

Pityrosporum orbiculare-Reactive T-Cell Lines in Atopic Dermatitis Patients and Healthy Individuals

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The yeast *Pityrosporum orbiculare* is one of the factors that may contribute to atopic dermatitis (AD). In the present study we compared the T-cell response to *P. orbiculare* in 12 AD patients with specific immunoglobulin (Ig)E antibodies (Ab) in serum against *P. orbiculare* with that of six non-atopic healthy controls. Freshly isolated peripheral blood mononuclear cells (PBMC) were cultured for 3 days in the presence of *P. orbiculare* extract. The proliferative response as measured by [³H]-thymidine incorporation was significantly higher in the AD patients than in the healthy controls ($P < 0.05$). Furthermore, significantly higher levels of interleukin (IL)-5 ($P < 0.05$), as analyzed by ELISA, were produced by PBMC from the AD patients compared to the healthy controls. *Pityrosporum orbiculare*-reactive T-cell lines (TCL) established by *P. orbiculare* stimulation of PBMC for 11 days produced significantly higher levels of IL-4 and IL-5 after stimulation with anti-CD3 Ab and showed a higher IL-4/interferon (IFN)- γ ratio ($P < 0.05$) in the AD patients compared to the healthy controls. The higher proliferative PBMC response to *P. orbiculare* and the Th2-like cytokine production by *P. orbiculare*-stimulated TCL from AD patients indicate that *P. orbiculare* may play a role in maintaining skin inflammation in AD.

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INTRODUCTION

The lipophilic yeast *Pityrosporum orbiculare*, also denoted *Pityrosporum ovale* or *Malassezia furfur* [1], is a member of the normal human cutaneous flora, preferentially colonizing the sebum-rich head, neck and upper trunk areas [2]. *Pityrosporum orbiculare* is associated with dermatological diseases such as pityriasis (tinea) versicolor, pityrosporum folliculitis, seborrhoeic dermatitis and atopic dermatitis (AD) [3]. Immunoglobulin (Ig)A, IgG and IgM antibodies (Ab) which are *P. orbiculare*-specific can be found both in healthy individuals and in patients with skin diseases caused by *P. orbiculare* [4]. In contrast, IgE Ab against *P. orbiculare* are only detected in atopic individuals, particularly in those with AD [5–7].

Atopic dermatitis, a chronic inflammatory skin disease of unknown aetiology, is often associated with increased levels of serum-IgE [8]. It is thought that T helper (Th) cells play an important role in the pathogenesis of the disease, and allergen-specific T cells from AD patients are mainly of the Th2 type,

producing interleukin (IL)-4 and IL-5 but little or no IL-2 and interferon (IFN)- γ [9]. We have previously shown that *P. orbiculare*-specific T-cell clones derived from *P. orbiculare* patch tested skin and lesional AD skin are mainly of the Th2 type and thus may play a role in maintaining an IgE-mediated skin inflammation [10]. Ketoconazole treatment in AD patients has resulted in improvement of the eczema and in decreased serum-IgE levels [11]. Thus *P. orbiculare* may be considered one of the factors that may contribute to AD.

The aim of this study was to investigate if *P. orbiculare*-reactive T-cell lines could be obtained, not only from patients with AD but also from healthy controls, and if so to compare the cytokine profile of the established T-cell lines.

MATERIALS AND METHODS

Subjects. The subjects included in the study were 12 patients (median age 30 years, range 15–63 years) with moderate to severe AD (diagnosed according to the criteria of Hanifin & Rajka, see Ref. 12) and six

