

Positive atopy patch test reactions to *Pityrosporum orbiculare* in atopic dermatitis patients

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Summary

Background *Pityrosporum orbiculare*, although a part of our normal cutaneous microflora, can cause skin infections and induce specific immunoglobulin (Ig) E antibodies in atopic dermatitis patients. *P. orbiculare* is therefore considered to be one of the trigger factors for atopic dermatitis.

Objective To investigate if *P. orbiculare* can induce an eczematous reaction in atopic dermatitis patients, seborrhoeic dermatitis patients and healthy controls.

Methods Fifteen atopic dermatitis patients, eight seborrhoeic dermatitis patients and eight healthy controls were patch tested with extract of *P. orbiculare* on non-lesional, tape-stripped skin of the back. NaCl was used as a negative control. The patch tests were evaluated after 24, 48 and 72 h. Skin biopsies were taken from *P. orbiculare* patch test sites at 24 h and 72 h, from NaCl patch test sites at 72 h, from non-lesional skin and, in the atopic dermatitis patients, also from lesional skin. The skin biopsies were investigated with immunohistochemical techniques. *P. orbiculare*-specific IgE in serum was analysed with RAST.

Results Specific IgE to *P. orbiculare* was found in serum from 13/15 atopic dermatitis patients and in eight of them a positive patch test reaction to *P. orbiculare* was observed, with a maximal reaction at 48 h. Significantly higher serum levels of *P. orbiculare*-specific IgE were detected in patch test-positive compared with patch test-negative atopic dermatitis patients ($P < 0.01$). The seborrhoeic dermatitis patients and healthy controls were RAST and patch test-negative for *P. orbiculare*. In the patch test-positive atopic dermatitis patients an infiltration of CD4⁺T cells and eosinophils was observed at the *P. orbiculare* patch test sites together with an upregulation of ICAM-1 and HLA-DR expression.

Conclusions *P. orbiculare* can induce an eczematous reaction in sensitized atopic dermatitis patients and may be an important trigger factor in these patients. The *P. orbiculare* patch test can be of diagnostic value in this subgroup of atopic dermatitis patients.

Keywords: eosinophils, immunoglobulin E, immunoglobulin G, lymphocyte proliferation, seborrhoeic dermatitis, skin biopsy specimen

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Introduction

Atopic dermatitis is a multifactorial, chronic inflammatory skin disease where CD4⁺T cells are considered to play an important role in its pathogenesis [1,2]. Allergen-specific T

cells from atopic dermatitis patients' skin and blood are mainly of the TH2 type and are thus of importance for immunoglobulin (Ig) E regulation [3–6]. Elevated serum IgE levels are found in 80 to 85% of the atopic dermatitis patients [1,7]. Many of the atopic dermatitis patients have specific IgE antibodies (Ab) to a variety of aeroallergens and food allergens [8]. Such allergens are considered to be trigger factors in certain atopic dermatitis patients [7]. The

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occurrence of IgE Ab to staphylococcal toxins and the yeast *Candida albicans* suggests that microbial allergens as well can be of importance in atopic dermatitis [9,10].

Positive patch test reactions to aeroallergens on non-lesional skin, atopy patch test reactions, have been observed in atopic dermatitis patients [11–14]. The presence of IgE on epidermal Langerhans' cells correlates with positive patch test reactions [15]. These reactions have immunohistochemical similarities to acute atopic dermatitis lesions [16].

Pityrosporum orbiculare (*P. orbiculare*, also denoted *P. ovale* or *Malassezia furfur*) is a lipophilic yeast that colonizes the sebum-rich head and neck skin of most healthy adults [17]. *P. orbiculare* can cause skin infections such as pityriasis versicolor and pityrosporum folliculitis [18] and is also involved in the pathogenesis of seborrhoeic dermatitis [19,20]. IgG antibodies to *P. orbiculare* have been detected in healthy individuals and patients with pityriasis versicolor and seborrhoeic dermatitis [21–23]. Serum IgE antibodies to *P. orbiculare* are often found in atopic dermatitis patients, but only occasionally in patients with atopic respiratory diseases without atopic dermatitis, and are not found in healthy controls [24–27]. *P. orbiculare* extract induces a significantly higher proliferative response of peripheral blood mononuclear cells (PBMCs) in atopic dermatitis patients than in healthy individuals [6,28,29]. In addition, ketoconazole treatment of *P. orbiculare*-sensitized atopic dermatitis patients has alleviated their eczema [30]. This suggests that *P. orbiculare* plays a role in atopic dermatitis.

The aim of the present study was to further evaluate the role of *P. orbiculare* in atopic dermatitis by investigating (1) the clinical and immunohistochemical patch test responses to extract of *P. orbiculare* in atopic dermatitis patients, patients with seborrhoeic dermatitis and healthy individuals, (2) the serum levels of *P. orbiculare*-specific IgG and IgE Ab in these subjects, and (3) the PBMC *in vitro* response to *P. orbiculare* in the atopic dermatitis and seborrhoeic dermatitis patients.

