

Assessment of Epidermal Barrier Function by Photoacoustic Spectrometry in Relation to Its Importance in the Pathogenesis of Atopic Dermatitis

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SUMMARY: With the use of the photoacoustic spectrometry system, in which a mixture of lipid- and water-soluble dyes is applied to the skin and then irradiated with light from a xenon lamp (425 nm and 550 nm), we measured photoacoustic signals of both dyes within the stratum corneum and their disappearance rate through the stratum corneum. The signal intensity was higher and dyes penetrated faster in clinically normal skin of patients with atopic dermatitis (AD) compared with healthy subjects, indicating an impairment of the *in vivo* cutaneous permeability barrier function against both lipophilic and hydrophilic chemicals. Furthermore, penetration rates of the hydrophilic dyes tended to increase in proportion to the severity of AD and significantly correlated with serum IgE levels in the severe AD group. Thus, abnormal barrier functions of clinically normal skin in AD may predispose inflammatory processes evoked by irritants and allergens, especially their water-soluble elements. (*Lab Invest* 2002, 82:1451–1461).

There is a general consensus that disruption of the barrier function of the stratum corneum is an essential etiologic factor for skin inflammation in patients with atopic dermatitis (AD) (Gloor et al, 1981; Ogawa and Yoshiike, 1993; Tupker et al, 1990). Dysfunction of the stratum corneum seems to be associated with the increased permeability and penetration of environmental irritants and allergens in clinically normal AD skin (Cooper, 1994; Deleuran et al, 1998; Fitzharris and Riley, 1999; Gloor et al, 1981; Ogawa and Yoshiike, 1993; Rothe and Grant-Kels, 1996; Tan et al, 1996; Tanaka et al, 1994; Tupker et al, 1990), which predisposes it to inflammation (Broberg et al, 1992; Lammintausta et al, 1993). The impaired barrier function of the stratum corneum in AD has been found to result from the decreased production of ceramides (Imokawa et al, 1991, 1994), which are generated from sphingomyelin by sphingomyelinase (Hara et al, 2000; Higuchi et al, 2000; Murata et al, 1996; Wood et al, 1996). However, the activity of sphingomyelin deacylase, which hydrolyzes sphingomyelin at the acyl site to yield sphingosylphosphorylcholine, is abnormally high in clinically normal and lesional skin in AD (Hara et al, 2000), resulting in the ceramide deficiency (Imokawa et al, 1991). The defect of the barrier function in AD skin has been generally evidenced by an

increased transepidermal water loss (TEWL) measured with an evaporimeter (which evaluates the evaporation rate of water through the skin) (Watanabe et al, 1991). Available data demonstrate a close correlation between the values of TEWL and the penetration of chemicals through the skin *in vitro* (Imokawa, 1993). It should be noted that the diffusion of evaporated water molecules through the stratum corneum represents only a facet of the barrier function and does not necessarily correlate with the diffusion of protein and chemicals that often affect inflammatory processes of AD. Re-evaluation of the barrier function of the stratum corneum on the basis of its *in vivo* permeability against reagents with different water solubilities may provide us further insights into the pathogenesis of AD. However, the lack of adequate instruments has hampered such study.

We have developed a new method, designated here as photoacoustic spectrometry (PAS) (Shioya et al, 2001), in which the percutaneous absorption of chemicals is evaluated by measuring PA signal changes over time. Those signals correspond to the amount of chemicals remaining in or penetrating the stratum corneum at a detection depth of approximately 14 μm . The reliability of this method for the *in vivo* measurement of the permeability of chemicals into the skin of normal individuals has already been established elsewhere (Takamoto et al, 1992, 1994). To ascertain the feasibility of our approach to measure the barrier function, it is important to apply the PAS method to skin with disrupted barrier function such as occurs in AD. In the present study, we determined the barrier function of the stratum corneum in normal individuals

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and in patients with AD. We demonstrate that PAS provides measurements of the barrier function distinct from those related to water evaporation. Comparison of the barrier function in normal individuals and in patients with AD suggests that the abnormal barrier function of clinically normal skin in AD as measured by PAS predisposes the skin to allergic inflammation in response to a variety of environmental irritants and allergens.

Results

The PA Signal, TEWL, and the Water-Holding Capacity in Human Skin

We used both lipophilic (Red 215) and hydrophilic (Yellow 4) dyes as penetrants because different aspects of the barrier function of the skin could be obtained. A time course study demonstrated that, after topical application of each dye solution, the intensity of PA signals obtained with Red 215 dye (lipophilic) or with Yellow 4 dye (hydrophilic) decreased in a time-dependent manner in healthy control skin, with the rate of decrease for the former being less than the latter (Fig. 1A). In clinically normal skin of patients with AD, there was a similar time-dependent decrease in the intensity of PA signals obtained with Red 215 or Yellow 4 (Fig. 1B). However, the rates of decreases for both reagents in clinically normal skin in AD were faster than in the healthy control skin, which suggests an impairment of the permeability barrier function in the clinically normal skin of AD patients. Consistent with our previous report for patients with AD (Imokawa, 1999), clinically normal skin in AD exhibited a significant increase ($p < 0.05$) in TEWL

(Fig. 2A) and a significant decrease ($p < 0.05$) in the water-holding capacity compared with healthy controls (Fig. 2B).

Distribution of Dyes Penetrating the Stratum Corneum

We then determined the distribution and penetration rate of dyes within the stratum corneum in two ways. First, after the dye solution was applied to the forearm skin for 2 minutes, the PA signal was measured at each of 15 tape stripping steps. In the healthy control and in the clinically normal skin in AD, the PA signals for both dyes decreased in proportion to the number of tape strippings. However, the signals became undetectable after 9 tape strippings in the healthy controls (Fig. 3A) but were still detectable even after 13 tape strippings in AD (Fig. 3B). In addition, the PA signal for Yellow 4 before tape stripping was higher in the clinically normal skin in AD than in the healthy control. Next, PAS analysis after application of dyes for 2 or 5 minutes under the closed patch condition revealed that there was a significant increase in the PA signals under both application conditions for the lipophilic (Red 215) dye ($n = 100-103$, $p < 0.001$; Fig. 4, A and B) and for the hydrophilic (Yellow 4) dye ($n = 100-103$, $p < 0.001$; Fig. 4, C and D) through the stratum corneum in clinically normal skin in AD compared with healthy controls ($n = 10$). These data indicate that the PA signals measured in this study reflect concentrations of dyes penetrating the stratum corneum and that the clinically normal skin of patients with AD has accelerated penetration rates for both types of dyes compared with healthy control skin.

