

Elevated Peripheral Allergen-Specific T Cell Response Is Crucial for a Positive Atopy Patch Test Reaction

Catharina Johansson^a Niklas Ahlborg^{c, d} Anna Andersson^a Lena Lundeberg^b
Maria A. Karlsson^b Annika Scheynius^a Maria Tengvall Linder^a

^aClinical Allergy Research Unit and ^bDermatology and Venereology Unit, Department of Medicine Solna, Karolinska Institutet and University Hospital, ^cMabtech AB, Nacka Strand, and ^dDepartment of Immunology, Stockholm University, Stockholm, Sweden

Key Words

Antigen-specific IgE • Atopic eczema • Atopic dermatitis • Atopy patch test • ELISpot • Interleukin-4 • Interleukin-5 • *Malassezia*

Abstract

Background: Atopic eczema is a chronic inflammatory skin disease in which several subgroups of cases can be identified. Atopy patch testing (APT) reveals allergen sensitization also in atopic eczema patients devoid of detectable allergen-specific IgE, suggesting the importance of factors other than IgE in the reaction. Here we investigate the relationship between APT reactions and allergen-specific peripheral IgE and T cell reactivity in atopic eczema patients. **Methods:** Adult patients with atopic eczema (n = 64) and healthy controls (n = 24) were analyzed for reactivity to *Malassezia sympodialis* extract by APT, measurement of specific plasma IgE and in vitro determination of the frequency of allergen-reactive peripheral blood mononuclear cells producing interleukin-4 and interleukin-5 using the ELISpot method. **Results:** When combining the results of the APT, IgE measurements and the ELISpot analyses, reactivity to *M. sympodialis* was found in a majority of the atopic eczema patients (69%), whereas the healthy controls were negative throughout. T cell reactivity to *M. sympodialis*, manifested by production of

both interleukins 4 and 5, was highly predictive for a positive APT reaction and displayed a strongly positive correlation with the APT score. In contrast, the allergen-specific IgE levels did not predict the APT outcome, and no correlation could be found between the IgE levels and the APT score. **Conclusion:** Peripheral allergen-specific T helper 2 cell-mediated reactivity appears to be required for a positive APT reaction to *M. sympodialis*. The diagnostic potential of measuring peripheral allergen-specific T cell responses should be considered in atopic eczema.

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Introduction

Atopic eczema (AE) is a highly pruritic chronic inflammatory skin disorder that affects 15–30% of children and 2–10% of adults [1]. The pathogenesis is not fully understood, but factors contributing to the symptoms include a genetic predisposition and environmental factors such as diet and presence of microorganisms and allergens [1, 2]. Genetic association has identified filaggrin to be a susceptibility gene for AE, supporting the importance of the epidermal barrier in AE [3]. Whereas the clinical picture of adult AE is rather similar, it is becoming more and more evident that several subgroups exist

Table 1. Characterization of the atopic eczema patients and healthy controls

	n	Gender (F/M)	Age, years	SCORAD [18]	Distribution to head and neck	Rhino-conjunctivitis and/or asthma	Total plasma IgE >122 kU/l ^a	Total plasma IgE level, kU/l ^a	Phadiatop [®] positive ^b
AE patients	64	31/33	30 (18–65)	46 (16–81)	46 (72)	48 (75)	40 (62)	340 (4.5–15,100)	54 (84)
Healthy controls	24	15/9	38 (21–64)	n.a.	n.a.	0 (0)	2 (8)	26 (3.2–140)	2 (8)

Data are numbers with percentages in parentheses or medians with ranges in parentheses. F = Female; M = male, n.a. = not appropriate.

^a Measured by ImmunoCap, reference range 1.6–122 kU/l.

^b Phadiatop[®] (Phadia AB), serum IgE to any of 11 common aeroallergens.

in terms of immunological reactivity and pathogenic mechanisms [1, 2]. Up to 80% of AE cases are assumed to be associated with atopy [2]. A distinction between an extrinsic (allergic) and an intrinsic (nonallergic) etiology has been proposed [4, 5]. Intrinsic AE has been suggested as a disease entity with a unique immunological pathogenesis [6–8], but it has also been claimed that patients suffering from intrinsic AE rather represent the ‘milder edge’ of an overlapping spectrum of clinical phenotypes in AE [9].

