Stratum corneum biomarkers for inflammatory skin diseases
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CHAPTER 3

Biomarkers for contact dermatitis, experimental studies
3.1

Effect of allergens and irritants on levels of NMF and corneocyte morphology

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ABSTRACT

Background: The irritant sodium lauryl sulfate (SLS) is known to cause a decrease in the stratum corneum level of natural moisturizing factor (NMF), which in itself is associated with changes in corneocyte surface topography.

Objective: To explore this phenomenon in allergic contact dermatitis.

Methods: Patch testing was performed on patients with previously positive patch test reactions to potassium dichromate (Cr), nickel sulfate (Ni), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI), or p-phenylenediamine. Moreover, a control (pet.) patch and an irritant (SLS) patch were applied. After 3 days, the stratum corneum from tested sites was collected, and NMF levels and corneocyte morphology, expressed as the amount of circular nanosize objects, quantified according to the Dermal Texture Index (DTI), were determined.

Results: Among allergens, only MCI/MI reduced NMF levels significantly, as did SLS. Furthermore, only MCI/MI caused remarkable changes at the microscopic level; the corneocytes were hexagonal-shaped with pronounced cell borders and a smoother surface. The DTI was increased after SLS exposure but not after allergen exposure.

Conclusions: MCI/MI significantly decreased NMF levels, similarly to SLS. The altered corneocyte morphology suggests that skin barrier damage plays a role in the pathogenesis of MCI/MI contact allergy. The DTI seems to differentiate reactions to SLS from those to the allergens tested, as SLS was the only agent that caused a DTI increase.
INTRODUCTION

Allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) are common inflammatory skin diseases that pose a major problem in public health because of the widespread use of skin irritants and/or contact allergens in occupational settings and in consumer products. Although these two forms of contact dermatitis have different pathogenesis, they show similar clinical features, including erythema, fissuring, and vesicles, and, in a more severe form, bullae (1, 2). The primary step in the development of ICD is, for most skin irritants, characterized by disruption of the skin barrier, which is followed by activation of the innate immune system without involvement of T cells (3). A genetic deficiency of the epidermal protein profilaggrin is a strong predisposing factor for ICD (4, 5). The odds ratio (OR) for filaggrin gene (FLG) mutations, adjusted for atopic dermatitis (AD), was 1.61, whereas individuals with a history of AD who are also carriers of an FLG mutation have a 4.7-fold risk for ICD (6). A history of AD increases the risk for ICD threefold. It has to be noted, however, that AD patients without FLG mutations also have reduced filaggrin expression caused by T helper 2-mediated inflammation in AD (7). Filaggrin and its degradation products, which are the main constituents of natural moisturizing factor (NMF), are responsible for a number of functions concerning skin barrier function in the stratum corneum, including mechanical properties, skin hydration, and the epidermal inflammatory response (8). Recently, it has been shown that various skin irritants significantly reduce the levels of NMF (3). NMF levels, in turn, showed a strong association with corneocyte surface morphology, expressed as the Dermal Texture Index (DTI), supporting the view that alterations in the skin barrier play a major role in ICD (9).

The effect of contact allergens on the skin barrier has not been extensively studied to date, and, if so, it has been mainly assessed with skin bioengineering techniques such as transepidermal water loss measurement (10). However, skin barrier defects arising from concomitant irritant properties of an allergen may play an important role in the activation of the adaptive immune response and the development of ACD (10-13). ACD is a type IV cell-mediated immune reaction, separated into two distinct phases: the sensitization phase, in which the immune system is primed to react to a given allergen (usually molecules with a molecular weight (MW) of <500), and the
elicitation phase, following re-exposure. In this process, the impaired skin barrier may facilitate sensitization in the first place, but also the allergic response as a result of the increased penetration of contact allergens (8, 11, 12). The structural components of the stratum corneum, such as the extracellular lipid matrix, the cornified envelope, and the corneodesmosomes, are primary targets for most irritants (13). Recently, it has been shown that the model irritant sodium lauryl sulfate (SLS) affects the expression of filaggrin (14, 15). Similar morphological changes have also been seen in mice when they are exposed to 2,4,6-trinitro-1-chlorobenzene, a potent contact allergen (16). Studies on the effect of contact allergens on corneocyte morphology in humans are lacking. Therefore, in the present study, we investigated the levels of NMF, the associated filaggrin-degradation enzymes bleomycin hydrolase (BH) and calpain-1 (C-1) and stratum corneum plasmin, as an indicator of skin barrier damage and corneocyte surface topography and morphology (9, 17, 18). We focused on the effects of skin exposure to clinically relevant allergens: potassium dichromate (Cr), nickel sulfate (Ni), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI), and p-phenylenediamine (PPD), and to the model irritant SLS.

