Characterization of a Hapten-Induced, Murine Model with Multiple Features of Atopic Dermatitis: Structural, Immunologic, and Biochemical Changes following Single *Versus* Multiple Oxazolone Challenges

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Atopic dermatitis (AD) is a chronic dermatosis bearing clinical, histological, and immunologic similarities to chronic allergic contact dermatitis (ACD). AD shows a Th2 cell-dominant inflammatory infiltrate, elevated serum IgE levels, a permeability barrier abnormality, and *Staphylococcus aureas* colonization. Repeated hapten challenges reportedly produce a Th2-like hypersensitivity reaction (Th2-like HR). Here, 9–10 challenges with oxazolone (Ox) to hairless mice also produced a chronic Th2-like HR. Permeability barrier function and expression of differentiation proteins, filaggrin, loricrin, and involucrin, became abnormal. CRTH-positive Th2-dominant inflammatory infiltrate, with increased IL-4 expression, and a large increase in serum IgE levels were observed. The barrier abnormality was associated with decreased stratum corneum (SC) ceramide content and impaired lamellar body secretion, resulting in abnormal lamellar membranes, as in human AD. Furthermore, as in human AD, epidermal serine protease activity in SC increased and expression of two lamellar body-derived antimicrobial peptides, CRAMP and mBD3, declined after Ox challenges, paralleling the decrease of their human homologues in AD. Thus, multiple Ox challenges to normal murine skin produce a chronic Th2-like HR, with multiple features of human AD. Because of its reproducibility, predictability, and low cost, this model could prove useful for evaluating both pathogenic mechanisms and potential therapies for AD.

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INTRODUCTION

Over the last decade, a variety of murine models have been developed and characterized that display certain features of atopic dermatitis (AD). These models include: (1) spontaneous mutants, such as the Nc/Nga mouse (Matsuda *et al.*, 1997; Aioi *et al.*, 2001); (2) transgenic mice that either over- or under-express selective cytokines, such as IL-4, IL-18, or thymic stromal lymphopoietic factor (Chan *et al.*, 2001b; Konishi *et al.*, 2002; Chen *et al.*, 2005); (3) mice genetically-engineered to over-express stratum corneum (SC) chymotryptic enzyme (kallikrein 7) (Hansson *et al.*, 2002); and (4) mice challenged repeatedly with applications of soluble or aerosol haptens, such as ovalbumen (Savinko *et al.*, 2005), mite antigens (Matsuoka *et al.*, 2003; Gao *et al.*, 2004; Kang *et al.*, 2006), or trinitrochlorobenzene (Matsumoto *et al.*, 2004). While repeated hapten challenges elicit an AD-like dermatosis with some features of AD (Matsumoto *et al.*, 2004), an analogous process occurs only rarely in otherwise normal humans (Werfel *et al.*, 1997), perhaps because barrier function is superior in human epidermis. The importance of a primary inherited defect in the barrier, with subsequent hapten ingress, for the pathogenesis of AD is becoming increasingly accepted, because inherited mutations in the gene that encodes the SC structural protein, filaggrin, have been reported in a plurality of European kindreds with AD (Marenholz *et al.*, 2006; Palmer *et al.*, 2006; Sandilands *et al.*, 2006; Smith *et al.*, 2006; Weidinger *et al.*, 2006).

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Abbreviations: ACD, allergic contact dermatitis; AD, atopic dermatitis; Cers, ceramides; HR, hypersensitivity reaction; SC, stratum corneum; SP, serine protease; TEWL, transepidermal water loss; Th2-like HR, Th2-like hypersensitivity reaction

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To date, hapten-induced, chronic ACD models, although having the unique advantages of being convenient, reproducible, and inexpensive, have not been sufficiently characterized to ascertain whether they are true analogues of AD. While the trinitrochlorobenzene model displays certain clinical and immunologic features of human AD (Matsumoto *et al.*, 2004; Matsukura *et al.*, 2005; Tagami and Kikuchi, 2006), whether epidermal structural, functional, and lipid biochemical abnormalities, characteristic of human AD, occur in this model, is not known. Without these types of essential data, hapten-induced models remain of limited use for further studies on either AD pathogenesis, or for the evaluation of barrier-based, therapeutic interventions of potential utility in AD. We describe here another hapten-induced chronic Th2-like hypersensitivity reaction (HR) model, in which simple applications of the sensitizer, oxazolone (Ox), previously utilized to elicit ACD (Sheu *et al.*, 2002; Fowler *et al.*, 2003), was utilized to generate a chronic Th2-like hypersensitivity reaction (Th2-like HR) model. Following 10 Ox challenges, a dermatosis emerges with not only the immunologic but also with epidermal structural, functional, and biochemical abnormalities that parallel human AD. Availability of this thoroughly characterized model should facilitate future studies on both AD pathogenesis, as well as the evaluation of emerging therapies for AD.