The use of the atopy patch test (APT), an epicutaneous patch test utilizing protein allergens known to evoke an IgE response, has been proposed for evaluating the relevance of sensitization to such allergens in AE [10–12]. The presence of IgE+ Langerhans cells in the skin is claimed to be essential for a positive APT [13]. Interestingly, APT reveals allergen sensitization also in AE patients devoid of detectable allergen-specific IgE, while often more than 50% of AE patients with allergen-specific IgE remain APT negative [11, 12, 14]. This implicates the involvement of other factors complementary to, or interchangeable with, IgE in the APT reaction. Characteristics for the acute AE lesion, as well as for the APT reaction, are an influx of eosinophils and T helper 2 lymphocytes (Th2 cells) [2, 15, 16]. Little attention has been paid to the peripheral blood levels of allergen-specific T cells in relation to the APT reaction.

An environmental trigger factor in AE is the yeast *Malassezia*, a member of the normal human skin flora. Approximately 50% of adult AE patients display IgE and/or APT reactivity to *Malassezia* [17]. One of the most frequently isolated species isolated from both AE patients and healthy controls is *Malassezia sympodialis* [18], which was used here as a model environmental allergen to investigate the relationship between peripheral IgE, T cell reactivity and APT reactions. Our results suggest a strong relationship between the peripheral allergen-specific T cell response and the reactivity manifested by a positive

APT reaction. In contrast, no evidence for a similar relationship between the levels of allergen-specific IgE and APT was found. Considering the relevance of allergen-specific T cell levels in AE may contribute to an increased knowledge of the pathogenesis of AE and facilitate a diagnostic in vitro alternative, or complement, to APT.

Materials and Methods

Subjects and Study Design

Sixty-four adult AE clinical out-patients, and 24 healthy controls (HC) were recruited in Stockholm, Sweden (table 1). Inclusion criteria for the AE patients were diagnosis, set by a dermatologist, according to the UK working party criteria [19]. The eczema was to be moderate-to-severe and not only restricted to the hands. Exclusion criteria were skin diseases other than AE, autoimmune diseases, immune deficiencies, malignant diseases, pregnancy or lactation, immunosuppressive treatment and an age below 18 or above 65 years. The HC were to have no clinical symptoms or history of allergy or skin diseases. Use of systemic glucocorticoids or systemic antifungal treatment was not allowed for 2 months before the investigation. UV therapy or topical corticosteroids were not allowed on the test sites for 1 week before the study. At the first visit the subjects were interviewed about their medical history, the severity of the eczema was assessed using SCORAD [20], a blood sample was drawn for plasma IgE and ELISpot analysis and the APT was thereafter applied. All participants gave their informed consent. The study was approved by the regional ethics committee.

M. sympodialis Extract and Specific IgE Analysis

Extract from *M. sympodialis* American Type Culture Collection strain number 42132 [21] was used to measure specific plasma IgE to *M. sympodialis* with ImmunoCAP[™] product code m70 (Phadia AB, Uppsala, Sweden), and used for the APT and ELISpot analysis. The endotoxin content was less than 0.2 ng/mg protein (Limulus Amebocyte Lysate test; Charles River Laboratories Inc., Charleston, S.C., USA).

Atopy Patch Test

APT was performed on nonlesional skin of the back. 30 µl of the extract or the vehicle phosphate buffered saline (PBS) was applied on paper discs in Finn chambers (12 mm; Epitest Ltd. Oy,

Tuusula, Finland) under coded conditions (individually randomized for each subject). The *M. sympodialis* extract was tested at 1 and 5 mg/ml. The patch tests were removed after 48 h and the skin reaction was evaluated by a physician, still under coded conditions. The test results were scored from 0 to 5+, where 0 = negative reaction, 1+ = erythema and infiltration, 2+ = erythema and up to 3 papules, 3+ = erythema and from 4 to many papules, 4+ = erythema and many papules or spreading papules, 5+ = erythema and vesicles [22].

