

Atopy patch test reactions are associated with T lymphocyte-mediated allergen-specific immune responses in atopic dermatitis

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Summary

Background Positive test reactions to epicutaneous application of aeroallergens served as a model of 'early' eczema in atopic dermatitis (AD) in a number of dermato-immunological studies. However, no quantitative evaluation has been performed so far comparing specific T-cell activation parameters in the peripheral blood of AD patients with positive or negative atopy patch tests (APT).

Objective The purpose of this study was to investigate specific immunological parameters in patients with atopic dermatitis showing positive or negative atopy patch tests reactions.

Methods APT results ($n = 96$) were compared with allergen-specific IgE, specific lymphocyte proliferation, and the expression of 'activation' markers on peripheral blood T-cells upon *in vitro* stimulation with house dust mite, cat or grass pollen allergens.

Results Only a subpopulation (48%) of patients sensitized to aeroallergens (i.e. specific IgE > 0.7 kU/L) developed APT-reactions to the corresponding allergen. APT reactions were, however, significantly associated with allergen specific lymphocyte proliferation ($p < 0.0001$), and a higher number of CD54⁺ or CD30⁺ T-cells ($p < 0.05$) upon *in vitro* stimulation.

Conclusion The association of delayed skin reactions with allergen specific T-cell parameters in the blood points to an immunologically mediated mechanism leading to positive reactions in the APT.

Keywords: atopic dermatitis, atopy patch test, cat epithelia, grass pollen, house dust mite

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Introduction

There is increasing evidence that T-cell responses to environmental allergens are important for the pathogenesis of atopic dermatitis (AD) [1–3]. In 1918, Walker first reported on several patients who had flare-ups of AD on exposure to timothy grass, ragweed pollen or horse dander [4]. Rost first observed improvement of AD by reduction of indoor allergens [5]. A marked improvement of AD upon elimination of house dust mite (HDM) was described for the first time in 1949 [6]. During the last decade the role of HDM for AD

was focused in many studies: the relative risk of severe AD appear to increase with the exposure of HDM [7,8]. It was speculated that the scales of atopic patients may have more nutritional value than scales from nonatopic patients [8]. In allergic contact dermatitis, it is well known that a close contact of allergens (haptens) to skin is a prerequisite for the sensitization. In contrast, it is not clear if sensitization to 'inhalant' allergens occurs in AD via their exposure to the airways or to the skin. There are several reports of improvement of skin conditions in open clinical studies after HDM reduction. Established and recommended avoidance procedures include encasing mattresses, pillows and blankets in impermeable synthetic material and hot washing of all bedding once a week. A previously published double-blind

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controlled trial confirmed the positive effect of HDM avoidance in AD [9].

An important finding pointing to significance of environmental allergens in AD was the observation that eczematous skin reactions can be induced by patch testing with protein allergens from HDM, pollen or animal dander. In 1982, Mitchell *et al.* reported that patch testing with mite resulted in an eczematous rash for the first time [10]. This finding was reproduced and extended by several groups and the term 'atopy patch test' (APT) was introduced for this skin test [11]. In most studies, nonatopic control individuals were found to be nonreactive. The proportion of positive patch test reactions vary from 15 to 70% in patients with AD in different studies. A recent study investigating the clinical relevance of patch test reactions with inhalant allergens points to a high frequency of positive results in patients with eczematous skin lesions that are predominantly on air-exposed skin sites [12].

Patch tests with environmental allergens have led to new and stimulating results relating to the pathophysiological role of different haematopoietic cell populations in AD. In eczematous reactions following patch testing with HDM, antigen bearing Langerhans cells, co-expressing IgE, were exclusively found in the epidermis after 6 h, and mainly in the dermis after 24 and 48 h [13,14]. A model has been proposed in which delayed hypersensitivity to aeroallergens is thought to be IgE-mediated with the binding of allergen to IgE-bearing epidermal Langerhans cells [15,16].

A significant immigration of eosinophils in the skin was detected after 2–6 h after application of allergens onto the skin in AD [17]. Twenty-four hours after allergen challenge eosinophils could be detected even in the epidermis and many activated eosinophils were seen in spongitic epidermal lesions. These findings supported the view that eosinophils are important effector cells in allergen-induced lesional skin of AD as well [18].

