Glutathione Metabolism in Mice Is Enhanced More With Hapten-Induced Allergic Contact Dermatitis Than With Irritant Contact Dermatitis

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Cutaneous inflammation induced by electrophilic compounds involves irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD). Reduced glutathione (GSH) and related thiols have been postulated to play important roles in detoxification of electrophilic xenobiotics, protection of tissues against reactive oxygen species, and modulation of immunologic functions in normal and diseased subjects. The dynamic aspects of GSH metabolism, however, and its significance in patients with ICD and ACD remain to be clarified. The current study was carried out to elucidate the pathogenesis and possible involvement of GSH in both types of inflammation. Normal mice and mice sensitized with dinitrochlorobenzene (DNCB) were challenged by cutaneous administration of DNCB, and changes in GSH metabolism in skin and liver were determined. Kinetic analysis revealed that 24 h after challenge with DNCB, levels of hepatic glutathione and its secretion increased more markedly in the sensitized mice than in the unsensitized animals. Administration of buthionine-L-sulfoximine (BSO), a specific inhibitor of GSH synthesis, inhibited the increase in glutathione levels in the liver and the skin of both groups. Histologic examination revealed that cutaneous inflammation was enhanced by BSO more significantly in mice with ACD than with ICD. These results suggest that GSH might play an important role in the suppression of the immune reaction in mice with ACD. ***Key words: immune reaction/oxidative stress/dinitrochlorobenzene. J Invest Dermatol 109:314–318, 1997***

The skin protects organisms from hazardous xenobiotics, but there are many compounds that induce inflammatory reactions. Reactive electrophiles bind to some nucleophilic residues in proteins, such as amino and free thiol groups, and function as haptens (Eisen *et al*, 1952). Reduced glutathione (GSH) is the most abundant intracellular nonprotein thiol that detoxifies these electrophiles by the mercapturic acid pathway (Inoue *et al*, 1984; Hinchman *et al*, 1993) and has been postulated to play an important role in inhibiting contact dermatitis. Moreover, GSH and related thiols play critical roles in the expression of immunologically relevant genes (Peristeris *et al*, 1992) and in the regulation of functions of lymphocytes (Suthanthiran *et al*, 1990; Dröge *et al*, 1991). Thus, GSH may also regulate immunologic reactions in the process of allergic contact dermatitis (ACD).

Metabolism of GSH occurs via inter-organ and intra-organ cycles in the liver, kidney, and small intestine. The liver is the major organ that synthesizes GSH and exports this tripeptide in plasma and bile. The secreted GSH is degraded to the constituent amino acids in tissues that have γ-glutamyltransferase, such as the kidney, small intestine, and the bile tree. These amino acids are taken up by the liver and other organs to synthesize GSH and proteins (Meister, 1985; Inoue and Hirota, 1989). Cutaneous levels of GSH in mice have been known to change markedly after epicutaneous application of various haptens (Schmidt, 1992), but the dynamic aspects of GSH metabolism in the skin and other tissues and its role in irritant contact dermatitis (ICD) and ACD remain to be elucidated. The current work describes the changes in GSH metabolism in skin and liver and their role in mice with ICD and ACD.

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Abbreviations: ACD, allergic contact dermatitis; BSO, buthionine L-sulfoximine; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; ICD, irritant contact dermatitis; SH, free thiol; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

MATERIALS AND METHODS

Materials Female BALB/c mice, 8–12 wk of age, were obtained from Charles River Laboratories (Kingston, NY). GSH, dinitrochlorobenzene (DNCB), and cysteine hydrochloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Buthionine-L-sulfoximine (BSO), glutathione reductase, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), ninhydrin, thiobarbituric acid, and ferricytochrome c were obtained from Sigma Chemical Company (St. Louis, MO). All other reagents were of analytical grade.

Animal Experiments The mice were fed laboratory chow and water *ad libitum*. Under mild ether anesthesia, the skin was shaved with electric clippers before the application of DNCB. Animals were sensitized by a topical application of 50 μl of 2% DNCB in acetone on the abdominal skin 5 d before the experiments. To induce ICD and ACD, the unsensitized and sensitized mice were challenged with 50 μl of 0.5% DNCB on the dorsal skin. Control animals were treated concurrently with 50 μl of acetone.

