

## ***Pityrosporum ovale* Extracts Increase Interleukin-4, Interleukin-10 and IgE Synthesis in Patients with Atopic Eczema**

SVEN KRÖGER, KARSTEN NEUBER, ELVIRA GRUSECK, JOHANNES RING and DIETRICH ABECK

Department of Dermatology and Allergology, University Hospital Hamburg-Eppendorf, Hamburg, Germany

Evidence for a possible role of the lipophilic yeast *Pityrosporum ovale* in the pathophysiology of atopic eczema has been found both in laboratory and therapeutical studies. Positive type I prick test reactions to *P. ovale* correlate with the intensity of eczematous skin lesions in the head and neck regions of patients with atopic eczema. Furthermore, antifungal treatment has been shown to be helpful in atopic eczema. In the present study the effect of *P. ovale* on IgE synthesis and cytokine production (IL-2, IFN $\gamma$ , IL-4, IL-10) was investigated in patients with atopic eczema, *in vitro*. Eight patients with atopic eczema were studied; of these, 5 patients had specific IgE antibodies against *P. ovale*, as determined by fluoroimmunoassay (RAST). The control group consisted of 5 healthy non-atopic, *P. ovale* IgE-antibody-negative volunteers. Freshly isolated peripheral blood mononuclear cells (PBMC) were incubated in the presence of different antigen concentrations (0.01, 0.1, 1.0, 10  $\mu\text{g/l} \times 10^6$  cells) of *P. ovale*. IgE contents in the cell culture supernatants were significantly elevated in RAST(+) patients with atopic eczema ( $p < 0.05$ ), compared with RAST(-) atopic eczema patients and healthy volunteers. Coincubation of *P. ovale*-stimulated PBMC with IL-4 (50 U/l  $1 \times 10^6$  cells) resulted in a significantly higher IgE synthesis only in the RAST(+) atopic eczema patients. Additionally, incubation of PBMC from RAST(+) patients with atopic eczema led to an elevated synthesis of the T<sub>H</sub>2 related cytokines IL-4 and IL-10. Within the atopic eczema group, two subgroups differed markedly in their response to *P. ovale* antigen stimulation with a good correlation to the presence of specific IgE in serum and *in vitro* IL-4 and IL-10 production. The data support the assumption that *P. ovale* antigens might play a role in skin inflammation in at least a subgroup of patients with atopic eczema characterized by the presence of specific IgE antibodies to *P. ovale*. **Key word:** *interferon-gamma*.

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K. Neuber, Department of Dermatology and Allergology, University Hospital Hamburg-Eppendorf, Martinistr. 52, D-20246 Hamburg, Germany.

The etiopathophysiology of atopic eczema (AE) is still largely unknown; yet there is some evidence that exogenous, immunological and non-immunological factors play a crucial role in the altered reactivity of AE skin to various stimuli (1). Hypersensitivity reactions against parts of the cutaneous microflora acting as permanent stimuli for allergic skin reactions via an IgE-mediated mechanism may play an important role in the pathogenesis of AE (2, 3). Among these the lipophilic yeast *P. ovale* has gained much interest in recent years, since a high prevalence (50 to 80%) of type I skin reaction to extracts of this organism has been reported in patients with AE (4). In addition, specific anti-*P. ovale* IgE has also been demonstrated in the sera of patients with AE (5, 6). Further evidence for the pathophysiological

role of *P. ovale* is provided by clinical observations with AE of the head and neck type that responded favourably to an antimycotic regimen (7). The link between AE of the head and neck type and *P. ovale* may be due to the preferential regions of the body where the yeast can be found, e.g. upper chest, face and scalp (8).

### MATERIALS AND METHODS

#### *Patients and controls*

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers ( $n = 5$ ; age:  $44.8 \pm 21.9$  years) and 8 patients (age:  $33.4 \pm 9.7$ ) suffering from atopic eczema (AE). Five AE patients had specific serum IgE for *P. ovale* (RAST+). The AE patients were classified according to the criteria of Hanifin & Rajka (9). The following features were present: a chronic or chronically relapsing eczema, flexural lichenification, pruritus and a personal or family history of atopy. None of the AE patients received systemic steroid treatment. As controls served healthy volunteers with no personal history of allergic diseases and serum IgE levels below 60 kU/l.

