Cutaneous Barrier Perturbation Stimulates Cytokine Production in the Epidermis of Mice

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Abstract

The disruption of the cutaneous permeability barrier results in metabolic events that ultimately restore barrier function. These include increased epidermal sterol, fatty acid, and sphingolipid synthesis, as well as increased epidermal DNA synthesis. Because tumor necrosis factor (TNF) and other cytokines are known products of keratinocytes and have been shown to modulate lipid and DNA synthesis in other systems, their levels were examined in two acute models and one chronic model of barrier perturbation in hairless mice. Acute barrier disruption with acetone results in a 72% increase in epidermal TNF 2.5 h after treatment, as determined by Western blotting. Furthermore, epidermal TNF mRNA was elevated ninefold over controls 2.5 h after acetone treatment. This elevation in TNF mRNA was maximal at 1 h after acetone, and decreased to control levels by 8 h. After tape stripping, a second acute model of barrier disruption that avoids application of potentially toxic chemicals, TNF mRNA was elevated fivefold over controls at 2.5 h. Moreover, the mRNA levels for epidermal IL-1 alpha, IL-1 beta, and granulocyte macrophage–colony-stimulating factor (GM-CSF) also were elevated several-fold over controls, after either acetone treatment or tape stripping, but their kinetics differed. GM-CSF mRNA reached a maximal level at 1 h after acetone, while IL-1 alpha and IL-1 beta were maximal at 4 h after treatment. In contrast, mRNAs encoding IL-6 and IFN gamma were not detected either in control murine epidermis or in samples obtained at various times after tape stripping or acetone treatment. The relationship of the cytokine response to barrier function is further strengthened by results obtained in essential fatty acid deficient mice. In this chronic model of barrier perturbation mRNA levels for epidermal TNF, IL-1 alpha, IL-1 beta, and GM-CSF were each elevated several-fold over controls. These results suggest that epidermal cytokine production is increased after barrier disruption and may play a role in restoring the cutaneous permeability barrier. (*J. Clin. Invest.* 1992. 90:482–487.) Key words: tumor necrosis factor • interleukin-1 • granulocyte macrophage–colony-stimulating factor • essential fatty acid deficiency

Introduction

The stratum corneum provides the cutaneous permeability barrier that is essential for terrestrial life (1). This permeability barrier resides in the extracellular lipid domains of the stratum corneum which arise from the exocytosis of the lipid enriched contents of epidermal lamellar bodies (1–3). Topical application of organic solvents or detergents or tape stripping removes lipids from the stratum corneum, thereby acutely disrupting the permeability barrier (1, 4). Such acute perturbations of the barrier initiate a chain of events that ultimately leads to the return of lipids to the stratum corneum and the restoration of barrier function (5). These events include the rapid secretion of preformed lamellar bodies by stratum granulosum cells (6, 7), the formation and accelerated secretion of newly synthesized lamellar bodies in the stratum granulosum cells (6, 7), an increase in epidermal sterol (8), fatty acid (9), and sphingolipid synthesis (10, 11), and an increase in epidermal DNA synthesis (12). In essential fatty acid deficient (EFAD)[1] mice, a dietary-induced model of chronic barrier disruption, epidermal sterol, fatty acid, sphingolipid, and DNA synthesis is similarly stimulated (9, 10, 12, 13).

Keratinocytes, which comprise 95% of the cells in the epidermis, have been reported to produce a wide array of cytokines, including tumor necrosis factor (TNF) alpha, colony stimulating factors, interleukins 1, 6, and 8, and growth factors (14–16). Moreover, increased synthesis of TNF alpha, IL-8, and other cytokines have been demonstrated in psoriatic skin and it has been hypothesized that changes in cytokine production may play a role in the pathogenesis of this disorder (17, 18). UV irradiation also induces the release of epidermally derived TNF, IL-1, and IL-6 (19–21). Both psoriatic and UV-exposed epidermis have defective barrier function, and it has been suggested that epidermal damage may initiate the psoriatic process (22). Finally, numerous in vitro studies have shown that cytokines regulate a wide array of cellular processes in keratinocytes (15). For example, both the growth and differentiation of cultured keratinocytes are profoundly altered by the addition of selected cytokines (23–25). Because cytokines are produced by epidermal cells and are important regulators of keratinocyte function, the present study was designed to determine whether perturbations of barrier function alter epidermal cytokine production.

