

Immunohistological analysis of 'negative' patch test sites in atopic dermatitis

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Summary

Background Variable results have been obtained when patients with atopic dermatitis (AD,) have been patch tested with allergens known to produce a positive prick test. The significance of patch test results in our understanding of the pathogenesis of AD therefore remains questionable.

Objective This study was designed to determine the relevance of either positive or negative patch test results in relation to the expression of cell mediated immunity to allergens in patients with AD.

Methods Thirty-five patients with AD exhibiting patch test positivity to one or more aeroallergens on 'tape stripped' areas of the back were retested without prior tape stripping. Nine patients again showed positivity to one or more allergens while 26 failed to show positive reactions. In six of the positive patients both positive and negative patch tests were observed. Skin biopsies were taken from these matched positive and negative patch test sites as well as from an area of uninvolved skin. Samples were frozen and cryostat sections were analysed with immunohistological techniques using monoclonal antibodies to investigate the distribution of immunocompetent cells.

Results All positive patch tests exhibited characteristics of a cell mediated immune response. The negative patch test sites were also found to contain evidence of mononuclear cell infiltration. Both negative and positive patch test sites showed significantly greater proportions of T cells compared to uninvolved skin. No increase in numbers of RFD1 positive and RFD7 positive macrophages were observed in either positive or negative patch test sites. Expression of CD23 by CD1 positive Langerhans cells was raised in both negative and positive patch tests compared to uninvolved areas. A significant increase in the population (RFD7+, CD23+) was seen in positive patch test sites compared to uninvolved skin. An increase in the proportion of RFD1 positive cells expressing CD23 was also seen in both negative and positive patch tests compared to uninvolved skin.

Conclusions This paper demonstrates that immunological reactions are promoted at 'non-tape stripped' patch test sites where no clinical evidence of reactivity is seen. Together the data demonstrate that the presence of systemic cell mediated immunity to specific allergens identified in patients by positive patch test, may also be present when no clinical signs are seen at the patch test site.

Keywords: atopic dermatitis, patch tests, acquired cell mediated immunity, allergens.

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Introduction

It is recognized that over 80% of patients with atopic dermatitis characteristically demonstrate positive

immediate type hypersensitivity skin-prick tests to multiple aeroallergens [1]. However, the pathogenic role of these allergens in atopic dermatitis is unclear. The eczematous lesions in AD do not have the morphology typical of an immediate hypersensitivity phenomenon but exhibit the cellular characteristics suggestive of a delayed-type hypersensitivity reaction [2–4]. Recently, several studies have demonstrated positive and supposedly specific reactions to epicutaneous patch tests with house dust mite antigens in atopic dermatitis [3–7]. Furthermore, many of these studies have also shown that the phenotype of the cellular infiltrate is consistent with a delayed hypersensitivity reaction. Earlier work in this laboratory showing the cellular infiltrate in the aeroallergen patch test reaction to be similar immunohistologically to lesional skin in both patients with AD and also in patients with nickel contact dermatitis supports this hypothesis, [8].

Hypersensitivity to allergens in any given patient with AD may be identified by history, positive skin prick test, RAST and positive patch test reactions. These patch test reactions may be performed on areas of skin 'stripped' using repeated application of Sellotape. It is well known that if non-stripped skin is used for the test there are times when no clinical reaction is seen in subjects who would otherwise give positive reactions when such tape stripping is applied [5]. This observation could be taken to indicate that positive reactions after tape stripping are artefactual, thus bringing in to question the significance of any relationship seen between the immunopathology of patch test reactions and that of lesional skin. However, the immunopathological similarity between patch test reactions and lesional skin [8] suggests that allergen contact could promote clinical lesions even though no definite evidence is available. At the very least, patch test positivity identifies the presence of acquired T cell mediated immunologic sensitivity to these allergens. With the well-recognized variability in patch test reactivity (see above) it is not known whether a negative result is proof of the absence of T cell mediated immunosensitivity.

