

Original article

Atopy patch test in patients with atopic eczema/dermatitis syndrome: comparison of petrolatum and aqueous solution as a vehicle

Background: The atopy patch test (APT) is an *in vivo* model to study the induction of eczema by inhalant allergens. This study was designed to compare two commonly used APT methods.

Methods: In the first method, the allergen is dissolved in aqueous solution, which is applied on tape-stripped skin. In the second method, the allergen is dissolved in petrolatum and applied without tape stripping. Thirteen patients with atopic dermatitis sensitized to inhalant allergens were patch tested using both methods. Reactions were evaluated macroscopically and microscopically after 48 h.

Results: Nine out of 13 patients displayed a positive reaction for both methods. One patient had a positive APT for the aqueous method alone and three for the petrolatum method alone. Reactions were significantly stronger when using the petrolatum method. Histological evaluation of the nine patients positive for both methods showed no significant differences in number of eosinophils, T-cells and neutrophils.

Conclusion: The APT using the petrolatum vehicle induces a higher number of positive reactions and is significantly stronger relative to the APT using allergen in aqueous vehicle. The cellular influx in both test methods is comparable. Both methods can be used to study the mechanisms in the induction of eczema by inhalant allergens.

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The atopy patch test (APT) is a frequently used *in vivo* model to study the mechanisms involved in the induction of eczema by inhalant allergens. Mitchell et al. first demonstrated that epicutaneous application of inhalant allergens could induce eczematous skin lesions in patients with a positive immediate skin reaction to the same allergen (1). Thereafter, many groups studied the APT as a model to investigate the role of aeroallergens and food allergens in atopic eczema/dermatitis syndrome (AEDS) (2–10). Allergic diseases such as AEDS are mediated by allergen-specific IgE and a crucial role has been ascribed to the Th2-cells responsible for a high production of IL-4, IL-5, IL-13 and a low presence of IFN γ . In AEDS patients, the majority of T-cells in acute lesional skin also appear to be of the Th0/Th2-type, producing IL-4 and IL-5 (11). The early APT reaction, up to 24 h after application, is likewise characterized by an increased IL-4 production by infiltrating T-cells, thereby resembling the T-cell phenotype of acute lesional skin (12). However, in chronic lesional skin, the majority of the lymphocytes show a Th1-cytokine profile,

producing IFN γ (13). Looking subsequently at the late APT reaction at 48–72 h, a switch from the original Th2/Th0 cytokine profile towards a Th1 skewed cytokine pattern was found, showing an increased level of IFN γ , resembling chronically inflamed skin in AEDS (14, 15). Furthermore, histological evaluation of the cellular infiltrate of the APT reaction shows close resemblance to lesional skin with a dermal infiltrate predominantly consisting of CD4+ T-cells and activated eosinophils (14, 16).

The mechanism involved in the induction of the eczematous reaction by the APT has been extensively studied (7, 12, 14, 17–22). The hypothesis of the reaction mechanism of the APT is that allergens penetrate the skin and bind to Fc ϵ RI-bound IgE on Langerhans cells. The Langerhans cell recognizes the allergen and presents the antigen subsequently to T-cells (22, 23). T-cells of the Th2 phenotype proliferate and move to the place of allergen exposure initiating local inflammation by release of IL-4 and IL-5.

Many different methods have been developed for APT. The differences in methodology include differences in the source of the allergens, vehicles, pretreatment procedures of the skin, application site and reading time of the patch

Abbreviations: AEDS, atopic eczema/dermatitis syndrome; APT, atopy patch test; GP, grass pollen; HDM, house dust mite.

test (2, 4, 8, 9, 24–26). Owing to these factors, large variations exist in the macroscopic outcome of the APT (27). For this reason, Langeveld-Wildschut et al. (25) and Darsow et al. (26) extensively studied the methodology of APT.

Darsow et al. advised performing the APT without pretreatment of the skin using petrolatum as the vehicle and a high allergen concentration. Reading time was recommended at 48 and 72 h (26).

