For IL-4, the capture antibody was made of a polyclonal goat anti-IL-4 antiserum (R & D Systems Europe Ltd, Abingdon, United Kingdom) and the tracer antibody of the anti-IL-4 rat

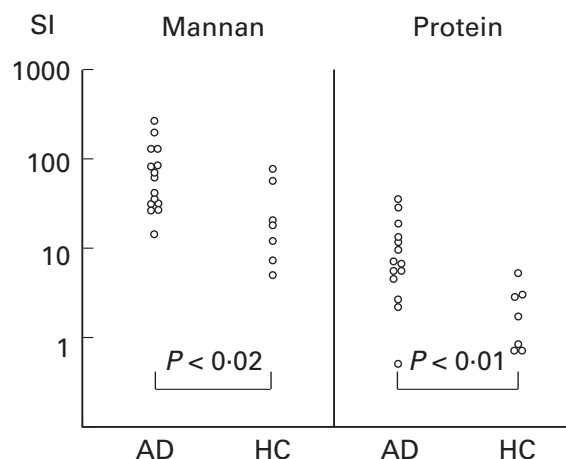
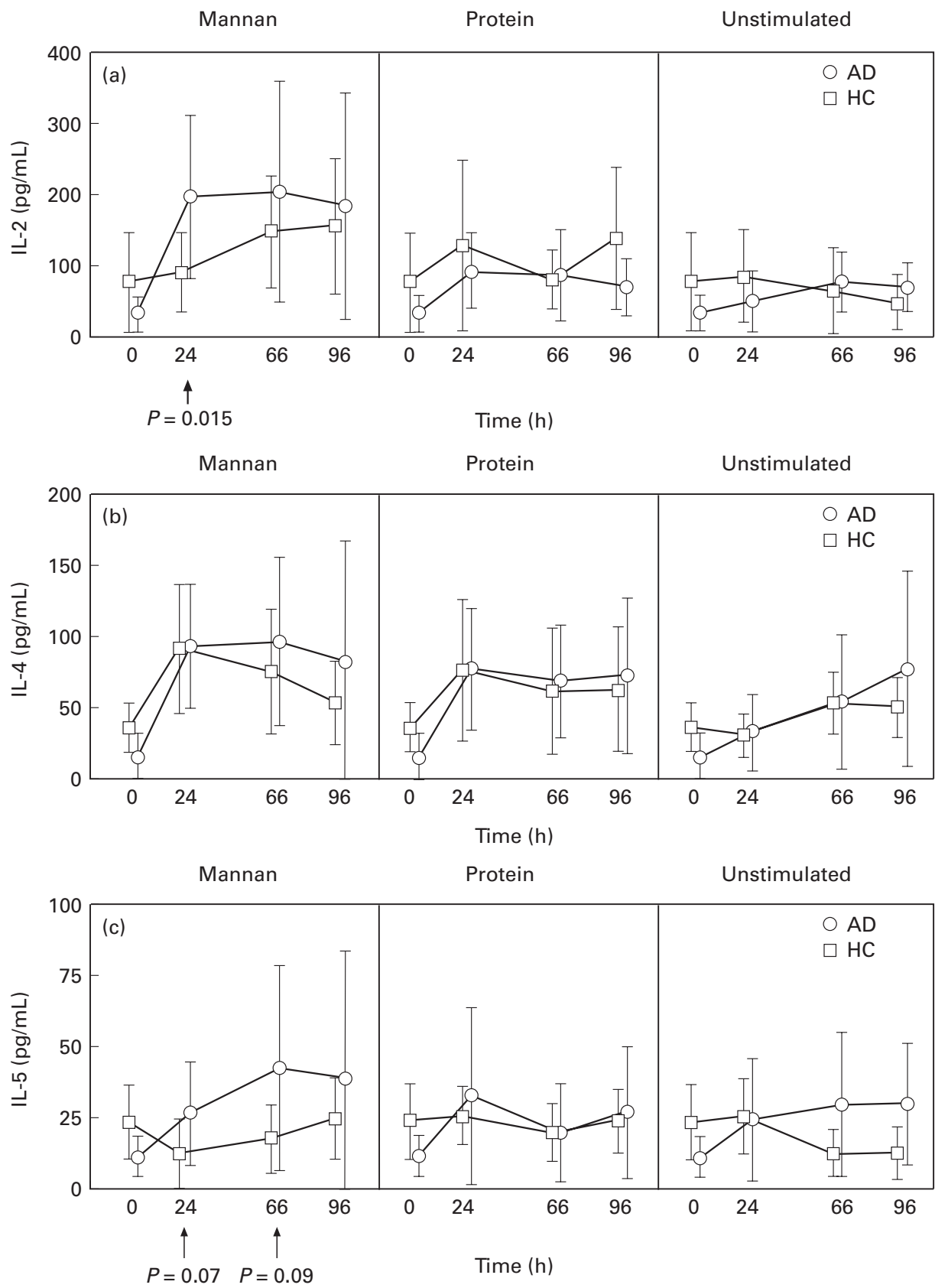