Analysis of positive and negative patch tests to white soft petrolatum and paraphenylene diamine in patients with allergic contact dermatitis [9] showed that despite an absence of erythema in some patients, there was consistent evidence of a migration of Langerhan cells from epidermis to the dermis and an increase in the number of dermal T cells and monocytes. Such observations would indicate that the difference between patch tests on stripped and non-stripped skin is quantitative rather than qualitative.

The purpose of this study was to determine whether a clinically negative response to epicutaneous aeroallergen challenge indeed reflected the absence of an immunopathological reaction in the skin. This has been

done by taking patients exhibiting positive patch tests on Sellotape stripped skin sites and challenging these individuals with identical patch tests on unstripped skin.

Materials and methods

Subjects

From an original panel of 102 patients with atopic dermatitis, 35 patients were selected who exhibited a positive patch test on Sellotape stripped skin (12 applications) to one or more of the allergens listed below. Patch tests were performed using Finn chambers (Epitest Ltd, Finland). All 35 patients fulfilled established clinical criteria [10]. Demographic and clinical details of these subjects are documented in Table 1. A panel of 11 allergens were used: *Cladosporium herbarium* (CL); *Alternaria tenuis* (AT); *Candida albicans* (CA); *Dermatophagoides farinae* (DF); *Cat dander* (C); *Dog dander* (D); *Plane tree extract* (PT); *Timothy grass pollen* (T); and *Rye grass pollen* (R).

Allergens were used at a concentration of 50 000 BU/ml. In all cases patch test positivity was only observed against allergen to which the patients showed immediate-type skin prick test positivity.

Study patch testing

Patch testing of the patients documented above was repeated on non-tape stripped skin using only the allergens to which a positive reaction had been recorded on 'stripped' skin. With the exception of not 'stripping' the skin, the patch tests were performed in an identical manner to that described above, using the same concentration of allergens. Patch tests were removed after 48 h and were exposed to air for 1 h prior to reading, to allow for non-specific irritant reactions (due to adhesives) to clear. Positive reactions were identified according to the International Contact Group guidelines [11].

Skin biopsy

In six of the nine patch test positive subjects, an example of a positive patch test and a negative patch test were recorded on non-stripped skin (Table 2). These were selected for immunological analysis; 5 mm punch biopsies were obtained from three sites:

- 1 site of positive patch test;
- 2 site of a patch test that was clinically negative; and
- 3 an area of uninvolved skin.

All biopsies were orientated on cork discs, covered in 'Cryoembed' medium (Brights Inst. Co., Huntingdon), and frozen in isopentane cooled in a bath of liquid

Table 1. Patients studied

Patient	Age	Sex	FH _x	+SPT	IgE KU/l	Severity	PTR
1	35	M	—	11	8800	Md	C,DP
2	35	M	—	9	1355	Mld	CL
3	29	M	+	4	2089	Md	R,T
4	20	M	+	9	19 400	Sv	C,PT,CL
5	41	M	—	8	5467	Sv	R,CL
6	46	F	—	3	1221	Mld	DP
7	29	F	+	5	1657	Sv	R
8	29	M	+	9	21 060	Sv	DP
9	49	F	+	6	1400	Sv	B
10	34	M	+	8	1000	Md	CL,DP,DF,B,R
11	29	F	+	6	13 760	Sv	DP,T,R
12	54	M	—	8	6830	Sv	DP,DF
13	48	M	—	11	410	Md	AT,DP
14	27	M	—	4	1170	Md	R
15	31	F	—	6	10 770	Md	CA
16	33	M	—	5	83 370	Md	C
17	22	M	+	8	114	Md	R,C,DF,DP
18	27	M	+	5	1770	Sv	DP,R,T
19	27	F	+	5	1375	Sv	DP,C,R
20	29	M	+	6	1338	Md	C
21	17	F	—	6	1073	Sv	DP
22	21	F	+	8	1889	Md	R
23	37	F	+	3	1917	Sv	R
24	34	M	—	5	684	Md	B,R
25	33	M	—	5	1647	Sv	DP,R,DF
26	29	F	—	7	1876	Md	CA,DP
27	48	F	—	6	2385	Sv	R,DP
28	26	F	+	7	54 660	Sv	DP,DF,B
29	38	M	—	6	927	Md	R
30	27	M	+	4	710	Md	PT,CA,DP
31	45	M	—	3	19 460	Md	CL
32	42	M	—	3	653	Md	B
33	39	M	—	6	18 406	Sv	DP
34	26	F	—	6	2034	Sv	DF
35	42	M	—	3	526	Md	B