Langeveld-Wildschut et al. recommended tape stripping the skin 10 times as pretreatment, using a high allergen concentration of 10 000 AU/ml dissolved in an aqueous solution. The optimal reading time was at 24 and 48 h (25).

Both methods recommend nonlesional skin of the back as the best application site. The aim of the present study is to compare the above-mentioned methods macroscopically and histologically.

Methods

Patients

Thirteen patients (seven women and six men; age range 19–49 years; mean age, 28.5 years) with the AEDS were studied (28). Of the 13 patients studied, three also had allergic rhinitis, three had allergic asthma, three had both allergic rhinitis and asthma and four no other allergic disorders apart from AEDS.

Patients had not taken oral immunosuppressive drugs, including oral corticosteroids, for at least 1 month before the APT was performed. Oral antihistamine drugs and topical treatment of the back with corticosteroid was discontinued for at least 14 days before the APT was performed. None of the patients were treated with ultraviolet light radiation for at least 4 weeks before testing.

Clinical activity of skin lesions was established using the objective SCORAD (29).

All patients had a score between 15 and 40. All patients had positive skin prick tests to at least one aero-allergen. The characteristics of all patients are shown in Table 1. All participants in this study gave their informed consent. This study was approved by the local medical ethical committee.

Table 1. Patient data

Patient	Age	AR	AA	SCORAD	SPT
1	26	+	–	nd	HDM2+
2	33	–	+	34.2	HDM3+
3	21	–	+	34	HDM3+
4	26	+	+	29.9	GP 3+
5	39	–	+	33.5	HDM 3+
6	35	–	–	31.1	HDM 3+
7	19	+	+	39.1	HDM 2+
8	28	+	+	23.5	HDM 3+
9	19	+	–	37.3	HDM 2+
10	25	–	–	28.7	GP 2+
11	23	+	–	29.2	HDM 3+
12	28	–	–	20.8	HDM 3+
13	49	–	–	27	HDM 3+

AR, allergic rhinitis; AA, allergic asthma; SPT, skin prick test; HDM, house dust mite; GP, grass pollen.

Atopy patch test

Atopy patch tests were performed on clinically uninvolved skin of the back with the two methods to be compared.

The aqueous method was previously described by Langeveld-Wildschut et al. (25). The test sites are stripped 10 times with adhesive tape (Transpor hypoallergenic tape; 3M Health Care, St Paul, MN) before application of aqueous allergen preparation. Commercially available allergen preparations were used (0.08 ml/test) from Haarlem's Allergen Laboratorium (HAL) (Haarlem, The Netherlands) containing *Dermatophagoides pteronyssinus* (HDM) and grass pollen (GP) in concentrations of 10 000 allergy units (AU)/ml. The allergens were dissolved in HAL buffer solution containing a buffered salt solution. The buffer solution was applied as a negative control in all subjects. The test was performed with Leukotest patches (Beiersdorf AG, Hamburg, Germany).

The petrolatum method was previously described by Darsow et al. (26). In brief, the allergens (HDM and GP) were dissolved in the petrolatum vehicle (Stallergènes S.A., France) and applied with large Finn chambers (Epitest Ltd., Oy, Finland; diameter = 12 mm). The allergenic potency was translated as an index of reactivity (IR) and was 200 IR/ml for all allergens. Petrolatum was applied as a negative control in all subjects. No tape stripping was done before allergen application.

The patch test reactions in patients positive for both methods were compared macroscopically after 48 h. Patch test reactions were recorded as positive when at least erythema and induration occurred and no reaction was observed in the control patch: 1+, erythema with slight induration; 2+, marked erythema and induration, papules; 3+, marked erythema and induration, papules and vesicles.

Biopsy specimens

Punch biopsy specimens (3 mm) were taken from the APT positive sites at 48 h after application of local anaesthesia (2% xylocaine). Biopsies were taken when the APT was positive for both methods. Biopsies were immediately fixed in 4% formalin and stored in small vials. Subsequently, biopsies were embedded in paraffin using an automatic tissue processor (Histokinette). Paraffin-embedded tissue was stored at room temperature until further handling. Sections (5 µm) were cut from the paraffin-embedded skin tissue and mounted on 3-aminopropyltriethoxysilane-coated (Sigma-Aldrich, Steinheim, Germany) glass slides.