Table 1. Characterization of patients and healthy controls

Subjects	Age years	Sex	IgE kU/l	<i>P. orbiculare</i>	<i>C. albicans</i>	SI* <i>P. orbiculare</i>	SI <i>C. albicans</i>
				RAST class/kU/l	RAST class/kU/l		
AD 1	20	F	456	2/2.8	0	1.6	1.2
AD 2	15	F	481	2/2.1	2/3.4	4.9	8.1
AD 3	24	F	678	3/6.2	2/1.6	6.4	9.0
AD 4	44	F	1208	2/1.5	0	3.3	6.0
AD 5	27	F	1788	2/1.4	3/8.8	1.9	5.4
AD 6	55	M	1974	3/3.5	3/3.5	6.1	8.7
AD 7	63	M	3643	2/2.8	3/13	2.5	5.4
AD 8	23	M	3887	3/6.3	5/60	1.7	5.1
AD 9	29	M	4370	4/24	3/7.9	10	3.5
AD 10	33	M	4943	2/1.6	2/2.8	5.7	0.8
AD 11	31	F	5960	3/9.7	3/4.3	1.2	7.5
AD 12	34	M	6609	4/24	4/26	4.0	1.1
HC 1	26	F	4	0	0	1.9	6.0
HC 2	34	M	5	0	0	1.9	9.2
HC 3	27	F	6	0	0	1.2	3.2
HC 4	26	F	11	0	0	1.4	3.7
HC 5	63	M	12	0	0	1.1	3.5
HC 6	38	F	14	0	0	2.5	5.5

* SI, stimulation index calculated as:

$$\frac{\text{mean cpm of PBMC} + P. orbiculare \text{ extract (10 } \mu\text{g/ml) or } C. albicans \text{ extract (10 } \mu\text{g/ml)}}{\text{mean cpm of PBMC} + \text{medium alone}}$$

PBMC were cultured in triplicate for 3 days and proliferation was measured by [³H]-thymidine incorporation. AD, atopic dermatitis patient; HC, healthy control; F, female; M, male.

non-atopic healthy subjects with no personal history of skin disorders (median age 31 years, range 26–63 years: see Table 1). The patients had widespread eczema, and all had a head and neck localization. The median serum-IgE level of the patients was 2808 kU/L (range 456–6609 kU/L, reference range 1.6–122 kU/L) (Pharmacia CAP System IgE FEIA: Pharmacia AB Diagnostics, Uppsala, Sweden) and of the healthy controls 8.5 kU/L (range 4–14 kU/L) at the time of the investigation. All patients were Phadiatop positive (Pharmacia CAP System RAST FEIA: Pharmacia AB Diagnostics) and RAST positive for *P. orbiculare*, RAST class 2–4 (Pharmacia AB Diagnostics: Table 1). A total of 10 patients were RAST positive for *Candida albicans* (*C. albicans*), RAST class 2–5 (Table 1). The healthy controls were Phadiatop negative and RAST negative for *P. orbiculare* and *C. albicans* (Table 1). No topical corticosteroids stronger than group 2 had been applied on the skin and no corticosteroids had been ingested for 4 weeks before the investigation. The study was approved by the Ethics Committee of the Karolinska Hospital, Stockholm, Sweden.

Cell medium and yeast extracts. As cell culture medium Iscove's modified Dulbecco's medium was used, supplemented with gentamycin 25 µg/ml, penicillin 100 IU/ml, streptomycin 100 µg/ml, L-glutamine 2 mM/L (Gibco BRL, Life Technologies Ltd, Paisley, UK), 2-mercaptoethanol 0.25 µM/L (Merck, Darmstadt, Germany) and 10% pooled, heat-inactivated human ABRh+ serum. For detection of cytokines AIM V serum-free cell medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with L-glutamine (2 mM) was used, referred to as AIM V

medium. Recombinant human IL-2 was purchased from Amersham Laboratories (Amersham, Buckinghamshire, UK). We prepared *P. orbiculare* and *C. albicans* extracts as previously described [13].

Preparation of peripheral blood mononuclear cells (PBMC) and T-cell lines (TCL). The PBMC were isolated from heparinized blood by gradient centrifugation on Ficoll Paque® (Pharmacia Biotech, Sollentuna, Sweden). To establish TCL, PBMC at 2.5×10^6 cells/well were cultured for 11 days in 12-well plates (Costar, Cambridge, UK) in 2.5 ml of cell culture medium in the presence of *P. orbiculare* extract (10 µg/ml), at 37°C and 6% CO₂. Interleukin-2 (50 U/ml) was added on day 5 and day 8. Additional cell medium (1.5 ml) was added on day 8.

Flow cytometry. To determine the phenotypes of the TCL phycoerythrin-conjugated anti-CD4 and anti-CD8 MoAbs (Dako A/S, Glostrup, Denmark) were used. Phycoerythrin-conjugated mouse IgG1 (Dako A/S) was used as a negative control. The TCL were analyzed in a Coulter EPICS Profile II (Coulter Inc., Hialeah, FL, USA). At least 3000 cells were examined for each sample.

