Prevention of Cutaneous Penetration and CD1c+ Uptake of Pollen Allergens by a Barrier-Enhancing Formulation

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Key Words

Laser scanning microscopy · Magnetic activated cell sorting · Atopic dermatitis · Skin barrier function

Abstract

Recent studies have shown that pollen proteins can penetrate the impaired skin barrier of atopic patients and exacerbate their disease. In the presented study the effect of a topically applied barrier-enhancing formulation was investigated for its preventive effect on the uptake of pollen allergens into CD1c+ epidermal cells. The pollen proteins were fluorescence labelled and applied on barrier-disrupted excised human skin. CD1c+ cells were selected after magnetic cell sorting and analysed using laser scanning microscopy. In untreated disrupted skin, 81% of the CD1c+ cells contained the fluorescence-labelled pollen allergens. In formulation-pretreated skin only 12% of the CD1c+ cells showed an uptake of pollen allergens. These results encourage the treatment of atopic patients with barrier-enhancing formulations to reduce the impact of pollen on air-exposed skin areas and hence the exacerbation of cutaneous symptoms.

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Introduction

Atopic dermatitis (AD) is a common skin disease with impaired skin barrier function [1, 2]. In previous studies it was found that pollen allergens may enhance AD [3, 4]. On a European scale, birch and grass pollen are the most dominant airborne pollen [5]. Recently, it was demonstrated that controlled exposure to airborne allergens of patients with extrinsic IgE-mediated AD induced a worsening of cutaneous symptoms [6]. Due to a filaggrin mutation, patients with AD have an impaired barrier function that allows proteins to penetrate into the viable epidermis, contrary to healthy epidermis that prevents allergen penetration beyond the stratum corneum [4, 7]. Furthermore, Nakamura et al. [8] demonstrated that allergens themselves can disrupt the barrier function of the skin by their proteolytic activity in vivo. Airborne proteins can cause increased local inflammation, itch sensation and IgE binding through their proteolytic activity, their activation of PAR-2 receptors on keratinocytes and unmyelinated C fibres, and their ability to act as an allergen via type I IgE-mediated hypersensitivity, as shown by Hostetler et al. [9]. Langerhans cells are considered to play a major role in IgE-mediated allergy; the skin of patients with AD exhibits an increased amount of Langer-

hans cells expressing the high-affinity IgE receptor FcεRI, contrary to skin not affected by AD [10].

In this context, hair follicles are discussed to play an important role as they are surrounded by a dense network of Langerhans cells, and stem cells are located in the bulge region of the hair follicle [11]. Previous investigations have shown that the penetration of pollen allergens into the hair follicles was significantly reduced when the skin had been treated with commercial care products [12]. Furthermore, previous studies revealed that pollen allergens are capable of passing through the skin barrier and reaching the Langerhans cells once the skin barrier is disrupted [7].

Therefore, the present study aimed at investigating whether skin care products can prevent the penetration of pollen allergens not only into the hair follicles but also into the viable epidermis. For this purpose, the uptake of topically applied pollen allergens by the Langerhans cells was investigated on barrier-disrupted untreated and lotion-pretreated excised human skin samples, respectively. The pollen allergens were labelled with a fluorescent dye and, hence, detectable by laser scanning microscopy.

Materials and Methods

Labelling of Pollen Allergens

Grass pollen allergens were labelled with a fluorescent dye as described in detail by Jacobi et al. [13]. Ampoules with the extract of grass pollen (Gräsermischung; ALK-Scherax, Hamburg, Germany) were dissolved in 1 ml PBS buffer (Sigma-Aldrich Chemie GmbH, Geisenhofen, Germany) and a 10-mM solution of fluorescein isothiocyanate (FITC; Sigma-Aldrich Chemie GmbH) was added to the pollen extract. This mixture was allowed to incubate in the dark for 2 h at 4°C. Subsequently, the FITC-labelled pollen protein was purified using membrane dialysis (Spectra/Por®, MWCO 6–8000; Theodor Karow GmbH, Berlin, Germany). An excess of L-alanyl-L-glutamine (Dipeptamin®, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) was added to the FITC-labelled protein and the dialysate in order to inactivate the bonded fluorescent dye. Both solutions were incubated for 2 h at 4°C. Aliquots were dried at 30°C using a lyophilisator (Concentrator 5301; Eppendorf, Hamburg, Germany) and lyophilisates were stored at –20°C in the dark. For application onto the excised human skin, one aliquot of the lyophilisates (450,000 SQ units) was dissolved in 240 µl phosphate buffer solution, resulting in a concentration of 1,875 SQ units/µl.