RESULTS

Repeated Ox applications provoke a pruritic dermatosis with increased epidermal hyperplasia and aberrant epidermal differentiation

While a single Ox challenge to hairless mouse skin produces an acute ACD-like, erythematous and edematous dermatosis over 6–12 hours (Figure S1; see also Sheu *et al.*, 2002; Fowler *et al.*, 2003), 9–10 Ox challenges over 22–25 days provoked a persistent, chronic dermatosis with evidence of moderate-to-severe pruritus (Figure S1).

Whereas single Ox challenges produce mild epidermal hyperplasia, this feature became more prominent with successive challenges (Figure S2), so that by 10 applications mean epidermal thickness was 2.06 ± 0.05 *vs* 0.67 ± 0.03 in vehicle-treated controls ($P<0.0001$). The development of epidermal hyperplasia could be attributed to a marked increase in DNA synthesis, demonstrated by increased numbers of proliferating cell nuclear antigen+ cells in the basal and first suprabasal epidermal layers (Figure S2). Finally, epidermal hyperplasia was accompanied by a prominent inflammatory infiltrate, even after one Ox challenge, but inflammation became more apparent after 10 Ox challenges (Figure S2). In parallel with the emergence of epidermal hyperplasia and inflammation, expression of three structural protein markers of differentiation (loricrin, involucrin, filaggrin) either declined in the outer nucleated layers, or extended proximally into suprabasal cell layers with repeated Ox challenges (Figure S3).

Abnormal epidermal function appears only after repeated hapten applications

We next assessed whether progression from acute to chronic ACD is associated with alterations in epidermal functional parameters, including changes in permeability barrier homeostasis, SC hydration, and SC acidification. While all of these functions remain normal after a single Ox challenge, basal permeability barrier homeostasis, assessed as rates of transepidermal water loss (TEWL) increased markedly after 10 Ox challenges (Figure 1c, >10-fold elevation; $P<0.0001$). At this time point, SC hydration also declined significantly (Figure 1a; $P<0.01$), while skin surface pH increased by about $\frac{1}{2}$ a unit (Figure 1b; $P<0.01$). Finally, barrier recovery kinetics, assessed as the rate of decline in TEWL over time, either accelerated modestly or remained unchanged in repeatedly Ox-challenged skin sites (Figure 1d), as in human AD (Seidenari and Giusti, 1995).

