

Penetration of Pollen Proteins into the Skin

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Key Words

Follicular penetration · Confocal laser scanning microscopy · Tape stripping · Atopic dermatitis

Abstract

Atopic dermatitis is a chronic remittent skin disease. In the extrinsic form of atopic dermatitis, type IgE-mediated reactions play an important pathophysiological role. The aim of the present study was to examine whether type I allergens can penetrate into the skin. Therefore, pollen proteins were labeled with fluorescein isothiocyanate (FITC), and their penetration profile was studied qualitatively. Solutions of FITC-labeled pollen proteins were applied in vitro on porcine skin and in vivo on human skin. In vitro, the FITC-labeled proteins were observed within the complete stratum corneum (SC) and inside the hair follicles even 15 min after application. They were also distributed inside the dermis around the hair follicles. In vivo, a similar pattern of distribution within the SC and the hair follicles was observed. These results indicate penetration via the SC lipid layers and a faster penetration via the hair follicles. The FITC-labeled proteins entered the dermis via the follicular pathway. Therefore, the follicular penetration should be considered in the development of skin protection strategies. To evaluate such strategies, the developed method can be used, and further studies in atopic dermatitis patients are necessary to determine whether the penetration of type I allergens is increased.

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Introduction

Atopic dermatitis is a frequently occurring chronic remittent skin disease [1, 2]. Its prevalence has continuously increased during the last decades and is currently estimated to be in the region of 10%. In the extrinsic form of atopic dermatitis, which affects up to 80% of atopic dermatitis patients, type I allergens play an important pathophysiological role [3]. Not only food allergens can lead to an aggravation of atopic dermatitis [4], but also pollen, which may be present in the air dust and can trigger skin disease through penetration into the skin [5]. Such clinical observations have been described and are clinically subgrouped into the airborne type of atopic eczema. Two phases – the sensitization and the induction phase – play a role in the development of type I allergies. Both phases can be started by direct contact of the skin with the according allergen. In a moist environment, pollens swell and release a mixture of proteins at different sizes within seconds [6–9]. Only a small number of these proteins function as allergens [6, 8].

The skin, and mainly the stratum corneum (SC), has an important barrier function and protects the organism against the penetration of exogenous substances [10–13]. Recently, mutations of the filaggrin structure protein have been described in atopic dermatitis patients, which may contribute to a disrupted skin barrier function [14]. Biological macromolecules, such as pollen proteins, cannot easily penetrate through the intact SC entering the

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1660–5527/07/0206–0297\$23.50/0

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epidermis and dermis [15, 16]. However, that a penetration of allergenic structures into the skin may occur is supported by several clinical observations. Erythema and edema can be caused by rub tests [17–19], and exanthematous skin lesions can be induced by the atopic patch test [20–23]. A disturbed skin barrier, as present in atopic dermatitis [15, 24], has been suggested to play a role in the eased penetration of proteins [16]. However, the role of the hair follicles as shunt routes into the skin has not yet been studied extensively [16].

Therefore, the aim of the present study was (1) to visualize the penetration of type I pollen allergens in atopic dermatitis patients into the skin, and (2) to investigate the different penetration pathways including the hair follicles. The experiments were performed with full awareness of the fact that real proteins can change their 3-dimensional structure or undergo biodegradation.

In order to overcome this problem, the used mixture of pollen proteins was labeled with fluorescein isothiocyanate (FITC). This labeling of a mixture of proteins with different and unknown structures occurs under mild conditions. Therefore, at the utmost, a minimal influence of the label on the native structure of the proteins can be expected. Furthermore, the possible biodegradation of the proteins would afford two smaller proteins, one labeled with FITC. In addition, a cleavage of the strong bond between the fluorescent marker and the primary amino group of the protein [25] cannot be expected because the analogous FITC-labeled dextrans have been successfully used for in vivo studies [26]. At least, the fluorescence of FITC can be detected beside the fluorescence of the skin itself [27] using an excitation wavelength of 488 nm and an emission wavelength of ≥ 600 nm.

The penetration of the FITC-labeled pollen proteins was studied in vitro and in vivo using the tape-stripping procedure and microscopic methods. In this manner, the penetration pathways of the proteins were determined qualitatively.