METHODS

Patients
The database of the dermatological outpatient clinic of the Zagreb University Hospital was screened for individuals with patch reactions (clinically graded according the ESCD/ICDRG guidelines, grading system for patch test reading, as 1+ or 2+) to one of four common contact allergens: Cr, Ni, PPD and MCI/MI (19). Patients with two or more 1+ or 2+ reactions to Cr, Ni, PPD or MCI/MI were preferred as multiple allergens could be tested in one individual. Patients with a 3+ reaction were not selected to avoid severe reactions which might hinder taking of tape strips. Patients with a history of atopic dermatitis were excluded. The experimental protocol followed the Declaration of Helsinki Principles and was approved by the Medical Ethics Committee of the University Hospital Centre Zagreb. Written informed consent was obtained from each participant.
Procedure
All participants were patch tested on the back with 1 or 2 allergens to which the participant had previously shown a 1+ or 2+ patch test reaction, and moreover to SLS and petrolatum. The tested substances were applied in the Van der Bend chambers (Van der Bend, Brielle, The Netherlands), namely, p-phenylenediamine 1% pet., potassium dichromate 0.5% pet. (Almirall Hermal, Reinbeck, Germany), nickel sulfate 5% pet. and Methylchloroisothiazolinone / methylisothiazolinone 3:1 at 0.01% aq. (Smartpractice Europe, Barsbuttel, Germany). To provoke irritant contact dermatitis, patches with 1% and 2% SLS aq. were used. A patch with the vehicle (100% petrolatum) was used as a control. Four identical patch series were applied: two series on the left and two series on right side of the upper back. Two identical series on the left side were for respective D2 and D3 assessment, enabling stripping could be performed on “fresh” non-stripped skin sites. The patches on the right side of the back functioned as a back-up for possible technical failures. On day (D)2 all patches were removed, patch sites were marked and the skin was allowed to rest for 30 min. On D2 and D3, respectively, the SC samples from the skin sites where the duplicate patches had been applied were collected using adhesive tapes (1.5 cm², D-Squame, CuDerm, Dallas, Texas, US)(20). On both D2 and D3, clinical reactions were graded according to ESCD/ICDRG guidelines (19). In total, 8 consecutive tape strips were taken from each patch application site for analysis. Different tapes were used for the various analyses; tape 3 was used for Atomic force microscopy (AFM), tape 4 for proteases and Scanning Electron Microscopy (SEM), and tape 5 for NMF analysis.

NMF and protease activity
NMF was defined as the sum of the concentrations of pyrrolidone carboxylic acid (PCA), urocanic acid and histidine. NMF was determined according to the method described in detail elsewhere (21). Briefly, the fifth tape strip was extracted with 0.5 ml 25% ammonia. The ammonia extract was evaporated and the residue dissolved in 250 µl of water before analysis by high-pressure liquid chromatography (HPLC). The NMF concentration was normalized for the SC protein amount determined with a Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, USA) to compensate for the variable amount of the SC protein on the tape strips. Enzymatic activities of bleomycin hydrolase (BH), calpain-1 (C-1) and plasmin were
determined in eight randomly selected subjects who were positive either for Ni or MCI/MI and their corresponding unpatched and petrolatum test sites. Analysis has previously been described in details by Raj et al. and Voegeli et al. (17, 22-25). Briefly, buffer extracts of the tape strips (250 µl) were combined with fluorogenic peptide substrates (1.25 µl) (BH-like activity: H-Cit-AMC, C1-like activity: Suc-Leu-Leu-Val-Tyr-AMC and for plasmin-like activity: MeOSuc-Ala-Phe-Lys-AMC) and agitated at 1000 rpm at 37°C. The reaction was stopped after 2 h by adding acetic acid (250 µl). The released AMC was quantified using reverse phase HPLC (excitation 354 nm, emission 442 nm) and results were corrected for SC protein content on the tape strips as determined by the 850 nm absorption infrared densitometer SquameScan 850A (Heiland Electronic, Wetzlar, Germany) according to the procedure described elsewhere (24).

Corneocyte morphology
Corneocytes from patients were analysed by AFM as described by Franz et al. (26). Briefly, the third consecutive tape strip was subjected to AFM measurements carried out with a Multimode atomic force microscope equipped with a Nanoscope III controller and software version 5.30sr3 (Digital Instruments, Santa Barbara, CA, USA). Silicon nitride tips on V-shaped gold-coated cantilevers were used (0.01 N/m, MLCT; Veeco, Mannheim, Germany). Imaging was performed at ambient temperature with forces less than 1 nN at one to three scan lines per second (1–3 Hz) with a resolution of 512 × 512 pixels. For texture analysis, subcellular scan areas of 20 × 20 µm² were recorded. For a larger overview, images of 70 × 70 µm² were recorded. Topographical data of the corneocyte surfaces were analysed with the nAnostic™ method, by the use of custom-built, proprietary algorithms (Serendip, Münster, Germany). The method evaluates each nanostructure protruding from the mean surface level, referred to as circular nanosize objects (CNOs). These are then automatically filtered according to their size and shape; in the present study, only structures of positive local deviational volume smaller than 500 nm in height and with an area of <1 µm² are considered. The DTI counts these features for an area of 20 × 20 µm² of cell surface per image (9). For MCI/MI and SLS, SEM was performed on the tape strips from 1 person. Fragments of D-Squame tapes were observed at a partial vacuum (0.133 kPa) without prior preparation of the samples (native state). Images of the removed corneocyte layers were recorded at 15 kV with the secondary electron detector of a Quanta 250 FEI scanning electron microscope.
Statistics
Data analysis was performed with graphpad prism® version 6.07 (GraphPad Software, La Jolla, CA, USA). Comparison of the NMF levels between different skin sites (allergen/irritant/unpatched) and their corresponding pet. controls in the same individual was performed with a paired two-tailed t-test. Comparison of the activity of BH, C-1 and plasmin between allergens and their pet. controls was performed with the Wilcoxon signed rank test if the distribution of data deviated from normal distribution as tested by the Shapiro–Wilk test, and with a two-tailed t-test when the data were normally distributed. The Spearman correlation coefficient was used to correlate the individual activities of the proteases with NMF levels. The Pearson correlation coefficient was used to correlate the individual patch test results (1+, 2+, and 3+) with NMF levels. One-way anova followed by Dunnett’s multiple comparison post hoc test was applied to the differences in DTI between SLS and individual allergens, pet., and unpatched skin sites. Data are shown as the mean value and standard deviation (SD) when distributed normally, and as median with interquartile range when non-normally distributed.