Fig. 2. The lymphoproliferative peak responses induced by purified *C. albicans* mannan and protein antigens in atopic dermatitis (AD) and healthy controls (HC). The data are presented as stimulation indexes (SI = stimulated proliferation/unstimulated proliferation). There was no difference in unstimulated proliferation between AD (Median 282 c.p.m., IQR 331 c.p.m.) and healthy controls (Median 283 c.p.m., IQR 231).



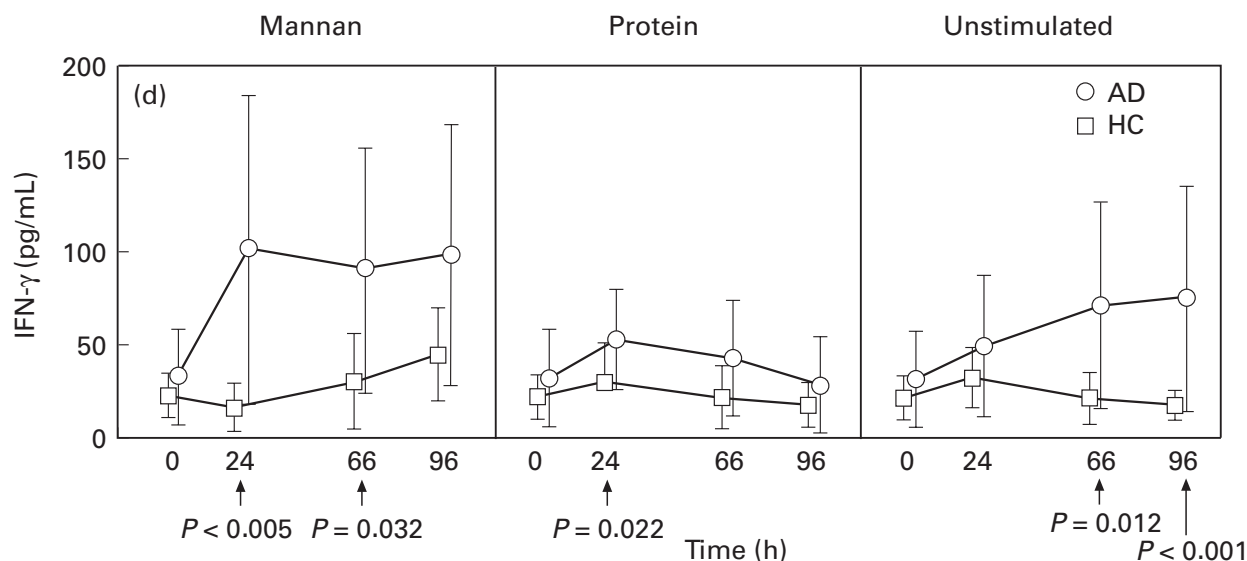


Fig. 3. The time-response curves of the *C. albicans* mannan and protein induced and spontaneous cytokine responses for (a) IL-2, (b) IL-4, (c) IL-5 and (d) IFN- γ in PBMC culture in atopic dermatitis (AD) patients and healthy controls (HC). Cytokines were measured from the culture supernatants with sandwich ELISAs. According to Kruskal–Wallis test, significant responses ($P < 0.01$) were seen with all IL-2 and IL-4 responses of AD patients and mannan-induced IL-5 and IFN- γ responses of AD patients.

MoAb 11B4 (Dr J Banchereau, Schering-Plough, Dardilly, France. Calibration curves consisted in serial dilutions of purified recombinant IL-4 (Genzyme Diagnostics, Cambridge MA, USA). A horse radish peroxidase-conjugated goat antirat Ig (Tago Inc. Burlingame, CA, USA) was used for labelling. ELISA for IFN- γ used the MoAb A35 (Dr J E DeVries, DNAX, Palo Alto CA, USA) and the biotinylated MoAb B27 as capture and tracer antibodies, respectively. Calibration curves were made with serial dilutions of purified recombinant IFN- γ (Genzyme Diagnostics). A streptavidin-alkaline phosphatase conjugate (Tago) diluted 1:5000 was used for labelling. IL-2 (Genzyme Diagnostics) and IL-5 (Pharmingen, San Diego CA, USA) concentrations were measured by commercially available antibody and standard kits for ELISA using the recommended procedures.

Statistics

Non-parametric one-way analysis of variance (Kruskal–Wallis test), Mann–Whitney *U*-test and Spearman's Rank Order correlation test were used for statistical analyses.

Results

IgE, IgG and proliferative responses

