Patients with Allergic and Irritant Contact Dermatitis are Characterized by Striking Change of Iron and Oxidized Glutathione Status in Nonlesional Area of the Skin

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To assess the consequences of oxidative stress in allergic and irritant contact dermatitis, we compared the iron level, unsaturated iron-binding capacity, total iron binding capacity, the percentage saturation of iron-binding capacity, the amount of diene conjugates as well as the amounts of total glutathione, reduced glutathione, oxidized glutathione, and the oxidized glutathione/reduced glutathione ratio in skin homogenate from lesonal and nonlesional skin. Lesional skin samples were obtained from positive patch test sites to 5% NiSO₄ in five subjects, and from chronic contact dermatitis lesions on the hands, which had exacerbated over 3–9 wk in six subjects. Contact dermatitis caused at least a 4-fold increase in the iron level in the lesional skin area compared with the nonlesional skin area (p < 0.02). The increase in the iron level depended on the duration of contact dermatitis and was accompanied by high unsaturated iron-binding capacity and total iron-binding capacity values in the positive patch test sites (p < 0.05), and by a high percentage saturation value in the chronic contact dermatitis lesions (p < 0.05). We found high indices for iron, total iron-binding capacity and diene conjugates in the apparently healthy skin of the patients with persistent contact dermatitis that significantly (p < 0.05) exceeded the corresponding values in the patients with only patch test reactions. In summary, we have succeeded in providing evidence that generalized oxidative damage of the skin occurs as a consequence of contact dermatitis in a restricted area.

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The skin functions as a biobarrier against hazardous environmental physical, chemical, and biologic agents. Inflammatory reactions triggered by exogenous and endogenous factors, including free radicals, are involved in the pathogenesis of dermatologic diseases (Harman, 1992; Jansen et al, 1993; Darr and Fridovich, 1994; Picardo et al, 1996; Lopez-Torres et al, 1998; Halliwell and Gutteridge, 1999).

In allergic and irritant contact dermatitis (ACD, ICD) an array of reactive oxygen species (ROS) is produced. ROS are set free by inflammatory mediators, generated directly by irritants or allergens (Corsini and Galli, 1998; Willis et al, 1998), and released during free radical chain reactions. The main source of ROS is inflammatory cellular infiltrate. Stimulated monocytes produce superoxide, the respiratory burst of infiltrating PMN in inflamed skin will produce high local levels of superoxide anion and hydrogen peroxide (Trenam et al, 1992a; Darr and Fridovich, 1994). Production of nitric oxide by nitric oxide synthase in keratinocytes also occurs (Halliwell and Gutteridge, 1999). Excessive production of ROS results in peroxidation of cell membrane lipids and damages of proteins and DNA (Bunker, 1992; Pantopoulos and Hentze, 1995; Picardo et al, 1996).

Iron and oxygen are at a metabolic crossroad where any mismanagement leads to toxicity, tissue damage, and severe inflammation. Therefore, biochemistry both of ROS and iron are closely related to each other (Trenam et al, 1992a; Morris et al, 1995; Halliwell and Gutteridge, 1999). ROS can release iron from intracellular iron storage protein, ferritin, and trigger a rapid reduction of ferritin synthesis (Trenam et al, 1992b; Pantopoulos and Hentze, 1995); and vice versa, iron promotes the formation of ROS that contribute to lipid peroxidation (LP) (Morris et al, 1995; Pantopoulos and Hentze, 1995; Van Lenten et al, 1995).

The skin possesses considerable endogenous protection against oxidative damage, as it is equipped with several antioxidants (Vessey et al, 1995; Kerb et al, 1997; Thiele et al, 1997; Kohen, 1999). Reduced glutathione (GSH) has gained attention as the central cellular antioxidant in the skin (Vessey et al, 1995; Picardo et al, 1996; Hirai et al, 1997; Shvedova et al, 2000) that also participates in the metabolism of xenobiotics and leukotriene synthesis (Halliwell, 1994; Hanada et al, 1997). The protective role of cutaneous GSH against UV injury has been thoroughly investigated on cultured human skin cells (Punnonen et al, 1991; Vile and Tyrrell, 1995), in the skin of mice (Hanada et al, 1997; Lopez-Torres et al, 1998), and in human volunteers (Kerb et al, 1997). The
The inhibitory role of GSH in contact dermatitis (CD) in mice has been exemplified by Hira et al (1997).

