

Murine model of atopic dermatitis associated with food hypersensitivity

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Background: Atopic dermatitis (AD) is an eczematous skin eruption that generally begins in early infancy and affects up to 12% of the population. The cause of this disorder is not fully understood, although it is frequently the first sign of atopic disease and is characterized by an elevated serum IgE level, eosinophilia, and histologic tissue changes characterized early by spongiosis and a CD4⁺ T_H2 cellular infiltrate. Hypersensitivity to foods has been implicated as one causative factor in up to 40% of children with moderate-to-severe AD.

Objective: The purpose of this study was to establish a murine model of food-induced AD.

Methods: Female C3H/HeJ mice were sensitized orally to cow's milk or peanut with a cholera toxin adjuvant and then subjected to low-grade allergen exposure. Histologic examination of skin lesions, allergen-specific serum Ig levels, and allergen-induced T-cell proliferation and cytokine production were examined.

Results: An eczematous eruption developed in approximately one third of mice after low-grade exposure to milk or peanut proteins. Peripheral blood eosinophilia and elevated serum IgE levels were noted. Histologic examination of the lesional skin revealed spongiosis and a cellular infiltrate consisting of CD4⁺ lymphocytes, eosinophils, and mast cells. IL-5 and IL-13 mRNA expression was elevated only in the skin of mice with the eczematous eruption. Treatment of the eruption with topical corticosteroids led to decreased pruritus and resolution of the cutaneous eruption.

Conclusion: This eczematous eruption resembles AD in human subjects and should provide a useful model for studying immunopathogenic mechanisms of food hypersensitivity in AD. (*J Allergy Clin Immunol* 2001;107:693-702.)

Key words: Atopic dermatitis, eczema, food hypersensitivity, murine model, T_H2-like cytokines, IgE-mediated hypersensitivity

Abbreviations used

AD: Atopic dermatitis
CM: Cow's milk
OVA: Ovalbumin

Atopic dermatitis (AD) is a form of eczema that generally begins in early infancy and is characterized by extreme pruritus, chronically relapsing course, and distinctive distribution. AD is often the first sign seen in a child who is prone to atopic disease, with 50% of all AD developing in the first year of life and 80% developing by 5 years of age.¹ In chronic skin lesions the epidermis has moderate-to-marked hyperplasia, with elongation of the rete ridges and prominent hyperkeratosis. Spongiosis is variable, and the numbers of mast cells and Langerhans cells are significantly increased.^{2,3} Eosinophils are sparse, although eosinophil products (major basic protein) are prominent.⁴ Although the pathogenic role of allergy in AD has been debated for over a century, clinical studies over the past 2 decades have supported the pathogenic role of food allergy in a subset of patients with AD.⁵⁻⁷ A recent study found that approximately 40% of children with moderate-to-severe AD attending a university dermatology clinic had food hypersensitivity.⁷ Other studies delineating the role of IgE-bearing antigen-presenting cells in establishing T_H2 lymphocytic responses⁸ and demonstrating the predominant T_H2-lymphocytic infiltrate in acute eczematous lesions⁹ have provided further support for the pathogenic role of allergy in AD.

Animal models provide powerful tools for dissecting pathogenic mechanisms responsible for various disorders. A number of murine models of asthma have been developed in the past several years that have contributed greatly to our understanding of the immunopathogenesis of allergen-induced asthma.¹⁰⁻¹² Models of antigen-associated AD have been reported in cats and dogs, but because of difficulties with consistent induction of AD-like eruptions and high expense, they have not been widely studied.^{13,14} A mouse strain, NC/Nga, has been reported that spontaneously has an AD-like skin lesion and IgE hyperproduction, presumably triggered by environmental factors.¹⁵ More recently, a murine model of AD was described in which BALB/c mice were sensitized to ovalbumin (OVA) by means of repeated application of OVA-impregnated patches.^{16,17} Biopsy specimens of lesional skin from sensitized mice revealed thickening

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and inflammation of the dermis and epidermis, mild spongiosis, a lymphocytic infiltrate consisting predominantly of CD4⁺ cells, and a large number of eosinophils. RT-PCR demonstrated increased expression of IL-4, IL-5, and IFN- γ . Here we report a mouse model of AD after oral sensitization with food allergens.

METHODS

Mice and antigen sensitization

Female C3H/HeJ mice were purchased from the Jackson Laboratory and maintained under specific pathogen-free conditions. Guidelines for the care and use of the animals were followed ("Guide for the Care and Use of Laboratory Animals," National Institutes of Health publication No. 86-23, as revised).