Analysis At the indicated times, the mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The dorsal skin including dermis and epidermis (about 200 mg) was quickly removed, weighed, cut

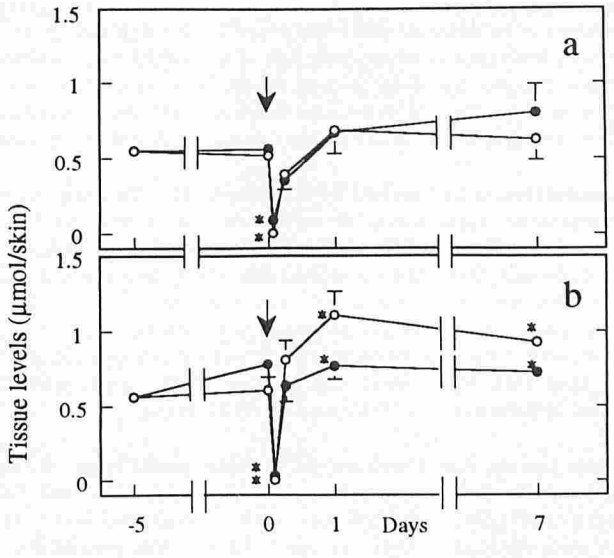

Figure 1. Changes in glutathione levels in the skin after challenge with DNCB. Both unsensitized mice (*a*, ICD group) and mice sensitized with 50 μmol of 2% DNCB (*b*, ACD group) were cutaneously challenged with 50 μmol 0.5% DNCB (↓) as described in the text. At the indicated times, the animals were anesthetized and their skin was excised and analyzed for free SH groups (○) and total glutathione levels (●). Six mice were generally used for each experimental group except for the two time points (at 0 h and after 7 d), in which four mice were used. Data represent the mean ± SEM. *, $p < 0.01$ compared to untreated initial levels.

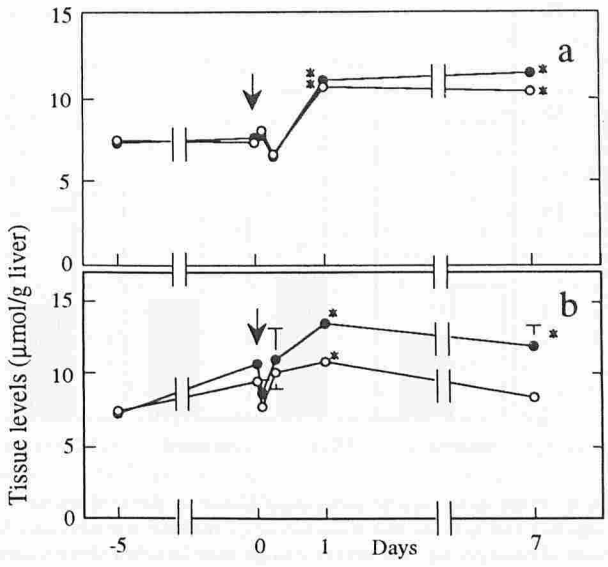

Figure 2. Changes in glutathione levels in the liver after challenge with DNCB. Both unsensitized mice (*a*, ICD group) and mice sensitized with 50 μmol of 2% DNCB (*b*, ACD group) were cutaneously challenged with 50 μmol 0.5% DNCB (→) as described in the text. At the indicated times, the animals were exsanguinated and their livers were excised and analyzed for free SH groups (○) and total glutathione (●). Data represent the mean ± SEM derived from four to six animals. *, $p < 0.01$ compared to untreated initial levels.

into small pieces, and pulverized in liquid nitrogen. The skin samples thus obtained were homogenized in 5 vol of 5% sulfosalicylic acid with a Polytron homogenizer at a maximum speed for 1 min. After perfusion with 10 ml of ice-cold saline solution, the excised liver was homogenized in 10 vol of sulfosalicylic acid. The homogenates thus obtained were centrifuged at 12,000 × *g* for 10 min, and the supernatant fractions were used for determination of total glutathione (GSH + 2GSSG [oxidized glutathione]) and free thiol (SH) levels. Total glutathione levels were determined by the method of Tietze (1969), which is based on the catalytic action of GSH reductase. Thiol levels were determined spectrophotometrically by reaction with DTNB (Elleman, 1959). Cysteine levels in the supernatant fractions were also determined spectrophotometrically using the acid ninhydrin reagent (Gaitonde, 1967). All procedures were carried out at 4°C.