FH_x = family history; SPT = skin prick test (number positive documented); Severity = MLD – Mild, MD – Moderate, Sv – Severe; PTR = patch test reaction; allergens producing PTR on tape stripped skin are identified (see text for explanation of abbreviations).

nitrogen. They were immediately transferred to pre-cooled universal tubes and stored in liquid nitrogen until used. Cryostat sections 6 microns thick were cut and collected onto poly-l-lysine coated microscope slides. They were air dried for at least 1 h, fixed in 1:1 chloroform:acetone for 5 min, wrapped in 'cling film' and stored at -20°C .

Immunohistology

The distribution of phenotypically distinct populations of immunocompetent cells was determined using

immunohistological techniques with the following panel of monoclonal antibodies (MoAbs): T lymphocytes, cocktail of CD2, CD3, CD7, CD8; Langerhan cells (CD1); antigen presenting cells (MoAb RFD1) [12]; and tissue macrophages (MoAb RFD7) [12].

In all cases, indirect immunoperoxidase methods were used [13]. The distribution of the low affinity IgE receptor was determined using MoAb CD23, using the alkaline phosphatase anti-alkaline phosphatase method, (APAAP) [14]. This procedure was used sequential to the immunoperoxidase method to reveal the phenotype of CD23+ cells.

Quantification

The relative distribution of specific cells sub-sets was quantified using computerized image analysis systems, (Seescan, Cambridge), in which positive cells were point counted in frame defined areas of the tissue sections [15]. In all cases control preparations omitting the first layer MoAb were used to reveal background colouration, and positive cells were only identified if the expression of the reaction product exceeded that in the control sections. All MoAbs were standardized on cryostat sections of palatine tonsil where specificity was determined by comparison with an irrelevant mouse anti-human MoAb of the same immunoglobulin class.

Statistics

Data within groups are presented as median and range. Significance was determined using the Mann-Whitney *U*-test for non-parametric data. Comparison of cellular distribution in tissue sections of paired biopsies from the same subject was performed using the Wilcoxon rank sum test for paired data, where $P \leq 0.05$ was considered significant. Where comparison was made between three groups, the Bonferroni correction was applied, where $P \leq 0.025$ was considered significant.

Results

Of 35 subjects tested, nine expressed positive patch tests on non-stripped skin (Table 2). Seven of the nine responded to one allergen, whereas the other two responded to two allergens. The expression of a positive

patch test on non-stripped skin was not related to any personal or family history of atopy, nor was it related to raised levels of IgE in the circulation.

Of the nine patients with positive reactions, five were defined clinically as having severe disease (55%), while of the other 26, 11 had eczema defined as severe (42%, Table 1). The group showing positive patch tests on non-stripped skin included one patient with mild disease and three with moderate disease (Table 2).

Skin biopsies of both positive and negative non-stripped patch test sites were taken from the six patients exhibiting this result (Table 2). Uninvolved skin was also biopsied (see methods).

Immunohistology

Using CD1 MoAb the number of Langerhan cells was determined in the epidermis and dermis of the three sites of biopsy. The results are presented in Table 3. No significant difference in the number of Langerhan cells was observed, either in the epithelium or the dermis, in the three sets of specimens studied. Small numbers of T lymphocytes were identified in biopsies taken from uninvolved areas (range 2.8–5.8 cells/unit area, Fig. 1). This number was seen to be significantly increased in both +ve and -ve patch tests, $P < 0.02$ in each case. No difference was observed between the -ve and +ve patch test sites (Fig. 1).