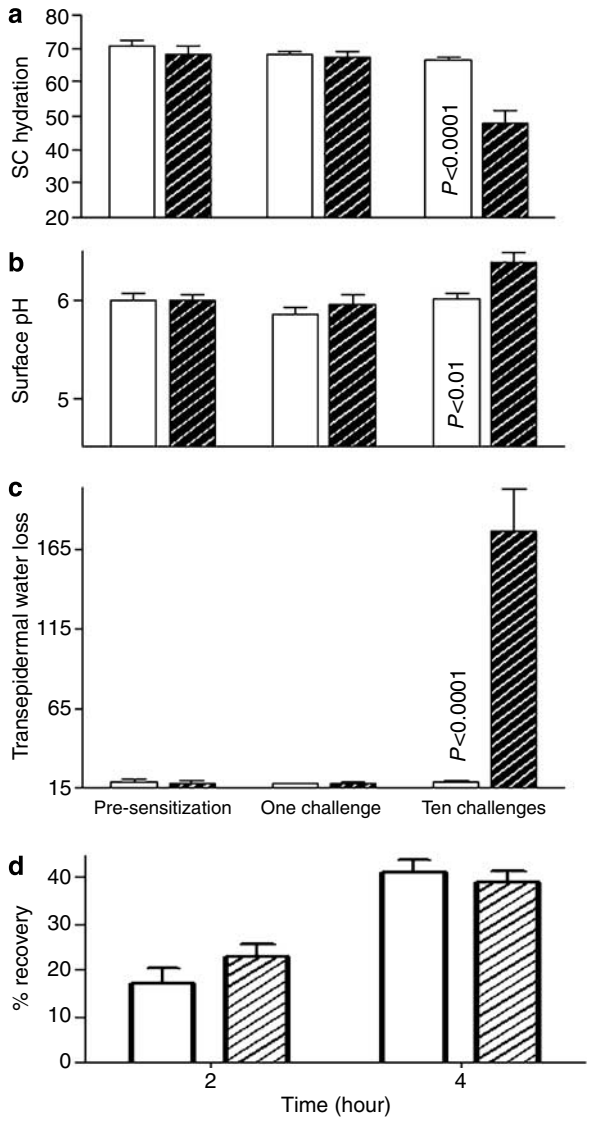

Figure 1. Functional abnormalities emerge after multiple Ox Challenges. (**a**) SC hydration assessed as electrical capacitance, in absolute units. (**b**) Surface pH assessed with a flat surface electrode. (**c**) Permeability barrier function, assessed as changes in basal TEWL with an electrolytic water analyzer (as mg/cm^2/hour). (**d**) Kinetics of permeability barrier recovery after acute barrier disruption by cellophane tape stripping (initial TEWL is increased to 20- to 30-fold above basal levels).

SC lipid abnormalities correlate with the barrier abnormality in Ox-challenged mice

Human AD is characterized by a decline in two key SC lipids, ceramides (Cers), and free fatty acids, with a further, selective decline in certain Cer species, most notably Cer 3 (Di Nardo *et al.*, 1998; Macheleidt *et al.*, 2002). Although total Cer content of SC in 10 Ox-challenged mice did not reduced, total free fatty acid content declined and that of cholesterol increased, as in human AD (Figure 2). Moreover, one of the five individual Cer species (murine SC displays only five Cer species because of lesser variation in sphingoid base and *N*-acyl fatty acid hydroxylation), Cer 3 declined significantly *versus* vehicle-treated controls (Figure 2; $P<0.001$), as observed repeatedly in human AD (Di Nardo *et al.*, 1998; Macheleidt *et al.*, 2002).

Ox-challenged mice display abnormalities in lamellar body secretion and post-secretory, lamellar membrane organization

The epidermis of human AD displays abnormalities in both lamellar body secretion (Fartasch *et al.*, 1992) and extracellular lamellar bilayer structure (Chamlin *et al.*, 2002), findings that correlate with reported lipid abnormalities (Chamlin *et al.*, 2002). While both lamellar body density and internal contents appeared normal in repeatedly Ox-challenged mice, secretion was impaired, as evidenced by retention of organelles in the peripheral cytosol (Figure 3a), coupled with reduced secretion at the stratum granulosum –SC interface (Figure 3c). Since considerable lamellar body contents become entombed within the corneocyte cytosol (Figure 3d, open arrows), rather than secreted, these contents would be unavailable to form lamellar membranes; hence, the decreased numbers of lamellar bilayers in repeated Ox-challenged mice (Figure 3d), as in human AD.

Ultrastructural evidence of impaired SC cohesion in Ox-challenged mice

The desquamation abnormality in repeatedly Ox-challenged mice could result, in part, from epidermal hyperplasia (Figure S2), but it also correlated here with multiple foci in the lower SC where corneocytes appeared to detach prematurely (Figure 3c). Extensive separation occurred both between adjacent corneocytes (Figure 3c, asterisks), and between the SC and the underlying nucleated cell layers (Figure 3c, open arrows). These ultrastructural findings are consistent with the visible scale in these mice, increased serine protease (SP) activity in these mice (see below), and the decreased SC cohesion in human AD (Cork *et al.*, 2006).