Homogenized cow's milk (CM; GAF Seelig Inc) and freshly ground whole peanuts were used as food allergens. Cholera toxin was purchased from List Biological Laboratories, Inc. To induce CM hypersensitivity-associated AD, 2- to 3-week-old mice in 2 separate experiments were sensitized intragastrically with CM alone (1 mg/body weight), boosted orally 5 times at weekly intervals, and maintained on a milk-containing mouse chow thereafter. To induce peanut antigen-associated AD-like lesions, 5-week-old mice were sensitized with peanut (1 mg per mouse) and cholera toxin (10 mg per mouse) and boosted thereafter 4 times at 2- to 3-week intervals. Intragastric feeding was performed as previously described.¹⁸ In earlier studies of CM hypersensitivity, we found that oral feeding of milk to 2- to 3-week-old mice induced moderate levels of IgE and immediate skin reactions (itching) after oral feeding of sensitized mice. In some cases the scratching persisted for more than 10 minutes. When mice with peanut allergy were generated, 5-week-old mice were used, and we also observed immediate skin reactions with itching after oral feeding of sensitized mice. Peanut is more allergenic than CM because we were unable to sensitize 5-week-old mice with CM, even in the presence of cholera toxin. Because of technical convenience, we used 5-week-old mice only for peanut sensitization. However, all these protocols for induction of food hypersensitivity were short in duration (5-6 weeks in our previous study). The finding that oral re-exposure to CM or peanut antigen resulted in immediate skin reactions prompted us to test the hypothesis that these sensitized mice might have chronic skin reactions similar to those induced by food antigens in AD after continuous exposure to the relevant food allergens. Thus we used the protocol used in a previous study for the initial sensitization in this study.

Microbiological examination

To exclude the possibility that AD-like lesions were associated with ectoparasites, hair was plucked from mice with AD-like lesions ($n = 4$) and naive mice ($n = 2$) and was examined by means of microscopy. To examine whether the lesional skin harbored bacterial infection, AD-like and normal control mice were anesthetized with a mixture of ketamine and xylazine (45 mg/kg and 10 mg/kg, respectively). Lesional skin of mice with AD-like lesions and normal skin of control mice were wiped with 70% alcohol, gently scraped with a scalpel blade, and then swabbed for bacterial culture. Each sample was cultured in thioglycolated enrichment media, TSA II 5% SB media, and Maccouny II media for detection of gram-positive, gram-negative, and anaerobic bacterial growth, respectively.

Histology

Skin biopsy specimens were obtained from the face or inguinal lesions of mice with AD-like eruptions and from the same sites of control mice 3 to 6 weeks after the skin symptoms developed. Skin samples were fixed in 4% phosphate-buffered formaldehyde (pH

7.2). Five-micrometer paraffin sections were stained with hematoxylin and eosin for identification of inflammatory cells or with toluidine blue or Giemsa stains for identification of mast cells. The total number of cells in the dermis, excluding the hair follicles, as well as the number of eosinophils and mast cells from each tissue biopsy specimen obtained, were determined. Cells were counted in 10 high-power fields in each of 3 to 5 sections from each biopsy specimen. The counting was performed with an Olympus BH2 microscope using a 40 $\times$ objective lens and a 10 $\times$ eyepiece containing a reticle, yielding a field of view of 0.22 mm by 0.22 mm. The data are expressed as cells per square millimeter.

Number of peripheral blood eosinophils

Blood was obtained from mice with AD-like lesions and normal naive control mice. Blood smears were stained with the Diff-Quik Stain Set (Dade Diagnostics of P.R. Inc). Differential cell counts of blood leukocytes were determined by means of microscopic evaluation. Two hundred cells per slide were counted.