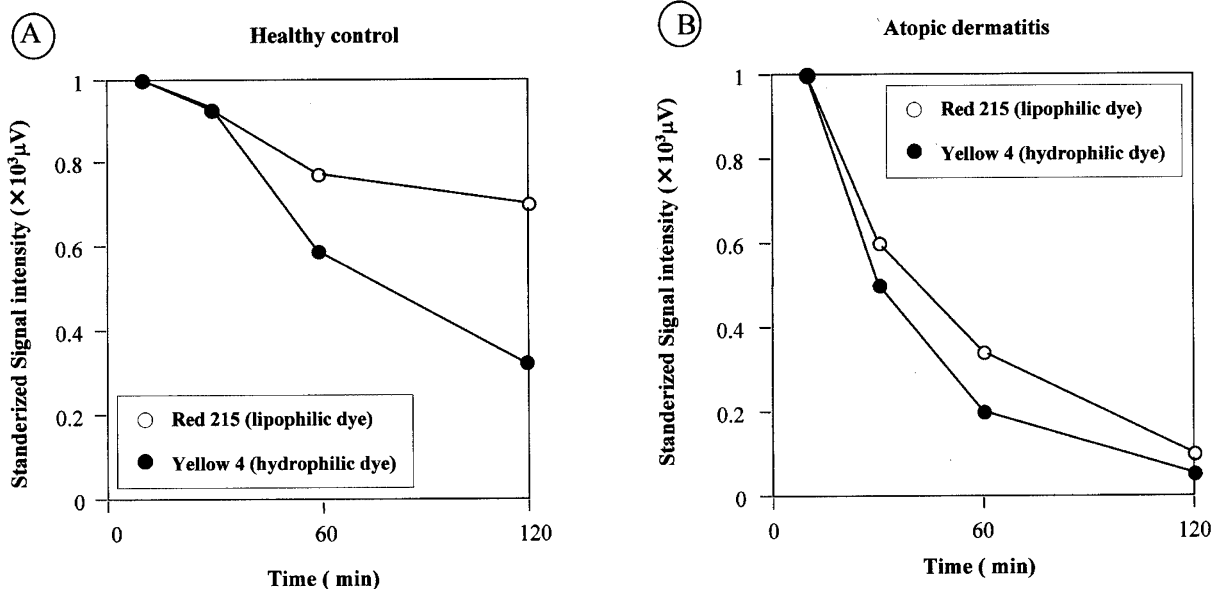


Figure 1.

Penetration profile of lipophilic and hydrophilic dyes through the stratum corneum of forearm skin. Red 215 and Yellow 4 were solubilized in 95% ethanol and 5% water at concentrations of 0.22 and 0.15 mM, respectively. Each solution (64 μ l) was topically applied to the forearm skin. Thirty seconds later, the portable open-ended photoacoustic (PA) cell was attached to the skin surface of the treated-sites for subsequent measurements. A, Healthy control subject (28 years old, male). B, Patient with atopic dermatitis (AD) (26 years old, female).

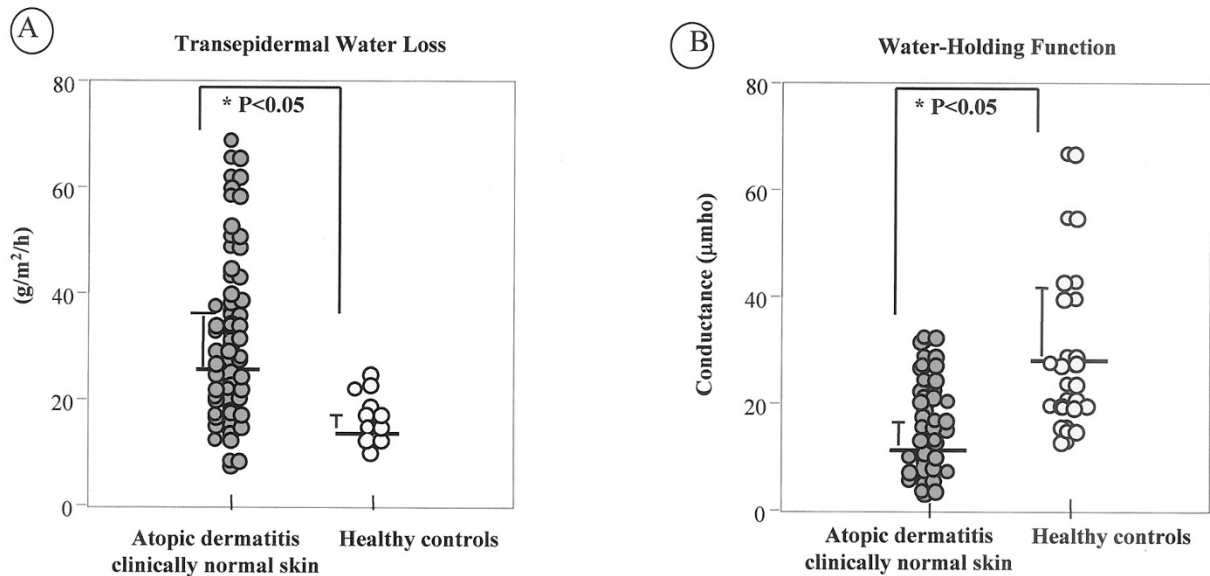


Figure 2.

Transepidermal water loss (TEWL) (A) and water-holding function (B) of the uninvolved skin of patients with AD. TEWL from the skin was measured in AD patients ($n = 104$, average age = 26.7 years for AD; $n = 18$, average age = 34 years for control) by detecting evaporated water according to a modified method using a crystal vibration microbalance system (Endo et al, 2001). The water-holding function was assessed by an impedance meter ($n = 86$, average age = 28 years for AD; $n = 16$, average age = 37 years for control).