SP activity increases in Ox-challenged mice

The elevation in surface pH that follows repeated Ox challenges (Figure 1b) could result in increased SP activity (Hachem *et al.*, 2003, 2005a). Such pH-induced changes in SP activity could, in turn, account for the abnormal corneocyte cohesion in Ox-challenged mice through degradation of corneodesmosomes. To assess this potential pathomechanism, we next assessed changes in SP activity and localization after Ox challenges by *in situ* zymography. SP activity was low both under basal conditions (Figure S4) and after single Ox challenges (Figure S4), and was restricted to a narrow region of the SC. In contrast, after 10 Ox challenges, SP activity increased throughout the SC, further impinging on the outer nucleated layers (Figure S4). The increase in SP activity correlates with the observed increase in pH of SC (c.f., Figure 1), providing a mechanistic basis for the alterations in desquamation in these mice.

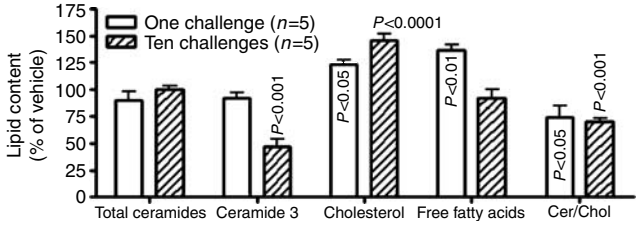

Figure 2. Repeated Ox challenges alter stratum corneum lipid content/distribution. Cohorts of hairless mice ($n=5$ each) were treated with vehicle alone, or Ox (1 or 10 applications). Lipids were extracted from isolated stratum corneum sheets with Bligh–Dyer solvents, fractionated, quantified, and the content of each fraction was expressed as μg/mg dry SC weight. Data were expressed as % change from vehicle-treated ± SEM.

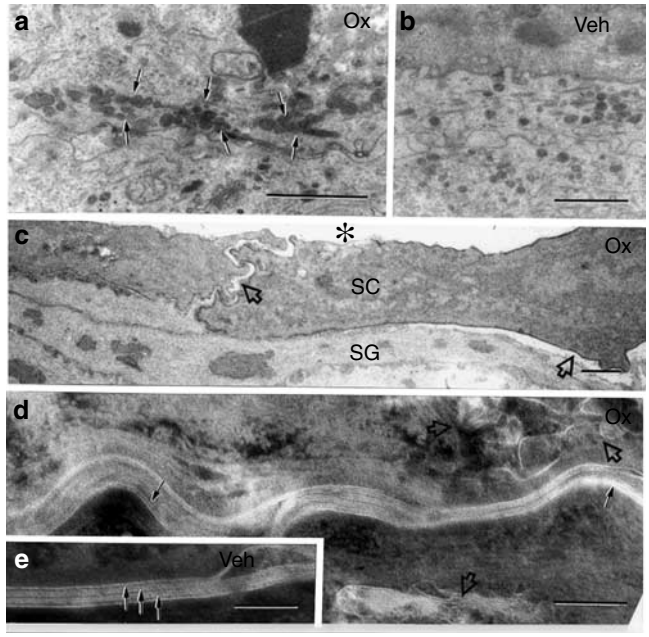

Figure 3. Repeat Ox-challenged SC displays entombed lamellar bodies, lamellar membrane disorganization, and corneocyte detachment. (**a**) Lamellar bodies (LBs) accumulate in the peripheral cytosol of granular cells in Ox-challenged mice (arrows), while (**b**) LB are more widely dispersed in preparation for secretion in vehicle (Veh)-treated mice. (**c**) Ox-challenged corneocytes (SC) focally detach from underlying stratum granulosum (SG) and lose contact between themselves (open arrows), sometimes leading to large cleavage planes (asterisk). Decreased quantities and poor organization of extracellular lamellar membranes are seen in Ox-challenged (**d**, single arrows) *versus* vehicle-treated (**e**, layered arrows) mice. Note entombed organelle remnants in corneocyte cytosol of Ox-challenged mice, indicating incomplete LB secretion (**d**, open arrows). (**a–c**) Osmium tetroxide post-fixation; (**d, e**) ruthenium tetroxide post-fixation. Bars = 1 μm (**a–c**); 0.1 μm (**d**, **e**).