Materials and methods

Subjects

Fifteen patients with moderate to severe atopic dermatitis [31] fulfilling the criteria of Hanifin and Rajka [32] (Table 1), eight nonatopic patients with seborrhoeic dermatitis and 14 nonatopic healthy controls were included in the study (Table 2). The subjects were clinically examined by a dermatologist and asked about their medical history. All atopic dermatitis patients had a past or present history of respiratory atopy. The intensity and severity of atopic dermatitis was assessed according to the SCORAD index [33]. All atopic dermatitis patients had a head and neck

distribution of their eczema and 13 had eczema in other areas as well. Serological data for the atopic dermatitis patients are given in Table 1 and for the seborrhoeic dermatitis patients and healthy controls in Table 2.

Exclusion criteria were diseases other than the investigated, except for respiratory atopy in the atopic dermatitis patients, pregnancy or lactation. Internal corticosteroids or UV therapy were not allowed in the 2 months before the investigation. No topical corticosteroids had been used at the patch test sites the week before the investigation. The study was approved by the Ethics Committee of the Karolinska Hospital. All participating subjects gave their informed consent.

P. orbiculare extract

The extract was prepared as described by Zargari *et al.* [34]. Briefly, the *P. orbiculare* yeast cells (American Type Culture Collection; no. 42132) were harvested after 4 days' culture and freeze-dried. The yeast culture medium contained: bacteriological peptone, glucose, glycerol monostearate, agar, Tween 80, olive oil and gentamicine. Immunoblotting tests of the medium with patient sera on SDS-PAGE did not give any reactive bands. Phosphate-buffered saline (PBS) was added to the yeast before sonication. The extract was incubated overnight at 4 °C and thereafter centrifuged. The supernatant was first filtered through a 0.8- μ m and then through a 0.2- μ m cellulose acetate filter (Advantec MFS, Inc., Pleasanton, CA, USA). The protein concentration of the extract was measured by BCA Protein assay Reagent (Pierce Chemical Company, Rockford, IL, USA) according to the manufacturer's instructions.

P. orbiculare atopy patch test

All atopic dermatitis and seborrhoeic dermatitis patients and eight of the healthy controls (three women and five men) were patch tested on non-lesional skin of the back. No signs of eczema were allowed at the patch test sites. The skin was tape-stripped 15 times with TransporeTM Tape (3M, Sol-lentuna, Sweden) as previously described [14]. Water-soluble extract of *P. orbiculare* diluted in physiological NaCl 0.9% (20 μ L) to six different concentrations (5–0.16 mg/mL) was applied on paper discs in Finn chambers (8 mm; Epitest Ltd Oy, Tuusula, Finland) which were held in place with Scanpore[®] tape (Norgesplaster A/S, Oslo, Norway). NaCl 0.9% was used as a negative control. The patch tests were removed after 24 and 48 h and the skin reactions were evaluated by a dermatologist. A further evaluation of the patch test that was removed at 48 h was performed at 72 h. The test results were scored as 0 to +++, where 0 = negative reaction, + = erythema, infiltration, possibly

papules; ++ = erythema, infiltration, papules and small vesicles and +++ = erythema, infiltration, papules and large vesicles [35].

Processing and immunohistochemical staining of skin biopsy specimens

Punch biopsies (4 mm) were taken under local anaesthesia (lidocain 5 mg/mL with epinephrine 5 µg/mL, Astra, Södertälje, Sweden) from *P. orbiculare* patch test sites at 24 and 72 h, from NaCl patch test sites at 72 h, from normal skin on the left buttock, and in 12 of the atopic dermatitis patients also from lesional skin. The biopsies were immediately frozen on dry ice and thereafter kept at -80 °C. Acetone-fixed, 6 µm thick cryostat sections of the biopsy specimens were used for routine haematoxylin and eosin staining and for peroxidase-antiperoxidase (PAP) staining [36]. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ and 0.1% NaN₃ in PBS [37] before the PAP staining. The following mouse monoclonal antibodies (mAb) diluted in 4% bovine serum albumin in PBS were used for the PAP stainings: Leu-4 (specificity CD3, dilution 1/16), Leu-3a (CD4, 1/128), Leu-2a (CD8, 1/8), and L243 (HLA-DR, 1/40) from Becton Dickinson (San Jose, CA, USA); EG₂ (activated eosinophils, 1/100) from Pharmacia & Upjohn (Uppsala, Sweden); AA1 (mast cell tryptase, 1/75) from DAKO (Glostrup, Denmark); 84H10 (CD54; ICAM-1, 1/80) from Serotec (Oxford, UK) and B3102E8 (IgE, 1/500) from Southern Biotech (Birmingham, AL, USA). No staining was observed when the primary mAb was omitted.