PBMC and TCL proliferation assay. We cultured PBMC (2×10^5 cells/well) or TCL (4×10^4 cells/well) for 3 days in 96-well round-bottomed plates (Costar) in cell medium (200 µl/well) with extracts of *P. orbiculare* (0.1–10 µg/ml) or *C. albicans* (10 µg/ml) at 37°C and 6% CO₂. For the TCL fresh autologous irradiated (30 Gy) PBMC (1×10^5 cells/well) were used as antigen-presenting cells. To ascertain that the T cells could proliferate, phytohaemagglutinin (PHA: 10 µg/ml) was added to cultures run in parallel. During the last 18 h of culture,

1 μCi [^3H]-thymidine (Amersham Laboratories) was added to each well and the thymidine incorporation was determined by scintillation counting. Triplicate cultures were set up and the results expressed as mean cpm $\pm$ SEM and as stimulation index (SI). The SI for PBMC was calculated as:

$$\frac{\text{mean cpm of PBMC + extracts of } P. \text{ orbicularis or } C. \text{ albicans (10 } \mu\text{g/ml)}}{\text{mean cpm of PBMC + medium alone}}$$

The SI for TCL was calculated as:

$$\frac{\text{mean cpm of TCL + autologous irradiated PBMC + } P. \text{ orbicularis extract (10 } \mu\text{g/ml)}}{\text{mean cpm of TCL + autologous irradiated PBMC + medium alone}}$$

Cultures for cytokine production. Cytokine production was measured in the supernatants of PBMC (2.5×10^6 cells/well) cultured for 11 days in 12-well plates (Costar) in cell culture medium (2.5 ml/well) in the presence of *P. orbicularis* extract (10 $\mu\text{g/ml}$) at 37°C and 6% CO_2 . Interleukin-2 (50 U/ml) was added on day 5 and day 8. Cell medium (1.5 ml) was added on day 8.

Cytokine production of the established TCL was measured in the culture supernatants after 20 h culture with or without immobilized anti-CD3 Ab (OKT3 hybridoma supernatant: Ortho Diagnostic Systems, Raritan Inc., Johnson & Johnson, New Brunswick, NJ, USA) [9]. Briefly, 12-well plates (Costar) were coated with goat-anti-mouse-IgG (Dakopatts, Copenhagen, Denmark: 20 $\mu\text{g/ml}$) in 0.05 M Tris buffer solution, pH 9.6, for 1 h at room temperature. The wells were washed three times with phosphate buffered saline (PBS), pH 7.4, and incubated thereafter with anti-CD3 Ab (dilution 1/100) in PBS, pH 7.4, supplemented with 10% human heat-inactivated ABRh+ serum for 1 h at room temperature. The medium was aspirated and TCL (2×10^6 cells in 2 ml of AIM V medium supplemented with IL-2, 20 U/ml) were added to each well and incubated for 20 h at 37°C and 6% CO_2 .

The TCL were also re-exposed to *P. orbicularis* extract and their cytokine production was analyzed. To prepare antigen-presenting cells irradiated (30 Gy) autologous PBMC, 2×10^6 cells per well in 1 ml of cell culture medium were incubated for 2 h at 37°C . The non-adherent cells were aspirated and the wells were washed twice with RPMI-1640 (Gibco Laboratories) and once with AIM V medium. Then TCL, 2×10^6 cells in 2 ml of AIM V medium supplemented with IL-2 (20 U/ml), were added to each well and incubated with or without *P. orbicularis* extract (10 $\mu\text{g/ml}$) for 20 h at 37°C and 6% CO_2 .