Enzymes The dissected skin and liver were stored at −80°C until used. The frozen samples were homogenized in 10 vol of 10 mM phosphate buffer, pH 7.4, for 1 min. Aliquots of the homogenates were used for determination of thiobarbituric acid reactive substances (TBARS) by the method of Ohkawa *et al* (1979). In brief, the assay mixture contained 0.1 ml of the homogenate, 0.3 ml deionized water, 0.1 ml 8.1% sodium dodecyl sulfate, and 1.5 ml of 0.4% thiobarbituric acid and 50% acetic acid in a final volume of 2 ml. The mixtures were heated at 95°C for 60 min. After cooling, 2.5 ml of n-butanol was added to extract lipid peroxides. The mixture was centrifuged at 1,250 × *g* for 10 min, and the butanol fractions were analyzed spectrophotometrically at 535 nm. The remaining homogenates were subjected to ultrasonic disruption at 4°C for 2 min. The resulting samples were centrifuged at 12,000 × *g* for 20 min. The supernatants were kept on ice and analyzed for enzyme activity and protein concentration. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured spectrophotometrically by the method of Floche (1984a, 1984b). In the SOD assay, the formation of superoxide anion is determined by cytochrome c reduction. The amount of SOD inhibiting the cytochrome c reduction rate by 50% is defined as one unit. In the GPx assay, one unit of GPx activity is equivalent to the oxidation of 0.5 μmol reduced nicotinamide adenine dinucleotide phosphate by GSH reductase under the defined conditions. The activity of Mn-SOD was also measured in the presence of 1 mM KCN. The enzyme activity was measured at 25°C. Protein concentration was measured by the method of Lowry *et al* (1951).

Histology Paraffin sections (5 μm) of the skin were prepared and stained with hematoxylin and eosin. Experiments were also carried out with animals that received oral BSO (2 mmol/kg/d), a specific inhibitor of γ-glutamylcysteine synthetase.

Statistical Analysis All data are expressed as mean ± SEM. Statistical analysis was carried out using analysis of variance.

RESULTS

Changes in Glutathione Levels in Skin and Liver When mice were challenged with DNCB, cutaneous levels of glutathione and free SH groups rapidly decreased in both the ICD and ACD groups **(Fig 1)**. The decreased glutathione and SH returned to their initial levels more rapidly in mice with ACD than with ICD. Levels of free SH in the ACD group increased further and remained at high levels for at least 7 d. Hepatic levels of glutathione and SH also decreased transiently after challenge with DNCB and increased further in both the ICD and ACD groups **(Fig 2)**. In contrast to the changes in the skin, total glutathione in the liver of the ACD group increased more markedly than did SH, suggesting an increase in

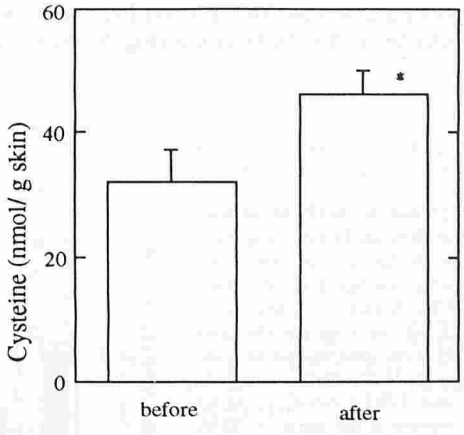

Figure 3. Increase in cysteine levels in mice with ACD. Before (n = 4) and 24 h after (n = 6) challenge with 50 μmol 0.5% DNCB, sensitized mice were sacrificed and cutaneous levels of cysteine were determined as described in the text. Data represent the mean ± SEM. *, $p < 0.05$.2