Evaluation of the immunohistochemical stainings

At least two sections per mAb and biopsy specimen were stained and examined under coded conditions, magnification ×400. A semiquantitative scale from 0 to 3 (0 = no, 1 = small, 2 = moderate, 3 = large cell infiltrates) was used for the evaluation of dermal infiltrates in the haematoxylin and eosin stainings and for the evaluation of the T-cell infiltrates (CD3, CD4, CD8). In addition, the number of CD3⁺, CD4⁺ and CD8⁺ cells in the dermal cell infiltrates was counted and expressed as the percentage of the total number of infiltrating cells. In biopsy specimens where no infiltrates were found, scattered cells were counted. A minimum of 100 cells was counted in each section, except in a few biopsy specimens where less than 100 infiltrating cells were found. The result is given as the mean value of two sections per specimen. The CD3⁺ epidermal cells were counted in two sections per biopsy specimen and the result is given as the mean value per field of view. For the evaluation of epidermal and dermal IgE, and dermal HLA-DR and ICAM-1 expression, a semiquantitative scale from 0 to 3 (0 = none, 1 = few, 2 = moderate,

3 = many positive cells) was used. Mast cells were counted in five or more fields of view in three sections per specimen and the results are expressed as the mean value per field of view. For EG₂⁺ cells, the total number was counted in three sections of each biopsy specimen and the results are expressed as the mean value per section. From one healthy control the biopsy specimen from the *P. orbiculare* patch test at 72 h could not be evaluated for technical reasons.

PBMC proliferation assay

The PBMC proliferation assay was performed as previously described [6]. In brief, PBMCs (2 × 10⁵ cells/well) from the 15 atopic dermatitis and eight seborrhoeic dermatitis patients were cultured for 3 days with extract of *P. orbiculare* (0.1–10 µg/mL) in cell culture medium supplemented with 10% pooled, heat-inactivated human ABRh⁺ serum. Phytohaemagglutinin (PHA, 10 µg/mL, Pharmacia Biotech, Uppsala, Sweden) was added to cultures run in parallel to ascertain that the PBMCs could proliferate. During the last 18 h of culture, 1 µCi [³H]thymidine (Amersham Laboratories, Amersham, UK) was added to each well and the thymidine incorporation was determined by scintillation counting. Triplicate cultures were set up and the results expressed as stimulation index (SI). The SI for PBMC was calculated as:

$$\frac{\text{mean c.p.m. of PBMCs + extract of } P. \text{ orbiculare} (10 \mu\text{g/mL})}{\text{mean c.p.m. of PBMCs + medium alone}}$$

Statistics

The McNemar change test was used to calculate differences in cell infiltrate scores between lesional and non-lesional skin in atopic dermatitis patients. The exact χ^2 -test was applied for analysis of difference in cell infiltrate scores between lesional skin in atopic dermatitis patients and non-lesional in seborrhoeic dermatitis and healthy controls. The Wilcoxon Signed Rank test was used for analysis within groups and the Mann-Whitney *U*-test for comparisons between groups. Associations were computed using the Spearman rank-order correlation coefficient (r_s). Analysis of variance was used for comparisons between more than two groups. $P < 0.05$ was considered statistically significant.

Results

P. orbiculare atopy patch test reactions

Eight out of 15 atopic dermatitis patients were patch test-positive to *P. orbiculare* (Table 1), while all the seborrhoeic dermatitis patients and the eight tested healthy controls were negative (Table 2). The clinical appearance of the

Table 1. Characterization of the atopic dermatitis patients and results of *P. orbiculare* patch tests and PBMCs proliferation assay

No.	Sex	Age (years)	Phadiatop*	IgE (kU/L*†)	<i>P. orbiculare</i> specific IgE (kU/L*‡)	<i>P. orbiculare</i> specific IgG (mg/L*§)	<i>P. orbiculare</i> patch test score			Lymphocyte stimulation test SI#
							24 h¶	48 h¶	72 h¶	
1	M	30	+	5500	43.0	24.3	+++	+++	++	6.0
2	M	28	+	498	4.4	4.2	++	++	++	2.3
3	F	31	+	6100	17.6	8.8	+	++	+	1.2
4	F	24	+	2300	56.2	13.4	0	++	+	19.4
5	M	23	+	2700	17.2	8.6	+	+	0	2.0
6	M	18	+	5400	25.1	8.9	0	+	+	2.4
7	F	25	+	649	4.2	7.7	0	+	+	3.3
8	M	22	+	1506	14.5	16.2	0	+	0	2.8
9	M	25	+	3400	17.0	9.4	0	0	0	1.4
10	M	28	+	5700	8.3	53.5	0	0	0	1.7
11	M	24	+	1590	2.3	12.3	0	0	0	2.6
12	F	25	+	1238	1.1	11.3	0	0	0	2.0
13	M	30	+	909	1.0	11.5	0	0	0	1.5
14	M	30	+	169	<0.35	8.1	0	0	0	9.1
15	F	23	-	21	<0.35	8.9	0	0	0	2.2
Median		25		1590	8.3	9.4				2.3
(range)		(18–31)		(21–6100)	(<0.35–56.2)	(4.2–53.5)				(1.2–19.4)

*Pharmacia & Upjohn CAP System, Pharmacia AB, Diagnostics, Uppsala, Sweden. †Reference range 1.6–122 kU/L. ‡The reference range for specific IgE is ≤ 0.35 kU/L for nonatopic individuals. §No reference values stated by the company. ¶The results of the patch tests are given for the highest concentration of *P. orbiculare* extract (5 mg/mL) as 0, +, ++ or +++ (see Materials and methods). #The SI was calculated as mean c.p.m. of triplicate cultures from *P. orbiculare* stimulated PBMCs/mean c.p.m. of PBMCs in medium alone.