In addition, first evidence for the presence of skin infiltrating, aeroallergen-specific T cells in AD came from the analysis of T-cell clones which had been generated from biopsies of patch test lesions in AD [19]. These T-cell clones proved to be grass pollen [20], house dust mite [21] or cat allergen [22] specific.

So far, no quantitative comparison between results of the APT and specific lymphocyte responses have been performed. Such responses are of interest since the maximum of APT reactions is often observed during the first 24 h of patch testing which resembles epicutaneous reactions to skin irritants. Moreover, doubts about the immunological specificity of positive APT reactions were raised by the observation of similar mononuclear cellular infiltrates both in positive and negative APT sites [23].

In this study, APT results were compared with allergen-specific IgE, specific lymphocyte proliferation, and the

expression of 'activation' markers on peripheral blood T cells upon *in vitro* stimulation with house dust mite, cat or grass pollen allergens.

Methods

Patients

Thirty-two patients with AD fulfilling the criteria of Hanifin and Rajka [24] were enrolled in the first part and 18 AD patients were investigated in the second part of this study. Anamnestic data concerning the possible clinical relevance of inhalant allergens for the course of AD were not criteria for the inclusion or exclusion of AD patients. The clinical severity of AD was evaluated by the SCORAD score which summarizes items by the intensity and extent of eczematous skin reactions [25]. From each patient, blood was obtained before skin tests were performed as described below. In some experiments, which are indicated in the results section, a second lymphocyte proliferation test was performed after the APT. Specific IgE was measured with the CAP FEIA system (Pharmacia, Freiburg, Germany). The investigated patients had either no or significant levels of specific IgE in their serum (i.e. IgE < 0.35 kU/L or specific IgE > 0.7 kU/L). Prick tests were performed with standardized extracts (SQ quality) from ALK Scherax (Hamburg, Germany). The patients gave their written consent for the study which was approved by the ethical committee of the Hannover Medical University (MHH).

Atopy patch test

The APT procedure of the first part of this study was part of a multicentre study of the German APT study group conducted by J. Ring (Munich). The clinical outcome of this study will be published in detail separately (Darsow *et al.*, manuscript in preparation). Patch tests were performed on the patients' back in the first part of the study, after the discontinuation of antihistamines and (topical) steroids for at least 7 days. The antihistamine astemizol with a long half life had not been taken by any patient. The test substances (HDM, grass pollen or cat allergens; Allergopharma, Reinbek, Germany) were diluted a concentration of 10 000 PNU/g in petrolatum. The corresponding concentrations of major allergens were 20 µg/g Der p 1 in the mite extract, 17 µg/g Fel d 1 in the cat allergen extract and 65 µg/g Phl p 5 grass pollen extract in the APT. The concentration was chosen due to former experiments [26] and preliminary results from the above mentioned multicentre study addressing the optimal allergen dose in the APT. The allergens were applied for 48 h in large Finn chambers (Epitest Ltd, Oy, Finland) on clinically uninvolved, untreated back skin. Petrolatum served as negative control which was performed

each patient and did not lead to any positive test reaction. Evaluation was performed after 48 and 72 h. Grading of positive APT reactions was principally similar to the criteria used in conventional contact allergy patch testing: erythema without infiltration represented a questionable reaction; all reactions associated at least with an infiltration were considered to be positive [26].

In the second part of the study allergens were replaced by a house dust mite extract of another source (house dust mite extract II, Smith Kline Bencard, Munich, Germany) in a concentration of 19 µg/g Der p 1 in the APT. In this part of the study, all patients were tested in parallel with test solutions contaminated with LPS in a concentration range between 1000 and 70 000 pg/mL measured by the limulus assay (Coatest Endotoxin, Pharmacia, Freiburg, Germany). No positive reaction to LPS contaminated solutions was visible.

Preparation of antigens for cell culture

Inhalant allergens were found to be contaminated with lipopolysaccharides (LPS) between 830 pg/mg (grass pollen) and 8.00 pg/mg (cat epithelia) by the limulus assay. LPS was removed from the protein fractions by polymyxin B coupled with a solid phase as described [27]. The protein fractions were incubated on a shaker for 24 h at 4°C with polymyxin B agarose (Serva, Heidelberg, Germany) in the presence of octyl-β-D glucopyranoside (Calbiochem-Novabiochem, Bad Soden, Germany). After centrifugation, the whole procedure was repeated and the supernatants were dialysed twice against pyrogen-free 0.9% NaCl. LPS concentrations were determined by the limulus assay according to the manufacturer's protocol. The detection limit was 50 pg LPS/mL.