Cytokine assays. The supernatants from the cell cultures were centrifuged at 300 g and thereafter kept at -20°C until assessed for the presence of IL-4, IL-5 and IFN- γ . The determination was performed by ELISA technique according to the manufacturer's instructions (IL-4 and IFN- γ , MabTech AB, Stockholm, Sweden; IL-5, PharMingen Research Products, San Diego, CA, USA). Briefly, 96-well flat-bottomed plates (Nunc, Roskilde, Denmark) were coated with the MoAbs anti-IL-4 Ab (IL-4-I, 1 $\mu\text{g/ml}$, 100 $\mu\text{l/well}$), anti-IFN- γ Ab (1-D1K, 1 $\mu\text{g/ml}$, 100 $\mu\text{l/well}$) or anti-IL-5 Ab (TRFK5, 4 $\mu\text{g/ml}$, 50 $\mu\text{l/well}$), overnight at room temperature, followed by treatment with 200 μl of PBS-Tween (PBS containing 0.5% Tween-20) for IL-4 and IFN- γ , and PBS containing 10% heat-inactivated fetal calf serum (FCS: Hyclone, Logan, UT, USA) for IL-5. Next, samples or standards (100 μl) were added to the wells in duplicate followed by addition of 1 $\mu\text{g/ml}$ of biotin-labelled anti-IL-4 (IL-4-II), anti-IFN- γ (7-B6-1) or anti-IL-5 (JES1-5A10) Ab. After incubation with streptavidin-alkaline phosphatase (MabTech AB, dilution 1/1000) the substrate (p-nitro-phenyl-phosphatase disodium: Sigma, St Louis, MO, USA), diluted in 1 M diethanolamine buffer, pH 9.8, was added. The absorbance at 405 nm was measured by an ELISA plate reader (Labsystem Multiscan, Helsinki, Finland). As standards the NIH (National Institute of Health, Bethesda, MA, USA) standard was used for

IFN- γ , recombinant human IL-4 from PreproTech Inc. (London, UK) for IL-4 and recombinant human IL-5 from PharMingen for IL-5. The detection limit for IL-4 was 0.02 ng/ml, for IL-5 0.06 ng/ml and for IFN- γ 0.03 ng/ml.

Statistics. Mann-Whitney *U*-test was used for comparison between the AD patients and the healthy controls. Wilcoxon signed rank test was used for analysis of differences in cytokine production between stimulated and unstimulated TCL. Correlations were computed using the Spearman rank-order correlation coefficient (r_s). We considered $P < 0.05$ to be statistically significant.

RESULTS

Proliferative response in PBMC

The PBMC response to *P. orbicularis* stimulation was significantly higher in the AD group than in the control group ($P < 0.05$), with an SI ranging from 2.5 to 10 in eight out of 12 patients (Table 1, Fig. 1). Only one of the healthy controls reached an SI above 2. Peripheral blood mononuclear cells from AD patients with SI values of 2.5 or more, but from none of the healthy controls, showed a dose-dependent proliferative response to *P. orbicularis* (Fig. 2). A correlation was not found between SI for *P. orbicularis* and total serum-IgE levels in the AD patient group ($r_s = 0.001$, $P = 1.0$) or between SI for *P. orbicularis* and specific IgE (kU/l) for *P. orbicularis* ($r_s = 0.16$, $P = 0.62$). We found that SI for *C. albicans* did not differ significantly between the AD patients and the healthy controls

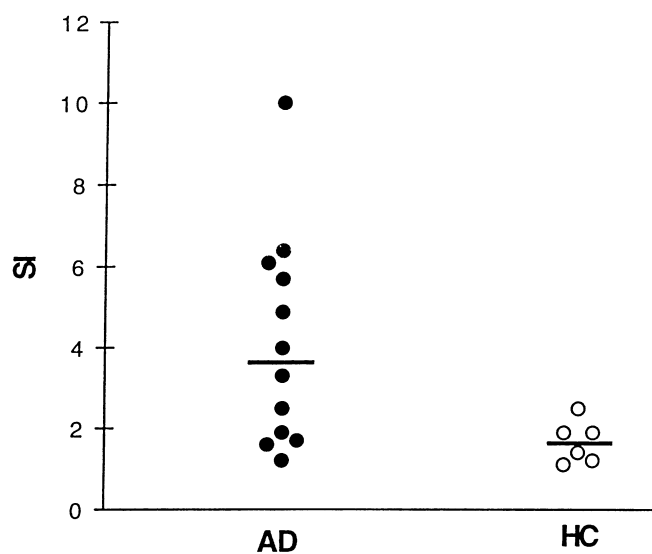


Fig. 1. Significantly higher proliferative response of freshly isolated PBMC (2×10^5 cells/well) in 12 AD patients compared to six healthy controls (HC) after stimulation with *P. orbicularis* extract (10 $\mu\text{g/ml}$) for 3 days ($P < 0.05$). The PBMC proliferation was determined by [^3H]-thymidine incorporation and presented as stimulation index (SI). The SI was calculated as mean cpm of triplicate cultures from stimulated PBMC/mean cpm from control PBMC. The background defined as PBMC cultured in medium alone was in all cases below 4105 ± 112 cpm. Bars indicate median values.