#### *Isolation of PBMC*

Isolation of PBMC was performed by centrifugation on a Ficoll-sodium metrizoate (Sigma, München, Germany) gradient according to Böyum (10). Briefly, heparinized venous blood (40 ml) was layered over Ficoll-sodium metrizoate (density = 1075 g/ml) and centrifuged at  $375 \times g$  for 35 min. Cells at the interface above the Ficoll-metrizoate were removed and washed three times with RPMI-1640.

#### *Culture conditions*

The basic culture medium was RPMI-1640 supplement with 2 mM glutamine, 100  $\mu\text{g/ml}$  streptomycin and 100 IU/ml penicilline. Medium containing 10% fetal calf serum is referred to as RPMI-1640 with 10% FCS.

Cell suspensions containing  $1 \times 10^6/\text{ml}$  viable cells in RPMI-1640 and 10% FCS were dispensed into each well of 24-well plates (Nunc, Roskilde, Denmark). Stimuli were added and the cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

#### *Culture of P. ovale*

An isolate of *P. ovale* purchased from the German Collection of microorganisms (strain DSM No. 6170) was grown for 4 days at 37°C on a glucose-peptone agar containing bacteriological peptone (1.0%), glucose (4.0%), yeast extract (0.01%), glycerol monosterate (0.25%), Tween 80 (0.2%), olive oil (2.0%) and agar (1.2%), as described by Faergemann & Fredriksson (12).

#### *Extracts of P. ovale*

The *P. ovale* cells were carefully harvested from the solid agar medium and a thick cell suspension was obtained. The cells were washed three times with Aqua dest., resuspended in 0.125 M NH<sub>4</sub>HCO<sub>3</sub> buffer, pH 7.8 and sonicated for 5 min (13, 14). The cell suspension was extracted overnight at 4°C and centrifuged at  $1,200 \times g$  for 10 min. Afterwards, the supernatant was membrane-filtered (pores: 0.45  $\mu\text{m}$ ). The protein concentration of the *P. ovale* extract was estimated by the method of Lowry. All extracts were lyophilized by vacuum-centrifugation at 4°C for 8 h and stored at -20°C until tested.