P. orbiculare positive patch test reactions was consistent with allergic contact dermatitis reactions. In general, the patch test reactions were most intense at 48 h (Table 1). Four of the eight patch test-positive atopic dermatitis patients were positive after 24 h; two of them showed positive patch tests to more than one concentration, while two only reacted to the highest concentration. Six of the eight patients with positive reactions at 48 h showed a dose-dependent positive response, reacting at two concentrations or more. Of the six patients with positive reactions at 72 h, three reacted at two concentrations or more. Two atopic dermatitis patients

showed an eczematous reaction spreading outside the patch test application site at 72 h. No reactions were seen at the NaCl patch test sites in any of the groups.

In one atopic dermatitis patient, the *P. orbiculare* patch test induced an intense local itch within 12 h. The remaining patch test-positive atopic dermatitis patients experienced a moderate local itch, which started 6–24 h after the patch test application.

Two of the originally recruited healthy controls were excluded from the study due to adverse reactions to the Scanpore® tape of the Finn chamber. The tape caused

Table 2. Characterization of SD patients and HC and results of *P. orbiculare* patch tests and PBMCs proliferation assay

	Sex	Age (years) median (range)	Phadiatop*	IgE (kU/L*†) median (range)	<i>P. orbiculare</i> specific IgE (kU/L*‡)	<i>P. orbiculare</i> specific IgG (mg/L*§) median (range)	<i>P. orbiculare</i> patch test score¶	SI# median (range)
SD	2F/6M	40 (28–46)	–	13 (2.3–109)	<0.35	7.0 (<2–29)	0	1.4 (0.8–3.3)
HC	8F/6M	31 (20–46)	–	13 (2–55)	<0.35	5.7 (2.1–30)	0††	ND

*†‡§¶# See Table 1. ††Eight HC (3F/5M) were patch tested. SD = seborrhoeic dermatitis, HC = healthy controls, ND = not done.

folliculitis in one of them and a contact dermatitis in the other.

Cell infiltrates induced by the *P. orbiculare* patch test

The *P. orbiculare* patch test-positive atopic dermatitis patients showed significantly increased dermal cell infiltration at the 24 h patch test compared with the NaCl patch test as judged from the haematoxylin and eosin stainings ($P < 0.05$) (Fig. 1a, c). The majority of the infiltrating cells were mononuclear lymphoid cells. The cell infiltrates mainly had a perivascular location in the upper part of dermis. In the epidermis of the patch test-positive atopic dermatitis patients, an exocytosis of mononuclear cells was the most prominent finding at the *P. orbiculare* patch test sites. The dermal cell infiltrates in lesional atopic dermatitis

skin were significantly larger than in non-lesional skin of atopic dermatitis patients, seborrhoeic dermatitis patients or healthy controls (Fig. 1d).

Immunohistochemical observations

The *P. orbiculare* patch test induced a dermal infiltration of CD3⁺ cells in the patch test-positive atopic dermatitis patients (Table 3). In these patients, a statistically significant correlation was found between the *P. orbiculare* patch test score and the size of dermal CD3⁺ cell infiltrates at 72 h (Table 4). The CD3⁺ cell infiltrates consisted mainly of CD4⁺ cells. About 10% of the CD3⁺ cells were CD8⁺, ranging from 3 to 24%. The proportion of infiltrating CD8⁺ cells was not influenced by the patch tests. A statistically significant increase in the number of epidermal CD3⁺ cells