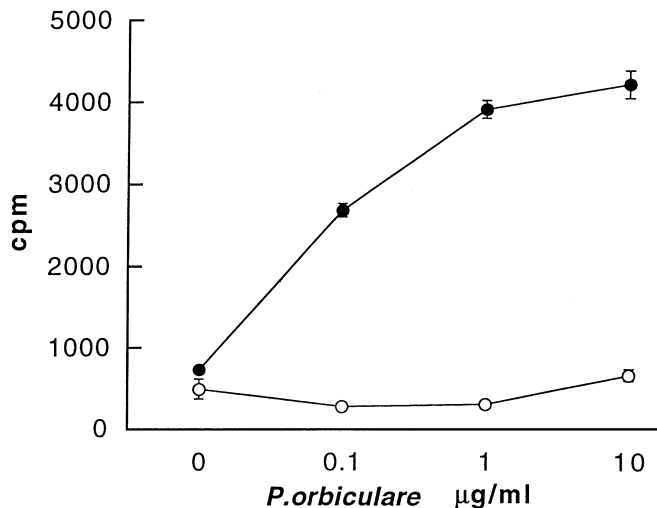


Fig. 2. Dose-dependent proliferative response of freshly isolated PBMC exemplified by AD patient number 10 (●) and healthy control (HC) number 4 (○). A total of 2×10^5 cells/well were cultured for 3 days with or without extracts of *P. orbiculare* (0.1–10 µg/ml). The PBMC proliferation was determined by [^{3} H]-thymidine incorporation and the results are presented as mean cpm $\pm$ SEM of triplicate cultures. As a positive control, PHA (10 µg/ml) induced a proliferative response of $159\,830 \pm 9076$ cpm in the AD patient and of $97\,269 \pm 2536$ cpm in the healthy control.

($P = 0.89$). In all, nine out of 12 AD patients and all healthy controls showed an SI above 2.5 to *C. albicans* extract (Table 1). There was no correlation between SI for *C. albicans* and total serum-IgE levels ($r_s = -0.45$, $P = 0.15$) or between SI for *C. albicans* and specific IgE levels for *C. albicans* ($r_s = -0.24$, $P = 0.45$) in the AD group. Stimulation with PHA resulted in PBMC proliferation in all subjects (range 50079–374988 cpm).

Proliferative response in TCL to *P. orbiculare* stimulation

It was possible to establish TCL from 11 AD patients and six non-atopic healthy controls. In both the AD group and the control group the TCL contained $\approx 90\%$ T cells, of which 80% (median value; range 72–91%) were CD4 $^+$ and 20% (range 9–28%) were CD8 $^+$. The CD4/CD8 ratio did not differ between the AD patients and the healthy controls.

Results after restimulation of the TCL with *P. orbiculare* extract for 3 days were obtained from 11 patients and five controls (Fig. 3). There was no significant difference in proliferative response expressed as cpm (Fig. 3) or SI (data not shown) of the TCL between the AD patient group and the control group ($P = 0.43$ and $P = 0.50$, respectively).

Cytokine production by PBMC

Interleukin-4, IL-5 and IFN- γ were measured in culture supernatants obtained from freshly isolated PBMC stimulated with *P. orbiculare* for 11 days from the six healthy controls and all