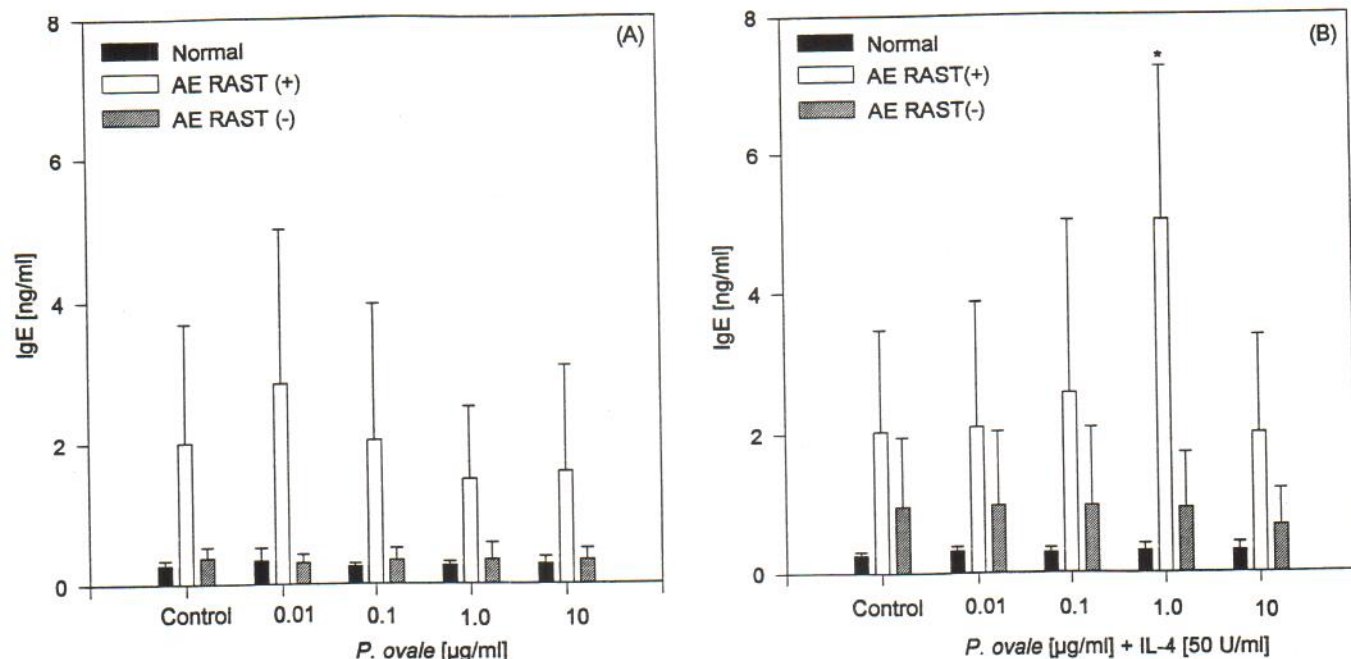


Fig. 1. Results of *P. ovale* (A) and *P. ovale* + IL-4 (B) stimulated IgE synthesis of PBMC from healthy donors, RAST(-) and RAST(+) AE patients. \* significantly enhanced compared with the unstimulated control and with the healthy donors as well as with RAST(-) AE patients ( $p < 0.05$ ).

#### Cell stimulation

Freshly prepared PBMC were resuspended in culture medium with different concentrations of the *P. ovale* extract: 0.01, 0.1, 1.0, 10.0  $\mu\text{g/l}$   $\times 10^6$  PBMCs. When cytokines were measured, cells were stimulated only with 1.0  $\mu\text{g/l}$   $\times 10^6$  PBMCs. This concentration revealed optimal stimulatory effects.

#### ELISA for IgE

The IgE content of culture supernatants was determined by an enzyme linked immunosorbent assay (ELISA) on day 7. Cell viability was assessed microscopically by trypan blue exclusion analysis. 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-human IgE (100  $\mu\text{l}$ , diluted 1:4000 in 0.1 M carbonate buffer, pH 9.6; DAKO, Denmark) for 1 h at 37°C. Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS, 0.05% Tween 20, (1 h at 37°C). A 100- $\mu\text{l}$  aliquot of the supernatant was added and incubated for 1 h at 37°C. As second antibody a peroxidase-conjugated rabbit-anti-human IgE (100  $\mu\text{l}$ , diluted 1:2000 in PBS, 0.05% Tween 20, pH 7.2; DAKO, Denmark) was used. In parallel, standard curves (0.18–200 ng/ml) were performed (Behring, Marburg, Germany). The specificity of the assay was confirmed by adding immunoglobulin of other isotypes to rule out the possibility of cross-reactivity.

Finally, o-phenylenediamine (OPD, Sigma, St.Louis, USA), 1 mg/ml in 33 mM phosphate-citrate buffer, pH 5.0, was added as substrate, and optical density was measured at 490 nm.

#### Cytokine production

The amounts of IL-2, IFN $\gamma$ , IL-4, and IL-10 were measured after 4 days of culture by quantitative sandwich enzyme immunoassay kits distributed by Hermann Biermann GmbH (Bad Nauheim, Germany). Briefly, samples and standards were incubated in microtiter wells coated with a monoclonal antibody specific for each cytokine. Afterwards, an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells. After a further incubation period, the excess detector complex was removed by washing and a substrate solution was added to the wells and colour developed in proportion to the amount of the cytokine. Absorbance was measured at 490 nm, 450 nm, and 405 nm, respectively.

#### Statistical analysis

All experiments were performed with five different donors in the control and eight different donors in the AE group, respectively. The data were calculated as means  $\pm$  standard deviation (SD). The significance was evaluated with Student's t-test for independent means.  $p < 0.05$  was considered as significant.

## RESULTS

#### IgE synthesis

Neither PBMC from normal donors nor from RAST(-) AE patients showed an increased IgE synthesis after incubation with *P. ovale* antigen (0.01, 0.1, 1.0, 10.0  $\mu\text{g/ml}$ ). However, IgE synthesis of PBMC from RAST(+) AE patients was significantly higher compared with the other donors (Fig. 1A). Addition of IL-4 and 1.0  $\mu\text{g}$  *P. ovale* antigen (Fig. 1B) resulted in a significantly enhanced IgE synthesis in RAST(+) patients with AE up to  $5.02 \pm 2.23$  ng/ml, compared to healthy donors and to RAST(-) AE patients, respectively.