ELISpot

Blood samples were obtained by venepuncture and collected in sterile heparinized plastic vials (Venosafe®; Terumo Europe N.V., Leuven, Belgium) and the peripheral blood mononuclear cells (PBMC) were separated by gradient centrifugation over Ficoll-Paque™ Plus (GE Healthcare Biosciences AB, Uppsala, Sweden). The PBMC were washed and resuspended in culture medium [RPMI-1640 supplemented with 10% heat-inactivated bovine growth serum (BGS), L-glutamine 2 mM, penicillin 100 IU/ml, streptomycin 100 µg/ml (HyClone, Logan, Utah, USA), gentamicin 25 µg/ml (Gibco BRL, Life Technologies Ltd., Paisley, UK) and 2-mercaptoethanol 50 µM (Merck, Darmstadt, Germany)]. The cell culture medium was supplemented with BGS and not with pooled human AB+ or autologous sera since human sera may contain heterophilic antibodies that can crosslink the capture and detection antibodies used in the assay and cause nonspecific staining of the ELISpot plate membranes, similar to the well established interference observed in ELISA [23].

ELISpot was performed using polyvinyl-difluoride plates pre-coated with capture monoclonal antibodies (mAb; Mabtech, Nacka Strand, Sweden). The mAb used were IL4-I (IL-4) and TRFK5 (IL-5). PBMC suspensions of 3×10^5 cells in 200 µl culture medium, stimulated with 10 µg/ml *M. sympodialis* extract, were added per well. For all individuals, stimulation with anti-CD3 mAb (CD3-2, 0.1 µg/ml; Mabtech) was used as a positive control. Cell-free culture medium was included in every plate as a control for artifacts. The cells were incubated at 37 °C and 6% CO₂ in humidified air for 40 h. After the incubation, the produced cytokine was visualized with biotinylated detection mAb (1 µg/ml in PBS/0.5% BGS); IL4-II (IL-4) and 5A10 (IL-5; Mabtech), followed by streptavidin-alkaline phosphatase conjugate diluted 1/1,000 (Mabtech) and finally developed with bromo-chloro-indolyl phosphate/nitro blue tetrazolium-plus substrate (Mabtech). The spots were counted using the AID ELISpot reader and software (Autoimmun Diagnostika GmbH, Strassberg, Germany).

The IL-4 and IL-5 values are presented as mean of triplicates with the background subtracted. The background, defined as spontaneous IL-4 production from PBMC cultured in medium alone, was below 6 IL-4+ cells/ 3×10^5 PBMC in all healthy controls and in 62 of the 64 AE patients. The 2 AE patients with a higher spontaneous IL-4 production had 12 and 19 spontaneous IL-4+ cells/ 3×10^5 PBMC and responded to *M. sympodialis* extract with 77 and 138 IL-4+ cells/ 3×10^5 PBMC, respectively. The spontaneous IL-5 production, assessed in the initial 25 AE patients and 14 healthy controls, never exceeded 4 IL-5+ cells/ 3×10^5 PBMC.

Statistical Methods

Correlations were calculated using Spearman rank correlation analysis. $p < 0.05$ was considered as statistically significant.

Results

Specific Plasma IgE and APT Reactions to M. sympodialis Extract

Thirty-four AE patients (53%) had *M. sympodialis* specific plasma IgE (m70+) and 19 patients (30%) reacted with a positive APT reaction to *M. sympodialis* extract (APT+). Fourteen of the patients (22%) were both m70+ and APT+ (fig. 1a). Five m70– patients were APT+ to *M. sympodialis* extract, 2 with a strong reaction (4+ to 5 mg/ml and 2+ to 1 mg/ml) and 3 with a weak reaction (2+ reaction to 5 mg/ml and negative to 1 mg/ml). Twenty-five of the 45 APT– AE patients were m70–. All healthy controls were negative in both tests (fig. 1a).