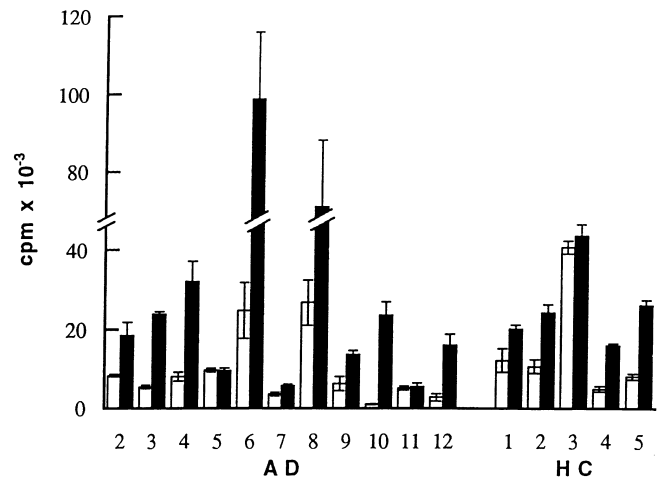


Fig. 3. Proliferative response of *P. orbiculare*-reactive TCL (4×10^4 cells/well) from 11 AD patients and five healthy controls (HC) was measured after restimulation with *P. orbiculare* extract (10 µg/ml; ■) or after culture with medium alone (□) for 3 days. Irradiated (30 Gy) autologous PBMC (1×10^5 cells/well) were used as antigen-presenting cells. The proliferation of the TCL was determined by [^{3} H]-thymidine incorporation and the results are presented as mean cpm $\pm$ SEM of triplicate cultures. The background defined as proliferation of irradiated PBMC + *P. orbiculare* extract (10 µg/ml) was below 615 ± 140 cpm in all individuals. Stimulation of the TCL with PHA (10 µg/ml) resulted in a proliferative response in the AD patients of 18 802–119 425 cpm and in the healthy controls of 46 191–155 503 cpm.

AD patients except number 1 (Table 2). There was a significantly higher production of IL-5 in the AD group than in that of the control group ($P < 0.05$). We found that PBMC from patients with a high SI for *P. orbiculare* produced higher amounts of IL-5 than PBMC from patients with a low SI and healthy controls, although no significant correlation was found between SI for *P. orbiculare* and IL-5 production ($r_s = 0.45$, $P = 0.07$).

No statistical difference was found between the AD group and the control group for IL-4 ($P = 0.21$) or IFN- γ ($P = 0.42$; Table 2).

Cytokine production by TCL

It was found that TCL stimulated with anti-CD3 Ab for 20 h produced significantly higher cytokine levels compared to TCL cultured in medium alone, except for IL-4 in the healthy controls (Table 2). The production of IL-4 and IL-5 after anti-CD3 stimulation was significantly higher in the AD group than in the control group ($P < 0.05$). High amounts of IFN- γ were produced by both AD patients and healthy controls, with no statistical difference between the groups ($P = 0.51$). The ratio between IL-4 and IFN- γ production of the TCL for 20 h was significantly higher in the AD group ($P < 0.05$) (Fig. 4). The IL-4/IFN- γ ratio after anti-CD3 stimulation correlated with total serum-IgE levels when both AD patients and healthy controls

Table 2. Cytokine production by PBMC and TCL

Source	Cultured with	IL-4 ^a ng/ml		IL-5 ^a ng/ml		IFN- γ ^a ng/ml	
		AD n = 11	HC n = 6	AD n = 11	HC n = 6	AD n = 11	HC n = 6
PBMC	<i>P. orbiculare</i> ^b	0.44 ^e (0.14–0.75)	0.25 (<0.02–0.75)	2.3 ^f (1.3–5.3)	0.8 (<0.06–2.7)	6.9 (0.4–78)	6.6 (1.5–32)
TCL	Anti-CD3 Ab ^c	0.52 ^{fg*} (0.17–3.7)	0.12 (0.04–0.76)	2 ^{fg**} (0.29–3.6)	1.1 ^{g*} (0.18–1.6)	32 ^{g**} (3.2–93)	41 ^{g**} (5.4–76)
TCL	Medium alone ^c	0.03 (<0.02–0.12)	<0.02 (<0.02–0.04)	0.47 (0.11–2)	0.3 (0.07–0.9)	0.85 (<0.03–32)	2.3 (0.27–22)
TCL	<i>P. orbiculare</i> ^d	0.03 (<0.02–0.74)	<0.02 (<0.02–0.05)	0.8 (0.09–1.9)	0.14 (<0.06–1.4)	2 (0.22–61)	4.6 (0.24–32)

^a Cytokines in the culture supernatants from PBMC or TCL were measured in duplicates by ELISA. The detection limit was 0.02 ng/ml for IL-4, 0.06 ng/ml for IL-5 and 0.03 ng/ml for IFN- γ .