Both mannan- ($P < 0.005$) and protein- ($P < 0.002$) specific IgE levels were elevated in atopic dermatitis patients as

compared with healthy controls (Table 2). In IgG responses no differences between the groups were observed with either antigen. On the other hand the antimannan response was nearly tenfold as compared with antiprotein response.

The dose–responses of the antigen concentrations used in lymphocyte stimulations are presented in Fig. 1. The mannan concentration shows a peak at 50–500 $\mu\text{g/mL}$ and the protein concentration at 5–50 $\mu\text{g/mL}$. In general it can be seen that mannan induced a tenfold stronger proliferation as compared with protein. In fact, at a high concentration the protein has a tendency to a suppression of the spontaneous proliferation. The lymphoproliferative responses induced by both mannan ($P < 0.02$) and protein ($P < 0.01$) were significantly increased in AD as compared with healthy controls (Fig. 2).

Cytokine responses

The kinetics of the spontaneous and antigen-induced cytokine responses in AD and healthy controls are presented in Fig. 3. In analysis of the kinetics of the cytokine production, mannan was used at a concentration of 250 $\mu\text{g/mL}$ and protein at 25 $\mu\text{g/mL}$, based on previous titrations. Mannan, but not protein, induced an elevated IL-2 response at 24 h of incubation in AD as compared with healthy controls ($P = 0.015$) (Fig. 3a). Both mannan and protein induced an IL-4 response but there were no differences in the responses between AD and healthy controls (Fig. 3b). There was a tendency towards an elevated mannan-induced IL-5

response at 24 and 66 h in AD patients, but the generally low levels of specific responses do not allow firm conclusions (Fig. 3c). Mannan induced an elevated IFN- γ production in AD in comparison with healthy controls at 24 h ($P < 0.005$) (Fig. 3d). There were also elevated IFN- γ responses in AD patients at 66 h which were explained by elevated unstimulated responses at 66 and 96 h.