Ox-challenged mice develop abnormalities in innate and adaptive immunity

We next determined whether repeatedly Ox-challenged mice develop an immunophenotype that is similar to human AD. After a single Ox challenge, the dermis displayed a modest inflammatory infiltrate (Figure S2), dominated by postglandin D receptor (CRTH2)-negative lymphocytes (Figure 4b) and most became CRTH2 positive (Figure 4c), with an increased density of mast cells, but few eosinophils (Figure S2). Over subsequent challenges, the lymphocyte-dominated infiltrate increased progressively, with some lymphocytes appearing to invade the overlying epidermis (Figure S2). The density of eosinophils in the dermis of 10 Ox-challenged mice also increased significantly (139.04 ± 28.13 *vs* 8.02 ± 5.71 in vehicle-treated, and 29.41 ± 18.46 after a single challenge; $P < 0.005$ for 10 *vs* 1 challenge). In addition, immunostaining for IL-4 also markedly increased in the dermis of 10x Ox-challenged mice (Figure S5). Finally, the increase in Th2 immunophenotype was paralleled by a progressive increase in serum IgE levels. While mean serum IgE levels were 29.7 ± 5.7 after one Ox challenge, they increased to $3{,}460 \pm 734$ *vs* 20.2 ± 3.5 in $10\times$ vehicle-treated mice ($P < 0.002$; $n = 5$ each). These results show that repeated Ox challenges yield a predominant Th2 phenotype, prominent eosinophils, and highly elevated IgE levels, mirroring humans with ''extrinsic'' AD.

In addition to the above-described changes in adaptive immunity, Ox-challenged mice rapidly developed alterations in antimicrobial peptide expression. While both the cathelicidin carboxyfragment, LL-37, and the human β-defensin 2, hBD2, typically upregulate during inflammation and/or wound healing (Sorensen *et al.*, 2003; Butmarc *et al.*, 2004), both of these peptides fail to upregulate in human AD (Ong *et al.*, 2002b). Hence, we next assessed changes in expression of the murine analogues of LL-37 and hBD2 (i.e., cathelin-related antimicrobial peptide (CRAMP) and mBD3, respectively) after one *vs* 10 Ox challenges. Basal protein levels of both peptides were demonstrable in the outer nucleated layers in untreated murine epidermis (Figure 5a and d), but both CRAMP and mBD3 immunostainable proteins declined markedly after a single Ox challenge (Figure 5b and e), a decrease that was sustained after 10 Ox challenges (Figure 5c and f). Thus, repeated hapten challenges induce changes in two innate immunity peptides that parallel human AD (Ong *et al.*, 2002b).

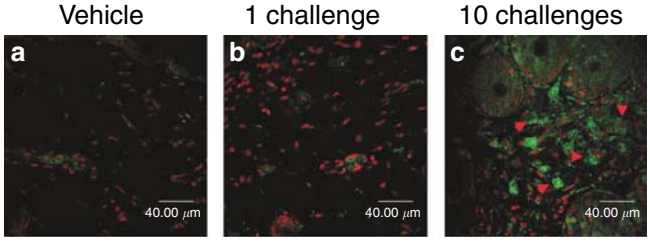

Figure 4. Th2 immunophenotype predominates after repeated Ox challenges. (**a**) CRTH2 immunostaining of Th2 lymphocytes is virtually absent in dermis after (**a**) vehicle treatment alone or (**b**) one Ox challenge, but density of CRTH2-positive cells increases significantly after 10 challenges (**c**, arrows). Merged images with propidium iodide secondary staining in 5 μm frozen sections. Bar = 40 μm.

DISCUSSION

The striking increase in the incidence of atopic disorders, including AD, observed in recent decades, has been ascribed to the migration of populations from rural to urban areas, where lack of early exposure to a variety of microbes purportedly results in reduced immune tolerance. Although the ''hygiene hypothesis'' may have some validity for mucosal atopy, the increased prevalence of AD in industrialized, urban areas instead results from increased exposure to indoor aeroallergens, such as dust mites (Sager *et al.*, 1992; Matsuoka *et al.*, 2003; Kiank *et al.*, 2006), and life-style changes coupled with frequent bathing, which could further degrade the barrier (Cork *et al.*, 2006). Moreover, aeroanti-