Methods

Materials. [$^{-32}$P]deoxycytidine 5′-triphosphate, tetra (triethylammonium) salt (3,000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Multiprime DNA Labelling System was obtained from Amersham Corp. (Arlington Heights, IL). Sephadex G-50 spin columns were from Worthington Biochemical Corp. (Freehold, NJ). Nylon membranes (0.45 μm Nytran) and nitrocellulose (0.2 μm)

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1. *Abbreviations used in this paper:* EFAD, essential fatty acid deficient; GM-CSF, granulocyte macrophage–colony-stimulating factor; TEWL, transepidermal water loss; TNF, tumor necrosis factor.

were obtained from Schleicher and Schuell, Inc. (Keene, NH). Oligo (dt) cellulose type 77F, was from Pharmacia (Uppsala, Sweden). X-OMAT AR film was purchased from Kodak (Rochester, NY). The Western Light Chemiluminescent detection system was obtained from Tropix Inc. (Bedford, MA). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific Co. (Fairlawn, NJ).

The mTNF alpha cDNA probe (B9) was obtained from Dr. Bruce Beutler (University of Texas, Southwestern Medical School, Dallas, TX). The mIL-1 beta and mIFN gamma-pms10 cDNA probes and mTNF alpha protein were kindly provided by Genentech (South San Francisco, CA). The rat IL-6-pRI16C.94 cDNA probe was obtained from American Type Culture Collection, Rockville, MD (37681). The granulocyte macrophage–colony-stimulating factor (GM-CSF) cDNA probe was obtained from Drs. N. Gough and Ashley R. Dunn (Royal Melbourne Hospital, Melbourne, Australia). The IL-1 alpha cDNA was kindly provided by Dr. J. Ansel (University of Oregon Medical Center). Rabbit anti-TNF alpha antibodies were generated against mTNF in rabbits by immunization of New Zealand White rabbits by standard techniques at Caltag Laboratories (South San Francisco, CA). A second polyclonal antibody against TNF was purchased from Genzyme Corp. (Cambridge, MA).

Disruption of the permeability barrier. Male hairless mice (Simonsen Animal Vendors, Gilroy, CA), 6–8-wk old, were treated with absolute acetone by gently rolling acetone-soaked cotton balls on the skin for 5–10 min (5, 9). Alternatively, barrier disruption was achieved by repeated applications of cellophane tape (five to eight times). Both procedures were terminated when the transepidermal water loss (TEWL) reached 6–10 mg/cm^2 per h measured with an electrolytic water analyzer (Meeco, Inc., Warrington, PA) as described (5, 9). Control mice were either untreated, or treated with sterile, endotoxin-free 0.9% sodium chloride. Essential fatty acid deficient mice, who have a chronic barrier defect (TEWL > 4 mg/cm^2 per h) were prepared as described previously (26).

Isolation of epidermis. Mice were killed by cervical dislocation. Skin was immediately excised from the entire torso and placed epidermal side down on petri dishes. Subcutaneous fat was scraped off with a scalpel, and skin was placed epidermal side up onto 10 ml of 10 mM EDTA in Ca, Mg-free PBS and incubated for 35 min at 37°C to separate epidermis from dermis (8, 9). The epidermis was removed using a scalpel and was immediately placed at −80°C for subsequent mRNA isolation.

mRNA isolation and Northern blotting. 300–400 mg of epidermis (from two mice) was processed for mRNA extraction by the method of Chomczynski and Sacchi (27). Poly(A)$^+$ mRNA was isolated by oligo (dt) chromatography according to Sambrook et al. (28). Typical yields were 18–20 μg per sample. 6–8 μg of poly(A)$^+$ mRNA was loaded per lane onto a formaldehyde/1% agarose gel which was prepared according to the method described by Davis et al. (29). After electrophoresis the gel was stained with acridine orange for visualization of the integrity of residual rRNA bands. Electrotransfer of RNA to a nylon membrane was performed in 1X 3-(4-Morpholino) propane sulfonic acid (MOPS) buffer, overnight, at 4°C. The nylon was then placed between two sheets of filter paper (Whatman Laboratory Products Inc., Clifton, NJ) and baked in a vacuum oven at 80°C for 2 h. cDNA probes were ^{32}P-labeled by the random priming method according to the manufacturer's instructions. Labeled probes were purified by column chromatography through G-50 mini spin columns. After the addition of 0.5 mg salmon sperm DNA, probes were denatured by boiling for 5 min, and then chilled on ice.