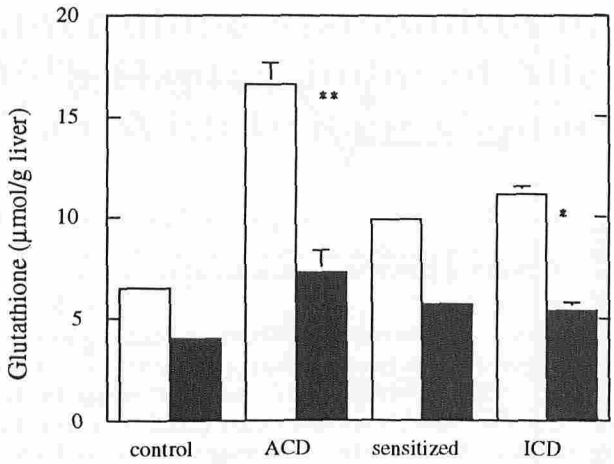

Figure 4. GSH turnover is enhanced more in ACD than in ICD. Both unsensitized and sensitized animals were injected intravenously with 0.4 mmol of BSO per kg 24 h after challenge with 50 μmol of 0.5% DNCB or control treatment (50 μl acetone). Hepatic glutathione levels were determined before *(open bars)* or 1 h after BSO injection *(closed bars)*. Data represent the mean ± SEM derived from four animals. *, $p < 0.05$; **, $p < 0.01$ compared to untreated groups.

GSSG. The levels of glutathione and SH remained unaffected in control animals treated concurrently with acetone (data not shown).

Increase in Cysteine Levels in the Skin Because cutaneous levels of the free SH group in ACD mice increased significantly after challenge with DNCB, we determined cysteine levels in the skin before and after the treatment **(Fig 3)**. One day after challenge with DNCB, the cutaneous level of cysteine in the ACD group increased by 144%, but the concentration of cysteine in the skin was less than 10% of that of free SH groups.

GSH Turnover Is Enhanced More in ACD Than in ICD To elucidate the dynamic aspects of GSH metabolism in mice with ICD and ACD, we administered BSO intravenously and determined changes in hepatic levels of glutathione **(Fig 4)**. BSO decreased the hepatic levels of glutathione in control mice by about 40%. Although hepatic levels of glutathione were significantly higher in the ACD and ICD groups than in the control group, BSO also decreased the levels in these groups. The extent of the BSO-induced decrease in glutathione levels differed markedly from one group to another. This result suggested that GSH turnover in these groups differed significantly (control < sensitized < ICD < ACD).

Levels of both glutathione and free SH groups in the skin also increased markedly in the ACD group **(Fig 5)**. Hepatic levels of these compounds also increased in both the ICD and ACD groups. Pretreatment of animals with BSO not only inhibited these increases in both groups of animals, but also resulted in a significant decrease. Despite the drop in total levels, however, there was no change in the absolute difference between SH and glutathione levels in the skin in the ACD group (about 0.3 μmol per g tissue).

Inflammation Is Enhanced by BSO in ACD To study the role of glutathione metabolism in mice with ICD and ACD, we measured the effect of BSO on the histology of the skin **(Fig 6)**. In control animals, BSO had no appreciable effect. In mice with ICD, mild infiltration of inflammatory cells was observed, which was slightly enhanced by BSO. In mice with ACD, infiltration of inflammatory cells was apparent and was enhanced significantly by BSO. Epidermal injury including necrosis of the upper epidermis was also apparent in the BSO-treated ACD group.

TBARS Levels Are Unchanged in Skin and Liver Because GSH status in the skin and liver of mice with ICD and ACD changed markedly after challenge with DNCB, oxidative stress might be increased in these animals. To test this possibility, we determined levels of TBARS in the skin and liver. Levels of TBARS have been used as an indicator of lipid peroxidation caused by free radicals. Cutaneous levels of TBARS before and after challenge with DNCB were 0.87 ± 0.04 (mean ± SEM) and 0.93 ± 0.06 nmol per mg protein in mice with ICD and 0.92 ± 0.12 and 1.18 ± 0.29 nmol per mg protein in mice with ACD, respectively. Hepatic levels of TBARS before and after challenge with DNCB were 0.49 ± 0.06 and 0.55 ± 0.03 nmol per mg protein in mice with ICD and 0.52 ± 0.04 and 0.59 ± 0.07 nmol per mg protein in mice with ACD, respectively. Thus, no significant change in TBARS levels in skin and liver was found between the animal groups.

Decrease in SOD and GPx Activities in the Skin Because GSH status in animals with ICD and ACD changed markedly, the enzymes involved in the metabolism of GSH and related compounds might be affected in these animals. To test this possibility, we measured cutaneous levels of SOD and GPx **(Fig 7)**. Administration of DNCB rapidly decreased the total activity of SOD in the skin of both animal groups. Differential analysis of SOD isozymes revealed that both Cu/Zn-SOD and Mn-SOD decreased instantaneously after challenge with DNCB. The decreased activity of SOD remained low for at least 1 wk, except for Mn-SOD in the ACD group. The activity of GPx also decreased rapidly by about 70% and returned to 70% of the control level in both groups. In contrast, the levels of these enzymes remained unaffected in control animals treated concurrently with acetone (data not shown).