Materials and Methods

Porcine Skin

Porcine ear skin is a frequently used in vitro model for human skin [28–30] showing a similar penetration for topically applied substances [31]. The studies were carried out using the ears of pigs (6-month-old, German domestic pig) slaughtered on the day of the experiments. The hair of the ears was carefully removed with scissors. The ears were washed with water and dried using soft tissue. Approval for these experiments had been obtained from the Veterinary Board of Control, Berlin Treptow-Köpenick.

Human Skin

The experiments, using confocal laser scanning microscopy, were performed on 2 healthy human female volunteers, aged 26 and 30 years. After topical application of the solutions, the volunteers rested without sweating and without covering the test area with textiles. Both volunteers had given signed informed consent. Approval had been obtained for these experiments from the Ethics Committee of the Charité University Hospital.

Labeling of the Pollen Allergens with FITC

An extract of grass pollen (2 ampoules with 1.26 mg proteins; Lieschgraspollen, 450,000 SQ units, ALK Scherax, Hamburg, Germany) was utilized. The extract contains purified allergens with protein sizes from 10 to 100 kDa and was solved in 1 ml PBS buffer (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). A 10-mM solution (2 mg in 1 ml PBS buffer) of FITC (Sigma-Aldrich Chemie GmbH) was added to the solution of the pollen extract. This mixture was incubated during 2 h at 4°C.

Purification of the FITC-labeled pollen proteins was performed using membrane dialysis (Spectra/Por 1, MWCO 6–8000, Theodor Karow GmbH, Berlin, Germany). The dialysis was carried out in 50 ml PBS buffer in the absence of light at 4°C. The PBS buffer was renewed 32 times after each hour and 4 times after 16 h. Absorption of the fluorescein chromophore of the proteins was measured at 430 nm before commencement of the dialysis and regularly during dialysis. The dialysis was finished, if the absorption measured had become constant and was less than 4% of the one measured before the dialysis (after approximately 92 h).

An excess of L-alanyl-L-glutamine (Dipeptamin®, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) was added to the FITC-labeled proteins and the dialysate in order to inactivate FITC not bonded. Both solutions were incubated for 2 h at 4°C.

Both solutions were aliquoted and dried at 30°C using a lyophilisator (Concentrator 5301, Eppendorf, Hamburg, Germany). The dry lyophilisates were stored at 20°C. Each lyophilisate of the pollen proteins contained 63 µg of the original allergens. The lyophilisates of the dialysate were used as control in the experiments.

Protocol of Topical Application

Four areas of 2×2 cm² were marked on the pig ear using a pencil (edding 140 S, OHP-marker permanent, edding, Ahrensburg, Germany). The pig ear was incubated at 37°C (HERA cell, Kendro Laboratory Products, Hanau, Germany) for 30 min before application. Thus, a skin surface temperature of approximately 32°C was realized. The FITC-labeled allergens were applied onto two areas. The control was applied onto the other two areas. The amount of 63 µg allergens was solved in 20 µl PBS buffer and applied using a pipette (10–100 µl; Eppendorf). Therefore, an amount of 15.75 µg pollen proteins was homogeneously distributed per cm² area using a spatula. The control was also solved in 20 µl PBS buffer and applied as described previously. The pig ear was stored at 37°C until the commencement of tape stripping.

Tape Stripping Protocol

After a penetration time of 15 or 45 min at 37°C, the SC was removed using the tape-stripping method as described in detail by Weigmann et al. [32]. The adhesive tape (tesa film No. 5529, Beiersdorf, Hamburg, Germany) was pressed onto the surface of