Measurement of antigen-specific IgE in sera

Blood was obtained from the tail vein at 3- to 4-week intervals from week 9 through week 18. Serum antigen-specific IgE levels were measured by means of ELISA, as described previously.¹⁹ Briefly, Immulon II 96-well plates (Dynatech Laboratories, Inc) were coated with 2 μ g/mL purified CM protein (Ross Laboratories) or 2 μ g/mL crude peanut extract in coating buffer, pH 9.6 (Sigma). After overnight incubation at 4°C, plates were washed 3 times with PBS/0.05% Tween-20 and blocked with 3% gelatin in PBS/0.05% Tween-20 for 1 hour at 37°C. After the plates were washed 3 times, serum samples (1:10 dilutions) were added to the wells and incubated overnight at 4°C. Plates were then washed extensively, and 100 μ L of goat anti-mouse IgE (0.3 μ g/mL) was added to each well. The plates were incubated for 2 hours at 37°C. Plates were then washed 3 times, and 100 μ L of biotinylated donkey anti-goat IgG antibody (0.3 μ g/mL) was added for an additional 1 hour at 37°C. After 6 washings, 100 μ L of avidin peroxidase (Sigma, 1:1000 dilution) was added, and plates were incubated for an additional 10 minutes at room temperature. After 8 washings, the reaction was developed with ABTS (KPL) for 30 minutes at room temperature and read at 405 nm. Quantities of antigen-specific IgE were calculated by comparison with a reference curve generated with the use of mouse mAbs (anti-DNP IgE, Accurate Scientific Inc), as described previously.^{20,21}

Intradermal skin testing

Mice with CM-induced AD-like lesions and normal control mice were tested for immediate active cutaneous hypersensitivity reactions at week 12 after the initial CM sensitization, as previously described.²²⁻²⁴ Briefly, under anesthesia, the skin of the belly was shaved 1 day before the test. For each skin test, 50 μ L of CM protein (4 mg/mL) was injected intradermally with a 30-gauge needle while the skin was stretched taut. Ragweed antigen was used as an irrelevant antigen control. The wheal reactions were assessed 30 minutes after antigen injection. A reaction was scored as positive if the wheal diameter was greater than 3 mm in any direction. In addition, biopsy specimens of the wheal sites, sham-injected skin, and naive skin were obtained, and mast cell degranulation was assessed by means of histologic examination.

Immunohistochemistry

Biopsy specimens (2-3 mm) of AD-like mouse lesional facial skin and naive mouse facial skin were placed into molds in Tissue-Tek OCT compound (Sakura Finetek USA Inc), frozen in liquid nitrogen, and stored at -80°C. Twelve-micrometer sections were prepared and either stained immediately or stored at -20°C. Before staining, slides were fixed in acetone at -20°C for 10 minutes and

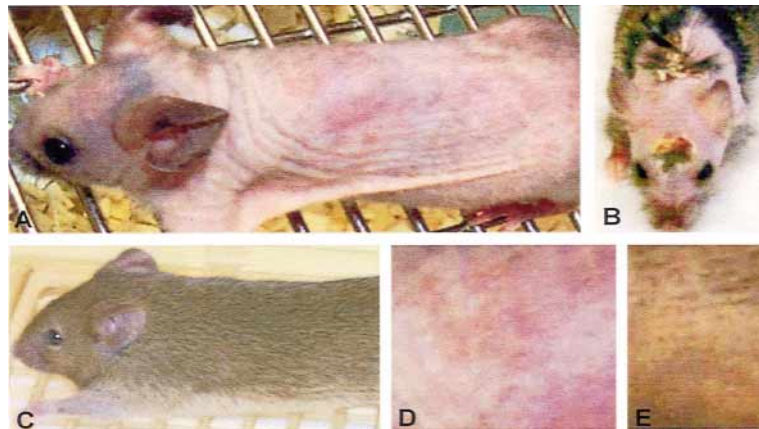


FIG 1. Expression of AD-like skin lesions. **A**, Extensive AD-like skin lesion with raised erythematous eruption, areas of hypopigmentation, and alopecia. **B**, AD-like lesion about the head and neck with alopecia and excoriations. **C**, Normal C3H/HeJ mouse. **D**, Close-up view of erythematous maculopapular eruption with areas of hypopigmentation and alopecia. **E**, Close-up view of normal skin from shaved naive mouse.

air-dried for 20 minutes. After washing with PBS to remove mounting media, sections were blocked with 2% normal goat serum for 30 minutes and incubated for 45 minutes at room temperature with rat anti-mouse mAbs: L3T4 or Ly2 diluted 1:250 or rat anti-mouse IgE diluted 1:100 (PharMingen) to detect CD4, CD8, and IgE⁺ cells, respectively. Rat IgG2a isotype antibodies were used as a control. After washing twice with PBS, specimens were incubated with biotinylated rabbit anti-rat IgG (Vector) diluted 1:200 in 2% normal goat serum for 30 minutes at room temperature. After washing twice with PBS, endogenous peroxidase was quenched with 3% H₂O₂ in methanol for 10 minutes at room temperature. After 2 washes with PBS, avidin-peroxidase complex (Zymed) was added for 15 minutes at room temperature. After 2 washes with PBS, diaminobenzidine (Vector) was added for 5 to 10 minutes at room temperature. Specimens were washed extensively with distilled H₂O and counter stained with hematoxylin. A cover slip was mounted with Advantage Aqueous Mounting Medium (Innovex). Additional specimens were counter stained with toluidine blue, dehydrated, cleared, and mounted with permanent mounting medium for the detection of IgE-bearing mast cells. Numbers of CD4⁺, CD8⁺ T cells and IgE⁺ cells, and IgE⁺ mast cells were counted, as described above.