Lymphocyte proliferation test (LPT)

LPT were performed prior to APT. No patient had been receiving steroids systemically 3 weeks before the tests were carried out. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by density centrifugation on Ficoll Hypaque (Nycomed Pharma AS, Oslo, Norway; $d = 1.077$ g/mL). PBMC (2×10^5) were cultured in Iscove's medium (Biochrom KG, Germany) supplemented with 4% heat-inactivated AB serum, glutamine, gentamycin, penicillin, streptomycin and nonessential amino acids in the presence or absence of antigen. If not otherwise indicated, inhalant proteins of the first part of this study (Allergopharma, Hamburg, Germany) were used at a concentration of 5 µg/mL, Dp-extract II (Smith Kline Bencard, Munich, Germany) was used at a concentration of 3 µg/mL, and tetanustoxoid (Behring, Marburg, Germany) at 10 µg/mL. Hexaplicate cultures

were performed. After 5 days the cultures were pulsed with ^3H -thymidine (0.5 µCi/well for 4 h), and the β decay was counted in a liquid scintillation counter. The stimulation index (SI) was defined by the ratio of mean counts per minute (c.p.m.) of stimulated to unstimulated cultures.

Immunofluorescence staining of PBMC and flow cytometry

PBMC were incubated in culture medium alone or with antigens/allergens, stained with a combination of labelled MoAbs and measured by flow cytometry as described [27,28]. Expression of surface antigens on PBMC was assessed by flow cytometry (FACSCAN, Becton Dickinson, Heidelberg, Germany) using labelled CD3 (Immunotech, Hamburg, Germany), CD30 (antihuman Ki1 antigen, DAKO), CD54 (anti-ICAM-1, Immunotech) or CD69 (anti-early activation molecule, Becton Dickinson) antibodies.

Statistical analyses

Statistical differences were assessed by using the Mann–Whitney *U*-test. The results of increases of CD54⁺ T cells on allergen-stimulated PBMC were normally distributed and were therefore compared by the Students *t*-test.

Results

Outcome of the clinical study

House dust mite (dp extract I), grass pollen and cat epithelium extract were tested epicutaneously on 32 patients with AD. Seventeen of 32 patch-tests were positive for mite, 12/32 for grass pollen and 6/32 for cat allergens after 48 h and/or 72 h. The maximum of the test reaction was observed after 48 h in the majority of the cases as published previously [13,26,29]. In three APTs, results were positive only after 48 h. All patients were asked about the clinical relevance of these allergens with respect to their skin condition. As shown in Table 1, the diagnostic sensitivity of the APT results based on the patients answers varied between 45 and 71% according to the allergen, and specificities between 76 and 95% were calculated. The specificities of the APT were markedly higher than those of skin prick tests (HDM 58.3%, cat epithelia 42.8%, grass pollen 27.3%) or RAST (HDM 54.5%, cat epithelia 61.9%, grass pollen 31.8%), calculated on the basis of patients' self assessments.

Twenty-two of 32 patients had at least one positive reaction in the APT. The clinical activity of AD prior to performance of the APT as assessed by SCORAD was not different in these patients as compared with patients not reacting at all in the APT (Table 2).

The APT separated sensitized AD patients as defined by positive prick test reactions or RAST results into two

Table 1. Sensitivity and specificity of the atopy patch test.

	Patch + reaction		Patch – reaction		Specificity ³ (%)	Sensitivity ⁴ (%)
	Relevance		Relevance			
	Yes	No	Yes	No		
	Yes	No	Yes	No		
Catepithelia	5	1	6	20	95.2	45.4
Grass pollen	7	5	3	16	76.1	70.0
House dust mite	15	2	6	9	81.8	71.4

¹ Positive skin reaction was defined as infiltration and erythema (optional also papules, papulovesicles or vesicles) after 48 and/or 72 h. ² The number of positive or negative reactions are shown independent of the clinical relevance which was determined by patients self-assessment. ³ The diagnostic specificity was calculated by the formula: (number of true negative results)/(number of true negative + false positive results) × 100. ⁴ The diagnostic sensitivity was calculated by the formula: (number of true positive results)/(number of true positive + false

groups: less than 50% of sensitizations were associated with positive APT reactions. On the other hand, more than 90% of negative sensitizations as defined by negative RAST or skin prick tests were associated with negative APT as well (Table 3).