^b The PBMC were cultured with *P. orbiculare* extract, 10 μ g/ml, for 11 days before cytokines were measured.

^c The TCL were cultured with medium alone or anti-CD3Ab for 20 h before cytokines were measured.

^d The TCL were cultured with *P. orbiculare* extract, 10 μ g/ml, and syngenic antigen-presenting cells for 20 h before cytokines were measured.

^e Values are given as medians with ranges in brackets.

^f Significantly higher than the corresponding value of the healthy controls: ^f = $P < 0.05$.

^g Significantly higher than the corresponding value of TCL cultured in medium alone: ^{g*} = $P < 0.05$, ^{g**} = $P < 0.01$.

were examined ($r_s = 0.65$, $P = 0.01$), but not with *P. orbiculare*-specific serum-IgE levels.

After restimulation with *P. orbiculare* extract for 20 h TCL, whether derived from AD patients or healthy controls, did not

induce significantly higher cytokine levels compared to TCL cultured with antigen-presenting cells in the absence of antigen (data not shown). Likewise there was no significant difference between the AD patients and the healthy controls in the cytokine production by TCL after *P. orbiculare* restimulation (Table 2).

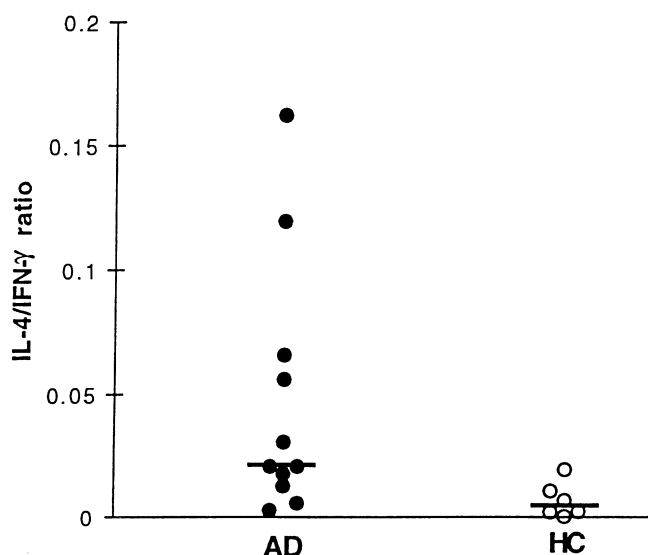


Fig. 4. Significantly higher ratio between IL-4 and IFN- γ production by *P. orbiculare*-reactive TCL in the AD patients compared to healthy controls (HC) ($P < 0.05$). Levels of IL-4 and IFN- γ were measured by ELISA technique in the supernatants of TCL (2×10^6 cells/well) cultured for 20 h in the presence of immobilized anti-CD3 Ab. The detection limit for IL-4 was 0.02 ng/ml and for IFN- γ 0.03 ng/ml. Bars indicate median values.

DISCUSSION

Both IgE-mediated [5–7] and cell-mediated [14–17] immune reactions against the yeast *P. orbiculare* have been found in patients with AD, indicating that *P. orbiculare* may be of importance for the pathogenesis of AD. In the present study, we found significantly higher SI of freshly isolated PBMC after *P. orbiculare* stimulation for 3 days in patients with AD and specific IgE Ab against *P. orbiculare* than in healthy controls. This is in accordance with a previous report by Rokugo *et al.* where AD patients showed significantly higher SI values than healthy controls after 5 days of stimulation with *P. ovale* [14]. In our study eight out of 12 AD patients showed SI values of 2.5 or more whereas only one healthy control reached an SI above 2. In two other studies it has been shown that most healthy controls respond with low SI values for *P. orbiculare* [15, 16]. In contrast higher SI values from healthy individuals after 6 days of stimulation with *P. orbiculare* extract were reported by Sohnle & Collins-Lech [17]. The higher SI values found by Sohnle & Collins-Lech might be explained by the use of an extract that was prepared in a different way than in the studies with lower SI.