An increase in RFD1+ antigen presenting cells was also observed when uninvolved skin was compared to patch test skin (Fig. 2). This increase was greatest in the sample from the +ve patch test site. However, on analysis no statistical difference was recorded

Table 2. Patch test results non-tape stripped skin sites

Subject	Disease severity	Reactions on stripped skin (all +ve, see Table 1)	Reaction on non-stripped skin	
			+VE	-VE
2	Mld	CL	CL	—
13	Md	AT,DP	AT	DP *
17	Md	R,C,DP,DF	C,DP	R,DF*
18	Sv	DP,R,T	R,DP	T *
19	Sv	DP,C,R	C	DP,R*
21	Sv	DP	DP	—
25	Sv	DP,R,DF	DP	DF,R*
27	Sv	DP,R	R	DP *
29	Md	R	R	—

Mld = mild disease; Md = moderate disease; Sv = severe disease.

* Patients biopsied at both +ve and -ve patch test sites.

For allergen abbreviations see text.

Table 3. Langerhan cell distribution

	Uninvolved skin	Negative patch test	Positive patch test
Epidermis	1.6 (0.9–4.6)	1.6 (0.8–3.5)	2.5 (1.1–3.9)
Dermis	2.9 (1.5–4.1)	3.04 (2.7–3.9)	2.1 (1.8–2.6)

Median values and ranges are shown, $P > 0.05$ in all cases.

($P > 0.025$). A similar picture was observed when numbers of RFD7+ tissue macrophages were quantified. Although an increase in median values was observed in both patch test reactions compared to uninvolved skin, these differences did not reach statistical significance (Fig. 3).

Expression of CD23

Using sequential techniques of immunoperoxidase and APAAP the proportions of CD1+, RFD1+ and RFD7+ cells expressing CD23 were determined on tissue from

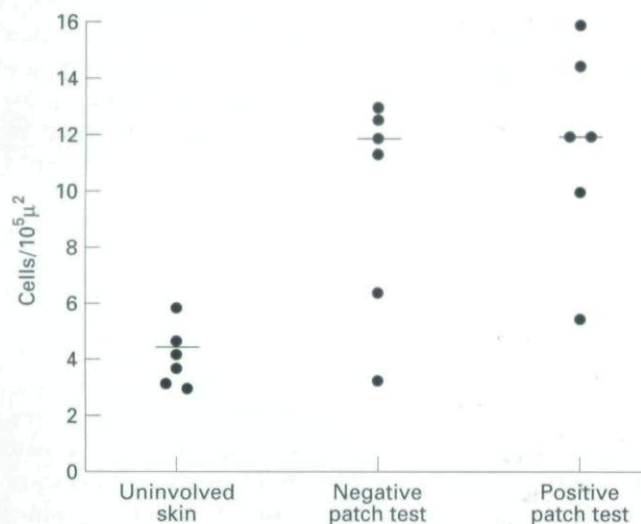


Fig. 1. The incidence of T cells in the dermis (10-cell depth from epithelium) in the three biopsies taken from each subject, quantified by point-counting lymphocytes showing positivity to a cocktail of anti-T cell monoclonals using the indirect immunoperoxidase method. Cell counts were performed within frame-defined areas using image analysis equipment (Seescan Cambridge). Five areas were measured on sections from each biopsy. Mean number of cells per unit area is given for each sample.