RESULTS

Clinical response
We included 27 patients (24 females), with an average age of 49.3 years (SD 12.6). A total of 34 positive 1+ or 2+, 3+ reactions to the investigated allergens were observed on D3: 11 for Ni, 11 for Cr, eight for MCI/MI, and four for PPD. Eleven patients had two positive reactions. Although selection was based on 1+ or 2+ reactions, several patients in the present study had 3+ reactions (one for Ni and Cr, and two for PPD). The clinical scores per allergen per patient are shown in Fig. 1.

Exclusion
Four patients, one for each allergen, did not show a positive reaction to the allergen to which they had a positive reaction in the past. Furthermore, one patient had a severe reaction to PPD and the tape strip samples could not be obtained. These patients (n=5) were excluded from the data analysis.
Fig. 1 shows the difference in the NMF levels (ΔNMF) between the allergens, SLS, unpatched sites, and their corresponding controls (pet.) on D3. A significant difference from the corresponding controls was observed for SLS (1% and 2%) and MCI/MI. The smallest effect was observed for Ni, for which none of the patients had ΔNMF lower than the median response after MCI/MI and SLS (Fig. 1). Although the difference with respect to the pet. control did not reach statistical significance for other allergens, several patients had negative ΔNMF values. For example, 3 patients for Ni and 1 for Cr and PPD showed NMF level decreases similar to the average decrease observed after MCI/MI and SLS (Fig. 1). Interestingly, those 5 patients had strong patch test reactions (3+ for Ni and Cr, and 2+ for PPD). To further explore possible associations between patch test readings and changes in NMF levels, we compared ΔNMF and patch test readings. The Pearson correlation coefficient amounted to -0.64 (p < 0.001), indicating a significant negative association between patch test reactions and decrease in NMF levels. The NMF levels after D2, determined in a limited number of patients, showed the same trend (data not shown). In each allergen group, 1 patient had no reaction to the allergen (denoted in Fig. 1. by an X symbol). As is evident from Fig. 1, the ΔNMF values in these subjects were close to those for the pet. control. There was no significant difference in the NMF levels between skin sites where no patches were applied and sites with petrolatum.
EFFECT OF ALLERGENS AND IRRITANTS ON NMF AND DTI

Fig. 1. The difference in the natural moisturizing factor levels (ΔNMF) between the skin sites tested with potassium dichromate (Cr), nickel sulfate (Ni), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI) or p-phenylenediamine (PPD), sodium lauryl sulfate (SLS), unpatched skin and their corresponding pet. controls on day 3. The individual patch test readings for each allergen are inserted as a table. As some patients had positive reactions to two allergens, the number of reactions (n = 34) is greater than the number of patients (n = 27). The ΔNMF of a positive reaction is indicated by a circle symbol, and that of a non-responder is indicated by a ‘X’ symbol. Non-responders were excluded from data analysis. The results are shown as mean of all subjects and standard deviation. The data of allergens, SLS and unpatched skin were compared with those of their corresponding pet. patches by use of a paired, two-tailed t-test. **p < 0.01; ****p < 0.0001.

Corneocyte surface morphology

The DTI values were determined from the AFM images of a 20×20-µm² area. As shown in Fig. 2, among all investigated compounds, only SLS led to a significant rise in the DTI, indicating increased numbers of CNOs, which can clearly be seen from Fig. 3g, representing a more detailed 20×20-µm² image of an SLS-tested skin site. The CNOs in the SLS image were also shown by SEM (Fig. 4). Larger overview AFM images (70×70 µm²) of the corneocytes from the skin sites tested with Cr, Ni, PPD, MCI/MI, SLS and pet. are shown in Fig. 3a–f. The images show that, at a microscopic level, the results for Cr, Ni and PPD resemble those for pet. MCI/MI differed, in that it caused distinct alterations in the structure; corneocytes were hexagonal-shaped and had pronounced cell borders (Fig. 3d). The surfaces were smoother, with a loss of corneocyte surface microtexture. This was also confirmed by SEM images showing loose lateral associations between the cells from the MCI/
MI-treated skin sites (Fig. 4). As indicated in Fig. 2, these microscopic alterations did not lead to an increase in cell surface CNOs; the average DTI value from MCI/MI samples was similar to that for other allergens and petrolatum.