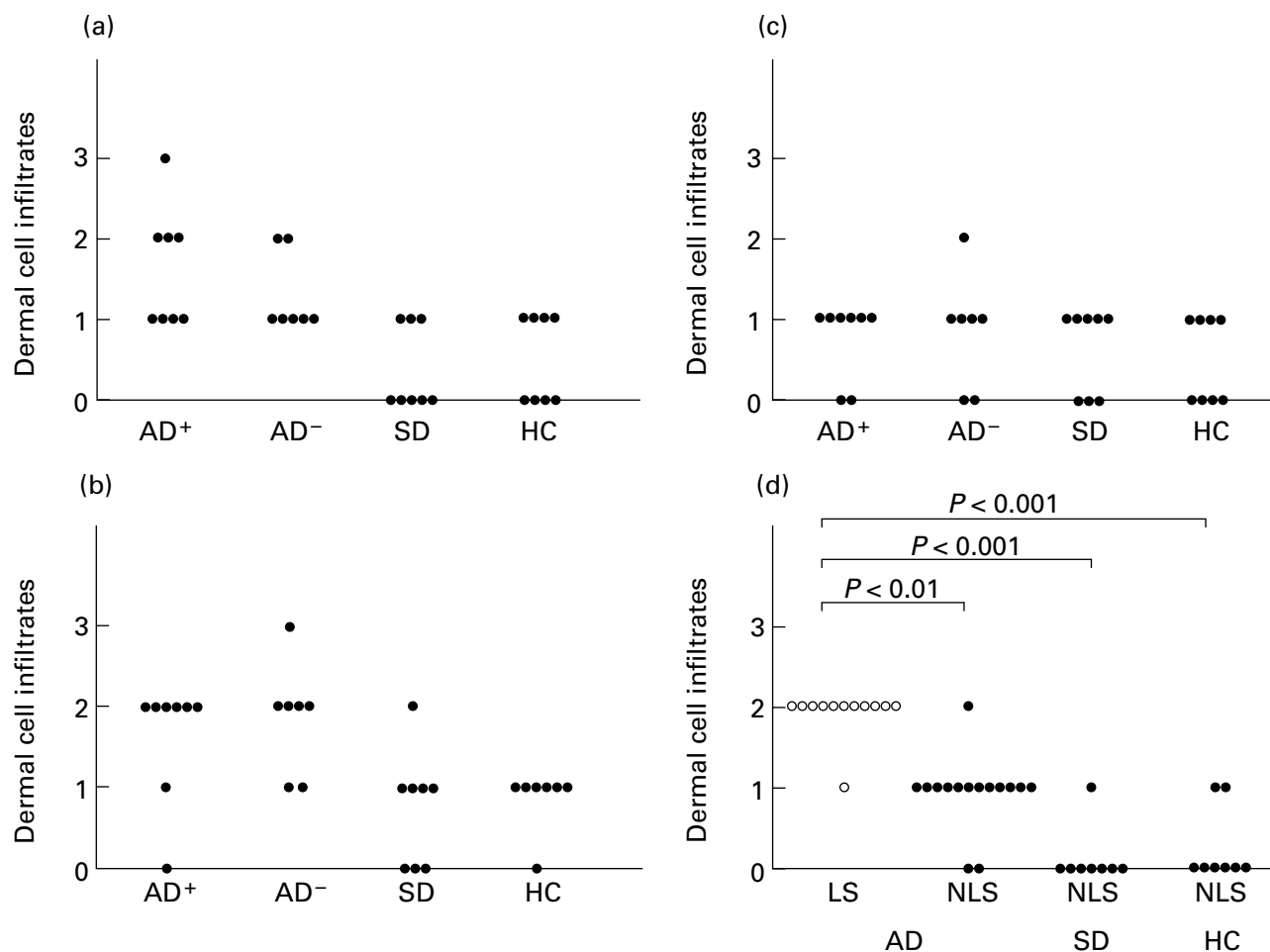


Fig. 1. The size of dermal cell infiltrates as judged from haematoxylin and eosin stainings in (a) the *P. orbiculare* patch test site at 24 h (b) at 72 h (c) the NaCl patch test site at 72 h, and (d) in lesional and non-lesional skin. A semiquantitative scale was used for the estimation: 0 = no; 1 = small; 2 = moderate; 3 = large cell infiltrates. Significant differences between groups are presented in (d). AD⁺ = *P. orbiculare* patch test-positive atopic dermatitis patients. AD⁻ = *P. orbiculare* patch test-negative atopic dermatitis patients. SD = seborrhoeic dermatitis. HC = healthy controls. LS = lesional skin. NLS = non-lesional skin

Table 3. Dermal CD3⁺ T-cell infiltrates in patch test sites, lesional skin and non-lesional skin

Biopsy site	<i>P. orbiculare</i> patch test positive AD <i>n</i> = 8	<i>P. orbiculare</i> patch test negative AD <i>n</i> = 7	SD <i>n</i> = 8	HC <i>n</i> = 8
<i>P. orbiculare</i> patch test 24 h	1.5 (1–2)*†	1 (0–2)	0 (0–1)	0.5 (0–1)
<i>P. orbiculare</i> patch test 72 h	52% (35–64%)*‡	38% (22–72%)	33% (18–56%)	33% (22–44%)
NaCl patch test 72 h	2 (0–2)	1 (1–2)	1 (0–2)	1 (0–1)§
Lesional skin	58% (34–74%)*†	52% (46–74%)*†	36% (28–64%)	48% (30–70%)
Non-lesional skin	1 (0–1)	1 (0–2)	0.5 (0–1)	0 (0–1)
	28% (22–42%)	36% (18–51%)	33% (15–47%)	33% (26–45%)
	2 (2)¶	2 (1–2)	ND	NA
	58% (44–64%)	51% (37–56%)		
	1 (0–1)	1 (0–2)	0 (0–1)	0 (0–1)
	38% (29–46%)	34% (22–48%)	28% (20–38%)	28% (20–44%)

*The T-cell infiltrates were evaluated by a semiquantitative scale: 0 = no, 1 = small, 2 = moderate, 3 = large cell infiltrates. Values are given as medians with ranges in brackets. †Significantly higher than the NaCl patch test ($P < 0.05$). ‡The number of CD3⁺ cells expressed as percent of the total number of infiltrating cells (see Materials and methods). § $n = 7$; ¶ $n = 5$. AD = atopic dermatitis. SD = seborrhoeic dermatitis, HC = healthy controls, ND = not done, NA = not applicable.

at the *P. orbiculare* patch test sites compared with the NaCl patch test sites was found in the patch test-positive atopic dermatitis patients at 24 and 72 h and in the patch test-negative atopic dermatitis patients at 24 h (Table 5). The majority of the epidermal CD3⁺ cells were CD4⁺.