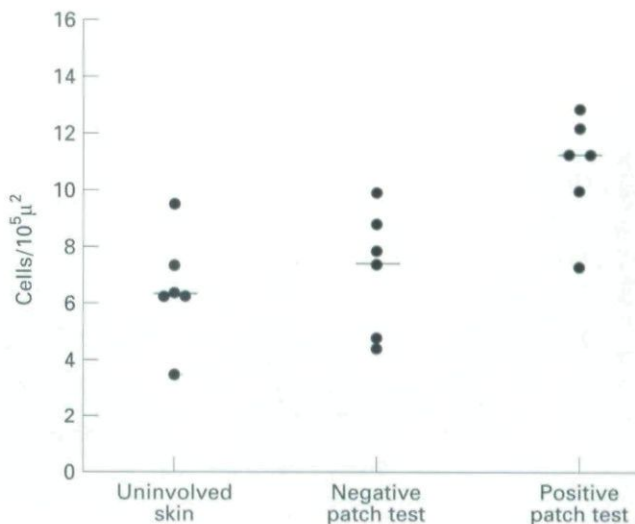


Fig. 2. The incidence of MoAb RFD1+ antigen presenting cells in the dermis of the three sites biopsied from each subject. Method of quantification as Fig. 1. Mean of a number of five readings given at each point.

each of the sites tested. The proportions of RFD7+ cells expressing CD23 in uninvolved skin showed a median of 28%; however, in negative patch test sites 22% of RFD7+ cells were CD23+ ($P = ns$). In positive patch test sites a median of 10.5% of RFD7+ cells expressed CD23. This reduction was significantly lower than either of the other two sites ($P < 0.02$ compared to negative

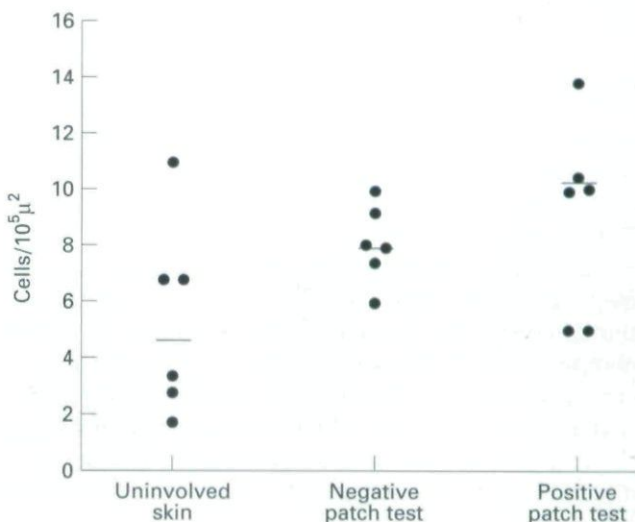


Fig. 3. The incidence of MoAb RFD7+ phagocytes in the dermis of the three sites biopsied from each subject. Method of quantification as for Fig. 1. Mean of a number of five readings given at each point.

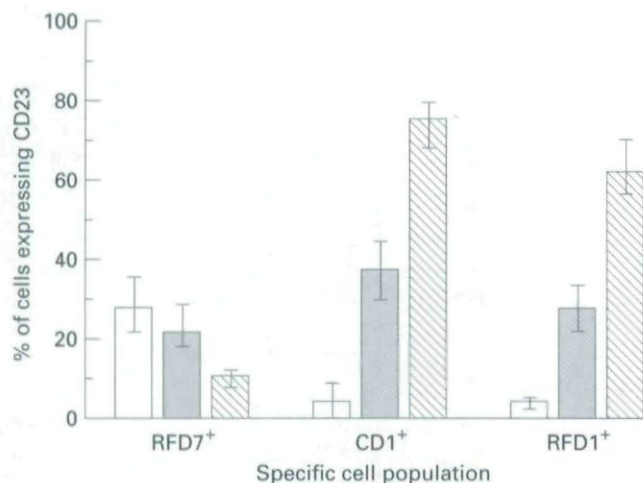


Fig. 4. The proportions of RFD7⁺, CD1⁺ and RFD1⁺ cells co-expressing CD23 in samples of uninvolved skin □, negative patch test ■ and positive patch test ▨. Sequential immunoperoxidase and APAAP methods. Proportions calculated from cell counts of five high power fields in each section using the formula:

$$\frac{\text{number subset}^+ \text{ CD23}^+}{\text{number subset}^+} \times 100$$

patch test site) (Fig. 4). The number of CD23⁺ CD1⁺ cells was significantly higher in both negative and positive patch test sites compared to uninvolved skin ($P < 0.02$, $P = 0.01$, respectively). A similar picture was seen on RFD1⁺ cells, where proportions expressing CD23 were again significantly increased in negative and positive patch test sites ($P < 0.01$, $P < 0.005$ compared to uninvolved skin) (Fig. 4).