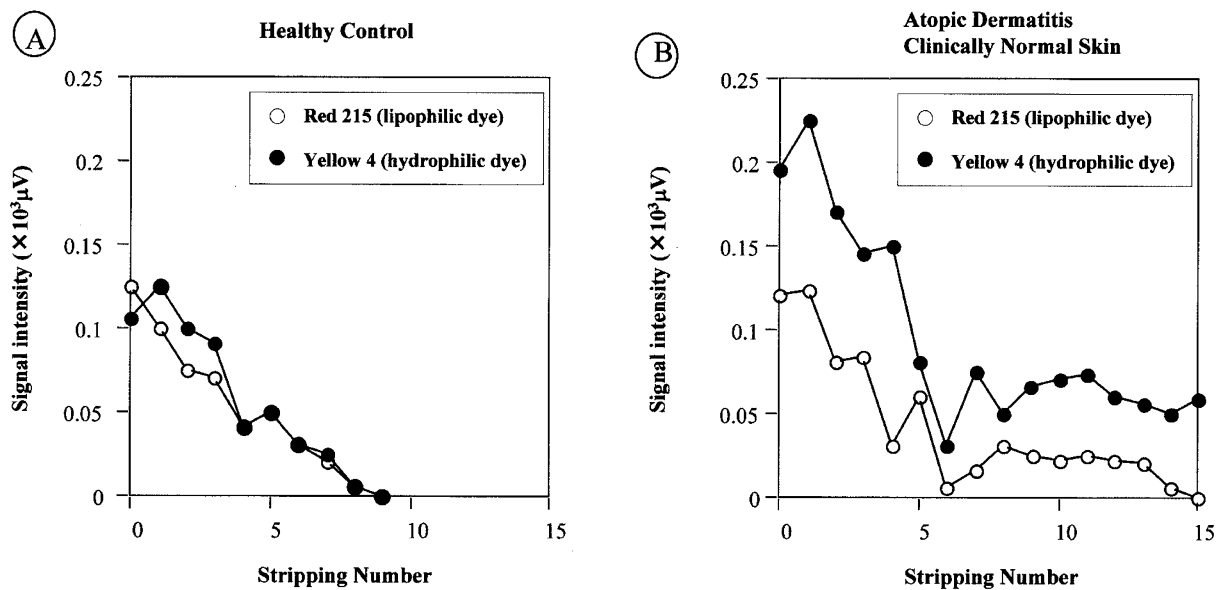


Figure 3.

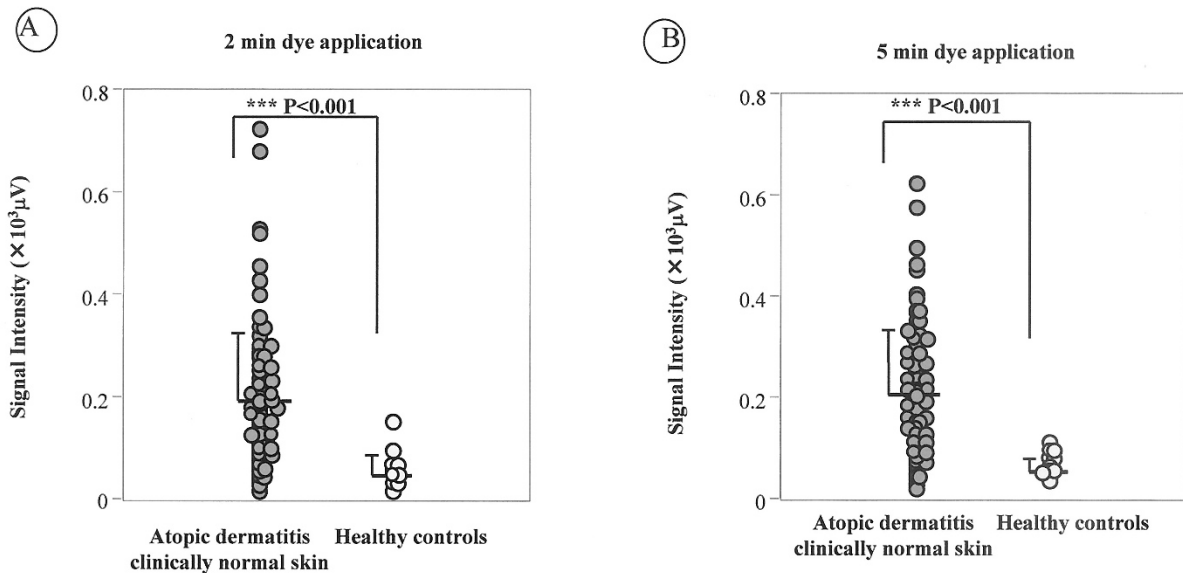
Distribution of dye concentrations penetrating the stratum corneum as assessed by tape stripping. Solutions containing Red 215 and Yellow 4 in 95% ethanol at 0.22 mm and 0.15 mm, respectively, were applied for 2 minutes to the forearm skin. PA signals were measured by the photoacoustic system (PAS) following each step of tape stripping, repeated 15 times. A, Healthy control subject (26 years old, male). B, Patient with AD (24 years old, male).

Relationship Between the PA Signal and TEWL

We determined whether the PA signal (as an indicator of chemical penetration) was correlated with TEWL (as an indicator of water diffusion) in the stratum corneum. In AD there was a very weak correlation between the PA signals with the hydrophilic dye and TEWL values at 2 minutes ($r = 0.309$, $p = 0.002$; Fig. 5A) and between the PA signals with the hydrophilic dye and

TEWL values at 5 minutes ($r = 0.224$, $p = 0.026$; Fig. 5B). In contrast, no correlation existed between the PA signals with the lipophilic dye and TEWL values at 2 minutes ($r = 0.073$, $p = 0.475$; Fig. 5C) or between the PA signals with the lipophilic dye and TEWL values at 5 minutes ($r = 0.086$, $p = 0.401$; Fig. 5D). These data suggest that the penetration behavior of the hydrophilic dye as measured with PAS is a better reflection

Red 215 (lipophilic dye)



Yellow 4 (hydrophilic dye)