Comparison of APT results with allergen-stimulated lymphocyte proliferation

LPS-contaminated extracts were detoxified with polymyxin-B agarose prior to *in vitro* testing. After this procedure no LPS contamination was detectable by the limulus assay. Detoxified extracts did not lead to proliferation of PBMCs from nonatopic donors (not shown).

When PBMCs from patients with AD and positive APT reactions were investigated, significantly higher proliferation rates were observed in the presence of the corresponding allergens compared with proliferative responses of PBMC from patients with negative APT results (Fig. 1).

Table 2. Skin condition of AD patients with positive or negative APT results.

	n	SCORAD ¹	
		Median	25–75%
APT [–]	10	34.8	29.2–39.8
APT ^{+,2}	22	34.1	25.7–36.7

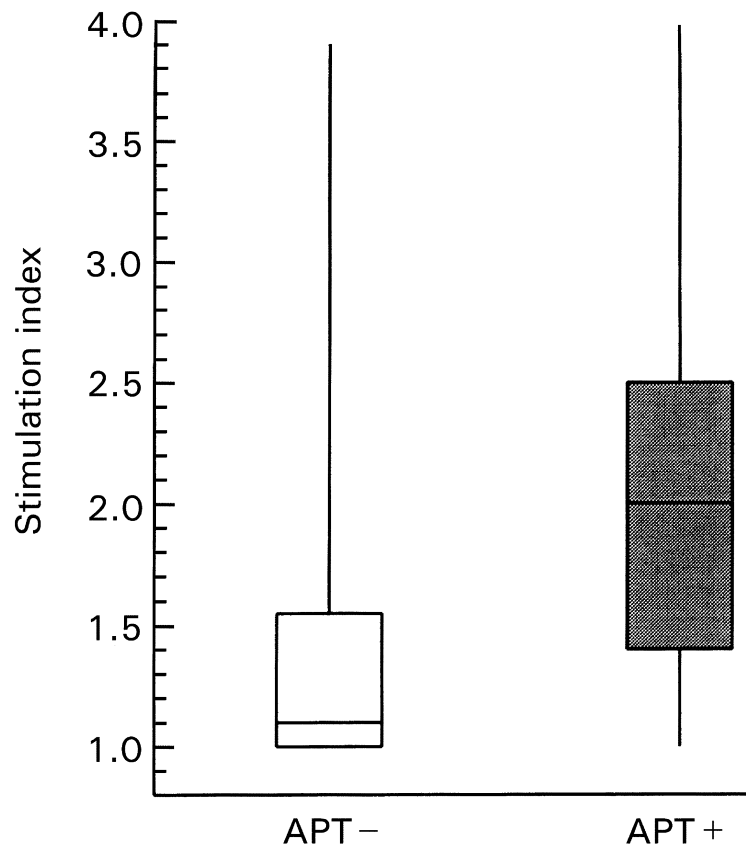
¹ The SCORAD was evaluated just before the application of allergens. ² Patients with at least one positive APT result were evaluated.

Table 3. Comparison: APT and specific IgE (RAST) and prick test.

	APT ^{+,1} (n)	APT [–] (n)	Sum (N)
<i>Cat epithelia</i>			
All patients	6	26	32
Prick test +	5	17	22
Prick test –	1	9	10
RAST + ²	4	14	18
RAST –	2	12	14
<i>Grass pollen</i>			
All patients	12	20	32
Prick test +	11	12	23
Prick test –	1	8	9
RAST +	12	14	26
RAST –	0	6	6
<i>House dust mite</i> (dp extract I)			
All patients	17	15	32
Prick test +	14	7	21
Prick test –	3	8	11
RAST +	15	6	21
RAST –	2	9	11
<i>All allergens</i>			
All patients	35	61	96
Prick test +	30	36	66
Prick test –	5	25	30
RAST +	31	34	65
RAST –	4	27	31

¹ APT + means: positive patch test reaction (i.e. erythema, infiltration, optional also papules, papulovesicles or vesicles) after 48 h and/or 72 h. ² RAST + means: more than > 0.70 kU/L of specific IgE (resp. > CAP RAST-class 2).