Only occasional eosinophils were found in the non-lesional skin of all groups and few were found in lesional atopic dermatitis skin. In the patch test-positive atopic dermatitis patients, a large number of eosinophils were recruited into the dermis of the *P. orbiculare* patch test sites at 24 and 72 h (Table 5). A few epidermal eosinophils were found in blisters at the *P. orbiculare* patch test sites.

Table 4. Correlations between the size of dermal CD3⁺ cell infiltrates or number of eosinophils and various variables in *P. orbiculare* patch test-positive atopic dermatitis patients at the *P. orbiculare* patch test sites ($n = 8$)

	24 h	72 h
<i>Size of dermal CD3⁺ infiltrates vs</i>		
No. of eosinophils	0.87*†	0.65
Dermal ICAM-1 expression	0.92*	0.76
Patch test score	0.23	0.81*
<i>No. of eosinophils vs</i>		
Dermal ICAM-1 expression	0.90*	0.59
Patch test score	0.60	0.85*

* $P < 0.05$; †values are given as the Spearman rank-order correlation coefficient (r_s).

An increase in eosinophils was also found in the patch test-negative atopic dermatitis patients at 72 h (Table 5). A correlation was found between the number of eosinophils at 72 h and the *P. orbiculare* patch test score at 72 h in the patch test-positive atopic dermatitis patients (Table 4). A correlation was also found between the number of eosinophils and the size of the CD3⁺ cell infiltrates at 24 h in these patients (Table 4).

The dermal tryptase positive mast cells ranged from 1.3 to 14.5 cells per field of view in all the skin biopsy specimens with no significant differences between the groups. *P. orbiculare* patch tests did not induce any significant change of the mast cell number within any of the groups.

In the *P. orbiculare* patch test-positive atopic dermatitis patients, a significantly higher number of HLA-DR⁺ dermal cells were found at the 72 h patch test site than at the NaCl patch test site ($P < 0.05$). No such difference in dermal HLA-DR expression was noted in the patch test-negative atopic dermatitis patients, the seborrhoeic dermatitis patients or in the healthy controls. HLA-DR expression on the keratinocytes was not observed in any biopsy specimen.

In the *P. orbiculare* patch test-positive atopic dermatitis patients, a statistically significant increased number of ICAM-1-expressing dermal cells was induced by *P. orbiculare* at 24 and 72 h compared with the NaCl patch test ($P < 0.05$). The dermal ICAM-1 expression in the responding atopic dermatitis patients correlated with the size of the dermal CD3⁺ infiltrates at 24 h and the number of eosinophils at the 24 h *P. orbiculare* patch test site (Table 4). In the atopic dermatitis patients, keratinocytes in lesional skin and

Table 5. CD3⁺ epidermal cells, dermal eosinophils, and IgE⁺ epidermal cells in patch test sites, lesional skin and non-lesional skin

Biopsy site	<i>P. orbiculare</i> patch test positive AD <i>n</i> = 8	<i>P. orbiculare</i> patch test negative AD <i>n</i> = 7	SD <i>n</i> = 8	HC <i>n</i> = 8
<i>CD3⁺ epidermal cells*</i>				
<i>P. orbiculare</i> patch test 24 h	2.8 (0–8.7)†‡	4.7 (0–30)‡	2.1 (0–19)	0.6 (0–4.4)
<i>P. orbiculare</i> patch test 72 h	1.6 (0.1–21.2)‡	3.3 (0.4–5.8)	0.6 (0–3.6)	0.8 (0.4–2.5)§
NaCl patch test	0.4 (0–2.5)	0.9 (0–11)	0.9 (0–3.4)	0.6 (0–2.7)
Non-lesional skin	0.3 (0–4.9)	0.4 (0–2.1)	0 (0–0.1)	0.05 (0–0.4)
Lesional skin	5.5 (0.7–10)¶	1 (0.4–4.1)	ND	NA
<i>Eosinophils#</i>				
<i>P. orbiculare</i> patch test 24 h	66 (0–230)‡	1.7 (0–166)	0 (0–2.5)	0.5 (0–42)
<i>P. orbiculare</i> patch test 72 h	99 (0–210)‡	15 (0–112)‡	0.5 (0–8.3)	0.7 (0–4.7)§
NaCl patch test	4.5 (0–8.3)	3.3 (0–23)	0.7 (0–23)	0.7 (0–8.3)
Non-lesional skin	0.3 (0–1)	0.3 (0–1.7)	0 (0–0.3)	0 (0–2.3)
Lesional skin	4 (0–27)¶	2.3 (0.7–22)	ND	NA
<i>IgE⁺ epidermal cells††</i>				
<i>P. orbiculare</i> patch test 24 h	1.5 (1–3)	2 (0–3)	0	0
<i>P. orbiculare</i> patch test 72 h	1 (1–2)	1 (0–3)	0	0§
Non-lesional skin	2 (1–3)	1 (0–2)	0	0
Lesional skin	2 (2–3)¶	1 (0–2)	ND	NA