Histochemical staining

Histochemical staining was done with haematoxylin, Congo red and Chromotrope 2R. For haematoxylin, the slides were incubated for 15 min in haematoxylin (Merck, Darmstadt, Germany). Subsequently, they were incubated for 10 min in tap water under flow, and then incubated for 2 min in eosin [0.5% in ethanol (96%)] (Chroma-Gesellschaft Schmid & Co, Stuttgart, Germany).

For Congo red, the slides were stained in haematoxylin (Merck) for 10 s and then incubated for 5 min in tap water under flow. Subsequently, the slides were stained in the solution containing 0.5% Congo red (Fluka A.G. Buchs, Switzerland) in ethanol/0.1 M glycine (1 : 1) for 20 min. The slides were then rinsed in 70% ethanol until the background became clear (30).

For Chromotrope 2R, the slides were stained in haematoxylin (Merck) for 30 s, incubated in tap water under flow for 5 min and then stained in 0.5% Chromotrope 2R (Sigma-Aldrich) containing 1% phenol crystals for 60 min (31).

Immunohisto/cytochemical staining

Monoclonal antibodies (mAb) against eosinophilic cationic protein in eosinophils (EG2, Pharmacia, Uppsala, Sweden), neutrophils (elastase, Dako, High Wycombe, UK) and T-cells (CD3, Becton Dickinson, San Jose, CA) were employed for immunohisto/cytochemical staining.

For EG2, slides were incubated with 1 : 50 diluted EG2 in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), subsequently Powervision Poly AP-Anti mouse (Immunologic, Duiven, The Netherlands) was added and incubated for 30 min.

Alkaline phosphatase activity was demonstrated using naphthol-ASMX phosphate (Sigma-Aldrich) as substrate and new fuchsin solution (Merck) as chromogen resulting in blue staining. Subsequently, the slides were stained with haematoxylin (Merck) for 10 s and rinsed in tap water for 5 min (32, 33).

For elastase, neutrophils were stained, using the same method as for EG2, using anti-elastase 1 : 25 in the incubation step. The incubation step for this procedure lasts 60 min.

For CD3, de-paraffined sections of 10 µm were heated at 95–100°C in EDTA-buffer solution for 10 min. T-cells were subsequently stained using the same method as for EG2, using monoclonal mouse antibody against CD3 1 : 50 dilutions in the incubation step. As the second antibody, goat-anti rabbit biotin 1 : 300 in 1% BSA in PBS was used. No Powervision was used in this procedure.

Quantification of staining

Skin sections stained with EG2 and Congo red were examined by light microscopy at 400× magnification by a blinded observer. Before evaluation, sections were compared with either the isotype-control-stained counter-sections or with the nonstained counter-sections. Positive cells in the dermis were counted in three different sections of 1–1.5 mm² per section and calculated as cells per square millimetre. In fields containing sweat ducts and hair shafts, only intervening dermal regions were counted. The number of CD3 positive and elastase positive cells was assessed using a three point scale: –, few positive cells/as in normal skin; +, moderate presence of positive cells; ++, pronounced presence of positive cells. The dermal and epidermal compartments were examined separately.

One out of every five analyses was controlled by a second independent observer. The mean interobserver coefficient of variation was approximately 10%.

Statistical analysis

Statistical analysis was performed using the program SPSS for Windows (version 10.0.5, 1999). The two groups were analysed by the nonparametric Wilcoxon signed rank test for paired samples in case of ordinal values and in case of categorical data the 'marginal homogeneity test' was performed.