The cell-mediated immune response against *P. orbiculare* seems to be separated from the IgE-mediated immediate type

reaction since no correlation was found between SI for *P. orbiculare* and serum-IgE and between SI and specific IgE for *P. orbiculare* in the AD patients. This is in accordance with the study performed by Rokugo *et al.* where no correlation was found between SI and skin prick test response at 15 min to *P. ovale* in AD patients [14]. Virtanen *et al.* found no significant correlation between IL-4-producing T cells from lesional AD skin and total serum-IgE, suggesting that infiltrating T cells of lesional skin do not play a major role in serum-IgE production [18].

In contrast to *P. orbiculare* stimulation of freshly isolated PBMC, SI for *C. albicans* did not differ significantly between AD patients and healthy controls. A decreased proliferative PBMC response to *C. albicans* in AD patients with serum-IgE Ab against *C. albicans* compared to healthy individuals, suggesting an anergic T-cell reaction in the AD patients, has previously been reported by Tanaka *et al.* [19]. The higher proliferative response to *C. albicans* compared to *P. orbiculare* in the control group could be due to a higher antigenicity of *C. albicans* compared to *P. orbiculare* [17].

Restimulation of the established TCL with *P. orbiculare* extract for 3 days induced a proliferative response of more than twice the background values in eight out of 11 AD patients and three out of five controls, but no significant difference was found between the groups (Fig. 3). Even though freshly isolated PBMC from the healthy controls were poor responders to *P. orbiculare*, the established TCL from the healthy controls showed a proliferation of 16 000 cpm or more (Fig. 3). The healthy controls thereby displayed an ability to react with T-cell proliferation against *P. orbiculare* extract *in vitro*. We have thus found that both AD patients and healthy controls are able to respond with T-cell proliferation after *P. orbiculare* stimulation *in vitro*, but the higher SI after *P. orbiculare* stimulation of PBMC in the AD group indicates that a majority of the patients had not only acquired a humoral immune response but also a cell-mediated immune response against *P. orbiculare* *in vivo*.

Significantly higher levels of IL-5 were found in the AD group when freshly isolated PBMC were stimulated with *P. orbiculare* extract for 11 days (Table 2), while no significant differences were seen for IL-4 and IFN- γ . In house dust mite-sensitized asthma patients, but not in healthy controls, increased IL-5 production from PBMC in response to allergen stimulation has been reported [20]. In a previous study *P. orbiculare*-specific T-cell clones from an AD patient produced IL-5, but hardly detectable levels of IL-4 and IFN- γ , after *P. orbiculare* stimulation for 12 days, and the IL-5 levels correlated with their SI for *P. orbiculare* [10]. In this study higher levels of IL-5 were produced by PBMC from AD patients who reacted with a high SI after *P. orbiculare* stimulation although no significant correlation was found ($P = 0.07$).

The *P. orbiculare*-stimulated TCL produced significantly higher levels of IL-4 in the AD patient group and of IL-5 and IFN- γ in both the AD group and the control group after anti-CD3 stimulation for 20 h (Table 2). In addition the IL-4 and IL-5 production was higher in the AD patients than in the controls,

while both groups produced high levels of IFN- γ . A similar cytokine response with production of high IFN- γ levels from allergen-stimulated TCL producing IL-4 and IL-5 has been found by Slunt *et al.* [21]. The IL-4/IFN- γ ratio was significantly higher in the AD group (Fig. 4), reflecting a more Th2-like cytokine profile in the AD patients. In contrast no significant increase in cytokine production was noticed in any of the groups when the TCL were restimulated with *P. orbiculare* extract for 20 h. The difference between *P. orbiculare* and anti-CD3 stimulation might be explained by the short stimulation time used, reflecting different kinetics in inducing cytokine production, as shown by McHugh *et al.* who found a slower cytokine response when PBMC were stimulated with an antigen compared to a stimulus acting directly on the T cell [22].

In conclusion we have shown that the proliferative PBMC response after *P. orbiculare* stimulation is significantly higher in AD patients with specific serum-IgE Ab against *P. orbiculare* than in non-atopic healthy controls. Furthermore, *P. orbiculare*-reactive TCL from the AD patients have a Th2-like cytokine profile indicating that *P. orbiculare* may play a role in maintaining skin inflammation in AD.

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