This study was performed to compare the iron status and glutathione redox status in ACID and ICD lesions with the values of apparently healthy skin of the same subjects.

MATERIALS AND METHODS

Patients With permission of the local ethics committee, two 4 mm skin punch biopsies were taken from 11 study participants under lidocaine + epinephrine local anesthesia. Lesional skin was biopsied in six patients (four female, two male, mean age 46.5 y, within the range of 22–84 y) with chronic ICD (three patients) or ACID (three patients) on their hands, which had exacerbated over 3–9 wk, and at the time of investigation was characterized by marked erythema, edema, and vesiculation. Simultaneously, the skin of symmetrical or functionally comparable unaffected sites was biopsied. These analyses served to provide control values. Skin from positive patch test sites to 5% nickel sulfate was obtained from the backs of five female patients (mean age 35.4 y, within the range of 19–67 y) on the second to the fifth day after the application. Positive test reactions were graded as +++ (extensive infiltrated erythema with coalescing vesicles) or as ++ (erythema, edema, and discrete vesicles). Their control biopsies were taken from the back or discrete vesicles). Their control biopsies were taken from the back or

Table I. Characteristics of OS in inflamed and apparently healthy skin

<table>
<thead>
<tr>
<th>Patients with positive patch test reactionsa</th>
<th>Patients with chronic CD on handsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparently healthy skin</td>
<td>Patch test site</td>
</tr>
<tr>
<td>Iron (µM)</td>
<td>1.000 ± 0.02</td>
</tr>
<tr>
<td>UIBC (µM)</td>
<td>157 ± 16</td>
</tr>
<tr>
<td>TIBC (µM)</td>
<td>256 ± 21</td>
</tr>
<tr>
<td>% saturation</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>DC (µM)</td>
<td>266 ± 97</td>
</tr>
<tr>
<td>TGSH (µM)</td>
<td>627 ± 400</td>
</tr>
<tr>
<td>GSSG (µM)</td>
<td>169 ± 61</td>
</tr>
<tr>
<td>GSH (µM)</td>
<td>457 ± 442</td>
</tr>
<tr>
<td>GSSG/GSH</td>
<td>0.76 ± 0.44</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD in positive patch test sites to 5% NiSO4 in five patients.

Iron, unbound iron-binding capacity (UIBC), and total iron-binding capacity (TIBC), are expressed as µM per g skin.

Total glutathione (TGSH), oxidized glutathione (GSSG), and reduced glutathione (GSH) are expressed as mg per g skin.

Significantly different (p < 0.05) by Student’s t test.

NS, not significant.

Sample preparation Epidermis and dermis together were frozen and stored at −80°C until used. Biopsies were kept for 5 d in liquid nitrogen to produce supernatants whose homogeneity was comparable with supernatants produced by sonification (Lopez-Torres et al, 1998). Biopsies were carefully homogenized in 1.15% KCl solution with special homogenizer and centrifuged at 10,000 × g for 10 min. The supernatants were kept on ice and used for antioxidant assays.

Level of iron, the unsaturated iron-binding capacity (UIBC), the total iron binding-capacity (TIBC), and the percentage saturation of iron-binding capacity For the assessment of these indices a special kit (Sigma 565, Sigma, St. Louis, MO) was used. All procedures were performed in triplicate. Briefly, for determination of the tissue iron level 0.250 ml of the tissue homogenate and 1.250 ml Iron Buffer Reagent (hydroxylamine hydrochloride, 1.5% w/vol in acetate buffer, pH 4.5) were mixed, centrifuged for 10 min at 3000 × g and initial absorbance was measured at 560 nm. Then 0.025 ml Iron Color Reagent, which forms water-soluble magnet complex with iron (Ferrone 0.85% w/vol in hydroxylamine hydrochloride solution) was added, mixed, incubated for 10 min at 37°C and centrifuged for 10 min at 3000 × g. Absorbance was measured at 560 nm versus water blank and the iron concentration was calculated by using a kit formula. Iron content was expressed as µM per g skin. To determine UIBC of the tissue 1.000 ml UIBC buffer reagent (TRIZMA, 0.5 M per liter, pH 1.81, 0.25 ml of the tissue homogenate and 0.25 ml iron buffer standard (0.500 mg per dl) were mixed, centrifuged for 10 min at 3000 × g and the initial absorbance was measured at 560 nm. The procedure was completed as described for total iron determination. UIBC (expressing the number of free iron-binding sites) was presented in µM per g skin.