Results

Of the 13 patients, nine were positive for both methods (eight HDM, one GP). The macroscopic outcome ($n = 13$) of both methods is shown in Table 2. The largest number of positive tests was demonstrated after applying the allergen by the petrolatum method (12 vs 10). One patient had a positive APT for the aqueous

Table 2. Macroscopic outcome

Patient	Allergen	Aqueous	Petrolatum
1	HDM	1+	2+
2	HDM	2+	2+
3	HDM	1+	2+
4	GP	1+	1+
5	HDM	2+	3+
6	HDM	2+	2+
7	HDM	1+	2+
8	HDM	1+	1+
9	HDM	2+	2+
10	GP	0	2+
11	HDM	0	1+
12	HDM	0	1+
13	HDM	1+	0

Reading time of the APT at 48 h after allergen application: 0, no reaction present; 1+, erythema and induration; 2+, erythema, induration and papules; 3+, erythema, induration and papules and vesicles.

method alone (HDM), whereas three showed positive reactions for just the petrolatum method (two HDM, one GP). Furthermore, more pronounced positive reactions were recorded after testing by the petrolatum method ($P < 0.05$). All reactions showed erythema and induration and 12 out of 22 positive reactions also showed papules, resembling lesional skin.

Microscopic evaluation was performed on biopsies of the positive APTs ($n = 18$) of patients who showed a positive reaction upon testing with either method ($n = 9$). All sections resembled lesional AEDS, showing hyperkeratosis and spongiosis of the epidermis and a cellular influx consisting of predominantly T-cells and eosinophils (see Fig. 1). Histological evaluation showed eosinophils in all sections. Although large individual differences were recorded, no significant difference was found in the number of eosinophils ($P = 0.77$) between the two tested methods. The mean number of eosinophils in the aqueous vs the petrolatum method was 124 ± 44 vs $153 \pm 41 \text{ mm}^{-2}$ (mean $\pm$ SEM) (Table 3). CD3+ cells were scored semiquantitatively. Large infiltrates were seen in all sections showing no significant differences between the two methods ($P = 0.317$). Neutrophils were not detected in 16 out of the 18 positive APT reactions. In patient 1, some neutrophils were detected in both APT reactions. No differences in neutrophil presence were found between the two methods.

Discussion

The aim of the present study was to evaluate two commonly employed APT methods as *in vivo* models to study allergic inflammation in AEDS. The first method uses an aqueous solution as a vehicle ('aqueous method') and the second petrolatum as a vehicle ('petrolatum method') (25, 26).

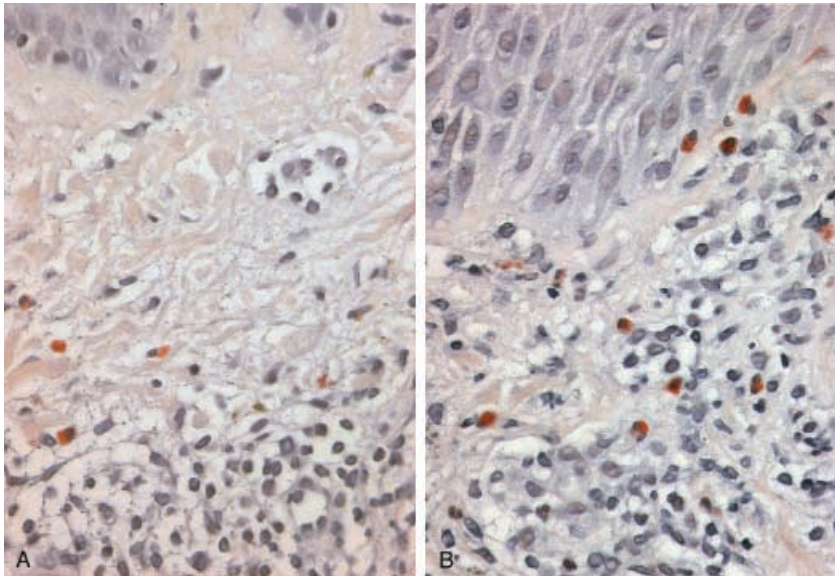


Figure 1. Patient 2: Congo red staining, 400× magnification. (A) Petrolatum method: histology showing a dermal infiltrate consisting of eosinophils, T-cells and no neutrophils. (B) Aqueous method: histology showing a dermal infiltrate consisting of eosinophils, T-cells and no neutrophils.