To elucidate the mechanism of the rapid decrease in enzyme activity, we studied the effects of DNCB on these enzymes *in vitro* **(Fig 8)**. DNCB rapidly inactivated all three enzymes. Among the

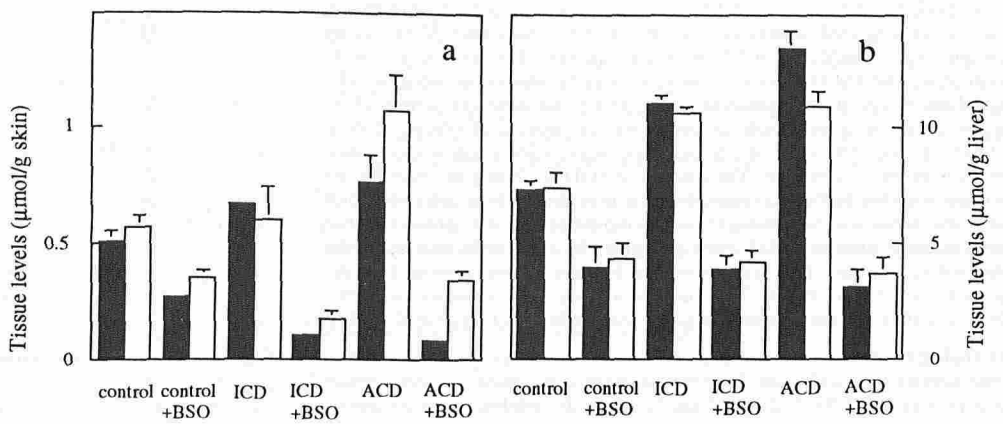

Figure 5. Increase in GSH is inhibited by BSO in skin and liver. BSO was administered orally for 6 d (2 mmol/kg/d). At 24 h after challenge with 50 μmol of 0.5% DNCB, levels of glutathione *(closed bars)* and SH *(open bars)* in the skin *(a)* and liver *(b)* were determined in control, BSO-treated, ICD, BSO-treated + ICD, ACD, and BSO-treated + ACD groups. Data represent the mean ± SEM derived from six animals.

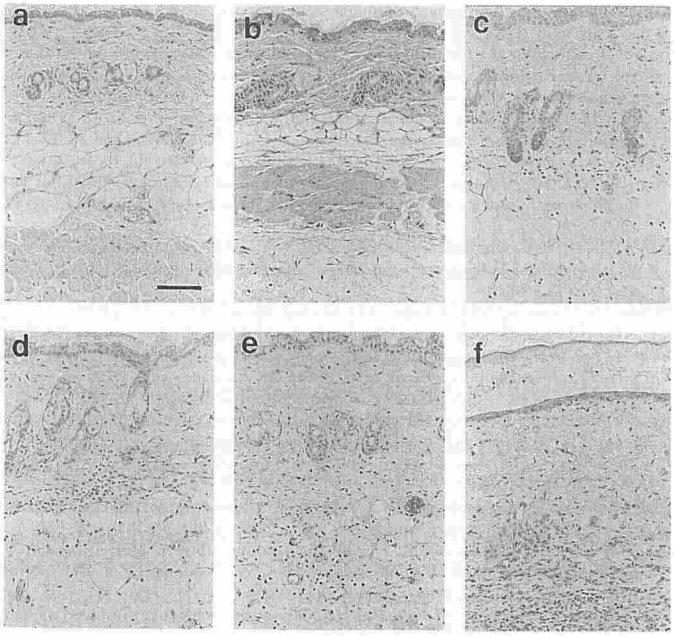

Figure 6. Inflammation is enhanced by BSO in ACD. BSO was administered orally for 6 d (2 mmol/kg/d). The skin was obtained from mice 24 h after challenge with 50 μmol of 0.5% DNCB or acetone. *(a)* Control; *(b)* BSO-treated; *(c)* ICD; *(d)* BSO-treated + ICD; *(e)* ACD; *(f)* BSO-treated + ACD groups. *Scale bar*, 50 μm.

three enzymes tested, the rate of inactivation was highest with Cu/Zn-SOD.