In vitro IL-4 and IL-5 Producing PBMC in Response to M. sympodialis Extract

The number of IL-4-producing cells in response to *M. Sympodialis* extract, as analyzed by the ELISpot method, ranged from 0 to 560 IL-4+ cells/ 3×10^5 PBMC in the AE patients and from 0 to 3 IL-4+ cells/ 3×10^5 PBMC in the HC (fig. 1b). Thirty-seven (58%) of the 64 AE patients responded with more than 3 IL-4+ cells/ 3×10^5 PBMC (3 IL-4+ cells/ 3×10^5 PBMC = $3 \times$ the mean response of the HC). 82% (28/34) of the m70+ AE patients responded with more than 3 IL-4-producing cells. Interestingly, more than 40 IL-4+ cells/ 3×10^5 PBMC was highly predictive for a positive APT reaction (fig. 1b).

In addition, IL-5-producing PBMC were analyzed in the first 25 AE patients and 14 HC investigated. There was a good correlation between IL-4+ and IL-5+ cells in response to *M. sympodialis* extract within the AE patients ($n = 25$, $r_s = 0.8$, $p < 0.0001$). Ten of the 12 APT+ AE patients, but none of the APT– patients, responded with more than 8 IL-5+ cells/ 3×10^5 PBMC (fig. 1c). All but 1 HC responded with 1 or no IL-5+ cells (fig. 1c). While already a moderate increase in the number of IL-4-producing cells was indicative of m70+, this was not the case for IL-5. Out of the 5 m70+/APT– patients, only 1 responded with more than 2 IL-5-producing cells/ 3×10^5 PBMC. The group of 6 individuals responding with more than 2 but less than 8 IL-5+ cells was heterogeneous and included, beside the already mentioned m70+/APT– patient, also 1 HC, 1 m70+/APT+ and 3 m70–/APT– patients.

Concordance between APT Reaction, Plasma IgE and PBMC Cytokine Production

In response to *M. sympodialis* extract, we found a remarkably good concordance between a high number of cytokine-producing cells in PBMC and the ability to re-