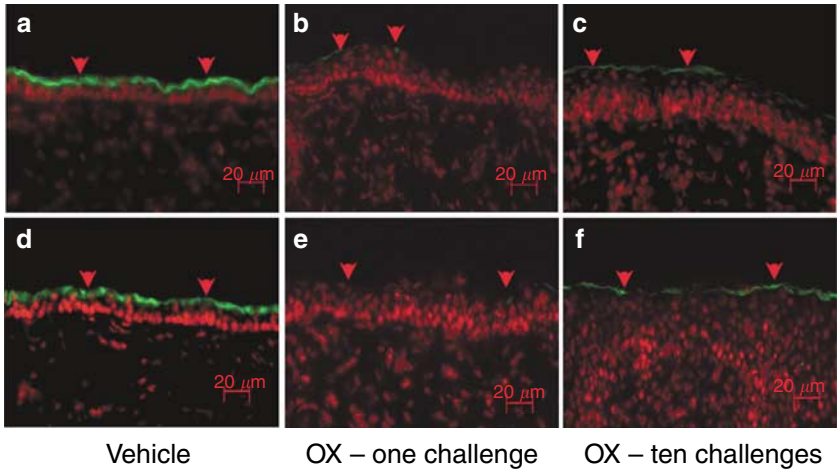

Figure 5. Antimicrobial peptide expression declines after one Ox challenge, and remains reduced after 10 challenges. Immunofluorescent staining for (**a–c**) cathelicidin-related antimicrobial peptide (CRAMP) and (**d–f**) mouse β-defensin 3 (mBD3). (**a, d**) With vehicle treatment; (**b, e**) with one Ox challenge; (**c, f**) with 10 Ox challenges. Propidium iodide counterstaining. Bars = 20 μm.

gens, responsible for mucosal atopy, can also gain access across the (defective) skin barrier in AD (Sager *et al.*, 1992). Furthermore, early and frequent use of topical glucocorticoids, as well as increased psychologic stress, which increases endogenous GC, may further degrade the barrier (Denda and Tsuchiya, 2000; Kao *et al.*, 2003; Choi *et al.*, 2005a, 2006), thereby increasing disease susceptibility.

Until recently, research on the etiopathogenesis of AD has focused primarily on immunologic abnormalities, including the potential provocative role of sensitized dendritic cells (reviewed in Novak and Bieber (2005), increased IgE production and/or receptor expression (Boguniewicz *et al.*, 2006), as well as the Th2 immunophenotype (Grewe *et al.*, 1998; Leung *et al.*, 2003, 2004), accompanied by production of cytokines that favor allergic responses and secondary pathogen colonization (Homey *et al.*, 2006). Without discounting the role of immunologic influences in the development of AD, evidence is accumulating that the primary abnormality in AD could result from inherited defects in SC structure and function (reviewed in Segre (2006). Inherited barrier abnormalities may predispose to AD by facilitating antigen egress. Hence, the focus has shifted, quite appropriately, from a primarily immunologic (''inside-outside'') to a more-probable, ''outside-inside'' paradigm for AD pathogenesis (eg, Elias *et al.*, 1996, 1996a, 1996b, 1999; Elias and Feingold, 1999). Accordingly, it becomes increasingly important to identify and characterize animal models that allow further investigations into the role of immunologic and skin barrier abnormalities in AD pathogenesis (Table S1).

The best-characterized murine model, the Nc/Nga mouse, develops a full panoply of atopic features, including a permeability barrier abnormality linked to Cer deficiency (Aioi *et al.*, 2001). AD-like disease only develops when mice are housed under conventional, ambient (i.e., ''dirty''), rather than sun protection factor conditions, but both environmental and topical haptens can provoke chronic Th2-like HR, even when Nc/Nga mice are housed in a sun protection factor facility (Matsuoka *et al.*, 2003; Gao *et al.*, 2004; Kang *et al.*, 2006). Although the specific abnormality in Nc/Nga mice remains unknown, they display mutations on chromosome 9, linked to increased IgE production, as well as Th2-type T cells, bearing CCR-4, within chronic Th2-like HR lesions (Matsuoka *et al.*, 2003; Gao *et al.*, 2004; Kang *et al.*, 2006). Coupled with further disadvantages of high costs, special housing requirements, and undependable elicitation of skin lesions, these mice have distinct disadvantages as an AD model. Likewise, published transgenic models, such as *Klk7* (Brattsand and Egelrud, 1999) and *IL-4* (Chan *et al.*, 2001a, b) overexpressing, and *IL-18* transgenic mice (Konishi *et al.*, 2002), while potentially useful for dissecting specific etiopathogenic steps, are of limited use for assessing alternate therapeutic approaches for AD, and none of these 3 models is commercially-available.