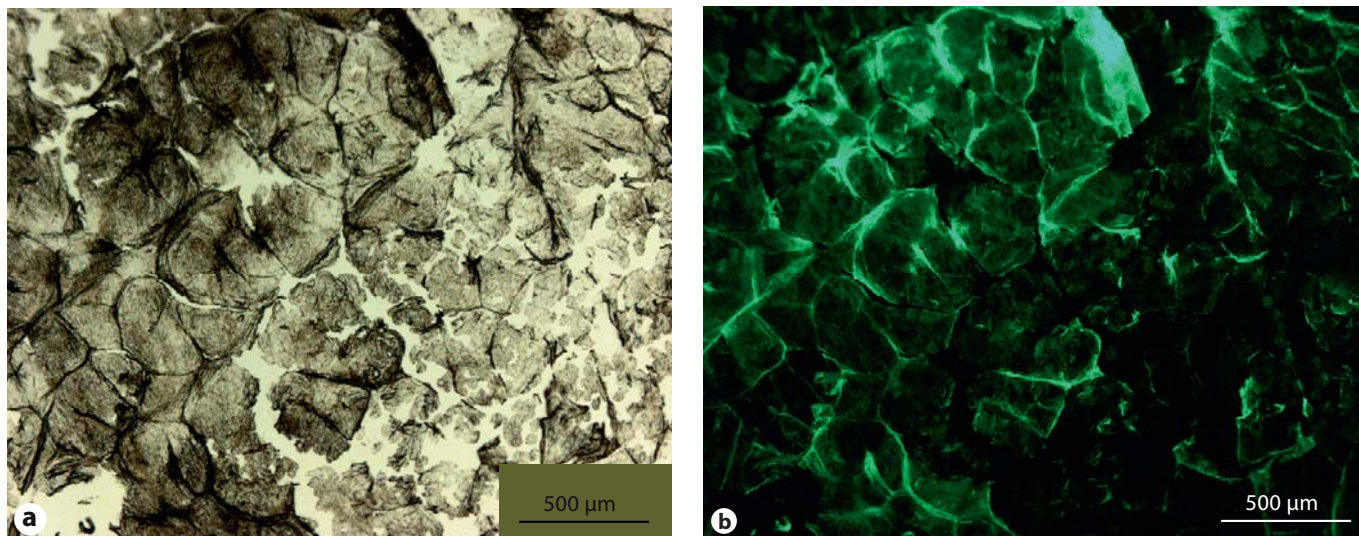


Fig. 1. Microscopic images of a tape strip, removed from porcine skin 15 min after application of the FITC-labeled pollen proteins. **a** Detected in the modus of phase contrast. **b** Detected in the modus of fluorescence.

the skin 10 times with a roller. A piece of paper was placed between the tape and the roller to avoid a transfer of the FITC-labeled proteins from the adjacent skin part to the adhesive film. The tape strip was removed with one quick movement and fixed to a plastic frame. The tape-stripping procedure was performed 50 times on the same skin area.

Determination of the Relative Concentration of FITC-Labeled Proteins within the SC

The area of the tape strips showing a fluorescence signal and covered by corneocytes was determined using fluorescence and light microscopy. A microscope with transmitted light (System microscope BX60, Olympus Optical Co., Hamburg, Germany) was used in the modus of phase contrast (objective UPLFL4X/0.13, Olympus Optical Co.). The modus of phase contrast offers the possibility to determine the covering density of the corneocytes on the removed tape strips as described in detail by Lindemann et al. [33]. The microscope was equipped with a digital camera (SIS ColorView12, Soft Imaging System GmbH, Münster, Germany) and the appropriate software program (analysis AUTO 3.1, Soft Imaging System GmbH). The size of the dark areas covered by the corneocytes (fig. 1a) was calculated using the software program analysis AUTO 3.1.

Then, the same area of the tape strip was studied using the fluorescence modus (filter MNIBA, Olympus Optical Co.). The excitation wavelength was 470–490 nm, while fluorescence was detected at a wavelength >550 nm. The time of measurement was 3 s per image. The size of the green areas shown by fluorescence (fig. 1b) was determined using the software program analysis AUTO 3.1 in the same manner as described previously for the corneocyte density.

Next, the ratio of the fluorescent area to the area covered with the corneocytes was calculated. This is necessary due to the inhomogeneous distribution of the corneocytes on the tape strip inside the small area investigated. These microscopic studies and

calculations were performed on 5 subareas of 3.68 mm^2 inside a total area of 4 cm^2 for each tape strip. An average value of the ratios was calculated for the 5 subareas of the same tape strip. This procedure was performed for all series of tape strips removed.

The ratios obtained for the tape strips removed from skin areas, where the control was applied, reflect both the background fluorescence of the porcine skin as well as the fluorescence of the control. Therefore, these ratios were subtracted from the ratios obtained for the application of the FITC-labeled pollen proteins under the same conditions. The resulting differences were used as relative concentration of the FITC-labeled proteins.