Northern blots were prehybridized for 1 h at 65°C in buffer (5× SSC, 2% SDS, 10% dextran sulfate, 2× SDS, 10% dextran sulfate, 2× Denhardt's, 0.1 mg/ml salmon sperm DNA). After the addition of ^{32}P-labeled probe, blots were hybridized overnight in the same buffer at 65°C. The first wash was carried out in 0.2× SSC, 0.1% SDS for 20 min at room temperature. The last wash was performed in buffer of the same composition at 65°C for 1 h. Blots were rinsed with diethyl pyrocarbonate-treated water and exposed to Kodak X-OMAT AR film at −70°C.

TNF-alpha Western blots. Mice were treated with absolute acetone as described above except that only one flank was exposed to solvent. The other flank served as control. Epidermis was isolated by placement on a drop of water and heat-splitting at 60°C for 55 s. A crude epidermal homogenate was obtained by a slight modification of the procedure described by Didierjean et al. (30). Freshly isolated epidermis (30–60 mg) was placed in 1.5 ml of extraction buffer (2% SDS, 62 mM Tris, pH 6.8, plus 17 μg/ml PMSF) and sonicated three times for 15 s each. After one spin at 16,000 *g* for 20 min the supernatant was removed and assayed for protein content. The samples were loaded onto 10% SDS-polyacrylamide gels (31). After electrophoresis, proteins were transferred from the slab gel onto nitrocellulose according to the method of Burnette (32). Immunoblotting to detect TNF alpha was performed with the Western Light Chemiluminescent detection system according to the kit instructions. Protein was determined using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL).

Densitometry. A densitometer from E-C Apparatus Corp., St. Petersburg, FL, was used for scanning films. The center of each lane was scanned on all films.

Statistical analysis. Statistical significance was determined using either a two-tailed Student's *t* test or a paired *t* test.

Results

We initially determined, using Western blots, the quantity of TNF in the epidermis of control mice vs. mice whose barriers had been disrupted by acetone treatment. As seen in Fig. 1 *A*, there is a prominent band at ~ 17,000 which corresponds to the molecular weight of TNF (33). A standard of pure murine TNF examined simultaneously (lane 5) comigrates with this band. Additionally, the use of a different antibody to murine

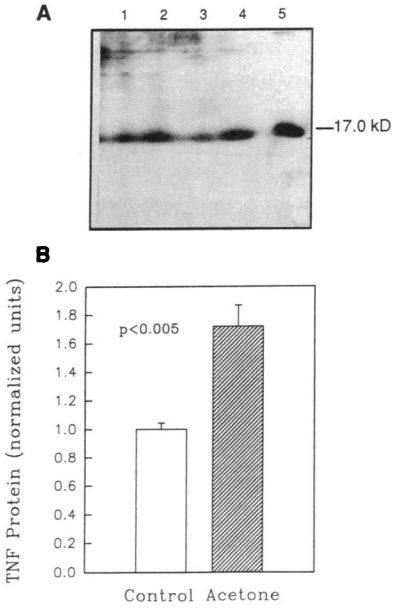

Figure 1. Western blot analysis of epidermal TNF alpha 2.5 h after barrier disruption with acetone. One flank from each of two hairless mice was treated with acetone as described in Methods. The other flank served as control. After 2.5 h mice were killed, epidermis was isolated from each flank, and a crude homogenate was obtained. Equal amounts of protein (300 μg) were loaded onto lanes of a reducing 10% SDS-polyacrylamide gel. After electrophoresis and transfer, immunoblotting was performed using polyclonal rabbit antiserum raised against TNF alpha. (*A*) Immunoblot: lanes *1* and *3*, control flanks; lanes *2* and *4*, treated flanks. Pure recombinant TNF alpha (20 ng) was loaded as a standard (lane *5*). (*B*) Quantification of epidermal TNF protein levels 2.5 h after barrier disruption with acetone. Densitometry was performed on Western blots obtained as described in *A*. Statistical significance was determined using a paired *t* test where control values were set to unity; $n = 6$. Data are mean±SEM.

TNF resulted in immunoreactivity with a similarly prominent 17,000 band. The intensity of the 17,000 band in the acetone-treated animals 2.5 h after barrier disruption is greater than in controls (Fig. 1 *A*), an impression supported by densitometry, which demonstrates a 72% increase in the amount of TNF in acetone-treated animals (Fig. 1 *B*). Little or no immunoreactive protein is detectable in either control or treated animals in the 24–26,000k region, where precursor TNF protein would be expected to migrate (34). These results show that barrier disruption results in an increase in TNF in the epidermis.