Table 3. Microscopic evaluation

Patient	Eosinophils		Neutrophils		CD3+ cells	
	Aqueous	Petrolatum	Aqueous	Petrolatum	Aqueous	Petrolatum
1	178	157	+	+	++	++
2	149	48	–	–	++	++
3	398	111	–	–	++	++
4	29	2	–	–	++	++
5	48	310	–	–	++	++
6	242	66	–	–	++	++
7	25	221	–	–	+	++
8	8	98	–	–	+	++
9	41	364	–	–	++	++
Mean ± SEM	124 ± 44	153 ± 41				

Microscopic evaluation of biopsies from positive APTs of the patients who showed a positive reaction to both methods. Eosinophils in count/mm². Neutrophils: –, no neutrophils present/as in normal skin; +, >10 neutrophils/field. CD3+: +, moderate infiltrate of positive cells; ++, large infiltrate of positive cells.

Macroscopic results showed erythema and induration in all cases and in 50% papules, which resemble lesional skin, in both types of application. All control patches with vehicle alone were negative, indicating that vehicle alone did not induce an irritant reaction. This moreover demonstrated the mild effect of the tape stripping done in the aqueous method.

The reactions recorded showed that the petrolatum method induced a higher number of positive reactions and that the reactions were significantly stronger than when the aqueous method was used. A possible explanation for this difference may be the reading time point. Reading time in this study was chosen at 48 h after application, since both methods showed positive

reactions at 48 h in previous studies (25, 34). However, the aqueous method has been previously shown to display a positive reaction at only 24 h which in most cases persists until 48 h or even 72 h, but in some cases has disappeared by 48 h (7, 25). This is in contrast to the petrolatum method, which shows a peak severity of the APT reaction at 48 h (26). Therefore, the reaction of the aqueous method recorded in this study could have been past its maximum and subsequently less pronounced or even absent at 48 h. Another explanation may be the allergen concentration used. The two types of application use different methods to identify the allergen concentration (index of reactivity vs allergy units). Therefore, a difference in allergenic capacity may be responsible for the stronger reaction recorded in the petrolatum method. Histological evaluation of both methods showed typical morphological characteristics of eczema with acanthosis, hyperkeratosis, spongiosis of the epidermis and a cellular influx consisting of eosinophils and T-cells. The average number of eosinophils counted in both methods did not differ significantly, although large individual variations were observed. The presence of eosinophils in all cases indicates that an allergic reaction occurs during the APT reaction. Moderate to mostly large T-cell infiltrates were seen in all positive reactions resembling lesional skin.

In eight to nine patients, no influx of neutrophils was detected in the two methods studied. In patient 1, a minor influx of neutrophils was seen. An influx of neutrophils can be the result of irritation or bacterial infection. However, no clinical signs of infection were seen at the tested site in the patient. In addition, control patch test reactions were negative, indicating that no irritation reaction due to occlusion of the vehicle had occurred. The overall low

number of neutrophils is in concordance with the histology of lesional AEDS-skin, where neutrophils are also sparsely present. This is in contrast to the late phase reaction after intracutaneous challenge where neutrophils are present in the infiltrate, possibly due to traumatic disruption or the direct degranulation of mast cells (16).

Following our data, both aqueous and petrolatum methods can be used as an *in vivo* model to study allergic inflammation in AEDS. The choice of the method mostly depends on the experience of the investigator. The petrolatum method is easier to perform; no pretreatment of the skin is needed and reactions are macroscopically more pronounced than the aqueous method. However, it is important to realize that large differences exist between reactions with different allergen extracts dissolved in petrolatum of different suppliers (35). The advantage of the aqueous method is that this method has been investigated more extensively as a pathophysiologic model of AEDS, showing good repro-

ducibility and specificity rates (25). It is stressed that allergen sources other than the currently described have to be compared in future before the same conclusions can be drawn.

Summarizing, the reactions to both APT methods studied show great similarity to lesional AEDS skin, macroscopically showing erythema, induration and papules. Furthermore, microscopically the dermal infiltrate consisted predominantly of T-cells and eosinophils with hardly any neutrophils, resembling lesional skin. In conclusion, both methods can be used as an *in vivo* model to study allergic inflammation in AEDS.

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