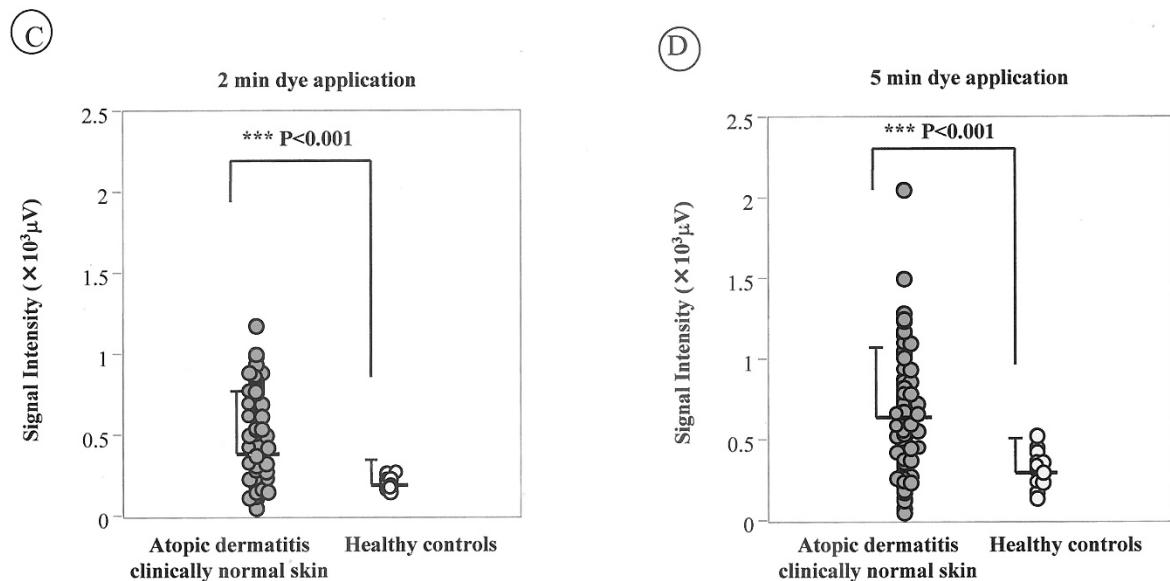


Figure 4.

Penetration rates of lipophilic (A and B) or hydrophilic (C and D) dyes through the stratum corneum. A patch chamber containing 640 μl of the mixed dye solution (Red 215: 0.12 mM, Yellow 4: 0.06 mM) was topically applied for 2 minutes (AD, $n = 103$, average age = 26 years; healthy controls, $n = 10$, average age = 35 years; A and C) or 5 minutes (AD, $n = 100$, average age = 27 years; healthy controls, $n = 10$, average age = 35 years; B and D) to the forearm skin under closed patch test conditions. Thirty seconds after removal of the patch, the portable open-ended PA cell was attached to the skin surface of the treated sites for subsequent measurements of PA signals.

of TEWL and that measurements of the PAS signal of hydrophilic and lipophilic molecules exhibit different facets of the stratum corneum barrier function.

Relationship Between the PA Signals of Dyes Through the Stratum Corneum and the Severity of AD

The penetration of various chemicals through the stratum corneum is distinct in AD skin compared with normal skin. Therefore, we examined the PA signals of

lipophilic and hydrophilic dyes in relation to the severity of AD. The difference between the PA signal of the healthy control group and the mild AD group with the lipophilic dye was significant ($p < 0.05$; Fig. 6, A and B) in contrast to no significant difference with the hydrophilic dye (Fig. 6, C and D). On the other hand, the intensities of the PA signals obtained with the hydrophilic dye differed significantly among the three groups of AD patients with different severity ($p < 0.05$

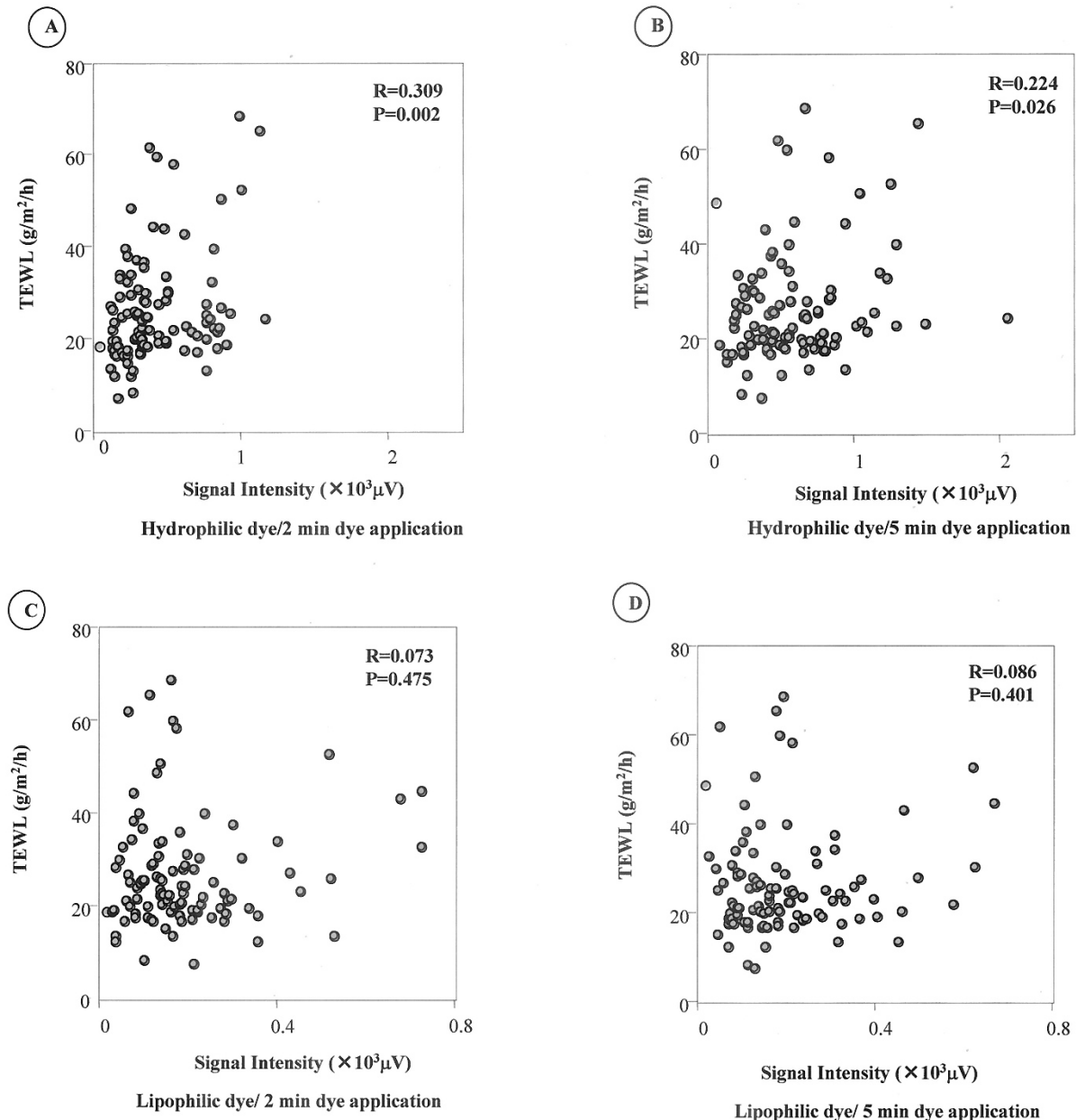


Figure 5.