Discussion

It has been previously reported that the immunopathology of a positive patch test site reflects that of lesional skin in patients with atopic dermatitis [16–18]. Thus the patch test reaction may be seen as a controlled model of the disease, at least in terms of the development of lesions, and may thus lead to the conclusion that allergens causing positive reactions *could* cause the manifestation of clinical eczema if contacted in sufficient concentration. If this were true one would predict that all AD patients would patch test positive providing the relevant allergen was available. This prediction is not fulfilled as this study and others have shown that only a minor proportion of atopic dermatitis patients are positive on patch testing, even when an exhaustive panel of aeroallergens are used. The fact that 'stripping' skin with adhesive tape which presumably allows greater entry of allergen, increases the proportions of

patients responding, suggests that these differences may be quantitative rather than qualitative.

The results presented in this study support this hypothesis. Immunohistological investigations of negative patch test sites have revealed changes in the disposition of T cells and macrophage populations to those recorded in positive patch tests. Interestingly, even the changing distribution of CD23 antigen from macrophages to dendritic cells, a phenomenon shown to distinguish aeroallergen patch tests from contact sensitivity reactions [8], was seen to occur in these negative patch test sites. This observation demonstrated (not surprisingly) that immunological interactions are manifest in the skin prior to the expression of frank erythema or induration. Of particular interest is the fact that the most significant changes observed between patch test sites and uninvolved skin came in the studies of CD23 expression [19]. This observation focuses attention on a possible cause and effect relationship between allergen exposure and changes to the distribution of this low affinity IgE receptor.

Relating these findings to overall pathogenesis, they offer cogent evidence to support the suggestion that an immune sensitivity to specific allergens may be present in AD patients despite the absence of a clinical response to patch testing. This result extends a previous report [9] showing an increased incidence of T cells and antigen presenting cells in 'negative' patch tests. The present report goes further in also showing a changing distribution of CD23 expression. Furthermore, it is clear that 'negative' patch tests may exhibit a cellular response *in situ* despite the absence of erythema, thus demonstrating sensitivity without overt clinical signs. It could be argued that cellular reactions not associated with clinical inflammation may be of no consequence. Certainly atopic individuals are often seen to respond to multiple allergens while not exhibiting clinical disease. It is notable, however, that no relationship was seen in patients between raised IgE levels and patch test reactivity (see Results). This is not surprising as cellular reactions generated by patch tests (including the clinically negative ones), bear the characteristics of T cell mediated cellular reactions and do not therefore reflect mechanisms of immediate type IgE mediated reactivity. As, however, all allergens used for patch tests had been shown to promote positive prick test reactivity, this study reveals that both IgE responses and T cell reactivity may be manifest by the same allergens. This observation promotes the interesting possibility that although distinct from one another it is the coincidence of immunological aberrations in T cell mediated and IgE mediated responses to the allergens, that promotes clinical disease. Such an hypothesis would fit with recent reports focusing on IgE/allergen complex mediated T cell activation in AD [20,21].

There is possible merit in identifying the allergens potentially responsible for exacerbation of atopic dermatitis in any given patient, in order to consider avoidance measures as part of management [22,23]. A recent report has clearly shown that reducing allergen contact can result in reduced severity of disease [24]. However, performing a biopsy on all patch test sites is impractical, thus the results presented in this study would support the use of Sellotape stripping, if patch tests are to be applied, on the grounds that positive results are more likely to be observed clinically.

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