TIBC is the sum of iron and UIBC values, and the percentage saturation indicates the percent of bound iron.

Lipid peroxidation The level of diene conjugates (DC) was measured as previously described (Ristimäe et al, 1999). Briefly, 0.15 ml of sample and 0.15 ml 0.9% NaCl (reagent blank contains isotonic saline) were incubated at 37°C for 30 min. Then 0.25% BHT (0.015 ml) was added, the samples were extracted with heptane/isopropanol (1:1, whole volume 1.8 ml) and acidified by 0.5 ml 5 N HCl. After extraction with cold heptane (1.6 ml), samples were centrifuged (for 5 min at 3000 × g) and absorbance of heptane fraction was measured at 234 nm.

Detection of total glutathione (TGSTH), oxidized glutathione (GSSG), and reduced glutathione (GSH) TGSH and GSSG were measured by the method of Griffith (1980), which we had slightly modified. The samples were deproteinized with 10% metaphosphoric acid (Aldrich, Cat. 43157). To measure glutathione content 0.005 ml 4 M triethanolamine (Aldrich, Steinhemm, Germany, Cat TS830–0) in water was added to the 0.1 ml of protein free sample and immediately mixed. The sample was diluted with 0.895 ml of 0.2 M sodium phosphate buffer (pH 7.5) containing 0.001 M ethylenediaminetetraacetic acid (buffer 1). For determination of GSSG, GSH was derivatized by adding 0.01 ml of 1 M 2-vinylpyridine (Aldrich, Cat 13229–2) in ethanol, mixed, and kept at room temperature for 1 h. To assay TGSH or GSSG, the samples were mixed with 0.5 ml of buffer 1 containing 0.5 U glutathione reductase (Sigma Cat G-7571), and 0.3 mM NADPH (Sigma Cat N7505). Reaction was initiated by the addition of 0.1 ml of 1 mM 5,5-dithio-bis-(2-nitrobenzoic acid) in buffer 1. The change in optical density was measured after 10 min at 412 nm. TGSH, GSH, and GSSG are expressed as µg per g skin. Glutathione redox status was expressed as GSSG/GSH.

Statistical analysis Statistical analysis was performed using the Student’s t test for unpaired samples. A difference was considered significant if p < 0.05.

RESULTS

Patients with CD are characterized by increased iron level in lesional areas of skin One of the features of OS in skin inflammation is altered iron metabolism (Trenam et al, 1992a; Morris et al, 1995). To evaluate the iron alteration in CD, comparative studies were carried out in lesional and nonlesional skin from 11 patients. As shown in Table I, both skin patch testing
and spontaneous CD caused at least a 4-fold increase in iron level in lesional skin compared with nonlesional area ($p < 0.02$). In patients biopsied from positive patch test sites the level of iron in lesional region was $741 \pm 396 \mu M$ per g skin and in apparently healthy skin $104 \pm 9 \mu M$ per g skin. UIBC and TIBC values were about six times higher in the patch test sites compared with healthy skin ($p < 0.05$). In chronic CD lesions the iron level was enhanced compared with the level in uninvolved skin ($1038 \pm 493$ and $322 \pm 183 \mu M$ per g skin, respectively, $p < 0.02$). There were no significant differences in UIBC and TIBC values, but the percentage saturation was higher in lesional skin ($p < 0.05$). All of these results are presented in Table I.