Relationship between PA signals and TEWL values. PA signals and TEWL were measured as in Figure 4. A, PA signal (at 2 minutes) with the hydrophilic dye/TEWL value (AD, $n = 103$, average age = 26 years). B, PA signal (at 5 minutes) with the hydrophilic dye/TEWL value (AD, $n = 100$, average age = 27 years). C, PA signal (at 2 minutes) with the lipophilic dye/TEWL value (AD, $n = 103$, average age = 26 years). D, PA signal (at 5 minutes) with the lipophilic dye/TEWL value (AD, $n = 100$, average age = 27).

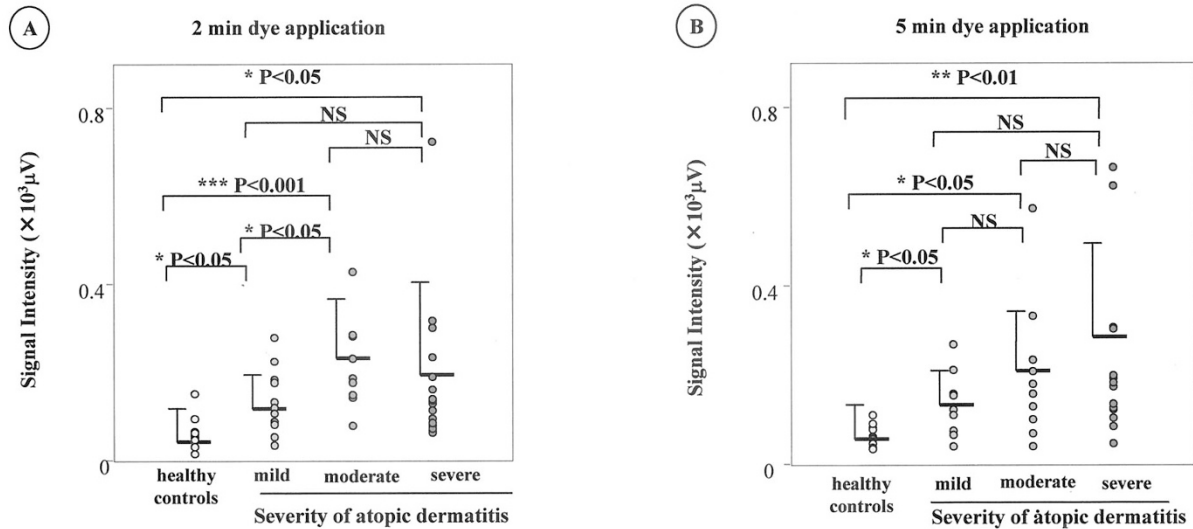
between the mild and moderate groups and between the mild and severe groups at 2 minutes [Fig. 6C] and $p < 0.01$ between the mild and severe groups at 5 minutes [Fig. 6D]). In contrast, there was a significant difference ($p < 0.05$) between only the mild and the moderate groups in the PA signals obtained with the lipophilic dye at 2 minutes (Fig. 6, A). A similar weak correlation for the severity of AD was also observed for TEWL, although there were no significant differences between the mild and moderate groups of AD and healthy controls (Fig. 7). These observations indicate that the signals obtained with the hydrophilic dye are

better suited to evaluate the severity of AD than are the signals obtained with the lipophilic dye.

Relationship Between PA Signals and Serum IgE Levels

Serum IgE levels represent a hallmark of the atopic diathesis, and about 80% of AD patients have increased levels of serum IgE (Leung, 2000). There was a strong correlation ($n = 7$, $r = 0.79$, $p < 0.05$ at 2 minutes; and $n = 7$, $r = 0.92$, $p < 0.005$ at 5 minutes) between the intensity of PA signals obtained with the hydrophilic dye (Yellow 4) and serum IgE levels in the

Red 215 (lipophilic dye)



Yellow 4 (hydrophilic dye)

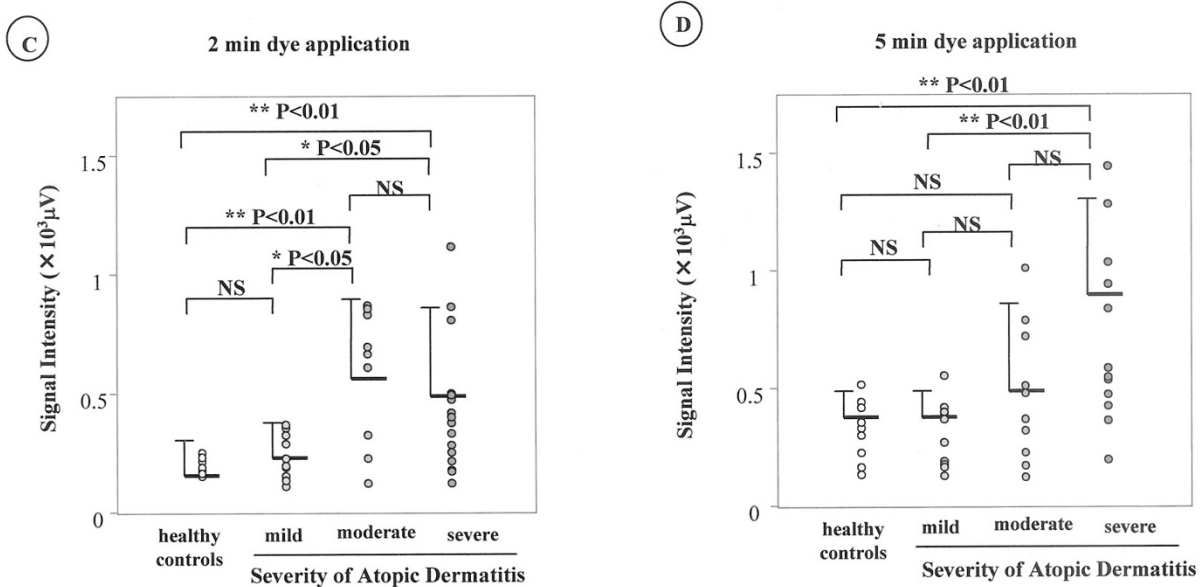


Figure 6.