#### IL-2, IFN $\gamma$ , IL-4, and IL-10 synthesis

PBMC of healthy donors showed a significantly ( $p < 0.05$ ) increased IL-2 and IFN $\gamma$  synthesis after addition of 1.0  $\mu\text{g/ml}$  *P. ovale* antigen extract (Fig. 2A,B). In contrast, IL-2 and IFN $\gamma$  production of PBMC from patients with AE was not stimulated significantly by the *P. ovale* antigen extract.

However, IL-4 and IL-10 synthesis of PBMC from RAST(+) patients with AE was significantly enhanced compared with healthy controls and RAST(-) AE patients after stimulation with 1  $\mu\text{g/ml}$  *P. ovale* antigen (Fig. 2C,D).

## DISCUSSION

In this study we have demonstrated that IgE synthesis and IL-4 as well as IL-10 production were increased after stimulation

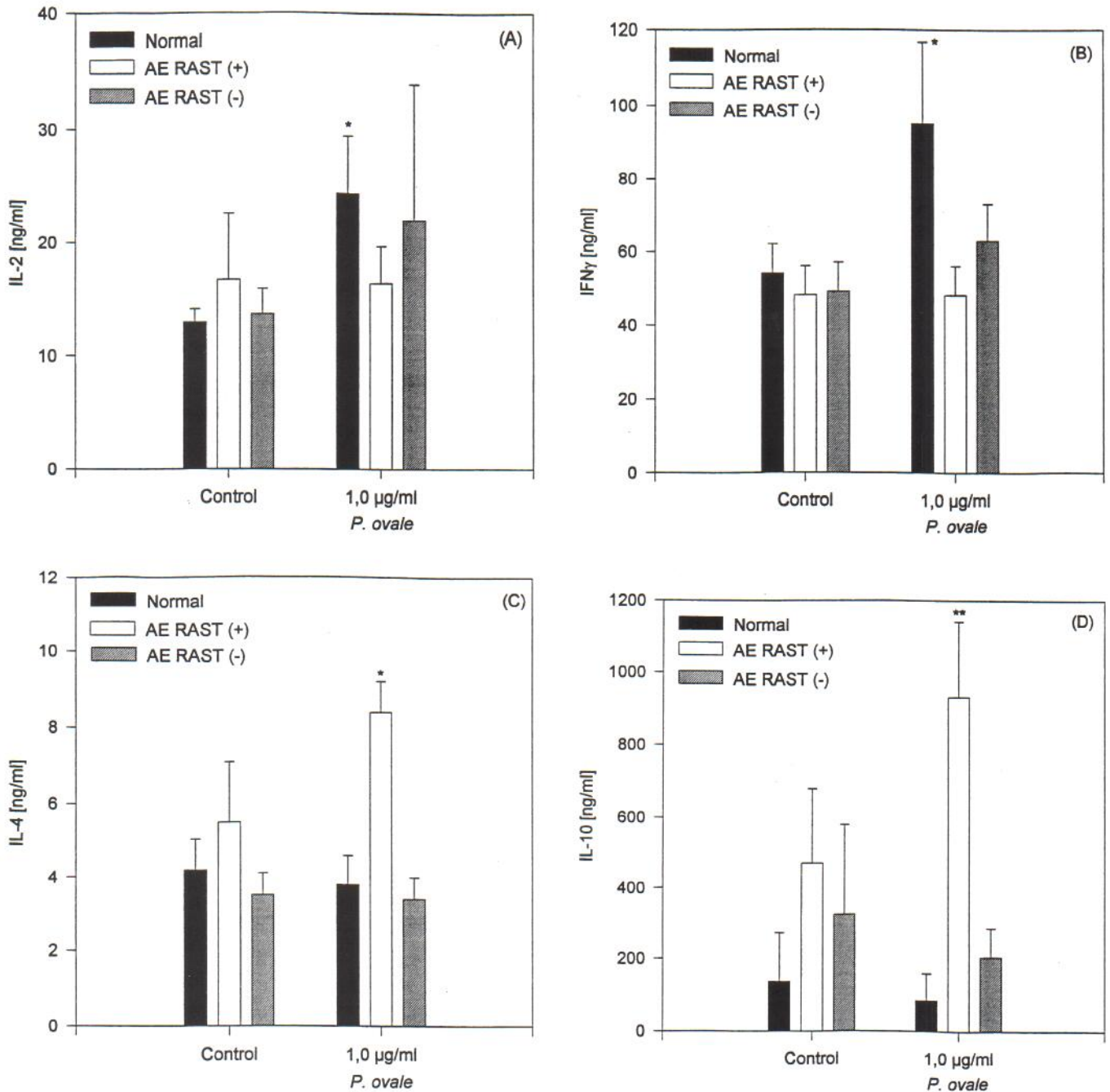


Fig. 2. IL-2 (A), IFN $\gamma$  (B), IL-4 (C), and IL-10 (D) production after stimulation with *P. ovale* antigen. \* significant compared with unstimulated PBMC (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ).

with *P. ovale* antigen only in AE patients with specific IgE antibodies (RAST +) to this lipophilic yeast.

The addition of *P. ovale* antigen and IL-4 to PBMC from RAST(+) patients with AE resulted in a dose-dependent increase of IgE synthesis. It is not clear whether IgE synthesis *in vitro* was specific or polyclonal. However, the fact that only PBMC from patients with specific serum IgE showed significantly elevated IgE contents in cell culture supernatant supports the assumption that specific IgE antibodies have been produced after stimulation with *P. ovale* antigen, *in vitro*.

Additionally, *P. ovale* antigen induced a markedly increased synthesis of IL-4 and IL-10 in RAST(+) patients with AE com-

pared with normal donors and RAST(-) AE patients, respectively. The increased production of IL-4 and IL-10 may be due to the presence of T<sub>H</sub>2 cells specific to *P. ovale* antigen in RAST(+) AE patients. T<sub>H</sub>2 cells are characterized by their ability to produce IL-4, IL-5 and IL-10, whereas T-cells from the T<sub>H</sub>1-type synthesize IL-2 and IFN $\gamma$  (15, 16, 17). Activation of T<sub>H</sub>2-cells via enhanced IL-4 production results in an increased IgE synthesis (18). In atopic disorders both in local inflammatory reactions and in peripheral blood, high IL-4/IFN $\gamma$  production ratios have been demonstrated indicating predomination of allergen specific T<sub>H</sub>2-cell subsets in atopic diseases, e.g. AE (19). IL-10 is produced under different conditions of immune activation by

the T<sub>H</sub>0 and T<sub>H</sub>2 subsets of helper T-cells, as well as by monocytes, macrophages, and B-cells (20). Human IL-10 inhibits IFN $\gamma$ , GM-CSF, TNF $\alpha$  and IL-3 production of activated PBMC (20). Additionally, antigen-specific T-cell proliferation is indirectly suppressed by IL-10 via monocytes (21–23). The role of IL-10 in AE is still unclear. In the presence of IL-4 a stimulatory effect on IgE synthesis has been described (20). In the present experiments significantly increased IL-10 amounts were observed after stimulation of PBMC from RAST(+) AE patients with *P. ovale*. Elevated IL-10 production may be due to a preferential activation of T<sub>H</sub>2 subsets in *P. ovale*-sensitized patients with AE. On the other hand, increased IL-10 levels may be partially responsible for low IL-2 production of PBMC from RAST(+) AE patients indirectly via downregulation of class II MHC expression on monocytes (21, 22) or directly via inhibition of IL-2 synthesis by T-cells (23).

Recently, similar results have been demonstrated for *Staphylococcus aureus* and *S. aureus*-derived enterotoxins (24–27). Thus, *S. aureus* and *P. ovale* may act as permanent allergic stimuli in AE by stimulation of T<sub>H</sub>2-cells and IgE synthesis.

Increased IgE, IL-10 and IL-4 production *in vitro* supports the assumption that *P. ovale* antigens play a role in skin inflammation in at least the subgroup of RAST(+) AE patients via penetration of the skin surface and binding to epidermal Langerhans' cells which may bear specific IgE antibodies against *P. ovale* antigens (28, 29).

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