Correlations of IgE, proliferative and cytokine responses

The significant correlations between protein-induced lymphocyte proliferation responses and IgE responses are shown in Fig. 4. Both serum total IgE ($r = 0.59$, $P < 0.01$) and protein-specific IgE ($r = 0.65$, $P < 0.005$) correlate significantly with the proliferation responses.

The significant correlations between cytokine responses and IgE and proliferative responses are shown in Fig. 5. The mannan-induced IL-2 responses correlate significantly with serum total IgE ($r = 0.71$, $P < 0.002$) as well as mannan-specific IgE responses ($r = 0.62$, $P < 0.01$) and furthermore with mannan-induced lymphocyte proliferation ($r = 0.51$, $P < 0.02$). Surprisingly also mannan-induced IL-4 and IFN- γ productions correlate ($r = 0.43$, $P < 0.05$).

Discussion

In this study we showed that in AD there were elevated IgE and lymphoproliferative responses against both *C. albicans* mannan and protein but antimannan IgE was more significantly associated with AD. The protein-induced lymphoproliferation also correlated with the serum total IgE and protein-specific IgE. In general mannan was a clearly stronger inducer of lymphoproliferation and cytokines as compared with protein. The mannan-induced IL-2 and IFN- γ responses were elevated in AD. The mannan-induced IL-2 correlated with the total and mannan specific IgE responses and mannan-induced lymphoproliferation.

Our major finding was that mannan induced TH1-type cytokines IL-2 and IFN- γ were elevated in AD patients. The

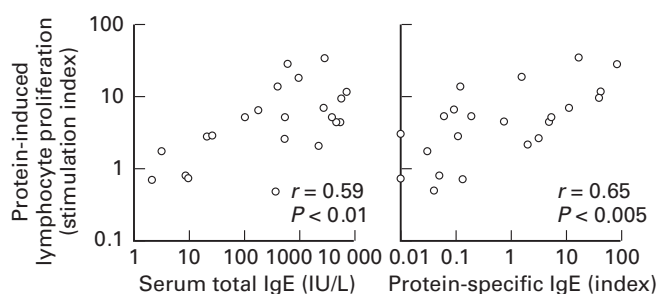


Fig. 4. Correlations between *C. albicans* protein-specific lymphoproliferation and serum total IgE and protein-specific IgE. (Spearman Rank Order Correlation.)