Fig. 2. The Dermal Texture Index (DTI; number of circular nanosize objects per 20-µm² area) measured in the stratum corneum collected on day 3. The results are averaged for all subjects, and are shown as mean values and standard deviation. The number of tested sites per group differed: Cr, n = 3; Ni, n = 5; methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI), n = 4; p-phenylenediamine (PPD), n = 3; sodium lauryl sulfate (SLS), n = 6; pet., n = 7; and unpatched, n = 4. The DTI values of allergens/pet./unpatched skin were compared with those of the SLS group; asterisks indicate level of significance. **p < 0.01 (one-way anova followed by Dunnett’s multiple comparisons test).
Fig. 3. (a–g) Atomic force microscopy images from stratum corneum samples collected on day 3. (a) Chromium. (b) Nickel. (c) p-Phenylenediamine. (d) Methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI). (e) Sodium lauryl sulfate (SLS). (f) Pet. (g) SLS, close-up. Images are three-dimensional representations; the brightness corresponds to the height of the imaged structures. At the macroscale (70 × 70 µm), distinct morphological changes are seen for MCI/MI and SLS. On a close-up view of an SLS sample (20 × 20 µm) (g), circular nanosize objects can be distinguished on the corneocyte surface.
Activities of stratum corneum BH, C-1, and plasmin

To explore whether allergens and SLS affect the activity of proteases that are involved in the degradation of NMF, we determined the activities of BH and C-1 in a limited number of samples. Furthermore, we included plasmin as an indicator of skin barrier damage. The activities of BH, C-1 and plasmin (Fig. 5a–c) were significantly higher in SLS-treated skin than in the corresponding pet. controls (BH, p < 0.01; C-1, p < 0.05; and plasmin, p < 0.01). The allergens did not produce significant differences from the pet. controls, although MCI/MI showed a trend of increasing values for all three proteases (each p = 0.13). The activities of all three proteases were negatively correlated with corresponding NMF levels. The respective Spearman correlation coefficients for BH, C-1 and plasmin amounted to −0.52 (p < 0.01), −0.47 (p < 0.01), and −0.58 (p < 0.001).

Fig. 5. Activities of bleomycin hydrolase (a), calpain-1 (b) and plasmin (c) in the stratum corneum samples of the skin sites tested with Ni (n = 4), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI) (n = 4), sodium lauryl sulfate (SLS) (n = 8) and their corresponding pet. controls (n = 8) and unpatched test sites (n = 8). Data for bleomycin hydrolase are shown as median with interquartile range; those for calpain-1 (b) and plasmin (c) are shown as mean ± standard deviation. *p < 0.05, **p < 0.01; (a) Wilcoxon signed rank test; (b, c), paired two-sided t-test.
DISCUSSION

In the present study, we observed different effects of contact allergens and SLS on relevant properties of the epidermal barrier: the stratum corneum NMF levels, corneocyte surface morphology, and stratum corneum protease activities. NMF levels have previously been used as a skin barrier biomarker in AD and ICD. To the best of our knowledge, this is the first time that NMF levels have been investigated in ACD (27, 28). Recent experimental studies showed that skin irritants with different physicochemical properties, such as SLS, NaOH, fruit acids and aliphatic alcohols, significantly decrease the stratum corneum NMF levels (28, 29). This is in accordance with the findings from the present study, which show a significant reduction in NMF levels after exposure to 1% and 2% SLS. SLS may potentially affect NMF levels in different ways. As an alkaline compound, SLS may lead to an increase in the stratum corneum pH, which might affect the activity of stratum corneum proteases, including those involved in filaggrin degradation into NMF components.

To explore this possibility, we measured, in a limited number of samples, the activity of the stratum corneum proteases BH and C-1, both of which known to be involved in breaking down filaggrin protein (30). The results suggest that it is unlikely that the decrease in NMF levels after SLS and MCI/MI treatment is caused by reduced activity of these enzymes, as their activities showed an opposite trend; protease activities were increased after SLS treatment, and an increasing trend was observed for MCI/MI (p = 0.07). The activities of these proteases were negatively correlated with NMF levels, so the increased activity might be a feedback reaction to the reduced NMF levels. SLS is known to denature proteins of the cornified envelope, which may lead to the leakage of NMF components from the corneocytes (31). This could also occur for the proteases, causing better extraction from the corneocytes and/or intercellular lipids. As the cornified envelope acts as an attachment point for the intercellular lipids, disruption of the cornified envelope additionally affects skin barrier function (32, 33). Further evidence that the reduction in NMF levels may be caused by skin barrier damage is provided by increased plasmin activity following SLS treatment (p < 0.01), which indicates a damaged skin barrier (18). A trend of increasing plasmin activity was also observed after MCI/MI treatment (p = 0.13).
Among the tested allergens, only MCI/MI caused a significant reduction in NMF levels. It is not likely that the NMF decrease after MCI/MI treatment is attributable to downregulation of (pro)filaggrin, as the stratum corneum samples originate from the upper part of the stratum corneum (approximately to the upper third of the stratum corneum depth). The deeper stratum corneum layers containing potentially downregulated expression of filaggrin would require a further 14 days to reach the more superficial part of the stratum corneum from which the samples originated (34). As NMF is mainly located within the corneocyte, where filaggrin degradation occurs, it might be speculated that MCI/MI, like SLS, damages the cornified envelope, resulting in leakage of NMF. MCI (the most abundant component in the 3:1 MCI/MI mixture) is a small lipophilic compound with favourable physicochemical properties for percutaneous penetration across the membrane (Kow = 2.5; MW 111). It has been shown that MCI has corrosive properties and is retained in the epidermis, probably because of binding to the epidermal proteins (35, 36). In the present study, MCI/MI treatment resulted in dramatic changes in the microscale corneocyte structure, characterized by the smoother corneocyte surfaces, the hexagonal shape, pronounced cell borders, and the absence of apparent fibrous structures, that were distinctly different from the effects of the other three allergens. As recently reviewed by Weidinger and Novak, a compromised barrier may facilitate sensitization and increased penetration of contact allergens (37). In murine studies, the irritant effect of an allergen was shown to determine the strength of the contact hypersensitivity response (11). The decrease in NMF levels observed in this study might at least partly have been caused by irritant characteristics of MCI/MI, and this is perhaps an explanation for its high allergenic potency. Although Ni and Cr did not lead to significant changes in NMF levels, individuals with the highest clinical scoring showed the lowest NMF levels, suggesting that allergen-induced inflammation and decreases in NMF levels are associated.