DISCUSSION

The current work demonstrates that GSH metabolism is enhanced more markedly in mice with ACD than in those with ICD. Cellular levels and turnover of GSH are regulated by the amounts of γ-glutamylcysteine synthetase and its substrates, feedback inhibition of the enzyme by GSH, extracellular secretion of GSH and its metabolites, and by conjugation with electrophiles (Inoue *et al*, 1984). Because both total glutathione and free SH groups in the skin and liver decreased almost instantaneously in ACD and ICD, this decrease might principally reflect the conjugation of DNCB with GSH. The initial GSH depletion and rebound observed in this study are typical of those observed in the liver after administration of toxicants (Nakagawa *et al*, 1984; Corongiu and Milia, 1987; Wallig *et al*, 1992). The amounts of hepatic glutathione decrease after challenge with DNCB in the ICD and ACD groups (1.2 and 2.0 μmol, respectively) were 10 times larger than the decreases in glutathione in the skin (0.1 μmol). This observation might suggest that DNCB first reacted with GSH in the skin, and the remaining ligand was transferred to the liver and metabolized enzymatically by conjugation with GSH. The total reduction of hepatocutaneous glutathione in the ACD group (2.1 μmol) was significantly greater than the dose of DNCB (1 μmol per mouse), whereas that in ICD mice was close to the dose of the ligand. This observation suggests that direct conjugation of DNCB with GSH occurred predominantly in ICD, whereas both conjugation and enhancement of extracellular secretion of GSH occurred in ACD. Consistent with this notion is the finding that the turnover of hepatic GSH was enhanced more in ACD than in ICD. Furthermore, the decreased glutathione and SH levels returned to normal more rapidly in the skin of the ACD group than that of the ICD group.

It should be noted that DNCB increased hepatic levels of GSSG in ACD, but not in ICD. It is known that glutathione conjugates competitively inhibit both biliary secretion of GSSG (Akerboom *et al*, 1982) and glutathione reductase (Bilzer *et al*, 1984). Thus, GSSG would have accumulated in the liver of ACD mice by means of formation of the DNCB conjugate of glutathione. Because delayed-type inflammation often induces oxidative stress, the inflammatory reaction might also participate in increasing GSSG in the liver of the ACD group. Although the mechanism by which hepatic GSSG increased in ACD mice is not clear, inflammatory metabolites and/or cytokines might be transferred from the injured skin to the liver and may have caused oxidative stress, but TBARS in the skin and liver remained unchanged after challenge with DNCB in both groups. These results suggest that lipid peroxidation may not play a critical role in the pathogenesis of contact dermatitis, although oxidative stress might enhance GSH metabolism in ACD (Jaeschke, 1992; Woods *et al*, 1992; Benard and Balasubramanian, 1993; Ochi, 1993; Shi *et al*, 1994). Moreover, inflammatory cytokines might participate in increasing GSH in the skin and liver in ACD. A recent study revealed that tumor necrosis factor-α (TNF-α) or interleukin-1β increased the activity and expression of γ-glutamylcysteine synthetase (Urata *et al*, 1996). The effects of inflammatory cytokines on GSH metabolism in mice with ACD should be studied further.

Histologic examination suggested that GSH might suppress immune reactions and oxidative stress in ACD rather than in detoxifying in ICD. In this context, nuclear factor-κB is known to trigger the expression of immunologically relevant genes for inflammatory cytokines such as TNF-α. TNF-α is produced in