Relationship between the penetration rates of lipophilic or hydrophilic dyes and the severity of AD. A, Lipophilic dye (Red 215) at 2 minutes (healthy controls, $n = 10$, average age = 35 years; AD mild, $n = 6$, average age = 25 years; AD moderate, $n = 5$, average age = 27 years; AD severe, $n = 10$, average age = 28 years). B, Lipophilic dye (Red 215) at 5 minutes (healthy controls, $n = 10$, average age = 35 years; AD mild, $n = 6$, average age = 25 years; AD moderate, $n = 5$, average age = 27 years; AD severe, $n = 10$, average age = 28 years). C, Hydrophilic dye (Yellow 4) at 2 minutes (healthy controls, $n = 10$, average age = 35 years; AD mild, $n = 6$, average age = 25 years; AD moderate, $n = 5$, average age = 27 years; AD severe, $n = 10$, average age = 28 years). D, Hydrophilic dye (Yellow 4) at 5 minutes (healthy controls, $n = 10$, average age = 35 years; AD mild, $n = 6$, average age = 25 years; AD moderate, $n = 5$, average age = 27 years; AD severe, $n = 10$, average age = 28 years). The penetration rates of lipophilic (A and B) and hydrophilic (C and D) dyes at 2 (A and C) and 5 (B and D) minutes are expressed as the PA signal measured by the PAS after the application of a patch chamber containing a mixed solution of Red 215 and Yellow 4, at a concentration of 0.12 and 0.06 mm, respectively, to the forearm skin.

severe AD group only (Fig. 8, A and B). In contrast, the intensity of PA signals with the lipophilic dye did not correlate with serum IgE levels in this group (data not shown). The mild and moderate AD groups did not show any correlation between the stratum corneum function and serum IgE levels (data not shown).

Discussion

In this study, we applied the PAS method for the first time to clarify the status of the in vivo skin barrier functions in AD. The results obtained with the externally applied dyes having different water solubility

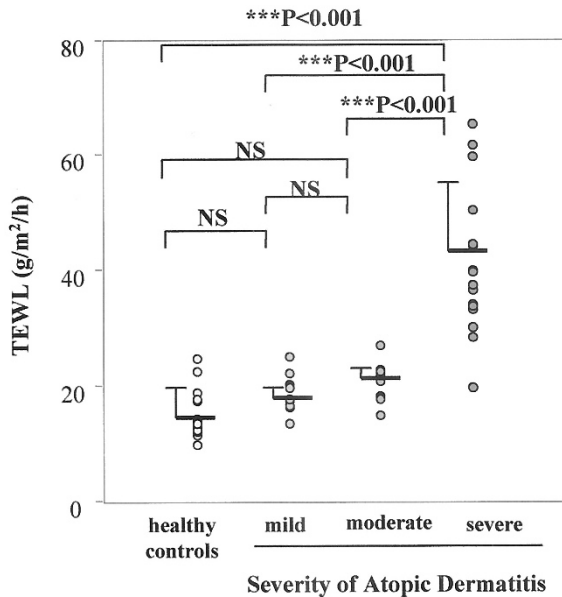


Figure 7.

Relationship between TEWL values and the severity of AD. TEWL from the skin was measured as in Figure 6 (healthy controls, $n = 10$, average age = 35 years; AD mild, $n = 6$, average age = 25 years; AD moderate, $n = 5$, average age = 27 years; AD severe, $n = 10$, average age = 28 years).

demonstrated that the stratum corneum of clinically normal skin in patients with AD had a higher permeability rate compared with that of healthy control skin. Since clinically normal skin in patients with AD has minimal or no inflammation (Wakita et al, 1994), this observation strongly suggests that defects in the permeability barrier function in AD are intrinsic and are not associated with inflammatory events. Although there was a significant increase both in PA signals and

in TEWL values in AD compared with healthy controls, the degree of barrier disruption in AD expressed as a relative ratio to healthy controls was greater with the PAS method (2.98- to 1.8-fold) than with the TEWL measurement (1.73-fold). This indicates that PAS is more useful than TEWL for evaluating the barrier function of skin.

Since AD could be considered a barrier disease in which antigens and irritants that have penetrated (or permeated) the skin trigger and worsen the dermatitis, it is of interest to compare the PA signal for dyes with the severity of AD (according to the criteria of Rajika and Langeland, 1989). Such comparisons revealed that the penetration of the hydrophilic dye through the stratum corneum correlated with the severity grade of AD. In contrast, there was no significant difference in the penetration rate of the lipophilic dye among the three AD groups, thus suggesting that there was no association of lipophilic penetration with the severity of that disease. This may support the notion that molecules with hydrophilic rather than lipophilic properties predominantly elicit and worsen skin inflammation in AD patients. Furthermore, the severity of disease seems to be determined in part by the amounts of irritants and allergens that have penetrated the stratum corneum. This is consistent with the clinical evidence that patients with severe AD have higher levels of IgE specific for house dust and mite antigens (Uehara, 1986). In those antigens, proteins are presumed to be solubilized by sweat and to then easily penetrate the stratum corneum in patients with AD. There is a large published literature assigning house dust or mite antigens, and metals, as possible pathogenic allergens and accelerators, respectively, for AD (Cooper, 1994; Deleuran et al, 1998; Fitzharris and