Prompted by the urgent need to develop a convenient, reproducible model, relevant for studies on both AD pathogenesis and assessment of alternate therapeutic approaches, we turned to a hapten-induced model, already employed to assess pathogenesis and potential therapies of acute allergic contact dermatitis (AACD), the Ox-sensitized mouse (Nakae *et al.*, 2001; Sheu *et al.*, 2002; Fowler *et al.*, 2003). We show here that with repeated hapten challenges over a 2- to 3-week period, this model shifts from typical delayed-type hypersensitivity, into a more chronic dermatosis, with multiple features of AD (Table S1), including the characteristic barrier (Seidenari and Giusti, 1995) and lipid abnormalities (Imokawa *et al.*, 1991; Bleck *et al.*, 1999).The short time required for disease induction, low costs, and reproducibility of disease expression are yet additional advantages of this model. Both the timing of disease development, and the cutaneous features of our model, are strikingly similar to the dermatosis that follows repeated applications of trinitrochlorobenzene (Kitagaki *et al.*, 1995; Matsukura *et al.*, 2005). Although such trinitrochlorobenzene mice demonstrate a shift from a Th1 to Th2 immunophenotype at sites of hapten application, neither epidermal structure, function, nor lipid biochemistry have been assessed in this model. Our studies show that initial hapten challenge, sufficient to elicit AACD, occurs while key epidermal functions still remain normal, emphasizing that inflammation alone does not suffice to produce epidermal functional abnormalities. Further hapten challenges, however, skew the AACD to a chronic ACD dermatosis, with a full panoply of features of AD, accompanied by the development of significant barrier dysfunction, likely allowing still more allergen to traverse the SC. The lesser competence of the permeability barrier in AD skin likewise presumably accounts for hapten ingress, sufficient to skew ACD toward chronic ACD, with features of AD. Although these results further underscore the potential importance of inherited abnormalities of the SC structural protein, filaggrin (Palmer *et al.*, 2006), and/or SP/anti-protease polymorphisms (Walley *et al.*, 2001; Vasilopoulos *et al.*, 2004) as primary provocateurs of AD, repeated hapten challenge can lead to Th2 cell activation, which could also reduce barrier function (Hatano *et al.*, 2005), linking immunologic changes to skin barrier abnormalities.

MATERIALS AND METHODS

Materials

Female hairless mice (hr/hr), aged 6–8 weeks old, were purchased from Charles River laboratories (Wilmington, MA) and fed mouse diet (Ralston-Purina Co., St Louis, MO) and water *ad libitum*. Ethanol and petroleum ether were purchased from Fisher Scientific (Fairlane, NJ). Ox, EDTA and trypsin were purchased from Sigma Chemical Co. (St Louis, MO). Affinity-purified, rabbit anti-mouse antibodies to loricrin, involucrin, and filaggrin were purchased from BabCo (Richmond, CA), and rabbit anti-mouse antibody against the postglandin D receptor, CRTH2/DP2, was from Cayman Chemical (Ann Arbor, MI). CRAMP antibody was a gift from Dr Richard Gallo, UCSD, and the mBD3 antibody was from Alpha Diagnostic (San Antonio, TX). Biotinylated goat anti-rabbit IgG antibody was purchased from Vector Lab (Burlingame, CA).

Experimental protocols and functional studies

All animal procedures were approved by the Animal Studies Subcommittee of the San Francisco Veterans Administration Medical

Center and performed in accordance with their guidelines. One group of animals was sensitized by one topical treatment with 10 μl of 5% Ox, while the ethanol-treated vehicle group served as the control. A week later, the Ox-treated group was treated topically with 60 μl of 0.1% Ox on both flanks, once every other day for an additional 2 weeks, with ethanol alone serving again as vehicle control. Both before challenge and at the end of the treatment period, basal TEWL, measured with an electrolytic water analyzer (Meeco, Warrington, PA), and SC hydration, assessed as capacitance, with a Corneometer CM820 (Courage & Khazaka, Germany), as described previously (Choi *et al.*, 2005b).