*The number of epidermal CD3⁺ cells given as the mean value per field of view (×400) (see Materials and methods). †Values are given as medians with ranges in brackets. ‡Significantly higher than the NaCl patch test ($P < 0.05$). § $n = 7$; ¶ $n = 5$. #The number of EG₂+ eosinophils given as the mean value per section (see Materials and methods). ††Semiquantitative scoring of epidermal IgE expression on a scale from 0 to 3 cells (see Materials and methods). AD = atopic dermatitis. SD = seborrhoeic dermatitis, HC = healthy controls, ND = not done, NA = not applicable.

skin patch tested with *P. orbiculare* and NaCl showed a patchy ICAM-1 expression in the basal layers, in some instances close to dermal mononuclear cell infiltrates. *P. orbiculare* patch tests did not induce any significant up-regulation of ICAM-1 on the keratinocytes.

In atopic dermatitis patients with strong patch test reactions (+++ or ++, Table 1) to *P. orbiculare*, larger T-cell infiltrates at the 72 h *P. orbiculare* patch test site and higher numbers of eosinophils and cells expressing ICAM-1 at the 24 and 72 h *P. orbiculare* patch test sites (data not shown) were found compared with atopic dermatitis patients with weak reactions (+, Table 1).

In non-lesional skin, all eight patch test-positive and 4/7 patch test-negative atopic dermatitis patients expressed IgE on epidermal dendritic cells. No epidermal IgE expression was found in the seborrhoeic dermatitis patients or healthy controls. *P. orbiculare* patch testing did not induce any significant increase or decrease in the epidermal IgE expression (Table 5). All atopic dermatitis patients showed a 2+ dermal IgE expression in non-lesional skin, except for patient numbers 14 and 15 who showed a 1+ expression. No increase or decrease in dermal IgE expression was found at the *P. orbiculare* patch test sites compared with

non-lesional skin in the atopic dermatitis patients. In four of the seborrhoeic dermatitis patients and four of the healthy controls a few IgE⁺ dermal cells were found. These cells had the morphology and distribution of mast cells and most of them showed a weak IgE-expression.

P. orbiculare-specific IgE and IgG

The atopic dermatitis patients with positive patch test reactions had significantly higher serum IgE levels to *P. orbiculare* than those with negative reactions ($P < 0.01$), while no significant difference in total serum IgE levels was seen (Table 1). The *P. orbiculare*-specific IgE but not the total IgE in serum correlated with the degree of patch test response at 48 h in the whole group of atopic dermatitis patients ($r_s = 0.74$, $P < 0.01$).

P. orbiculare-specific IgG levels in serum did not differ significantly between the groups (Tables 1 and 2). No correlation was found between *P. orbiculare*-specific serum IgG and patch test reactions. For the atopic dermatitis patients, the SCORAD results did not correlate with the patch test results, total serum IgE, IgE expression in the skin, *P. orbiculare*-specific IgE or IgG levels in serum.

Proliferative response in PBMCs

The atopic dermatitis patients showed a higher PBMC response (SI median value 2.3, Table 1) to *P. orbiculare* than the seborrhoeic dermatitis patients (SI median value 1.4, Table 2), although the differences were not statistically significant ($P = 0.09$). The median SI value in the patch test-positive atopic dermatitis patients was not significantly different from that in the patch test-negative atopic dermatitis patients (2.6 vs 2.0, respectively). The SI values from the atopic dermatitis patients did not correlate with total serum IgE, *P. orbiculare*-specific IgE, *P. orbiculare*-specific IgG or patch test results.

Discussion

In this study, we have shown that *P. orbiculare* extract can induce an eczematous reaction in atopic dermatitis patients with specific serum IgE to *P. orbiculare*, and that the reaction is related to the levels of *P. orbiculare*-specific IgE.