To elucidate the basis for this increase in TNF, the level of epidermal TNF mRNA was determined by Northern blot analysis (Fig. 2 *A*). In both acetone-treated and control epidermis a single band is seen at 2.0-kb which corresponds to the size of TNF mRNA (35). Moreover, the intensity of this 2.0-kb band is increased in animals whose barrier has been disrupted by acetone treatment. Quantification by densitometry (Fig. 2 *B*) demonstrates a ninefold increase in epidermal TNF mRNA in the acetone-treated versus control animals. Fig. 3 shows the time course of the increase in epidermal TNF mRNA following acetone disruption of the barrier. At 1 h after disruption, TNF mRNA levels are maximally increased and the levels gradually decrease thereafter, reaching control levels by 8 h. Thus, acute disruption of the barrier by acetone treatment causes a rapid increase in epidermal TNF mRNA levels which return to normal in parallel with barrier recovery (5, 6, 8).

We next determined whether other experimental manipulations that disrupt the barrier also affect TNF mRNA levels (Fig. 4). Acute disruption of the barrier with tape stripping also results in an increase in epidermal TNF mRNA levels at 2.5 h (4.4-fold increase). In essential fatty acid deficiency, a chronic model of barrier disruption, epidermal TNF mRNA levels are increased ~ 6.5 fold (Fig. 4). Thus a variety of models that produce perturbations in barrier function by entirely different

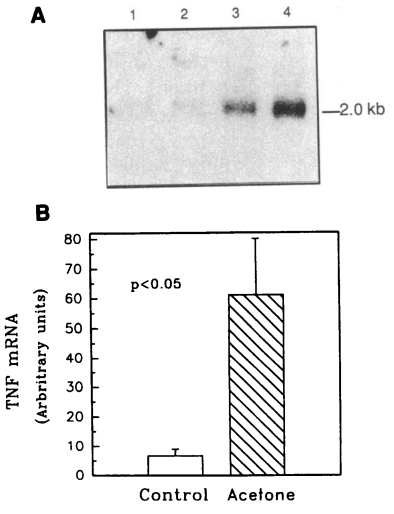

Figure 2. Northern blot analysis of epidermal TNF alpha mRNA 2.5 h after barrier disruption with acetone. The entire torsos of mice were treated with acetone as described in Methods. After 2.5 h treated and control mice were killed, epidermis was isolated, and RNA was prepared. Poly A^+ mRNA (6 μg) was loaded onto lanes of a 1% agarose gel. After electrophoresis and transfer, the blot was hybridized with a ^{32}P-labeled cDNA probe encoding TNF alpha. (*A*) autoradiograph: lanes *1* and *2*, control; lanes *3* and *4*, acetone treated. (*B*) Quantification by densitometry of epidermal steady-state TNF mRNA levels 2.5 h after barrier disruption with acetone. Densitometry was performed on Northern blots obtained as described in *A*. Statistical significance was determined using a Student's *t* test; $n = 4$. Data are means±SEM.

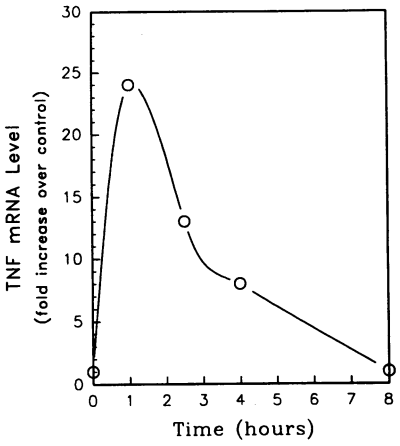

Figure 3. Levels of epidermal TNF alpha mRNA at various times after acetone treatment. Mice were treated with acetone as described in Methods. At 1 h, 2.5 h and 4 h after acetone treatment, the mice in each group were killed. Control mice were treated with saline, left undisturbed for 2–3 h, and then killed. The 8-h time point was examined with simultaneous controls in a separate experiment. Extraction, blotting, autoradiography, and densitometry were performed as in Fig. 2. Control levels were set at unity. Data points are plotted as mean values. SEM, which are small, are enclosed within the symbols; $n = 3$ for t = 0, 1, 2.5, and 4 h. $n = 4$ for t = 8 h and control.

mechanisms also display an increase in the levels of TNF mRNA in the epidermis.