At the topographical scale, quantified by the number of CNOs (expressed as the DTI), MCI/MI did not differ from the other allergens, and SLS was the only substance showing increased DTI values. Increases in DTI have recently also been found for other skin irritants, such as NaOH and lactic acid (C. Riethmuller, et al. pers. comm. 2016), suggesting that an elevated DTI is characteristic of skin irritation. The mechanisms that underlie the development of CNOs are not yet clear. In
another study by Riethmuller et al., AD patients with compound heterozygote or homozygote loss-of-function mutations in the filaggrin gene were shown to have increased numbers of CNOs (9). These patients lack filaggrin, which aggregates keratin filaments within the corneocyte and is also present in the cornified envelope. It might be suggested that, owing to the lack of filaggrin, the cornified envelope is more fragile and becomes more prone to structural changes resulting from osmotic pressure within the corneocytes caused by reduced NMF levels. Interestingly, this study shows that allergen-induced inflammation does not result in the formation of CNOs, regardless of the low NMF levels, indicating that their formation is multifactorial.

If the finding that the DTI does not change in ACD, as we show for four clinically relevant allergens, can be generalized to other allergens, the DTI might aid in differentiating ACD from ICD. However, it has to be noted that, in the occupational setting, mixed exposure to allergens and skin irritants is common, so results might be less clear than in this controlled study. Moreover, many allergens have irritant properties, so an increased DTI does not necessarily exclude ACD. Nevertheless, the investigated parameters can provide more insights in the aetiology of ICD and AD and the intrinsic irritant properties of contact allergens, which might support more targeted prevention in occupational settings.

CONCLUSION

Skin barrier characteristics, for example NMF levels and the number of nanosize objects (DTI) on the corneocyte surface, are useful for studying the effects of skin irritants and contact sensitizers on the epidermis. In contrast to the other allergens investigated, MCI/MI showed distinct effects on the skin barrier in terms of a significant decrease in NMF levels, similarly to SLS, and MCI/MI also had profound effects on corneocyte morphology; collectively, these findings suggest that skin barrier damage plays a role in the pathogenesis of MCI/MI contact allergy. The DTI seems to differentiate reactions to the tested allergens and to SLS, as the latter was the only agent that caused an increase in the DTI. Whether the effects on NMF levels and the DTI can be generalized to other skin irritants should be confirmed in further studies including irritants with different physicochemical properties.


3.1

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3.2

Stratum corneum profiles of inflammatory mediators in patch test reactions to common contact allergens and sodium lauryl sulfate

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ABSTRACT

Background: Recent studies have demonstrated allergen-specific differences in the gene expression of inflammatory mediators in patch tested skin.

Objectives: To determine levels of various inflammatory mediators in the stratum corneum (SC) after patch testing with common contact allergens and the skin irritant sodium lauryl sulfate (SLS).

Methods: In total, 27 individuals who had previously patch tested positive to nickel, chromium, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) or para-phenylenediamine were retested and then patch tested with SLS and petrolatum, with petrolatum serving as the patch test control. At 72 h, the test sites were clinically graded and the SC samples collected on adhesive tape.

Results: The levels of 18 of the 32 quantified mediators differed significantly from that of the control patches for at least one of the tested substances. SLS and MCI/MI induced the largest number of immunomediators. Interleukin (IL)-1α levels were significantly higher in patch test reactions in all allergens than they were in the controls, while no significant difference was detected for SLS. Furthermore, a strong negative correlation was found between strength of patch test reaction and IL-1α levels.

Conclusions: Cytokine profiles in the SC of patch tested skin did not show a distinct allergen-specific pattern. However, MCI/MI induced a larger and wider immune response than the other allergens, perhaps due to its potency as an irritant. The levels of IL-16 were significantly increased in patch test reactions to allergens but not to SLS; thus, they may help clinicians to differentiate between allergic contact dermatitis and irritant contact dermatitis.
BACKGROUND

Allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) are frequent, especially in occupational settings. (1-3) Although they share a similar clinical aspect, their pathogenesis and immunological mechanisms are quite different. ICD is the result of tissue damage by irritants, followed by an immunological response induced either by the direct effect of the irritant on keratinocytes or by the impairment of the skin barrier, resulting in the release of pro-inflammatory cytokines and chemokines. (4) Individuals with a skin barrier dysfunction, such as mutations in the filaggrin gene (FLG) or atopic dermatitis, are more susceptible to developing ICD. (5) The barrier dysfunction facilitates the easy disruption of the stratum corneum (SC). (6) ACD is a type IV hypersensitivity reaction of the skin. It results from the activation of a previously sensitized immune system by a hapten. (7) After penetrating the SC, haptens bind to epidermal proteins or peptides, which enables them to interact with dendritic cells (DCs), resulting in the activation of mainly CD4+ T cells. Metals such as nickel, cobalt and palladium are capable of directly activating DCs via Toll-like receptor-4, thereby inducing inflammatory signalling via nuclear factor-κB. (8, 9) A complex range of inflammatory mediators is involved in both ICD and ACD. Many allergens can also induce skin irritation, which is associated with the activation of the innate immune system. (10) It is likely that the early stages of ACD and ICD share cytokines associated with the innate immune system, such as interleukin (IL)-1 and tumour necrosis factor (TNF)-α. (11-13) However, in the effector stage of ACD the adaptive immune system is mobilized and associated cytokines are upregulated. (14) Vestergaard et al. have found that ICD and ACD show distinct histological responses, such as follicular spongiosis, which is present in early ICD but absent in early ACD reactions. (15) Furthermore, differences in cytokines profiles between various allergens have also been reported, indicating that ACD cannot be regarded as a single entity. (7) An evaluation of the differences in inflammatory profiles between ICD and ACD may give further insight into their pathogenesis and may help to differentiate between them. In addition, the detection of allergen-specific profiles may contribute to the development of more targeted anti-inflammatory drugs for treating ACD. Therefore, the aim of this study was to determine the SC profiles of inflammatory mediators induced by the common contact allergens nickel, chromium, methylchloroisothiazolinone / methylisothiazolinone (MCI/MI),
para-phenylenediamine (PPD) and an irritant frequently used in clinical research, sodium lauryl sulfate (SLS).

PATIENTS AND METHODS

Selection of patients
The experimental protocol followed the Declaration of Helsinki Principles and was approved by the Medical Ethics Committee of the University Hospital Centre, Zagreb. Patients with a previously positive (1+ or 2+) patch test reaction to either chromium, nickel, MCI/MI or PPD were identified from the database of the Department of Dermatology of the University Hospital Centre, Zagreb. Patients with a history of atopic dermatitis were excluded, and those with a positive reaction to more than one of the allergens studied were preferred over those with a single positive reaction. Eligible patients were invited to participate in the study and written informed consent was obtained from each participant. In total, 27 patients were finally included (24 female, average age 49 years).

Patch test and tape stripping
Each patient was patch tested with 8 × 8-mm Van der Bend patch test chamber® (Van der Bend, Brielle, the Netherlands) with: (i) one or two allergens dissolved in petrolatum (chromium, nickel, PPD) or water (MCI/MI); (ii) a 2% SLS solution (in water); and (iii) petrolatum. Petrolatum is used as a control although it is known that, very rarely, petrolatum can induce an allergic reaction and give a false-positive result when it is used as a vehicle. An SLS concentration of 2% was chosen to ensure a strong irritant reaction, comparable with a 2+ contact allergic reaction expected in the allergens group. The following allergen preparations were used: PPD 1%, potassium dichromate 0.5% (both Almirall Hermal, Reinbek, Germany), nickel sulfate 5% and MCI/MI 0.01% (both SmartPractice Europe, Barsbuttel, Germany (MCI/MI ratio 3:1)). After 48 h the chambers were removed. The strength of the patch test reaction was graded after 72 h (1+, 2+ or 3+). Each skin site was tape stripped using adhesive tape (1.5 cm², D-Squame, CuDerm, Dallas, TX, U.S.A.). Eight consecutive tapes from each skin site were collected. For the analysis, the sixth and seventh tape strips were used. The samples were extracted from the tapes
by 0.5 mL of phosphate buffered saline containing 0.05% Tween20 and sonicated for 15 min, as described previously (19). After vortexing, the extract aliquots were distributed in vials and stored at −80°C until analysis.

**Multiplex analysis**

The analysis of inflammatory mediators from the extracts was performed using a MESO QuickPlex SQ 120 assay (MSD, Rockville, MA, U.S.A.). The following cytokines and chemokines were included: eotaxin-1/CCL11, eotaxin-3/CCL26, granulocyte–macrophage colony-stimulating factor (GM-CSF)/CSF2, IL-1α, IL-1β, IL-1 receptor antagonist (RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-8 ha, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17a, IL-18, interferon (IFN)-γ-induced protein (IP)-10/CXCL10, monocyte chemoattractant protein (MCP)-1/CCL2, MCP-4/CCL13, macrophage-derived chemokine (MDC)/CCL22, macrophage inflammatory proteins (MIP)-1α/CCL3, MIP-1β/CCL4, thymus and activation regulated chemokine (TARC)/CCL17, TNF-α, TNF-β and vascular endothelial growth factor (VEGF). These inflammatory mediators were analysed using off-the-shelf panels (Human V-Plex Proinflammatory Panel 1 Kit, human IL-18, vascular and growth factor panel and a cytokine panel (MSD), according to the manufactures’ instructions. All these kits use human antibodies. As the amount of SC on the tape varies, the concentrations of the mediators were normalized for the total amount of protein on the tape, which was determined with a Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, U.S.A.) (20).

**Statistics**

Data were analysed by GraphPad prism® v. 6.07 (GraphPad Software, La Jolla, CA, U.S.A.). Data are shown as the mean ± SEM. The concentrations of the mediators on each skin site investigated were compared with the corresponding petrolatum control using a Wilcoxon matched-pairs rank test. Subsequently, a Kruskal–Wallis test followed by a Dunn’s multiple comparison test was used to test the difference between allergens, which was also used to test the difference between allergens and SLS. The association between the clinical severity of the response and the levels of inflammatory mediators was assessed using Spearman’s correlation test.
Power analysis

This was an exploratory study and the levels of most of the inflammatory mediators included in the SC have never previously been measured in ACD. In our previous study on cytokine and chemokine levels in patients with atopic dermatitis, we found 0.16 mean values (SD 0.18) pmol mL$^{-1}$ of the proinflammatory cytokine IL-1$\beta$ (21). For the power analysis, we assumed a twofold increase. On basis of these measurements, 10 participants per allergen were required for the generally recommended level of 0.80 (22).