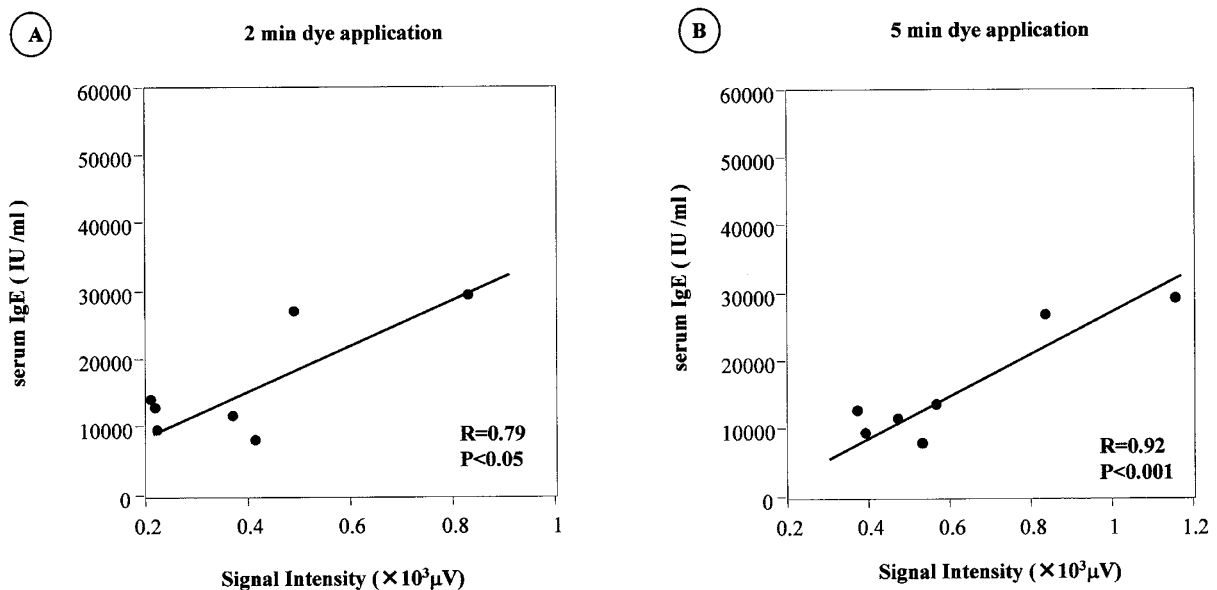


Figure 8.

Correlation between the penetration rates of the hydrophilic dye (Yellow 4) and serum IgE Levels. The penetration rates of the hydrophilic dye at 2 (A) and 5 (B) minutes is expressed as in Figure 4. Serum IgE levels were measured by ELISA using peripheral blood of patients with AD. Severe AD group, $n = 7$, average age = 27 years.

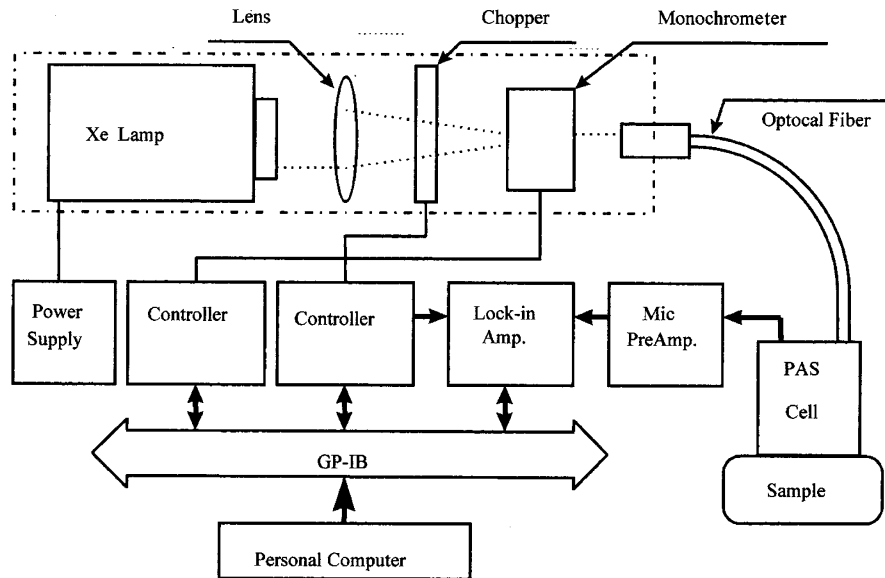


Figure 9.

Diagram of the PAS with an open-ended PA cell. The PAS consists of an Xe lamp, lamp housing, power supply, lens, chopper/wheel, chopper controller, monochromator/grating, monochromator controller, optical fiber, lock-in-amp, microphone, microphone pre-amp, and microphone power supply.

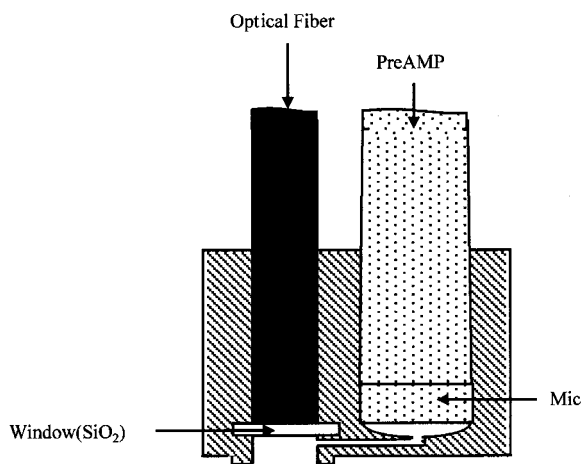


Figure 10.

Diagram of portable open-ended PA cells for in vivo measurement on skin. After application of the portable open-ended PA cell to the forearm skin, the PA signal detected by the cell within approximately a 14- μm depth from the skin surface was analyzed using a lock-in amplifier (NF, 6610A) and was recorded using a chart recorder.

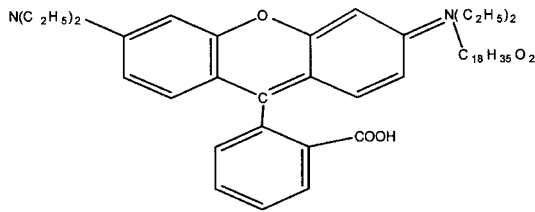
Riley, 1999; Gloor et al, 1981; Ogawa and Yoshiike, 1993; Rothe and Grant-Kels, 1996; Tan et al, 1996; Tanaka et al, 1994; Tupker et al, 1990). In accordance with the evaluation by PAS using the hydrophilic dye, TEWL values were significantly accentuated only in the severe AD group. Our data showed no strong correlations between the PA signals obtained with hydrophilic or with lipophilic dyes and TEWL values in AD patients and healthy controls, which suggests that each value represents a different barrier function against different substances, including water molecules. Furthermore, estimation of the barrier function by TEWL values would provide false results in terms of evaluating the penetration of lipophilic substances.

Collectively, these findings suggest that the barrier function of the skin as assessed with hydrophilic substances reflects the severity of AD, whereas the barrier function against lipophilic substances might provide a diagnostic aid for mild AD.