To determine whether epidermal production of other cytokines might be stimulated by barrier abrogation, we used cDNA probes to IL-1 alpha, IL-1 beta, GM-CSF, IL-6, and interferon gamma to measure the epidermal mRNA levels of these cytokines after acute barrier disruption with acetone. As shown in Fig. 5 *A*, after acetone treatment there is a rapid increase (by 1 h) in GM-CSF mRNA levels in the epidermis, which returns to baseline within 4–8 h. Epidermal mRNA levels for IL-1 alpha and IL-1 beta also are increased but, in contrast to TNF and GM-CSF, the increase in IL-1 alpha and beta mRNA levels are maximal at 4 h (Fig. 5, *B* and *C*). Neither IL-6 nor interferon gamma mRNA levels were detected in either control epidermis or at any time after disruption of the barrier with acetone (data not shown). Thus, acetone disruption of the barrier results in a selective increase in the mRNA levels of several cytokines.

The effect of other methods of disrupting the permeability barrier on IL-1 alpha, IL-1 beta, and GM-CSF mRNA levels in the epidermis is shown in Fig. 6. Acute disruption of the barrier with tape stripping increases IL-1 alpha, IL-1 beta, and GM-CSF mRNA levels. Chronic barrier disruption induced by an essential fatty acid deficient diet also increases IL-1 alpha, IL-1

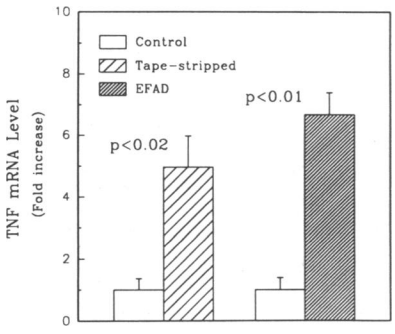

Figure 4. Levels of epidermal TNF mRNA in tape-stripped and EFAD mice. Northern blots containing epidermal samples from tape-stripped and EFAD mice were obtained and data analyzed as described in Fig. 3. For the tape-stripping experiment, $n = 5$, and for the EFAD experiment, $n = 4$.

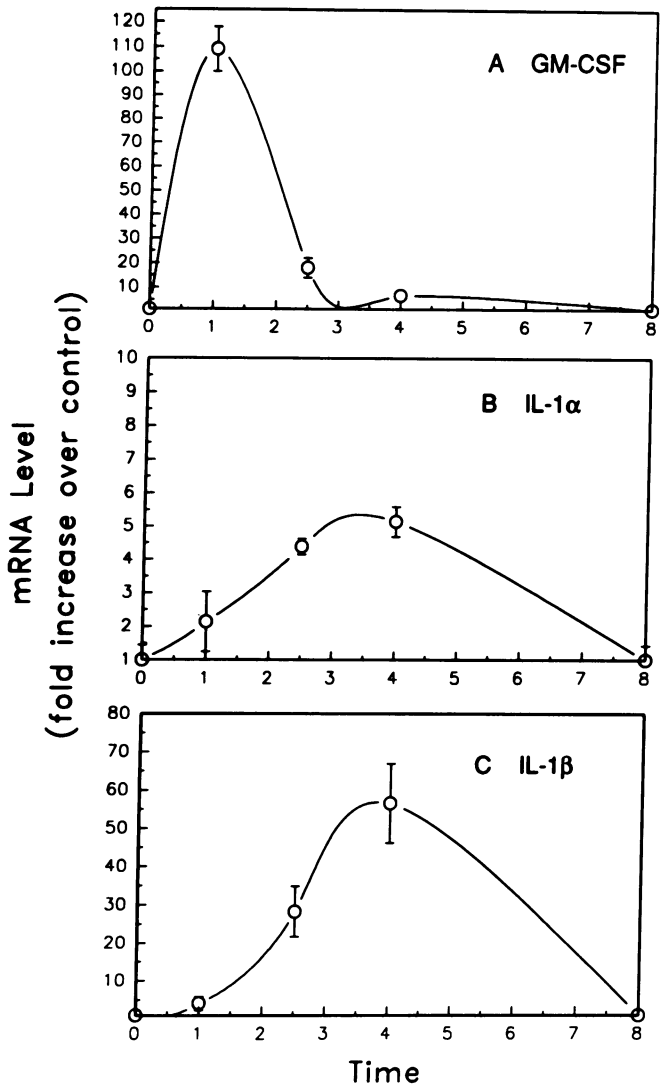

Figure 5. Levels of epidermal cytokine mRNA's at various times after acetone treatment. The Northern blots were prepared as described in Fig. 3 and probed with ^{32}P-labeled cDNA's encoding the indicated cytokines. Data are presented as mean±SEM. n = 3 for t = 0, 1, 2.5, and 4 h. n = 4 for 8 h and simultaneous control.