### Increase of iron level in lesional and nonlesional skin depends on duration of dermatitis

The effect of disease duration on iron status in inflammatory skin condition caused by ACD or ICD is given in Fig 1. The iron level and the percentage saturation value in lesional skin were higher in the patients with chronic exacerbated CD in comparison with the data, obtained from the positive patch test sites. The difference between the saturation indices was significant ($p < 0.05$). The UIBC value, on the contrary, was significantly higher in the patch test areas ($p < 0.05$). Surprisingly, in nonlesional skin of the patients with chronic CD the indices for iron and TIBC were about three times higher ($p < 0.05$) as in nonlesional skin of the patients whose skin symptoms were caused by patch testing and thus were of a shorter duration (Fig 2). The measured iron was nonhaem iron, as the iron measured in our samples was nonhaemic (mainly ferritin iron) as the control test with red blood cells, treated under the same conditions as skin samples, did not result in detectable iron.

### DC level does not differ in lesional and nonlesional skin areas but is increased time-dependently

ROS-mediated OS may induce an increase in iron-caused LP (Bunker, 1992; Trenam et al., 1992a, b; Morris et al., 1995). To assess LP level in patients suffering from ACD or ICD, the amount of DC was measured. The increase of DC level in the positive patch test sites to 5% NiSO$_4$ test concentration, the TIBC level, and the DC quantities were significantly higher in comparison with the normal skin values in the patients with only patch test reactions ($p < 0.05$). In chronic CD the, iron concentration, the TIBC level, and the DC quantities were significantly higher in comparison with the normal skin values in the patients with only patch test reactions. The difference in the UIBC values was not significant. The data shown are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.1$.

### Oxidized glutathione level in CD is increased both acutely and time-dependently

GSSG and glutathione redox status (GSSG/GSH) are well-known indices for cellular OS. GSSG and redox status values were increased in the lesional area of skin compared with the nonlesional skin area (Table I); however, due to significant individual variability of glutathione amounts a statistically significant difference was established only for GSSG in patients biopsied from positive to 5% NiSO$_4$ test patch test sites during the early stage of inflammation (Table I). Nonetheless, it should be underlined that the level of GSSG in the nonlesional skin area of patients suffering from chronic CD was even higher than in the positive to 5% NiSO$_4$ patch test sites.

### DISCUSSION

In this study, we have provided evidence that CD, restricted to a small area, is not a local problem as it is often thought to be, but its consequences are present in whole skin.

CD causes a potent increase of iron level not only in acute or chronic lesional area, but also in apparently healthy skin. In prolonged duration of inflammation the increase is even more evident.

Iron is a biologically important trace element. As iron catalyzes the formation of highly toxic hydroxyl radical, and peroxidation of lipids, excessive iron is stored in Fe(III) form. In cells, iron is bound to ferritin, extracellularly to transferrin and lactoferrin, and in circulation to hemoglobin (Halliwell, 1994; Morris et al., 1995; Pantopoulos and Hentze, 1995; Van Lenten et al., 1995). The iron measured in our samples was nonhaemtic (mainly ferritin iron) as the control test with red blood cells, treated under the same conditions as skin samples, did not result in detectable iron. The possibility that some of the iron is of hemosiderin and low-molecular weight chelators origin, in chronic inflammation in particular, cannot be excluded.

The high iron content in lesional skin might be explained by inflammation-induced OS. A chronic exposure of hairless mice to low levels of UVB has been reported to have increased the nonhaem iron content of the skin (Bissett et al., 1991). Trenam et al. (1992b) have shown that intradermal injection of H$_2$O$_2$–producing enzyme glucose oxidase rapidly produces a cellular infiltrate of monocytes and neutrophils, and significantly increases the levels of iron in the skin of rats both with or without prior iron loading. Despite not being species-specific, these is the best evidence of inflammation-induced changes in iron content of the skin so far.