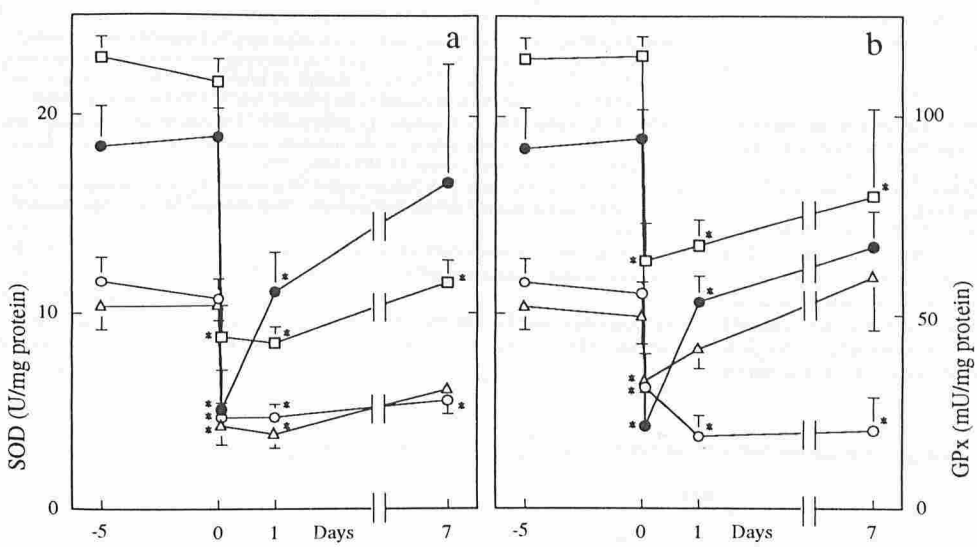

Figure 7. Decrease in GPx and SOD activities in the skin. Both unsensitized mice (*a*, ICD) and mice sensitized with 50 μl of 2% DNCB (*b*, ACD) were cutaneously challenged with 50 μl of 0.5% DNCB as described in the text. At the indicated times, skin was obtained and analyzed for enzyme activities. ○, total SOD; □, Mn-SOD; △, Cu/Zn-SOD; ●, GPx. Data represent the mean ± SEM derived from four to six animals. *, $p < 0.01$ compared to untreated initial levels.

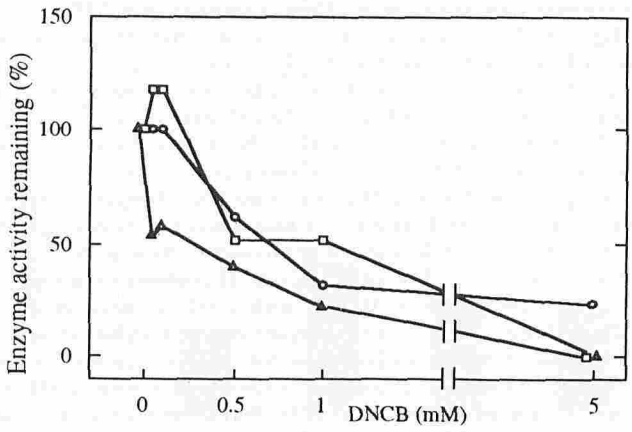

Figure 8. Inactivation of GPx and SOD by DNCB. The incubation mixture contained 0.1 M phosphate buffer, pH 7.4, 200 mg of the supernatant fractions of the skin per ml, and varying concentrations of DNCB. After incubation at 37°C for 5 min, the remaining enzyme activity was determined as described in *Materials and Methods*. ○, GPx; □, Mn-SOD; △, Cu/Zn-SOD.

keratinocytes and induces ACD-like inflammation (Piguet *et al*, 1991). Hence, depletion of cutaneous GSH by BSO might increase oxidative stress and stimulate the production of TNF-α, thereby enhancing the inflammatory reaction in mice with ACD. Consistent with this hypothesis is the report that N-acetylcysteine inhibited the expression of mRNA for TNF-α and decreased infiltration of inflammatory cells in ACD (Senaldi *et al*, 1994).

After challenge with DNCB, cutaneous levels of free SH groups increased markedly in the skin of ACD mice, but not in ICD mice. Although cutaneous cysteine increased in ACD mice, this increase was significantly smaller than the increase in free SH groups. Thus, low-molecular-weight thiols other than cysteine might be predominantly responsible for the increase in SH groups in the skin of ACD mice. Chemical properties of the increased thiols should be studied further.

DNCB directly inhibited the activities of SOD isozymes and GPx in the skin of both ICD and ACD mice. Among the three enzymes incubated with DNCB, inactivation was greatest with Cu/Zn-SOD. This might be due to the presence of lysine residues, which attract superoxide anions to the catalytic site of SOD (Birder and Johansen, 1980; Steinman, 1982). Inactivation of these enzymes might also increase oxidative stress in the skin, thereby enhancing inflammatory reactions, although the inhibitory effect of DNCB on the enzymes might occur independent of the change in GSH metabolism.

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