Determination of the Corneocyte Amount Removed

UV/VIS-spectroscopic measurements were performed using a modified double-beam spectrometer (Lambda 20, PerkinElmer, Überlingen, Germany) with a quadratic beam of $1 \times 1 \text{ cm}^2$. These measurements were used to determine the pseudo-absorption of the corneocytes at 430 nm, as a standard value for the amount of corneocytes removed [32, 34, 35]. These values were cumulated reflecting the SC depth from where the tape strips were removed [36, 37]. The removed tape strips were measured using an empty tape as reference.

Determination of the FITC-Labeled Proteins within the Epidermis and Dermis

Slices of the porcine tissue were removed from the areas where the allergen and control solutions were applied for 45 min followed by the SC removal. The tissue was removed parallel to the skin surface using a scalpel. The procedure was started outside the treated areas to avoid a transfer of the FITC-labeled proteins from the adjacent skin part to the stripped area. The slices were fixed on object slides. Images were taken using light and fluorescence microscopy (System microscope BX60, Olympus Optical Co.).

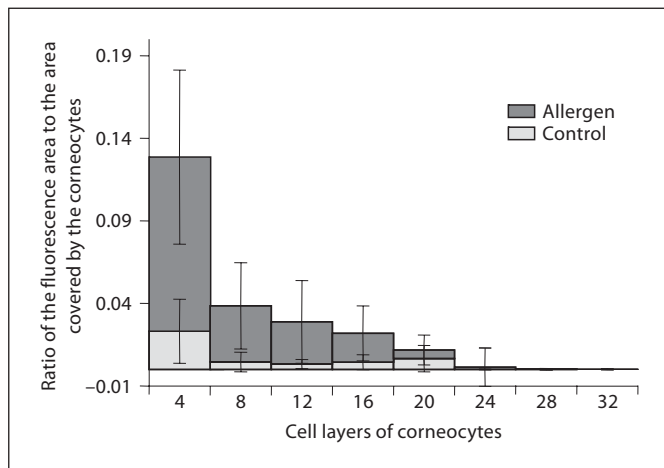


Fig. 2. Distribution of a relative amount of FITC-labeled proteins within the SC, 45 min after application (n = 5).

In vivo Studies Using Confocal Laser Scanning Microscopy

The allergen and control solutions were each applied on 2×2 -cm² areas of the human forehead. The fluorescence was detected 45 min after application of the solutions using a dermatological laser scanning confocal microscope (Stratum, OptiScan Ltd., Melbourne, Australia). The radiation of an Ar⁺ laser at 488 nm [38] was used to excite the topically applied FITC-labeled proteins. Fluorescence was detected at a wavelength of 505–585 nm on a measuring area of $200 \times 200 \mu\text{m}^2$. The skin was scanned in real time.

Statistics

Mean values and standard deviations were calculated using the software program Microsoft® Excel 97. Further statistical analysis was performed with the software program SPSS®. The Wilcoxon test was utilized to analyze the obtained fluorescence values dependent on the SC depth (n = 5, 2-sided, 3 samples, pairwise).

Results

The protein mixture of grass pollen was labeled with the fluorescence marker FITC. The dialysate obtained by dialysis of the FITC-labeled proteins was applied as control solution.

Determination of Pollen Proteins within the SC

The solution of the FITC-labeled proteins in buffer and the dialysate were applied to porcine skin. The procedure of tape stripping was used to remove the SC from both treated skin sites 15 and 45 min after application of the solutions. The area of fluorescence was determined and related to the area covered with corneocytes using a

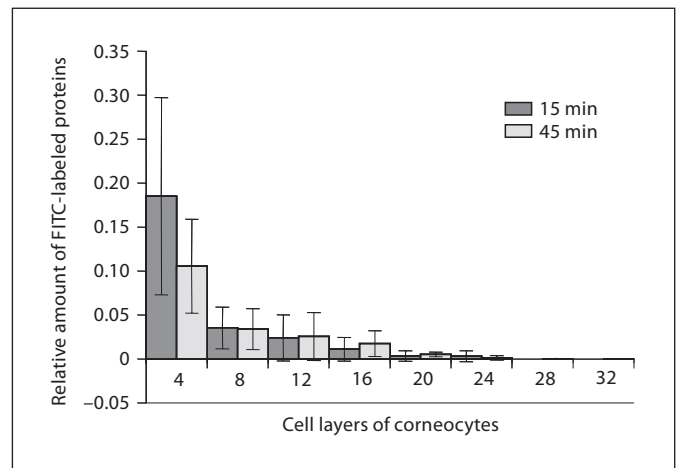


Fig. 3. Distribution of a relative amount of FITC-labeled pollen proteins within the SC, 15 and 45 min after application (n = 5).

fluorescence and light microscope. These ratios were correlated with the SC depth determined via their pseudo-absorption of corneocytes.