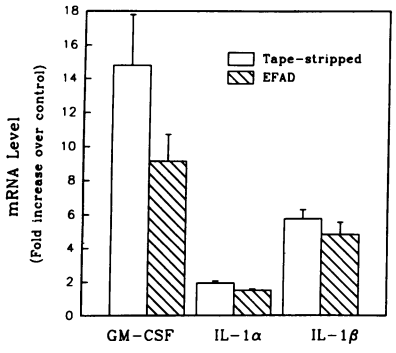

Figure 6. Levels of epidermal cytokine mRNA's in tape-stripped and EFAD mice. Northern blots were prepared as described in Fig. 4 and probed with ^{32}P-labeled cDNA probes encoding the indicated cytokines. Densitometry was performed and data analysis was as described in Fig. 3. Data are presented as mean±SEM. n = 5 for the tape-stripping experiments and n = 4 for the EFAD experiment. The levels of significance in the tape-stripped experiment are $P < 0.02$, $P < 0.005$, and $P < 0.01$, for GM-CSF, IL-1 alpha, and IL-1 beta, respectively. The levels in the EFAD experiment are $P < 0.02$, $P < 0.02$, and $P < 0.005$, for GM-CSF, IL-1 alpha, and IL-1 beta, respectively.

beta, and GM-CSF epidermal mRNA levels. Neither tape stripping nor essential fatty acid deficiency produced detectable levels of IL-6 or interferon gamma epidermal mRNA. Thus, in a variety of models of barrier disruption, epidermal mRNA levels for multiple cytokines are increased.

Discussion

The present study demonstrates that both acute disruption of the cutaneous permeability barrier by acetone treatment or tape stripping, or chronic disruption of the permeability barrier by feeding an essential fatty acid deficient diet increase the epidermal mRNA levels of TNF alpha, IL-1 alpha; IL-1 beta, and GM-CSF. In contrast, neither IL-6 nor interferon gamma mRNA levels are increased. It is unlikely that the increase in cytokine mRNA levels in the epidermis is a nonspecific toxic effect because three completely different models of barrier disruption, chemical, mechanical, and dietary, all result in a comparable profile of increased cytokine mRNA levels. The time course of the increase in the mRNA of these cytokines in response to acute barrier perturbation differs, with TNF and GM-CSF reaching maximal levels 1 h after disruption of the barrier while IL-1 alpha and IL-1 beta mRNA levels peak at 4 h. The magnitude of the increase in IL-1 alpha mRNA levels in response to barrier disruption is relatively small but this may be due to the fact that IL-1 alpha mRNA levels in controls are relatively high, whereas the baseline levels for TNF, GM-CSF, and IL-1 beta are very low in controls.

The mechanism accounting for the barrier disruption-induced increase in cytokine mRNA levels in the epidermis is unknown. In other cells, such as macrophages, increases in cytokine mRNA levels have been shown to be due to increased gene transcription (36, 37). Because of the small amount of epidermis that can be obtained from hairless mice, it is not possible to isolate sufficient quantities of nuclei to directly measure gene transcription. However, it is interesting to note that the mRNA's for TNF, IL-1 alpha, IL-1 beta, and GM-CSF all contain an eight nucleotide sequence in the 3′ untranslated region which controls mRNA stability (38, 39). Thus it is possible that mRNA stability could be altered by a common mechanism thereby leading to increased mRNA levels. The difference in the time course and the magnitude of the increases in mRNA levels for the various cytokines argues somewhat against alterations in mRNA stability alone being the primary mechanism for the increase in mRNA levels. Unfortunately, currently available, direct techniques for the measurement of mRNA stability are not applicable to in vivo studies of the epidermis. Nevertheless, our studies clearly demonstrate that cytokine mRNA levels in the epidermis are increased following barrier disruption.