In CD at least four potential mechanisms involved in storage and reduction of Fe(III) to Fe(II) may contribute to the rise of iron: (1) The mobilization of redox active iron from storage protein, e.g., ferritin, is related to the oxidative burst of infiltrating PMN with
superoxide radicals formation, and degradation of ferritin in macrophages (Trenam et al., 1992a; Morris et al., 1995). (ii) Inflammatory mediators (e.g., TNF-α and IL-1) generated by monocytes and keratinocytes under the influence of irritants and allergens (Barker et al., 1991; Howie et al., 1996) or staphylococcal superantigens in chronic lesions (Leung et al., 1998), and ROS themselves bring about an increase in the iron-containing acute phase reactants such as ferritin and lactoferrin (Morris et al., 1995). (iii) Acidic pH in the activated phagocytes and on the skin may contribute to the release of active iron (Trenam et al., 1992a). (iv) Inflammation-induced hyperproliferation of keratinocytes (Barker et al., 1991) may increase the epidermal excretion of iron (Trenam et al., 1992a; Morris et al., 1995).

In acute inflammation, the values reflecting sequestered iron (UIBC) and possible total binding capacity of sequestrers (TIBC) were high in the tissue samples in our study. Furthermore, the increase of iron in the patch test sites concurred with the increase in UIBC, indicating a rapid response to inflammation. Chronic CD was characterized by an elevated percentage saturation value showing that more iron-binding sites are occupied with iron. In addition, restricted to the hands chronic CD influenced the iron status in whole skin, evidenced by high TIBC values in the apparently healthy skin of our patients. Therefore, it can be stated that the iron level response to dermatitis involves whole skin, i.e., it is a generalized response. The presence of considerable amounts of PUFA in the skin makes it particularly vulnerable to ROS (Trenam et al., 1992b), especially in the case of inflammation when excessive formation of ROS occurs. Some studies have reported signs of LP in a model of human skin (Poddà et al., 1998), in the skin of mice with ICD and ADC (Hirai et al., 1997), and after UV irradiation of cultured keratinocytes (Punnonen et al., 1991; Stewart et al., 1996). Lipoperoxidation of skin surface lipids has also been described (Giral et al., 1996). We could not establish a statistically significant difference in the quantities of DC between the inflamed and the apparently healthy skin of the same subject; however, the level of DC was dependent on the duration of the disorder. This was less obvious between lesions of acute versus chronic CD, but statistically significant in apparently healthy skin. As the consequence of chronic inflammation, the level of DC in the unaffected skin of patients suffering from chronic CD was significantly higher in comparison with the values in lesions of acute CD (patch test sites). It refers to the fact that an increased iron level is accompanied by increased LP and continuous inflammation, even if inflammation is restricted to such a small area as the hands, will result in potentially oxidatively stressed whole skin.

ROS biochemistry and both iron (possible pro-oxidant) and glutathione (scavenger of ROS) biochemistry are known to be intimately related. The observation that localized CD causes a generalized OS is further supported by our data about glutathione. Glutathione is known to be the major cellular antioxidant (Halliwell, 1994). In this context, the low GSSG/GSH ratio in our patients may be accounted for by the study groups compared, both consisting of CD patients: an asymptomatic group and a symptomatic group. Besides, the level of glutathione reductase in the course of differentiation of cultured human keratinocytes is known to decrease (Vessey et al., 1995), reducing the GSH level.

Nevertheless, the glutathione system reacts efficiently with ROS generated by dermatitis. (i) We found a significant increase of the GSSG level only in the positive to 5% NiSO₄ patch test sites, i.e., in short-time CD. (ii) Although chronic CD caused an increase of GSSG levels in lesional as well as apparent healthy skin indicating to the severity and generalized nature of OS, these differences were because of a concomitant increase of the GSH values below statistical significance.

Skin disease, especially when restricted to a small area, is often considered to be a local problem. For that reason, it may be of particular interest to evaluate the degree of OS in urticaria, where the ability of the skin to respond to the stimulus as a uniform organ is much more obvious. Further research may be directed to the possible involvement of the skin antioxidant capacity by the addition of topical and systemic antioxidants and iron chelators to the treatment schedule of patients with CD that might facilitate a faster and more complete improvement of the patient’s state. In summary, we have provided evidence that generalized severe OS of the skin is expressed as a consequence of CD in a restricted area.

REFERENCES

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