In figure 2, the correlation of the mean value of the ratio of the fluorescence area to the area covered with corneocytes is shown for the application of the pollen proteins and the control for 45 min. For the complete SC studied, the ratio was higher, if the solution with the pollen proteins was applied compared with the application of the control. This significant difference is reflected by the value $p < 0.05$. Similar results were obtained when both solutions had been applied 15 min before the tape-stripping procedure. In general, the highest amounts of fluorescence were detected on the surface and in the superficial SC layer, localized around the corneocytes (fig. 1b).

The ratio obtained for the control solution reflects the background signal caused by skin fluorescence and the FITC-labeled dipeptide of the control solution (light areas in fig. 2). The relative concentration of the FITC-labeled pollen proteins alone was calculated as the difference of the ratios obtained for the application of the allergen versus the control solution. The resulting relative concentrations for both times of application (15 and 45 min) are depicted in figure 3. There is a difference in the amount of pollen proteins detected on the skin surface and in the superficial SC layer. The concentration detected 15 min after application is nearly twice as high compared with the concentration detected 45 min after application. For the deeper SC layers, the concentrations were nearly identical ($p > 0.05$).

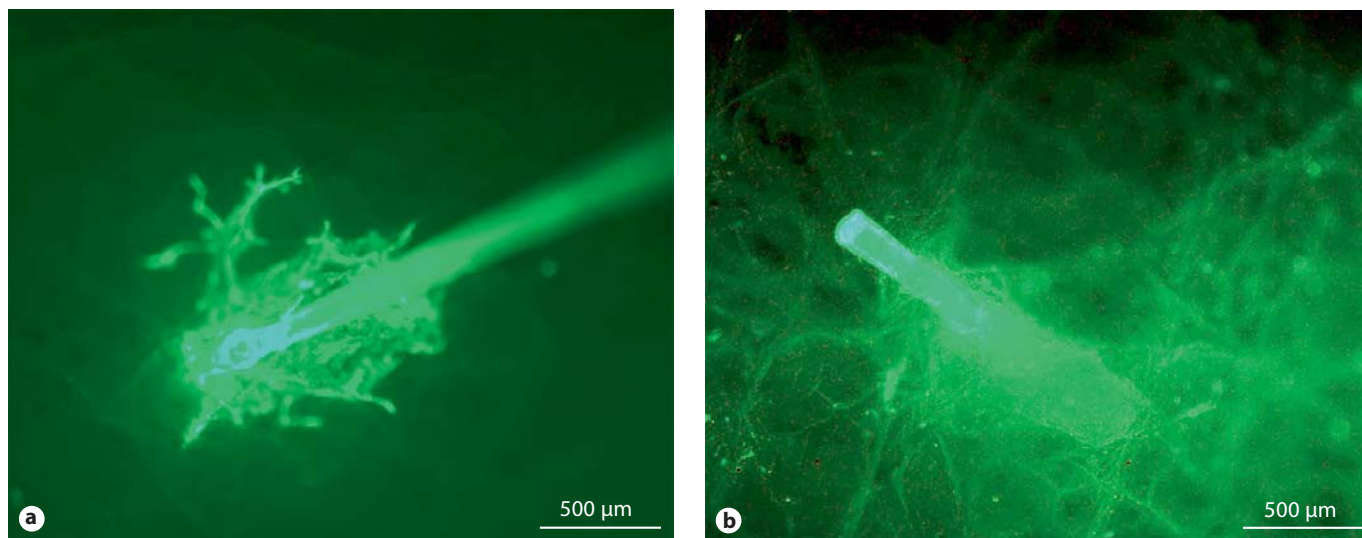


Fig. 4. Fluorescence images of a horizontal slice, removed from porcine skin 45 min after application followed by removal of the SC. **a** After application of the FITC-labeled pollen proteins. **b** After application of the control.