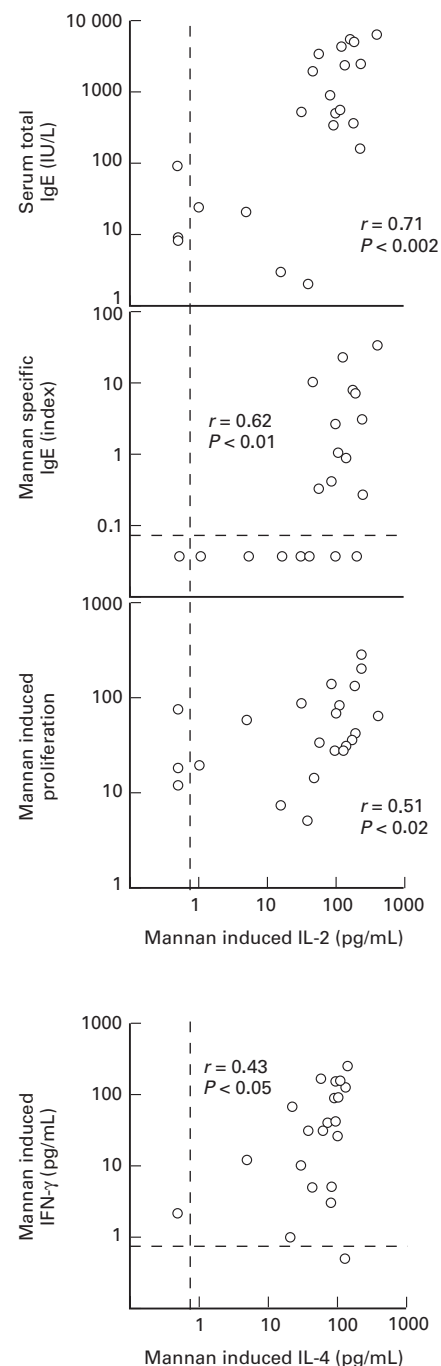


Fig. 5. Correlations between *C. albicans* mannan-induced IL-2 production and mannan-induced proliferation, mannan-specific IgE and serum total IgE. At the bottom the correlation between *C. albicans* mannan-induced IL-4 production and *C. albicans* mannan-induced IFN- γ production. (Spearman Rank Order Correlation.)

IL-2 responses furthermore correlated with IgE responses. The role for IL-4 in atopic dermatitis was suggested in the first studies on atopic dermatitis utilizing T-cell clones [10] [11]. More recent studies have shown that other cytokines are also involved and that in different steps of the inflammatory process, different cytokines are involved. The early phase of acute skin inflammation is associated with a predominance of IL-2 and IL-4 and maintenance of chronic inflammation is associated with IL-5 and IFN- γ [13,27]. Thus, as it appears that IL-2 and IL-4 initiate the inflammatory process, it may be speculated that mannan, as a strong inducer of IL-2, is involved in the process. This is further supported by the fact that the total serum IgE correlates with mannan-induced IL-2.

Mannan induced IFN- γ was also increased in AD as compared with controls. Furthermore, rather surprisingly the mannan induced IL-4 and IFN- γ correlated with each other. This correlation is likely to reflect two parallel independent phenomena, which both may contribute to the inflammatory process although no difference in IL-4 production was seen between the study groups. The mannan-induced IFN- γ may be connected to the later phase of the inflammation as has been suggested in the patch test studies [13]. Also elevated IFN- γ has been found in eczematous skin lesions and stimulated mononuclear cells from peripheral blood in atopic dermatitis [12,15].

There was an elevated lymphoproliferative response induced by both mannan and protein in AD as compared with controls. This was contradictory to previous observations showing frequent delayed skin reactions in healthy individuals and reduced delayed skin reactivity and *Candida*-induced lymphocyte proliferation in AD [28–30]. The lymphoproliferation induced by proteins correlated with protein-specific and serum total IgE and may be implied in the immunopathogenesis of the dermatitis. The mannan-induced proliferative response was seen in a wide range of antigen concentrations up to 1 mg/mL, in accordance with previous observations [31]. The mannan-induced response was also tenfold stronger than the protein-induced response. The lymphocytes may in fact be already presensitized to mannan by the saprophytic growth in the gastrointestinal tract, as the immune system is in activated state in AD.

The *in vitro* lymphoproliferative response by a purified polysaccharide of *C. albicans* requires the presence of autologous or allogeneic HLA-compatible macrophages or B cells [32–34]. It has also been suggested that in addition to recognition of mannan in association with HLA molecules, the induction of T-cell proliferation might happen by cross-linking of the T-cell receptor by multivalent mannan [35]. A 65-kDa mannoprotein of *C. albicans* has been shown to induce, most likely through interaction

with the antigen receptor complex, lymphoproliferation and production of the cytokines IL-1 β , IL-2, IL-6 and IFN- γ , but not IL-4 [19,20]. We could demonstrate, in addition to IL-2 and IFN- γ , mannan-induced IL-4 production.

C. albicans mannan appeared to be an immunologically active component in AD. It was able to elicit both IgE and lymphoproliferative responses as well as cytokine responses. The mannan-induced IL-2 response and IFN- γ responses appeared to be clearly connected with AD. Mannan also induced significant IL-4 responses but these could not be clearly connected to atopic dermatitis. As mannan has previously been shown to be the major cross-reacting allergen in AD, it seems that the immunomodulatory properties of *C. albicans* mannan make a demanding subject for future research within the immunopathogenesis of atopic dermatitis.

Acknowledgements

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