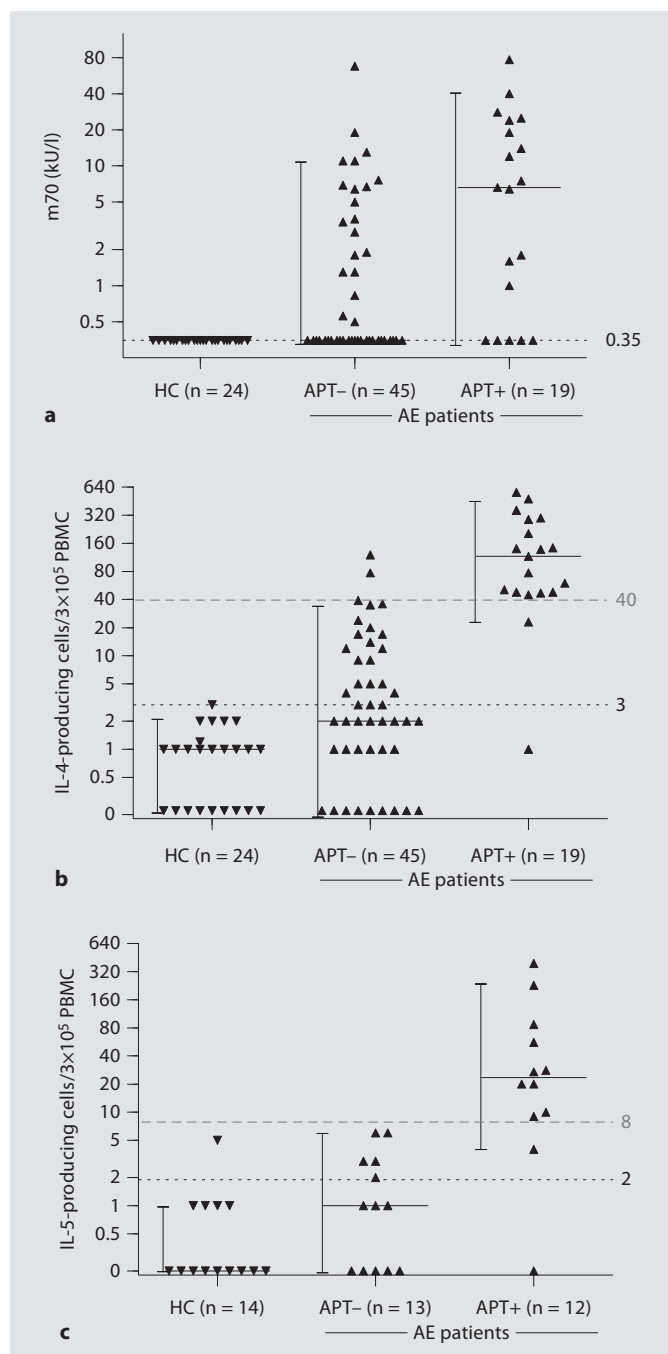


Fig. 1. Reaction to *M. sympodialis* extract in AE patients and healthy controls. **a** Specific plasma IgE levels (m70), reference range <0.35 kU/l. The black dotted line indicates the reference range. Number of IL-4 (**b**) and IL-5+ (**c**) cells/ 3×10^5 PBMC presented for the healthy controls (HC) and the AE patients with negative or positive APT reaction to *M. sympodialis*. The horizontal bars indicate medians and the vertical bars indicate 10th–90th percentiles. The black dotted lines (**b**, **c**) indicate $3 \times$ mean of the healthy controls. The gray dashed lines indicate the optimal cutoff level to distinguish between APT– and APT+ AE patients. A log₂ scale is used on the y-axes to visualize both low and high values.

spond in the APT (94 and 92% for IL-4 and IL-5, respectively; fig. 2a and b). In contrast, specific IgE to *M. sympodialis* (m70, cutoff level 0.35 kU/l) was in agreement with the APT reaction in only 61% of the patients (fig. 2c).

Specific IgE to *M. sympodialis* (m70, cutoff level 0.35 kU/l) showed a 77% concordance with the IL-4 response when using the cutoff 3 IL-4+ cells/ 3×10^5 PBMC (data not shown). 18% (6/34) of the m70+ AE patients were found among the AE patients with less than 3 IL-4-producing cells. As indicated above, the presence of IL-5-producing cells seems to be a poor indicator of allergen-specific IgE. The concordance between m70 (cutoff 0.35 kU/l) and IL-5 (cutoff 2 IL-5+ cells/ 3×10^5 PBMC) was only 56% (data not shown). 50% concordance is equal to no predictive value, while less than 50% concordance would indicate an inverse relationship.

Correlation between APT Reaction, Plasma IgE and PBMC Cytokine Production

To further explore the relationship between humoral and cellular sensitization with the APT reaction to *M. sympodialis* extract, we compared the reactions within the 44 AE patients (69% of all AE patients) who were APT+ and/or m70+ and/or responded with >3 IL-4+ cells/ 3×10^5 PBMC. Strong positive correlations were found between the APT score and the number of IL-4+ cells or IL-5+ cells (fig. 3a, b). In contrast, no correlation was found between the APT score and m70 levels (fig. 3c). The m70 levels showed a weak correlation with the number of IL-4+ cells (fig. 3d), but not with the number of IL-5+ cells ($r_s = 0.3$, $p = 0.245$, $n = 21$; data not shown).

Discussion

When combining APT and in vitro peripheral blood analysis on 64 AE patients, we found that a majority of the AE patients reacted to the investigated allergen *M. sympodialis* in accordance with previous reports [17, 24]. The number of peripheral T cells responding to *M. sympodialis* by IL-4 production was correlated with the APT score and was, if above a certain level, predictive for a positive APT reaction. In contrast, the levels of *M. sympodialis*-specific IgE antibodies did not correlate with the APT score. These findings implicate a T cell mediated reactivity, rather than allergen-specific IgE, as the critical parameter for a positive APT reaction.