Fig. 1. LPT upon stimulation with LPS-depleted inhalant allergens. LPTs were performed with PBMC of APT⁺ and APT⁻ patients using lipopolysaccharide-depleted inhalant allergens (5 µg/mL) as stimuli (house dust mite extract I, grass pollen or cat epithelia extract). Stimulation indices obtained with PBMCs from different groups of patients are shown (box plots, *n* = 96). Boxes contain 50% of all results. Vertical lines display the range of the results (the maximum of the APT group was 12.5). Horizontal lines show the median of the results. For statistical analysis, the Mann–Whitney rank sum test was performed (*P* < 0.0001).



In addition, lymphocyte proliferation was significantly higher in 'APT-positive' patients when the results for each allergen were analysed separately (Table 4). In contrast, lymphocyte proliferation was similar when tetanus-toxoid

was used as a stimulus in APT⁺ and APT⁻ patients (median stimulation index: 16.0 vs 16.5).

Table 4. Specific lymphocyte proliferation in patients with AD and positive or negative APT reactions.

	<i>n</i>	LPT (SI) Median (50% CI)	<i>P</i> -value
<i>Cat</i>			
APT +*	6	2.4 (1.3–3.4)	<0.05
APT–	26	1.1 (1.0–1.5)	
<i>Grass pollen</i>			
APT +	12	2.0 (1.6–2.8)	<0.005
APT –	20	1.1 (1.0–1.3)	
<i>House dust mite (extract I)</i>			
APT +	17	1.9 (1.35–2.18)	<0.05
APT –	15	1.3 (1.0–1.8)	

* APT-reactions to 10 000 PNU/g of mite, grass pollen or cat epithelium-extract were evaluated 48 and 72 h after allergen application. *P*-values calculated using the Mann–Whitney *U*-test.

Flow cytometric analysis of allergen-stimulated PBMCs from APT⁺ and APT⁻ patients

The rate of lymphocytes expressing the 'early' activation marker CD69 was higher on PBMCs stimulated with allergens leading to positive patch test reactions or to the recall antigen tetanustoxoid compared with PBMCs stimulated with allergens which were not associated with a positive patch test reaction in a given patient (Fig. 2). As described previously [28], non-T cells were found to be CD69⁺ as well. The increases of ICAM-1⁺ (CD54⁺) allergen-stimulated T cells were significantly higher when APT⁺ patients were compared with APT⁻ patients (Fig. 3). In contrast, a similar increases of CD54⁺ T cells were shown for all (i.e. APT⁺ and APT⁻) patients when tetanustoxoid was used as a stimulus (not shown). Figure 4 shows representative results obtained with PBMC from a patient who reacted to house dust mite (dp extract I) but not to cat epithelium in the APT. Significantly higher increases in CD30⁺ T cells were also observable upon allergenic stimulation of PBMCs from patients with positive APT as compared with negative