Applied Formulation

Eucerin® pH5 Lotion F (Beiersdorf AG, Hamburg, Germany) was used as a barrier-enhancing lotion. Based on a water-in-oil emulsion, it contained glycerine, panthenol, sodium citrate, and citric acid as active ingredients. The formulation had been tested on patients exhibiting sensitive skin associated with an atopic diathesis and was found to be suitable for specific, barrier-invigorating skin care in these subjects [14].

Skin Samples

The human skin samples, which were obtained from healthy non-atopic volunteers undergoing plastic surgery, were used within 48 h after excision. The tissue sampling was approved by the institutional Ethics Committee of the Charité – Universitätsmedizin Berlin in accordance with the rules stated in the Declaration of Helsinki principles.

Application of Allergens and Lotion

The excised skin was carefully cleaned with some buffered saline, and the subcutis was separated from the cutis. The skin was checked for scratches or lesions and fixed onto a board. An application area of 16 cm² was delineated with a skin surface marker on each skin sample. Subsequently, cyanoacrylate skin surface stripping was performed using superglue (UHU GmbH; Bühl/Baden, Germany). A drop of cyanoacrylate was applied onto the skin surface and an adhesive tape was fixed on top of it [15]. By stripping the tape, the hairs and approximately 30% of the stratum corneum and the follicular casts were removed. Afterwards the barrier-enhancing formulation was applied by a massage with a PC60 Petra Electric massage device (Petra Electric GmbH, Günzburg, Germany) to half of the skin samples (2 mg/cm²) and the skin samples were incubated for 1 h. To avoid the spreading of the formulation a fence was built before application. Onto the treated and untreated skin samples, 10 µl/cm² of pollen allergens in PBS were applied and incubated for 15–16 h at 37°C in a humidified chamber. Skin samples from 6 different donors were investigated.

Magnetic Activated Cell Sorting: Separation of Epidermal Cells

To separate the epidermal cells from the dermis, magnetic activated cell sorting (MACS) was used. The epidermal cell suspensions were generated as described previously [7, 16]. To separate the epidermis from the dermis, digestion was performed for 3 h at 37°C. The epidermal layers were treated with trypsin (0.025%), 1.5 mM CaCl₂ in PBS and a pH of 7.4 for 10 min at 37°C to isolate the epidermal cells. After re-suspending the cells, MACS separation with anti-BDCA-1 (anti-CD1c) antibodies was performed twice according to the manufacturer's recommendation (Dendritic Cell Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany). This separation of the epidermis from the dermis ensured that the obtained dendritic cells were mainly Langerhans cells.

Laser Scanning Microscopy

The separated MACS epidermal cells were investigated for their uptake of pollen allergens using confocal laser scanning microscopy. For the detection of the FITC-labelled pollen allergens, an excitation wavelength of 488 nm was used. The emission signal was detected cell by cell in the spectral range between 590 and 650 nm. For the determination of the mean intensity of the images, the programme ImageJ was used. To determine the signal of the cells, the signal of the background was subtracted. The background algorithms were evaluated in the range from 0 to 5 in arbitrary units, and the fluorescence in the range between 25 and 60 arbitrary units. For each skin sample, three fields of view were analysed.

Statistics

The results are from related sample tests. SPSS 19.00 for Windows (SPSS Inc., Chicago, Ill., USA) software was used. Data were

Fig. 1. Laser scanning microscopy images in the transmittance mode (**a, c**) and fluorescence mode (**c, d**) of dendritic cells. The cell in **c** shows a clear fluorescence in **d**, whereas for the cell in **a** no fluorescence was found, as demonstrated in **b**. In total, 211 MACS-separated CD1c+ epidermal cells were identified after the application of FITC-labelled pollen allergens to the barrier-disturbed skin that had not been pre-treated with lotion. In 40 cells identified in the transmittance mode, no fluorescence was observed; 171 of the 211 cells showed a clear fluorescence, typical for fluorescein after excitation at 488 nm. The individual relative numbers of cells are given in table 1.

analysed using non-parametric tests. First, the related sample McNemar test was performed. Differences among the means of groups were analysed by the Wilcoxon test, considering a significance of $p < 0.05$.

Results

If the fluorescence-labelled pollen allergens were taken up by CD1c+ they could be identified by laser scanning microscopy in the fluorescence mode (fig. 1). The total number of cells was counted in transmittance mode (fig. 1a, c). The cells containing fluorescence were identified in the fluorescence mode (fig. 1b, d). The individual relative numbers of cells are given in table 1.

From the lotion-pretreated skin samples, in total 181 CD1c+ epidermal cells could be identified, of which 159 did not show any fluorescence and 22 exhibited a clear FITC fluorescence. The results show that the individual skin samples yielded different relative amounts of CD1c+ epidermal cells. The mean values are shown in figure 2.