T-cell proliferation assays

Mice with AD-like lesions were injected intradermally with CM extract (100 μ L of 1:10 wt/vol, Center Labs). Forty-eight hours later, a 2- to 3-mm² skin biopsy specimen was taken from the injection sites, and dermal cells were prepared as described previously.²⁵ Briefly, the epidermis and dermis were separated by overnight incubation at 4°C in Dispase (2.4 U/mL, Boehringer Mannheim). The dermal tissue was then incubated in Hanks solution (Gibco) containing 0.1% collagenase D (Boehringer), 20 μ g/mL DNase (Sigma), 0.1% hyaluronidase (Sigma), 0.003% Versene (Gibco), and 10% FCS (Gibco) for 5 hours at 37°C. The cell suspensions were washed and resuspended in RPMI culture medium containing 1% penicillin-streptomycin, 1% amphotericin B (Gibco), and 1 μ g/mL gentamicin (Gibco). Cells (1×10^4 /well) were cultured in the presence or absence of CM antigen (50 μ g/mL, Ross Laboratories). Four days later, the cultures were pulsed with 1 μ Ci per well of tritiated thymidine for 16 hours. The cells were harvested, and the incorporated radioactivity was counted in a β -scintillation counter. The results were expressed as counts per minute. Cells were cultured in duplicate, and each experiment and all assays were repeated at least twice.

TABLE I. Food allergens inducing AD

Sensitization	Experiment	Incidence, n/total (%)
CM	1	1/5 (20)
CM	2	3/6 (50)
Peanut + CT	3	2/7 (29)
Naive	1-3	0/8 (0)

Mice were sensitized with CM or peanut plus cholera toxin, as described in the "Methods" section in 3 separate experiments. Some of the mice had eczematous skin lesions 9 to 14 weeks after the initial CM or peanut sensitization. The incidence and percentage of AD were calculated.
CT, Cholera toxin.

RT-PCR

Total mRNA was isolated from biopsy specimens of the lesional skin of mice with AD-like lesions and the normal skin of naive mice at week 20 by using Trizol reagent (Gibco BRL), as described by the manufacturer. The reverse transcription was performed by using the Superscript Amplification System kit for cDNA synthesis (Gibco BRL), as described by the manufacturer.²⁶ Briefly, 12 μ L of RNA (1 μ g)/Oligo(dT) (1 μ L) mixture was incubated at 70°C for 10 minutes and then incubated on ice for 2 minutes. Seven microliters of reaction mixture (1 $\times$ PCR buffer, 5 mmol/L MgCl₂, 0.5 mmol/L deoxyribonucleoside triphosphates, and 0.02 mol/L dithiothreitol) was added to the RNA/primer mixture and incubated at 42°C for 5 minutes. One microliter (200 U) of Superscript II reverse transcriptase was then added, and the mixture was incubated at 42°C for 50 minutes. The reaction was terminated by incubating the mixture at 70°C for 15 minutes followed by the addition of 1 μ L of RNase H. First-strand cDNAs were either stored at -20°C or used for the PCR step.

PCR was performed as described previously,²⁷⁻²⁹ with slight modification. Briefly, PCR reactions (50 μ L total volume) were carried out in 2 mmol/L MgCl₂, 1 $\times$ PCR buffer, 2.5 U of AmpliTaq DNA polymerase, 2 μ L of 10 μ mol/L antisense and sense primer pairs, and 2 μ L of cDNA. PCR was carried out for 35 cycles beginning with 95°C for 2 minutes followed by the following temperature profile: denaturation at 94°C for 45 seconds; primer annealing at 60°C for 45 seconds; and primer extension at 72°C for 90 seconds. The final extension was at 72°C for 10 minutes. Once the PCR reactions were complete, 10 μ L of the reaction mixture was separated by means of electrophoresis through a 2.5% agarose gel and visual-