A positive APT reaction is characterized by infiltrating lymphocytes, resembling the early stages of AE lesions [2, 16]. Wistokat-Wülfing et al. [25] in 1999 had al-

		APT		
		+	-	
ELISpot (IL-4 >40)	+	17 (27%)	2 (3%)	
	-	2 (3%)	43 (67%)	
		94% concordance (+/+ or -/-)		a

		APT		
		+	-	
ELISpot (IL-5 >8)	+	10 (40%)	2 (8%)	
	-	0 (0%)	13 (52%)	
		92% concordance (+/+ or -/-)		b

		APT		
		+	-	
IgE (m70 >0.35)	+	14 (22%)	20 (31%)	
	-	5 (8%)	25 (39%)	
		61% concordance (+/+ or -/-)		c

Fig. 2. Concordance between APT reaction, PBMC cytokine production and specific plasma IgE. **a, b** The concordance between APT reaction and in vitro PBMC cytokine production in IL-4 (**a**) with a cutoff level of 40 IL-4+ cells/ 3×10^5 PBMC, and IL-5 (**b**) with a cutoff level of 8 IL-5+ cells/ 3×10^5 PBMC. **c** The concor-

dance between specific plasma IgE levels (m70, reference range <0.35 kU/l used as cutoff level) and the APT reaction. All tests are measured in response to *M. sympodialis* extract and the concordance calculated within all tested AE patients (n = 64 for APT, IL-4 and m70, n = 25 for IL-5).

ready shown a significantly higher allergen-induced proliferation of PBMC from AE patients with positive APT reactions to cat, grass pollen and house dust mite allergens. However, in that study the possible association with allergen-specific IgE was not considered. Later we showed a significantly higher *M. sympodialis*-specific proliferation and a Th2-like cytokine response in PBMC from APT-positive versus APT-negative patients (with both patient groups having elevated levels of allergen-specific IgE) [26]. Moreover, in a study to explore the relevance of an in vivo topical challenge method for house dust mite hypersensitivity, Shah et al. [27] found that the same 30% of adult IgE+ AE patients that revealed eczema upon in vivo challenge also responded with significantly higher PBMC proliferation upon in vitro allergen challenge. Altogether these findings support the hypothesis that a high allergen-specific cellular response, in peripheral blood, is a critical feature for a positive APT reaction.

However, several lines of evidence suggest an impact of IgE in the APT reaction. In the skin of AE patients, in particular in skin lesions, the high-affinity IgE receptor (FcεRI) on epidermal Langerhans cells is up-regulated in comparison to the skin of healthy donors, where it is absent or expressed at very low levels [1, 13, 16]. Due to an enhanced allergen uptake and presentation as a result of the crosslinking of IgE on FcεRI on Langerhans cells, a cascade of immunological events is initiated that eventually may be manifested as a skin reaction [1, 2, 28]. But in contrast to this proposed mechanism, the relationship between AE patients' allergen-specific plasma-IgE levels and their skin reactivity is not clear. Positive APT reactions are found both in patients with intrinsic AE [14, 29]

and in extrinsic AE patients lacking detectable IgE against the allergen in question [11, 14, 16, 24, 29]. This could possibly be explained by the need for a minimal amount of circulating allergen-specific IgE, and/or local IgE production. However, the fact that often less than 50% of the AE patients with detectable plasma IgE for a specific allergen respond with a positive APT [14, 24] argues against that. Instead, it suggests that non-IgE mediated mechanisms may also be critical for a skin reaction, in line with the lack of correlation between the IgE levels and APT score observed herein. Our data indicate allergen-specific T cells as the critical factor for a positive APT reaction. The importance of IgE on Langerhans cells, as shown in earlier studies [6, 13, 16], may be to act as an amplifier of the reaction. The different roles of allergen-specific T cells and IgE in the APT reaction may offer an explanation for the higher predictive value of allergen-specific T cells for a positive APT reaction.