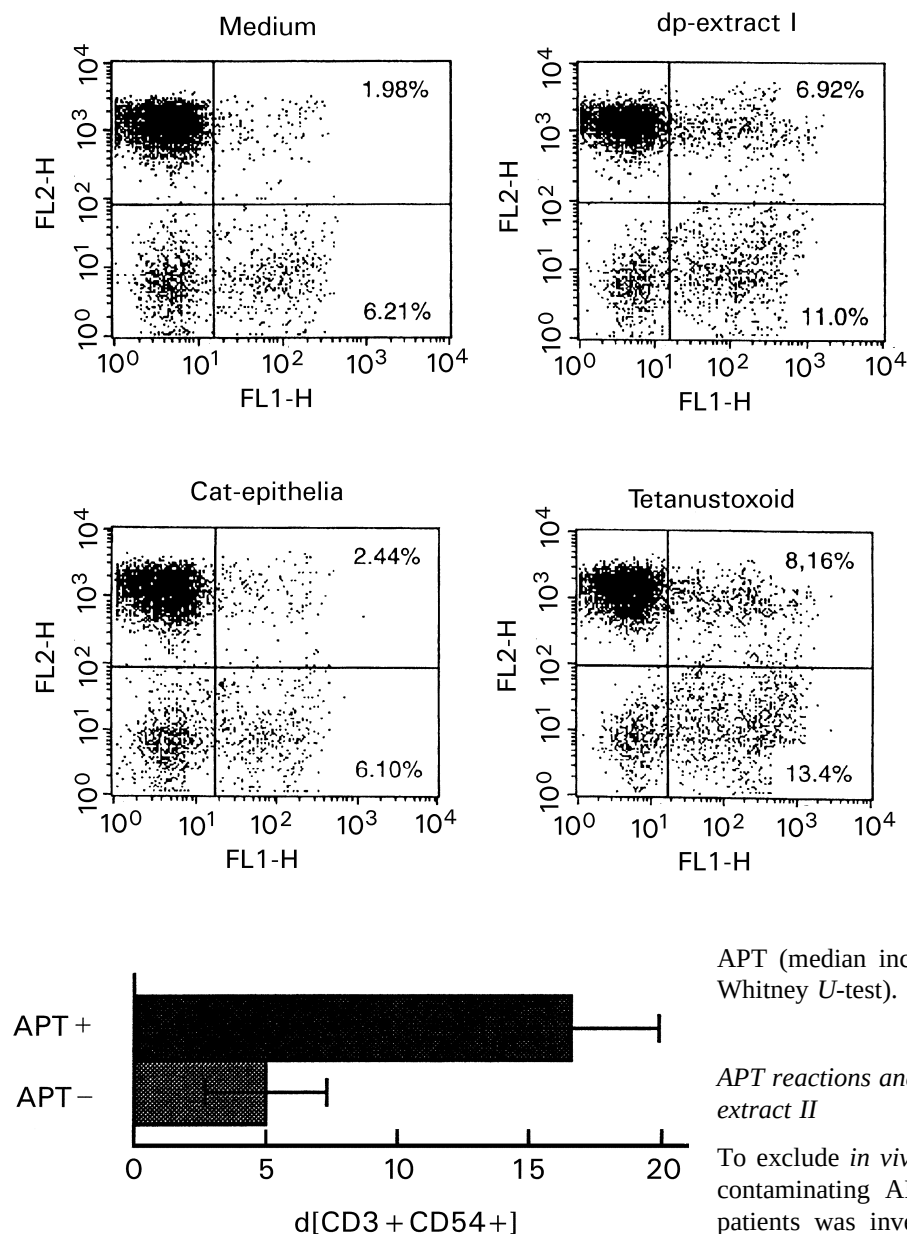


Fig. 3. Increased expression of ICAM-1 (CD54) on allergen-stimulated PBMCs from APT⁺ patients as compared with APT⁻ patients. PBMCs from APT⁺ or APT⁻ AD patients were incubated for 7 days with LPS-depleted inhalant allergens which had led to positive ($n = 17$) or negative ($n = 21$) epicutaneous test reactions. After double staining with labelled monoclonal antibodies, the samples were evaluated by flow cytometric analysis including blast cells after setting a large analysis gate. The difference (in percentage) of CD54⁺ T cells after incubation with medium alone or with allergen portion (d[CD3⁺CD54⁺]) is shown. The results in patients with positive resp. negative APT were significantly different ($P < 0.05$, t -test).

Fig. 2. Increased expression of CD69 on PBMCs stimulated with mite or tetanustoxoid as compared with cat epithelia in an individual AD patient with positive LPT and APT to dp-extract I and positive LPT to tetanustoxoid. The result for cat epithelia-extract was negative in APT. FL1-H shows the fluorescence of gated lymphocytes after binding of FITC-labelled CD69-antibodies to PBMCs, which were incubated with the given stimuli for 3 days. FL2-H shows the fluorescence after binding of PE-labelled CD3-antibodies to the cells. A similar result was found with cells from another patient.

APT (median increase 3.4% vs 0.1%, $P < 0.05$, Mann-Whitney U -test).