RESULTS

Overall, 25 individuals had a total of 34 positive patch reactions: 11 to nickel, 11 to chromium, eight to MCI/MI and four to PPD. One PPD patient had a severe reaction, so that sampling was impossible. Four of the patients tested with chromium (n = 1), MCI/MI (n = 1), nickel (n = 1) and PPD (n = 1) did not show an allergic response to the relevant allergen. The average strength of the patch test reactions was 1.5 + nickel, 1.5 + chromium, 1.8 + (MCI/MI), 2.5 + (PPD) and 1.6 + (SLS). Compared with the corresponding petrolatum controls, 18 of the 32 quantified inflammatory mediators showed significant levels of change for at least one of the investigated allergens or SLS (Fig. 1 and 2). In general, the largest changes compared with petrolatum controls were observed for chemotactic cytokines and T-(memory) cell recruiters (Fig. 2). The levels of TARC/CCL17, MDC/CCL22, MCP-1/CCL2 and MIP-1$\beta$/CCL4 in patch test reactions to both SLS and allergens were elevated, although a level of significance of $P < 0.05$ was not attained in all cases. In contrast to chemokines, IL-1 cytokines and other mediators of the innate immune system showed the opposite trend. The concentration of IL-1$\alpha$ was significantly decreased in test reactions to SLS as well as to allergens, with the exception of PPD, to which, however, only three individuals were positive. Other mediators with decreased levels were IL-1$\beta$ (SLS and nickel), IL-1RA (SLS), IP-10 (SLS), IL-2 (SLS), IFN-$\gamma$ (MCI/MI) and IL-7 (MCI/MI).
Fig. 1. Concentration of interleukin (IL)-1 and innate immunity related cytokines and chemokines of skin sites tested with chromium, nickel, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), paraphenylenediamine (PPD), sodium lauryl sulfate (SLS) and petrolatum. The results of petrolatum and SLS are averaged for all patients (n = 25). Data are given as mean with standard error of means (SEM). A Kruskal–Wallis test followed by Dunn's multiple comparison test was used to investigate differences between allergens as well as between allergens and SLS. Significant differences between allergens or SLS and their corresponding petrolatum controls, are given directly above the SEM error bar (Wilcoxon matched-pairs signed-rank test). *P < 0.05, **P < 0.01, ***P < 0.001. IFN-γ interferon-γ, TNF-α, tumour necrosis factor-α.
Fig. 2. Concentration of chemotactic and T-cell proliferation related cytokines and chemokines from the skin sites tested with Cr, Ni, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), para-phenylenediamine (PPD), sodium lauryl sulfate (SLS) and petrolatum. The results of petrolatum and SLS are averaged for all patients (n = 25). Data are given as mean with standard error of means (SEM). A Kruskal–Wallis test followed by Dunn’s multiple comparison test was used to investigate differences between allergens as well as between allergens and SLS. Significant differences between allergens or SLS and their corresponding petrolatum controls, are given directly above the SEM error bar (Wilcoxon matched-pairs signed-rank test). *P < 0.05, **P < 0.01, ***P < 0.001. TARC, thymus and activation regulated chemokine; VEGF, vascular endothelial growth factor.
### Table 1. Correlation of patch test reaction and the levels of inflammatory mediators

<table>
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<tr>
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<th>Spearman’s correlation coefficient, r</th>
<th>P-value</th>
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<tr>
<td>Eotaxin-1/CCL11</td>
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<tr>
<td>Eotaxin-3/CCL26</td>
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<td>IL-1RA</td>
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<td>IL-1β</td>
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<td>IL-2</td>
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<td>IL-4</td>
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<td>IL-6</td>
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DISCUSSION

The present study shows that contact allergens and SLS induce changes in a large number of immunomediators in the SC, which can be detected by a relatively noninvasive procedure. Overall, the profiles of most mediators in the patch test reactions were similar among the investigated allergens and SLS, reflecting common inflammatory pathways. However, the levels of IL-16 seemed to be indicative for ACD, as they were significantly increased in patch test reactions to all allergens, but not to SLS. Here, it may be noted that the increase of IL-16 levels did not reach significance for PPD, probably due to small number of patients in this study (n = 3). IL-16 plays a major role in skin hypersensitivity by the chemoattraction of CD4+ T cells (23). Masuda et al. showed that IL-16 is produced by epidermal cells, especially keratinocytes, during the sensitization and elicitation phases of hapten-induced contact hypersensitivity. In agreement with the results of the present study, Masuda et al. found that the application of haptens and not of primary irritants or of the control vehicle induces IL-16 production in the skin. Interestingly, Reich et
al. showed that polymorphisms in gene-encoding IL-16 influence susceptibility to contact allergy (24).