In the present study our earlier observation of a strong relationship between a Th2-like in vitro PBMC response and a positive APT reaction to *M. sympodialis* [26] is confirmed in a second and larger cohort of AE patients. Furthermore, we show that in vitro analysis of 1 single cytokine may be as specific as the more complicated APT. It was possible to set a detection limit with 94% concordance between the number of IL-4 spots and the APT reaction. Still, it might be that IL-4 is not the main player in the APT reaction, but merely serves as an indicator of other important factors. Such a candidate might be IL-5, a potent stimulator of eosinophils [2] frequently found in early APT lesions [15, 16]. In the first 25 patients, we investigated the IL-5 response to *M. sympodialis* and found

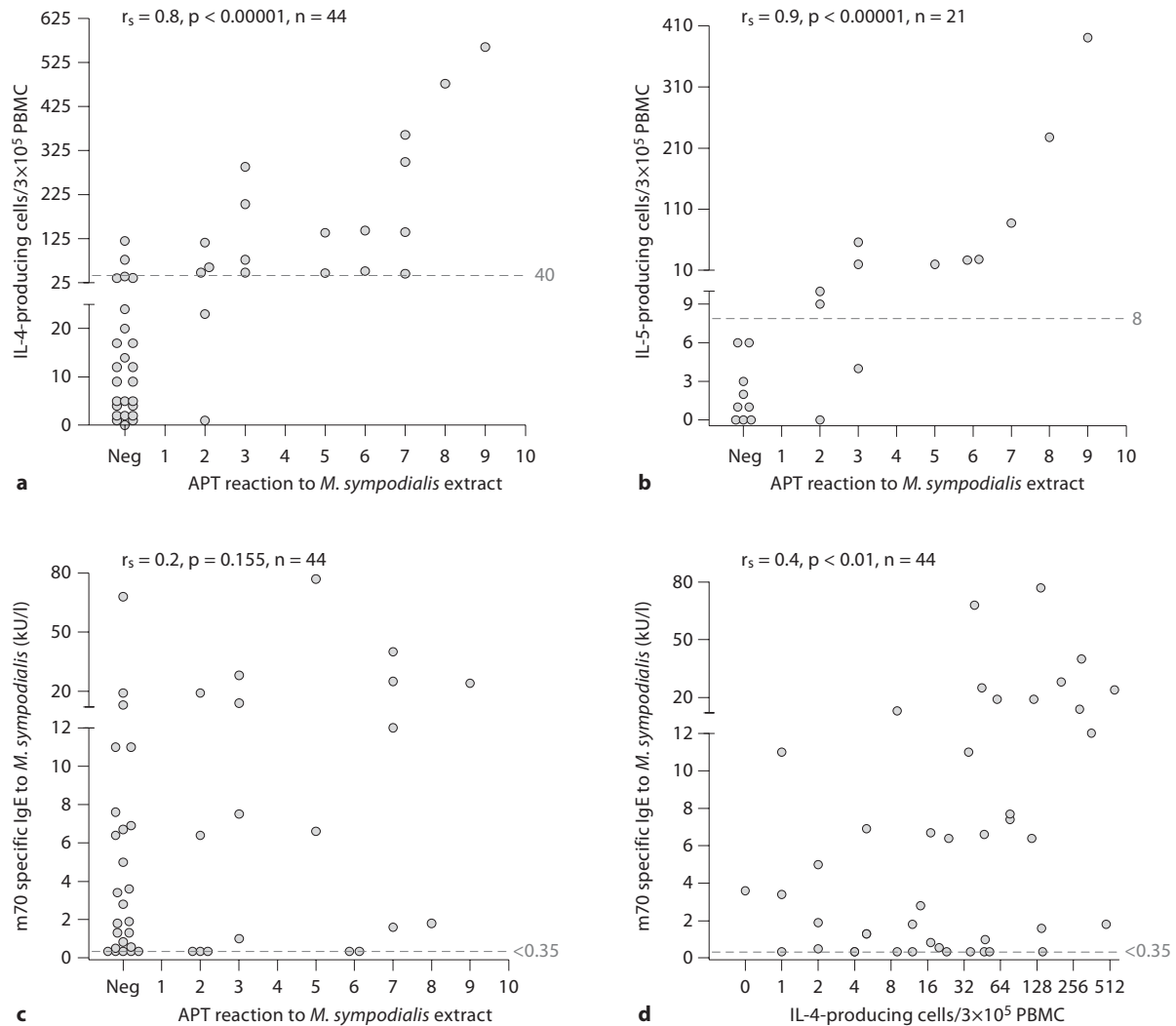


Fig. 3. Correlation between APT reaction, PBMC cytokine production and specific plasma IgE within the *M. sympodialis* reactive AE patients. **a–c** Comparison between APT score and the number of IL-4 (**a**) or IL-5 (**b**) producing cells in response to *M. sympodialis* extract or specific plasma IgE for *M. sympodialis* (m70; **c**). **d** The comparison between the number of IL-4 producing cells and specific plasma IgE (m70). The APT reactions were scored, ranging from 0 to 5+, and the accumulative score for the 2 APT reactions with 1 mg/ml and 5 mg/ml *M. sympodialis* ex-

tract, respectively, is presented. Data on the 44 AE patients reactive to *M. sympodialis* in APT reaction, specific plasma IgE and/or >3 IL-4/ 3×10^5 PBMC is presented and correlations were calculated using Spearman rank correlation analysis. In **a** and **b** the dashed lines indicate the optimal cutoff level to distinguish between APT- and APT+ AE patients. In **c** and **d** the dashed lines indicate the m70 reference range <0.35 kU/l. A log₂ scale is used on the y-axes and on the x-axis in **d** to visualize both low and high values.

a good correlation both with the APT reaction and the IL-4 response. Notably, an elevated IL-5 response to *M. sympodialis* was, in contrast to IL-4, restricted to the APT+ AE patients.

Since IL-4 promotes isotype switching to IgE and enhances IgE production [2], one could have expected a better concordance between the *M. sympodialis*-specific IgE levels and the frequency of allergen-specific IL-4-producing cells than that which was observed. We have seen a similar discrepancy for several other allergens (unpublished data). However, it may well be that the balance between Th2 and Th1 as well as the presence of Th17 and regulatory T cells [1, 2, 30], rather than the actual frequency of those cells, controls the production of IgE.