Immunohistochemistry and immunofluorescence

Immunohistochemical staining for assessment of changes in epidermal differentiation was performed as described earlier (Demerjian *et al.*, 2006). Briefly, 5 μm paraffin sections were incubated with the primary antibodies overnight at 4°C. After washes $\times 3$, sections were incubated with the secondary antibody for 30 minutes. Staining was detected with ABC-peroxidase kit from Vector Lab, and sections were then counterstained with hematoxylin. For immunohistochemistry for proliferating cells, changes in overall morphology were visualized after hematoxylin and eosin staining of 5 μm paraffin-enabled sections, and proliferating cells were detected by proliferating cell nuclear antigen staining. Briefly, 5 μm paraffin sections were incubated with biotinylated monoclonal antibody against proliferating cell nuclear antigen (CalTag Laboratories, Burlingame, CA) overnight at 4°C, and staining was detected by the ABC-peroxidase method (Vector).

Immunofluorescence was used to evaluate changes in T helper cell subpopulation expression. 5 μm frozen were incubated with the rabbit anti-mouse CRTH2/DP2 antibody overnight at 4°C, followed by incubation with fluorescence conjugated goat anti-rabbit antibody for 30 minutes at room temperature. Both CRAMP and mBD3 protein levels were assessed to determine whether antimicrobial peptide expression changes in epidermis, as described for human AD (Ong *et al.*, 2002a, b). For immunohistochemical staining for IL-4, we employed the method of Fan *et al.* (2001). Sections were examined with either a Zeiss fluorescence microscope (Jena, Germany), or on a confocal microscope, and digital images were captured with AxioVision software (Carl Zeiss Vision, Munich, Germany).

Eosinophil density measurement

The areas of dermis in hematoxylin and eosin-stained sections first were quantitated in a digital microscope equipped with AxioVision software. The depth of the measured areas extended to ≈ 170 μm below the basement membrane. The density of eosinophils was then expressed as the number of eosinophil per mm^2 ($n = 15$ for $10\times$ challenged, $n = 12$ for single challenge, and $n = 11$ for vehicle-treated samples).

SP activity

SP activity was assessed in freshly obtained skin samples by *in situ* zymography, as previously described (Hachem *et al.*, 2003, 2005b). 5 μm frozen sections were incubated with BODIPY-FI0-casain for 2 hours at 37°C. After 3x washing with 1% Tween 20 solution, sections were counter-stained with propidium iodide for 1 minute. Sections then were examined with the Zeiss or confocal microscopes, as above.

Serum IgE measurements

Blood samples were collected from mice tails after one Ox challenge, 10 Ox challenges, as well as in controls after one and 10 vehicle treatments alone. Serum IgE concentration was determined with a mouse IgE ELISA quantitation kit from Bethyl Laboratories (Montgomery, TX), following instructions provided by the manufacturer.

SC lipid content

After 2 weeks of treatment with Ox or vehicle, fresh SC sheets were collected after separation of dermis and epidermis by 10 mM EDTA preincubations, followed by trypsinization, as described (Mao-Qiang *et al.*, 1996). SC lipids were extracted by Bligh–Dyer solvents, and quantitated both as total lipids per mg protein and mg dry SC weight. Lipid extracts were further fractionated into cholesterol, free fatty acid, and Cer subfractions by high-performance thin layer chromatography, as described previously (Uchida and Hamanaka, 2006).

Electron microscopy

Skin biopsies of both vehicle and Ox-treated mice were fixed in Karnovsky's fixative overnight, and post-fixed with either 0.25% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide, as described previously (Hou *et al.*, 1991). Ultrathin sections were examined using an electron microscope (Zeiss 10A, Carl Zeiss, Thornwood, NY) operated at 60 kV.

Data are expressed as means $\pm$ SEM. A two-tailed Student's *t*-test was used to determine significant differences, and a further analysis of variance analysis was performed when three or more groups were compared.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Table S1. Described pathophysiological features of human AD and AD model mice.

Figure S1. Repeatedly Ox-challenged mice develop a pruritic dermatosis.

Figure S2. Repeatedly Ox challenges cause epidermal hyperplasia and chronic inflammation.

Figure S3. Alterations in epidermal differentiation after multiple Ox challenges.

Figure S4. SP activity increases and extends into nucleated layers after 10 Ox challenges.

Figure S5. IL-4 increase levels in repeatedly Ox-challenged mice.

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