When comparing allergens, patch test reactions to MCI/MI consistently showed the largest difference compared with the petrolatum controls, although similar patch test reactions were induced by the allergens investigated. Of the 32 mediators that could be determined in the samples, 11 showed a significant difference in MCI/MI. Most of these belong to the keratinocyte-derived cytokines (IL1-α, IL-16, TARC/CCL17, MCP-1/CCL2, IL-8/CXCL8, eotaxin-1/CCL11, IP-10/CXCL10). Among Th2-related mediators, concentrations of MDC/CCL22 and TARC/CCL17 were significantly higher than the petrolatum controls in patch test reactions to MCI/MI (MDC/CCL22) and MCI/MI and chromium (TARC/CCL17). In a recent study by Dhingra et al. increased mRNA expression of MDC/CCL22 and TARC/CCL17 was detected in biopsies from patch test reactions to nickel, rubber, fragrance mix, cobalt and potassium dichromate (7). An amplification loop has been suggested for TARC/CCL17 and MDC/CCL22, as both are regulated by IL-4 and increase their production by recruiting IL-4 releasing Th2 cells (14). In the present study, we found an increase in levels of TARC/CCL17 in patch test reactions to MCI/MI and chromium but not to nickel, as shown by Dhingra et al. (7). This discrepancy could be explained by the lower reaction severity to nickel in the present study, compared with that of Dhingra et al. (1·5+ vs. 2·1+, respectively) (7).

The levels of monocyte-derived chemotactic mediators (MCP-1, MIP-1a, MIP-1b) were increased in patch test reactions to allergens as well to SLS. Although not all these differences reached a level of significance, this may indicate there are common inflammatory pathways for allergens and irritants. While the concentrations of most mediators were increased in patch test reactions compared with their corresponding petrolatum controls, decreased values were found for several mediators, mainly those that are representatives of innate immunity, including IFN-γ (MCI/MI), IL-1α (chromium, MCI/MI, SLS), IL-1β (nickel, SLS), IL-7 (MCI/MI), IP-10 (SLS) and IL-1RA (SLS). IL-1α levels in the SC have previously been shown to decrease after exposure to various skin irritants, which is in agreement with the effect of SLS found in the present study (25, 26). The opposite, however, an increase of IL-1α, has been found in the epidermis (27). This discrepancy in the pattern of IL-1α levels.
between these two skin strata should probably be sought in the contribution of the preformed pool of IL-1α in the SC. Damage of the SC causes the release of these primary cytokines stored in the corneocytes and their gradual depletion in the SC intercellular matrix. This is supported by the negative correlation between IL-1α and the strength of patch test reactions. Therefore, it may be speculated that the significant decrease of IL-1α in patch test reactions to chromium and MCI/MI may, at least partly, be caused by the inherent skin damaging properties of these allergens (28).

The magnitude of changes of the inflammatory mediators in patch test reactions to MCI/MI as compared with other allergens may also be explained by its skin damaging properties, which are associated with skin barrier damage and the induction of innate immune responses. Esser et al. have shown a correlation between the ability of an allergen to activate innate immune responses and its allergenic potency (29, 30). MCI is a lipophilic molecule and is small enough to cross the epidermal barrier (Kow = 2.5; MW 111 Da). Furthermore, in a previous study we demonstrated that MCI/MI induces morphological cell changes that are distinctly different from that of other allergens (Koppes et al., submitted for publication) suggesting it has effects on the integrity of the skin barrier.

Recently, the important role of innate immune system in ACD has been revealed. Interestingly, metals such as nickel, cobalt and palladium are capable of directly activating innate immune signalling pathways via TLR-4 and nuclear factor-κB.(8, 9) However, not all metals are able to do this, for instance, chromium and iron cannot.(9) The present study did not detect major differences between nickel and other allergens; however, this may be due to the fact that sampling was limited to the SC. To gain more insight into the mechanism biopsy samples would be more suitable.

Some limitations of the present study should be considered. The levels of inflammatory mediators are probably time and concentration dependent. For example, an initial decrease of IL-1α levels is induced by damage to the skin barrier and the gradual depletion of the preformed pool of IL-1α in the SC. However, as a consequence of inflammation, de novo synthesis of IL-1α will occur in epidermis,
which will result in an increase in IL-1\(_{\alpha}\) levels. Thus, the actual concentration of IL-1\(_{\alpha}\) depends on the kinetics of these two processes. A longer study period and the measurement of cytokine profiles at more time points and with a series of concentration dilutions would allow for the detection of possible temporal and concentration variations existing between allergens as well as between individuals. To compensate for interindividual variation in immune response to various allergens it would be interesting to study polysensitized patients.

Moreover, we looked at the concentrations in the upper part of the skin, the dead corneocytes, and not in the viable epidermis where many inflammatory mediators are formed and where they primarily exert their activity; although, as shown here, the SC sampling method offers a non-invasive approach to measuring a wide range of inflammatory mediators in vivo in the skin. Lastly, objective assessment of patch test reaction, for example, by using skin bioengineering techniques (such as transepidermal water loss, erythema meter, imaging techniques, etc.) may improve the interpretation of the results. Nevertheless, current guidelines on allergy patch testing are based on clinical scoring performed by a trained dermatologist (17).

In conclusion, this study suggests that differences in SC profiles of inflammatory mediators exist between ACD and ICD as well as between the allergens investigated, although most of them show similar patterns. MCI/MI induces larger and broader immune responses than the other tested allergens. The decreased levels of IL-1\(_{\alpha}\) in patch test reactions to MCI/MI and chromium may suggest their inherent irritant capacities play a role. Of the mediators investigated, IL-16 is the most promising marker in the SC for differentiating between ICD from ACD. Additional investigations are needed to evaluate the possible influence of allergen or irritant concentration and interindividual variations.
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