Presence of Pollen Proteins within the Hair Follicles

After removal of the SC, a slice of the tissue with a thickness of approximately 1 mm was removed parallel to the surface using a scalpel. Images of this thin tissue were taken using the light and fluorescence microscope. On the areas treated with the FITC-labeled pollen proteins, nearly 60% of the hair follicles showed a high fluorescence signal (fig. 4a). In addition, the FITC-labeled proteins were distributed inside the dermis around the hair follicle. A weak fluorescence was detected in the hair follicle and around it on the areas treated with the control solution (fig. 4b).

Allergen Penetration in vivo

The solution with FITC-labeled pollen proteins was applied on the forehead of 2 nonatopic human volunteers. The fluorescence of the FITC-labeled proteins was analyzed in vivo using a confocal laser scanning microscope. The proteins were determined around the corneocytes (fig. 5a) and inside the hair follicle (fig. 5b). No fluorescence was detected after application of the control solution. Similar results regarding the distribution of the FITC-labeled proteins around the corneocytes were obtained studying the in vitro model of porcine skin (data not shown).

Discussion

Porcine skin was used as an in vitro model for human skin. It has a similar cutaneous structure and shows a similar penetration behavior for topically applied substances [31]. Under the conditions used for microscopic detection, porcine skin shows a weak fluorescence after application of the control solution. The effect of this fluorescence was avoided by the subtraction of this background from the fluorescence signal obtained after application of the FITC-labeled pollen proteins. The difference obtained was the relative concentration of FITC-labeled proteins.

The allergens were detected in vitro and in vivo within the superficial SC layer but also inside the hair follicles. In the SC, the proteins were located around the corneocytes (fig. 5a) in decreasing amounts with an increasing depth of the SC. This result indicates a penetration via the lipid layers of the SC.

The increase in penetration time from 15 to 45 min results in a smaller amount within the superficial SC layer. This decreasing amount might be caused by an ongoing penetration of the proteins into and through the SC. Otherwise, the reservoir for the proteins within the superficial SC layer could be emptied via the follicles. The content of the follicles cannot be removed by tape stripping [39].