P. orbiculare-specific IgE is almost exclusively found in atopic dermatitis patients [24–27] and in our study we detected *P. orbiculare*-specific IgE in all atopic dermatitis patients except for two patients showing low total serum IgE levels (Table 1). We also found a correlation between the levels of *P. orbiculare*-specific serum IgE and the patch test scores at 48 h. Such an association has previously been demonstrated for house dust mite and pollen in atopic dermatitis [12–14], but not for *P. orbiculare* [28]. We found IgE⁺ dendritic epidermal cells, probably Langerhans' cells, in all eight patch test-positive atopic dermatitis patients and in 4/7 negative atopic dermatitis patients. Mudde *et al.* have demonstrated that binding of IgE to Langerhans' cells is required for presentation of house dust mite to allergen-specific T cells *in vitro* and that this finding correlates with the presence of a positive patch test reaction *in vivo* [15]. Furthermore, the presence of IgE on dendritic cells results in a 100- to 1000-fold increase in their capacity to present the antigen to antigen-specific T cells [38]. Even though we could show that IgE was present on epidermal dendritic cells in non-lesional skin in the patch test-positive patients, we could not demonstrate any increase or decrease in the epidermal or dermal IgE expression at the patch test sites. This can probably be explained by the fact that most of the atopic dermatitis patients in the study had high total serum IgE levels, and were most likely expressing specific IgE to several allergens in their skin. A possible change in the dermal or epidermal *P. orbiculare*-specific IgE expression could thereby be concealed by high levels of IgE with other specificities.

Positive *P. orbiculare* atopy patch test reactions were found in 53% of the atopic dermatitis patients and in 62% of the *P. orbiculare*-sensitized atopic dermatitis patients in

our study. Studies of patch test reactivity to *P. orbiculare* have previously been done by Kieffer *et al.* who found only 13% reactive atopic dermatitis patients [25] and Rokugo *et al.* who found a reaction in 64% of the atopic dermatitis patients [28]. The variation in the percentage of positive atopy patch test, also found in studies with other allergens [11–14] probably reflects the selection of patients and the choice of extracts and methods. Since tape stripping has been shown to increase the number of positive atopy patch test reactions by a slight reduction of the corneal layer [14] we decided to tape strip the skin 15 times with medical tape. In our study the patch test reaction kinetics differed between the patients. Those with the strongest reactions responded at 24, 48 and 72 h. All the remaining patients had a response at 48 h (Table 1). As in other studies where aeroallergen or mites have been studied [13,14] the maximal atopy patch test reaction was found at 48 h. The clinical morphology of a patch test reaction can be a poor guide to whether the response is allergic or not, especially when the responses are weak. However, in our present study the clinical appearance was consistent with an allergic contact dermatitis reaction.

In the *P. orbiculare* patch test-positive atopic dermatitis patients, the *P. orbiculare* extract induced an infiltration of CD4⁺ T cells and eosinophils that was already detectable at 24 h but more pronounced at 72 h. A dermal cell infiltration was found at 72 h in both patch test-positive and -negative atopic dermatitis patients, suggesting an immunological response to *P. orbiculare* also in the patch test-negative atopic dermatitis patients. These patients may lack factors of importance for a positive patch test reaction, such as sufficient amounts of specific IgE and/or eosinophils in the patch tested skin area. Eosinophils are known to take an active part in the atopy patch test reaction [39]. Few eosinophils are present in chronic atopic dermatitis lesions but dermal deposits of eosinophilic cationic protein and major basic protein in lesional atopic dermatitis skin also suggest a role for eosinophils in this disease [40]. Eosinophils are recruited already 2–6 h after application of an atopy patch test [39]. We found a higher number of infiltrating eosinophils at 72 h than at 24 h, suggesting a prolonged cell recruitment. The number of mast cells does not seem to be influenced by patch tests with *P. orbiculare* or other allergens [16].

The role of the IgG response to *P. orbiculare* in the pathogenesis of *P. orbiculare*-associated diseases is unclear. Silva *et al.* have found elevated levels of *P. orbiculare*-specific IgG in patients with pityriasis versicolor and seborrhoeic dermatitis compared with healthy controls [23], whereas Bergbrant and Faergemann [21], and Ashbee *et al.* [22] did not. We found *P. orbiculare*-specific IgG in all participating individuals, with no significant difference between the groups. *P. orbiculare*-specific serum IgG-levels in the atopic dermatitis patients did not correlate with the

patch test responses in the atopic dermatitis patients. The determination of *P. orbiculare*-specific IgG seems to have no value in the diagnosis of sensitization to *P. orbiculare* in atopic dermatitis patients.

In this study we found a higher but not significantly different proliferative PBMC response (SI) to *P. orbiculare* in the sensitized atopic dermatitis patients than in the seborrhoeic dermatitis patients. We have previously demonstrated significantly higher SI values in atopic dermatitis patients than in healthy controls indicating an *in vivo* immunization in the atopic dermatitis patients [6,29]. The blood lymphocyte response in the atopic dermatitis patients does not appear to reflect the skin responsiveness to *P. orbiculare* since no correlation between the proliferative lymphocyte response to *P. orbiculare* and the *P. orbiculare*-specific serum IgE [6,29] or patch test response to *P. orbiculare* was found.

In conclusion, we have found that *P. orbiculare* can induce an eczematous reaction *in vivo* in already sensitized atopic dermatitis patients and that the level of specific serum IgE is of importance. Thus, *P. orbiculare* could be an important trigger factor in a group of atopic dermatitis patients. The *P. orbiculare* patch test can serve as a diagnostic tool in these patients.

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