It has been reported that serum IgE levels correlate with the severity of AD (Beck and Leung, 2000; Leung, 2000; Schafer et al, 1999; Uehara, 1986). The distinct relevance of the hydrophilic barrier function to the severity of AD allowed us to determine whether the PAS signal obtained with the hydrophilic dye is associated with the serum IgE level. Interestingly, we found that there was a close relationship ($r = 0.787$ at 2 minutes, $r = 0.915$ at 5 minutes) between PAS signals and serum IgE levels in the severe AD group, whereas no relationship existed among other parameters, including TEWL values, in the mild or moderate groups of AD. In a previous study (Kondo et al, 1998), we found that allergic reactions induced percutaneously with mite antigens are dominated by T helper 2-type immunity and lead to increased serum IgE levels in mice. Taken together, the close relationship between the hydrophilic barrier function and the serum IgE level in the severe AD group suggests that AD patients who have profound dysfunction of the hydrophilic barrier may be highly susceptible to percutaneous sensitization by the more water-soluble allergens derived from environmental antigens such as house dust mites. This would then result in increased serum IgE levels as a result of inflammatory reactions specific for observed allergens.

In conclusion, the present study demonstrates that a variety of barrier functions are disclosed by PAS in conjunction with the use of molecules having different water solubility. Furthermore, our data indicate for the first time that the clinically normal skin of patients with

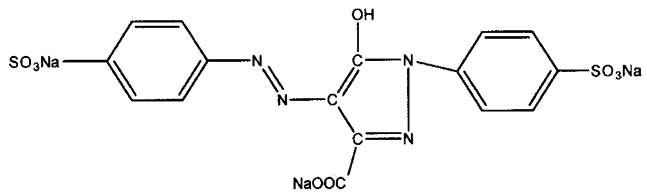
Red 215 (Lipophilic dye)



Molecular Weight 423

Solubility 0.2 % (ethanol)
0.2 % (water)

Yellow 4 (Hydrophilic dye)



Molecular Weight 524

Solubility 0.08 % (ethanol)
0.5 % (water)

Figure 11.

Chemical structure and solubility of Red 215 and Yellow 4.

AD has a potential impairment in the in vivo cutaneous permeability barrier against both lipophilic and hydrophilic reagents. Those abnormal barrier functions may predispose the skin to inflammatory processes evoked by irritants and allergens in AD.

Methods

Diagnosis

A total of 27 patients with AD (aged 19–44 years; 10 men, 17 women), and 18 healthy non-AD volunteers with low serum IgE levels (less than 250 U/ml) (aged 20–56 years; 7 men, 11 women) were enrolled in this study. The diagnosis of AD was made according to the criteria of Hanifin and Rajika (1989). The severity of AD was evaluated by the criteria reported by Rajika and Langeland (1989) and was classified into mild, moderate, or severe. Serum IgE levels of the patients ranged from 7670 to 53300 IU/ml and averaged 18254 IU/ml. All patients were referred by previous doctors who prescribed their treatment schedules. None had taken oral steroids, and none had evident systemic disease. Measurements in this study were performed after informed consent had been obtained.

Measurement Sites of the Skin

The volar surface of forearm skin was principally subjected to all measurements. In AD, clinically normal areas on the volar site were chosen to measure the PA signal, TEWL, and conductance. The patients did not apply any ointment or cream on the examined sites for at least 5 days before the measurements. Concerning

the inflammatory state at the clinically normal tested sites, we have previously demonstrated that clinically normal skin from patients with AD has no histologic evidence of inflammation (Wakita et al, 1994), suggesting that in the current study, no substantial inflammatory changes were present in the nonlesional skin.

TEWL and Water-Holding Property

TEWL was measured by detecting evaporated water according to a modified method (Gfesser et al, 1996; Nilsson, 1977) using a crystal vibration microbalance system (Endo et al, 2001) and is expressed as g/m²/hr. The amount of skin surface water was evaluated by an impedance meter (Werner, 1986; Yoshizuka et al, 1983) and is expressed as conductance (μ mho).

PAS

A PAS with an open-ended PA cell (Takamoto et al, 1992, 1994) was established in our laboratories (Shioya et al, 2001), as shown in Figure 9. The system consists of an Xe lamp (L2274; Hamamatsu Photonics, Hamamatsu, Japan); lamp housing (no. 66005; Oriel, Stratford, Connecticut); power supply (no. 68805; Oriel); lens (no. 66xxx, 3 inch F0.7; Oriel); chopper/wheel (no. 75152/no. 75166; Oriel); chopper controller (no. 70103; Oriel); monochromator/grating (no. 77298/no. 77919), monochromator controller (no. 77325, no. 20050), optical fiber (OF160-1.5 type; Tokyo Instruments); lock-in-amp (5610B; NF circuit block); microphone (4166; B&K, Alleroed, Denmark); microphone pre-amp (4166; B&K); and microphone power supply (5935; B&K). A diagram of the portable

open-ended PA cell for in vivo measurements of the skin is shown in Figure 10. After the portable open-ended PA cell was attached to each measurement site, the PA signal detected by the cell (within approximately 14- μ m depth from the skin surface) was analyzed using a lock-in amplifier (NF, 6610A, Kanagawa, Japan) and was recorded using a chart recorder. The wavelength of the light source was adjusted to 550 for Red 215 and to 425 nm for Yellow 4. The PA signal is expressed as microvolts (μ V) for the short time measurement study or as a standardized signal intensity for the time course study calculated as follows: standardized signal intensity = observed signal intensity – signal intensity before application/initial signal intensity – signal intensity before application.

Sample Application and Measurements

We used Red 215 and Yellow 4 (chemical structures are depicted in Fig. 11) as reagents for the penetration measurement, which are lipophilic and hydrophilic, respectively. Red 215 and Yellow 4 were solubilized in a solution of 95% ethanol and 5% water at concentrations of 0.18 to 0.23 and 0.064 to 0.150 mM, respectively. For the time course study, each solution containing Red 215 at 0.22 mM and Yellow 4 at 0.15 mM was topically applied to the forearm skin. For the short time measurement study, a patch chamber containing 640 μ l of the solution (containing both Red 215 at 0.118 mM and Yellow 4 at 0.06 mM) was topically applied for 2 or 5 minutes to the forearm skin under closed patch test conditions. The patches were removed before measurements were taken.

For determining the distribution of dyes within the stratum corneum, 95% ethanol solutions containing Red 215 at 0.22 mM and Yellow 4 at 0.15 mM were applied for 2 minutes to the forearm. Measurements were taken after each step of tape stripping that was repeated 15 times. For measurements, excess solution was wiped off with a towel, and 30 seconds later, the portable open-ended PA cell was attached to the dye-treated skin surface.

Statistics

The level of significance of differences was calculated by the Student's *t* test.

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