The relevance of food allergens, suspected of provoking flares of AE in sensitized infants, is today evaluated with controlled oral food challenge. Using the oral food challenge as a gold standard, APT with food allergens displays a higher specificity but lower sensitivity than skin prick test or specific serum IgE [31, 32]. Because it has low sensitivity, takes considerable time to perform and requires highly experienced investigators, APT with food allergens is not recommended for daily clinical practice [32, 33]. In contrast to food allergy, a gold standard of provocation in aeroallergen-induced AE has not been established. However, as in conventional patch testing, the prospectively obtained history of allergen-induced exacerbations of AE has been used to evaluate the clinical relevance of APT results, revealing a higher specificity despite a lower sensitivity in the APT compared to IgE measurements [11, 12, 14]. APT can thus add valuable information in the diagnosis of AE and identification of relevant allergens. A standardized protocol for APT has been proposed by the European Task Force on Atopic Dermatitis [14, 34]. In the present study this guideline was

followed with the exception of allergen concentration and vehicle, where our established protocol for *M. sympodialis* extract was followed [17, 24, 26]. Nevertheless, even with a standardized protocol and evaluation by experienced dermatologists the performance and evaluation of APT may be challenging, limiting the usefulness of this in vivo test for diagnostic purposes. An in vitro method with a high specificity similar to that of APT and with the same, or preferably higher, sensitivity would be beneficial.

In conclusion, contrary to the poor relationship between the allergen-specific IgE levels and the APT reaction observed, our results suggest a strong relationship between peripheral allergen-specific T cell reactivity and the ability to react with a positive APT reaction. A better understanding of the allergen-specific T cell reactivity involved in AE may contribute to an increased knowledge of the pathogenesis in AE and may, in addition, provide a diagnostic in vitro alternative to APT.

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Niklas Ahlberg is an employee of Mabtech AB, the Swedish biotech company from which monoclonal antibody reagents used in the study were obtained, but he owns no shares or stocks in the company. The other authors have declared no conflict of interest.

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