In the case of TNF, the increase in epidermal mRNA levels is associated with a 72% increase in the active 17,000 form of TNF protein in the epidermis (33, 34). The precursor 24–26,000 form of TNF is not increased after barrier disruption, indicating that TNF is being rapidly processed to the active 17,000 protein that is secreted from cells (34). The relatively small increase in TNF protein compared to the severalfold increase in TNF mRNA levels is likely due to the secretion of TNF by keratinocytes. After UV irradiation of the skin, which stimulates epidermal TNF production, circulating levels of TNF are increased, indicating that systemic circulation occurs

(40). In addition to systemic appearance, much of the secreted TNF that remains in the epidermis may be degraded by receptor-mediated mechanisms or by extracellular proteases, known to be present in large quantities in epidermis (41). Thus, it is not surprising that TNF protein levels are not markedly increased when examined using Western blots. Whether the increase in mRNA levels for IL-1 alpha, IL-1 beta, and GM-CSF also results in an increase in the expression of these proteins and whether these proteins are processed to active forms or remain in the membrane bound precursor forms are currently under investigation. Prior studies have suggested that keratinocytes, because of the absence of a cleavage enzyme, are unable to process IL-1 beta to the 17,000 active form that is secreted (42).

One can only speculate on the potential role of the increase in epidermal cytokine production and secretion in the repair of the barrier. Barrier repair involves a large number of metabolic alterations that affect all layers of the epidermis. For example, lamellar body secretion and formation is stimulated in the upper stratum granulosum cells (6, 7), DNA synthesis is increased in the lower basal cell layer (12), and lipid synthesis is enhanced in all cell layers (43). We would hypothesize that the increased production of cytokines after barrier disruption orchestrates in a paracrine and/or autocrine fashion the metabolic responses required to reform the stratum corneum and repair the permeability barrier. Studies by this laboratory have demonstrated that several cytokines, including TNF, IL-1, IL-6, and interferon alpha, stimulate lipogenesis in liver cells (44–46). The increase in fatty acid synthesis in the liver induced by TNF, IL-1, and IL-6 is due to an elevation in hepatic levels of citrate (47), an allosteric activator of acetyl CoA carboxylase (48), a rate-limiting enzyme of fatty acid synthesis. The increase in cholesterol synthesis in the liver induced by TNF is due to an increase in the activity of HMG CoA reductase (44). Previous studies by our laboratory have shown that the increase in epidermal cholesterol synthesis after barrier disruption is also due to a stimulation in HMG CoA reductase activity (49). The mechanism by which barrier disruption stimulates epidermal fatty acid synthesis is currently unknown. It is possible that the increase in cytokine production that occurs in response to barrier disruption plays a role in increasing epidermal lipid synthesis.

A primary insult to the barrier could also contribute to the inflammatory component associated with a wide variety of dermatoses, including such unrelated disease entities as psoriasis and irritant dermatitis; i.e., cytokines generated in the epidermis could modulate not only physiologic (appropriate) responses in the epidermis, but also pathophysiologic (excessive or inappropriate) responses in the subjacent dermis. Traditionally, epidermal hyperplasia and barrier dysfunction has been considered secondary to an initiating blood-borne factor (e.g., circulating T cells in psoriasis), or primary dermal irritation (50, 51). Our studies suggest an alternative hypothesis that links dermal inflammation (including potential recruitment of blood-borne factors) to a primary insult in the epidermal barrier. Superficial trauma is widely appreciated to be an antecedent of psoriasis (Koebner or isomorphic phenomenon) (50, 52). Likewise, clinically uninvolved skin in atopic dermatitis displays a defective barrier (53). Applications of topical irritants, such as retinoids and trinitrochlorobenzene, are associated with altered barrier function (54, 55). Urushiol and trinitrochlorobenzene are known to stimulate the production of TNF alpha from keratinocytes, which in turn is thought to induce other cytokines in surrounding cells (56, 57). Studies are underway in both acute models of barrier disruption to determine whether barrier abrogation alone leads to a dermal inflammatory response, and to characterize the cell types involved in such a response.

In summary, the present study demonstrates that three different models of barrier disruption stimulate cytokine production in the epidermis suggesting that the repair of the barrier may be coordinated by cytokines. In addition, if pulsatile generation of particular cytokines are important in maintaining and repairing the barrier, this could explain the association of certain inflammatory disease states with altered barrier function.

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