Table 1. Relative number of CD1c+ cells with and without fluorescence

Subject	Without pretreatment		Pretreated with lotion	
	no FL	FL	no FL	FL
1	25	75	97	3
2	0	100	100	0
3	4	96	84	16
4	19	81	83	17
5	17	83	89	11
6	15	85	82	18

FL = Fluorescence.

The McNemar test showed highly significant differences ($p < 0.001$) between the lotion-pretreated and untreated skin samples. While the untreated samples showed an 81% uptake of pollen allergens in the CD1c+ cells, the lotion-pretreated skin reduced the penetration and uptake to 12%.

Fig. 2. Relative amount of CD1c+ epidermal cells (mean $\pm$ SE) with and without fluorescence counted by laser scanning microscopy after the topical application of fluorescence-labelled pollen allergens on lotion-pretreated and untreated skin.

Discussion

Allergens cannot affect atopic skin unless they penetrate into the viable epidermis. Therefore, it was investigated whether a barrier-enhancing lotion could reduce or prevent the pollen penetration and consecutive uptake by epidermal Langerhans cells. For this purpose we performed experimental analyses of topically applied fluorescence-labelled pollen allergens followed by MACS separation with anti-BDCA-1 (anti-CD1c) antibodies. Because the epidermis was separated from the dermis, most of the isolated CD1c+ cells were Langerhans cells. Nevertheless, the detection of other dendritic cells or keratinocytes cannot be fully excluded.

Experiments using laser scanning microscopy showed that fluorescence-labelled pollen allergens could be detected in CD1c+ cells when the skin barrier was disturbed by cyanoacrylate skin surface stripping and no lotion was applied. This high penetration rate of pollen allergens was strongly reduced when a lotion was applied subsequent to cyanoacrylate skin surface stripping and prior to pollen allergen application. This result corresponds with the investigations of Meinke et al. [12] on porcine ear skin showing that the penetration of pollen allergens into the

hair follicle could be reduced depending on the applied formulation. A lipophilic water-in-oil emulsion was more effective than a hydrophilic oil-in-water formulation. In that study the water-in-oil formulation, applied also in the present experiments, increased the share of protected hair follicles from 4 to 61%. The hair follicles are an important target for allergens as they are surrounded by a dense network of dendritic cells. This finding would be even more pronounced *in vivo*, as the hair follicles are contracted in excised human skin [17]. Additionally, it was demonstrated that the hair follicles represent a long-term reservoir for substances that come into contact with the skin [18]. In contrast to the storage time of topically applied substances in the stratum corneum, the retention time in the hair follicles can extend to 1 week. Hence, there is a considerable risk that allergens penetrating into the hair follicles may be effective there for an extended period of time, thus inducing skin reactions.

A subpopulation of AD patients may have a reaction to common airborne proteins exacerbating their disease. This hypothesis is supported by data of Fölster-Holst et al. [7] who found a deterioration of subjective AD symptoms, especially in uncovered skin areas, during the high-density pollen season and a certain delay in the recovery from symptoms after the pollen burden had ceased. Interestingly, it was recently shown by Dittlein et al. [19] that pollen-induced inflammasome activation in keratinocytes is further enhanced by UVB, implying that the ‘head and neck’ type of AD might not only be due to contact to pollen but further aggravated by sun exposure.

Krämer et al. [20] reported that among 39 children affected by AD, 18 children exhibited more symptoms in summer and especially during days with high grass pollen exposure when the itch was 16% higher and the extent 19% greater. This effect was stronger for children sensitized against pollen.

Such patients would benefit not only from treatments avoiding pollen allergen exposition, regular washing/bathing, textile covering, etc. but also from regular application of barrier-enhancing formulations that reduce the invasion of pollen allergens and improve the skin barrier. Barrier repair is a fundamental therapeutic goal for all patients affected by AD [21–23] and is of particular importance for patients with airborne protein-driven AD as it can reduce the penetration of these proteins. The tested formulation was proven effective in this respect in the presented *in vitro* study.

Conclusion

The results demonstrated that pollen proteins are capable of penetrating impaired skin and reaching the dendritic cells in the living epidermis. In a subpopulation of AD patients this may lead to exacerbating their disease. The application of a barrier-enhancing formulation reduced the penetration of pollen allergens and might therefore reduce the deteriorating effects of airborne pollen allergens in AD patients if applied before going outside and consecutive exposure to pollen allergens.

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Statement of Ethics

The tissue sampling was approved by the institutional Ethics Committee of the Charité – Universitätsmedizin Berlin in accordance with the rules stated in the Declaration of Helsinki principles.

Disclosure Statement

The authors declare no conflicts of interest.