APT reactions and lymphocyte response to house dust mite extract II

To exclude *in vivo* and *in vitro* responses due to possibly contaminating APT substances, a second group of AD patients was investigated with another extract from the house dust mite *Dermatophagoides pteronyssinus* (dp extract II) by APT and by LPT. The difference in the proliferation rate between APT⁺ and APT⁻ patients was even more pronounced when this extract was tested on PBMCs from these patients (Table 5).

Discussion

Positive test reactions after epicutaneous application of aeroallergens served as a model of 'early' eczema in AD in a number of dermato-immunological studies. However, no quantitative evaluation has been performed so far comparing specific T-cell activation parameters in the peripheral blood of AD patients with positive or negative APT. In

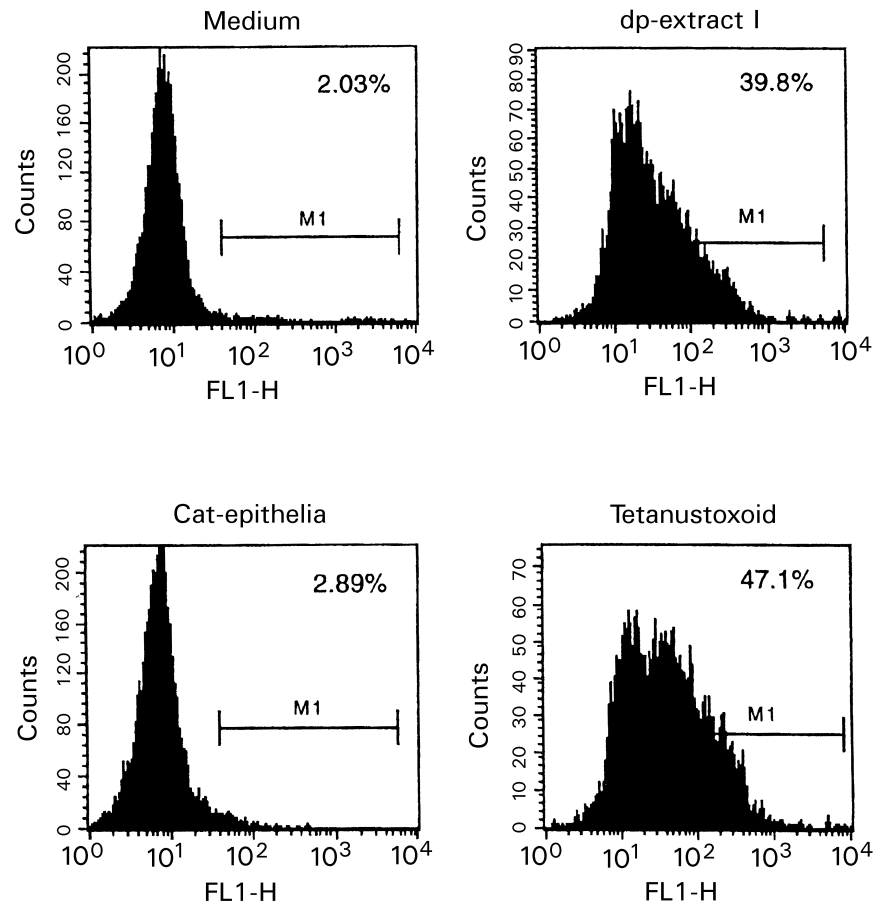


Fig. 4. Increased expression of ICAM-1 (CD54) on PBMCs stimulated with house mite or tetanustoxoid as compared with cat epithelia in an individual AD patient with positive LPT and APT to dp-extract I and positive LPT to tetanustoxoid. The result for cat epithelia extract was negative in APT and LPT. PBMCs were incubated with the above-mentioned stimuli for 1 week. After double staining with labelled monoclonal antibodies, the samples were evaluated by flow cytometric analysis including blast cells after setting a large analysis gate. The binding of FITC-labelled CD54 antibodies to stimulated CD3⁺ T cells is shown. The area 'M1' standing for the cell-fraction above the histogram was defined by the isotype-control where it included less than 1% of the cells.

this study, we compared allergen-specific IgE, specific lymphocyte proliferation and the expression of 'activation' markers on peripheral blood T cells upon *in vitro* stimulation with house dust mite, cat or grass pollen allergens to corresponding APT results.