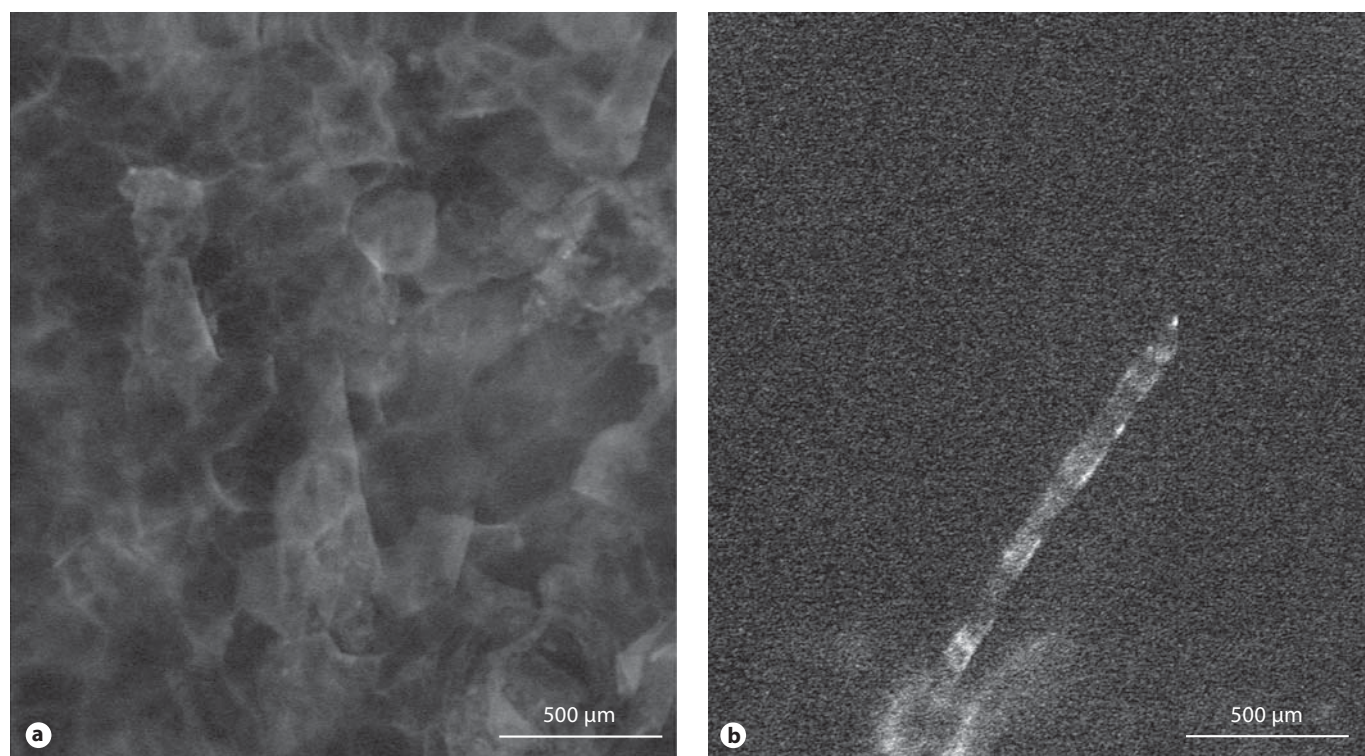


Fig. 5. Fluorescence images of human skin using the confocal laser scanning microscope 45 min after application of the FITC-labeled pollen proteins (forehead). **a** Corneocytes. **b** Hair follicle.

Indeed, a high amount of FITC-labeled proteins was detected in the hair follicles, removed with a thin slice of epidermal and dermal tissue. Nearly 60% of the follicles showed this behavior. Similar results concerning a limited number of follicles with penetrated substance were obtained in *in vivo* studies on humans. There, nearly every tenth follicle showed a penetration for microparticles [39, 40] while the fluorescent dye penetrated into 40–83% of the vellus hair follicles [41]. This phenomenon was related either to hair growth or the sebum production [42]. It is possible that similar processes had caused the selective *in vitro* penetration into the porcine hair follicles. The skin cells were still living under the conditions of postmortal storage used [42, 43]. In addition, the follicular penetration of the FITC-labeled proteins into a hair follicle was also detected *in vivo* in humans (fig. 5b).

A similar behavior of follicular penetration is reported in the literature for topically applied substances. Hair follicles were discussed as an important penetration pathway due to a higher penetration rate into skin with a high density of follicles, compared with a skin site with or without a low density of follicles [44–48]. Penetration

into the follicles seems to be the faster process compared with that via the SC [45, 49]. Recently, the follicular penetration has been determined for organic dyes [41, 42], DNA [50] and topically applied nanoparticles [40].

Presumably, one should distinguish between two main penetration pathways for exogenous substances. The substance can penetrate via the SC, including the corneocytes and the lipid layers, or via the follicles. Skin reactions were observed after contact with substances containing proteins up to a molecular weight of 75 kDa [16, 51, 52]. Bos and Meinardi [53] argue that the molecular weight of a compound must be below 0.5 kDa to allow skin absorption in healthy skin, whereas for atopic skin, also molecules of slightly over 0.8 kDa can be absorbed. This phenomenon is discussed as either due to the skin barrier disruption [15, 24], a cleavage of the proteins by enzymes [16], or both.

In the present study, the follicular route was observed as a pathway for the penetration of applied proteins. The FITC-labeled proteins were detected in the dermal tissue around the hair follicle. This result indicates that the penetration of the proteins into the skin can occur starting

from the hair follicle. This theory correlates with the structure of human skin. The highest number of Langerhans cells is located around the hair follicles [54]. The reaction of an allergenic protein with these cells is the first step in the immune reaction of the body [55, 56]. Indeed, skin reactions associated with protein allergens are often localized in skin areas with a high density of follicles, or around them.

Therefore, protection strategies against protein penetration should include the blocking of the follicular penetration pathway. The efficacy of the applied methods can be studied using the presented method. Otherwise, this pathway could be used for the fast delivery of proteins to the dermis.

Proteins that can function as allergens were detected in the SC and in hair follicles, both in vivo and in vitro. The follicular pathway seems to be faster than the one via the lipid layers. A distribution of the FITC-labeled proteins in the dermal tissue was observed around the hair follicles. For this reason, the follicular penetration of proteins should be considered in the development of protection strategies against allergens or the drug delivery to the dermis.

Acknowledgements

We thank Mrs. Heike Richter and Mrs. Sabine Schanzer for their technical assistance.

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