Table 5. *In vitro* parameters in patients with AD and positive resp. negative APT reactions to house dust mite extract II.

	HDM-specific IgE (kU/L)		LPT with HDM-extract (SI) ¹	
	Median	KI ²	Median	KI
APT–	0	0–12.6	1.0	1.0–1.9
APT+ ³	99.8	52.9–499*	4.4	2.6–7.1*

¹ The LPT was performed with PBMCs and 3 µg/mL HDM-extract II prior to the APT. ² 50% interval of confidence. ³ APT + means: positive patch-test reaction (i.e. erythema, infiltration, optional also papules, papulovesicles or vesicles) after 48 and/or 72 h. The level of statistical significance (*P*) was proved with the Mann–Whitney *U*-test. * *P* < 0.05.

APT reactions were found in a higher percentage in patients who attributed a clinical relevance to the corresponding allergen for the individual course of AD. With respect to the self-assessment data, the diagnostic specificity of the APT was higher than the specificity of skin prick tests or RAST. This confirms results of a clinical study from Darsow, Vieluf and Ring [12] who found a higher rate of APT reactions to grass pollen in those AD patients who suffered from an air-exposed eczema in summer as compared with other AD patients. These results suggest that the APT may detect relevant trigger factors in AD with a higher specificity than available tests.

The outcome of the APT does not reflect the skin condition of AD patients: both in children [30] and in adults (this study) similar SCORAD values were calculated for patients with positive or negative APT reactions. As described previously [26], only a subpopulation (48%) of patients sensitized to aeroallergens (i.e. specific IgE > 0.7 kU/L) developed APT reactions to the corresponding allergen. On the other hand, all except five APT reactions observed in this study were associated with circulating specific IgE which confirms the results of a number of previous studies [13,14,26,29]. Thus, the APT appears to

select a subgroup of sensitized AD patients which have to be characterized by further *in vivo* or *in vitro* parameters.

Since eczema is mainly a T lymphocyte-mediated reaction we concentrated on T-cell parameters in the further course of this study. Prior to their application in *in vitro* assays, we eliminated LPS from allergen extracts as published before [27] to avoid unspecific cellular responses. Using these LPS-free allergen extracts, APT reactions were associated with higher allergen-induced lymphocyte proliferation and higher rates of CD69⁺, CD54⁺ or CD30⁺ lymphocytes upon *in vitro* stimulation with corresponding allergens.

After 3 days, a higher percentage of CD69⁺ lymphocytes was found in APT⁺ patients. The increase of CD69⁺ on allergen-stimulated cells from APT⁺ patients was observed both on T lymphocytes and on non-T cells. In a recent study we described how IL-2 from allergen-specific T cells probably induces CD69 on the membranes of surrounding PBMCs including NK cells [28]. CD54, which is easily inducible by interferon-gamma from T cells and CD30 which was previously postulated to be a TH2-like marker [31] were maximally expressed on allergen-stimulated PBMC cultures after 7 days. In this respect, allergen induced lymphocyte responses resembled the well-established response to the recall antigen tetanustoxoid.

Previously, Santamaria-Babi *et al.* investigated proliferative responses in circulating T-lymphocyte subpopulations, observing a mite- (but not tetanustoxoid) specific response in patients with AD sensitized to HDM in the subset expressing the skin homing molecule CLA. In contrast, in sensitized patients with allergic bronchial asthma HDM-dependent proliferation was found in the CLA-subset [32]. This observation points to an immunological mechanism which targets aeroallergen-specific T cells to the skin in AD. In addition, the immunological specificity of inhalant allergen-induced AD has been suggested by a number of studies on skin-derived T-cell clones before [19–21,33].

The immunological specificity of the APT in AD is obviously supported by our observation of higher cellular responses to HDM, grass pollen or cat antigens in patients with positive patch test reactions to corresponding allergens than in nonreactive patients with AD. The data support the concept of an IgE-dependant immune response which is associated with an allergen-specific cellular reaction in the blood (and probably in the skin) in AD. The association of delayed skin responses with specific reactions of blood T cells to inhalant allergens suggests that the APT may serve as a 'new' diagnostic tool for the identification of clinically relevant allergens in AD patients.

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