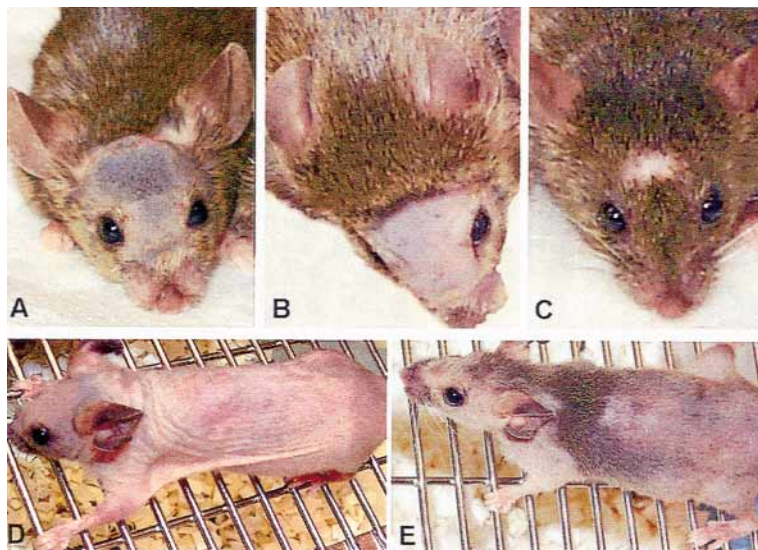


FIG 2. AD-like skin lesions before and after therapy with topical corticosteroids. **A**, Dermatitis and alopecia limited to the facial area. **B**, Dermatitis and alopecia limited to the facial area before topical corticosteroid therapy. **C**, Same mouse depicted in **B** after 2 weeks of topical corticosteroid therapy. No scratching behavior was noted after a few days of therapy, and facial hair had almost completely grown back. **D**, Extensive AD-like involvement with patches of erythema and hypopigmentation, dry scaly patches, lichenification, and alopecia. **E**, Same mouse depicted in **D** after 2 weeks of topical corticosteroid therapy. The mouse had partial hair regrowth and markedly reduced scratching behavior. Nevertheless, the skin retained patchy areas of dry scaly skin and hypopigmentation.

ized with ethidium bromide staining and UV irradiation. Gel images were captured by using a Gel Doc Image Analysis system (BioRad), and PCR product quantitation was performed by using densitometry with Quantity One Software (BioRad) and standardized against β -actin from the same mRNA preparation. Results were expressed as an OD ratio (cytokine/ β -actin). Before analysis, the PCR product band intensities were checked to ensure that they had not reached saturation. All reactions were repeated at least 2 to 3 times. Oligonucleotide primers for IFN- γ , IL-4, IL-5, and β -actin used in the PCR were purchased from Clontech (Clontech Laboratories), and IL-13 was obtained from Life Technologies.

Statistical analysis

All statistical analyses were performed with SigmaStat software (SPSS Inc). Data were expressed as means $\pm$ SEM, except for incidence of AD-like lesions and results of intradermal skin testing. The Mann-Whitney rank sum test was used for comparing differences between the total number of cells, intact and degranulated mast cells in the skin, and differences in peripheral blood eosinophils between mice with AD-like lesions and naive mice. The Student *t* test was used for comparing differences between serum IgE levels; T-cell proliferation; OD ratios of IL-4, IL-5, and IL-13; and IFN- γ versus β -actin between mice with AD-like lesions and naive mice. A *P* value of less than .05 was considered statistically significant.

RESULTS

Expression of AD-like skin lesions

Thirty-five percent of CM-sensitized mice and 29% of peanut-sensitized mice in 3 separate experiments had eczematous skin lesions 9 to 14 weeks after the initial CM or peanut sensitization (Table I). The clinical symptoms included pruritus, as demonstrated by scratching of the forehead, ears, legs, and inguinal regions and lichenified

plaques (Fig 1, A). As previously reported in other murine models of AD, various degrees of hair loss were a characteristic finding. Although alopecia was most commonly present on the forehead (Fig 1, A and B), varying degrees of hair loss were also present on the back and abdominal regions (data not shown). In the most severe cases the entire body was involved (Fig 1, A and D). Lesional regions were characterized by erythema, scaling and dryness, and hypopigmentation (Fig 1, A and D).

Because topical glucocorticoids are the cornerstone of therapy for eczema in human subjects, we examined the effect of topical glucocorticoids on the skin lesions in this AD model. Two mice with CM-induced AD-like lesions, one with severe (100% surface area involved) and one with moderate (less than 20% surface area) skin involvement, were treated with a topical steroid (Derma-Smoother, Fluocinolone Acetonide, 0.01% topical oil) once daily for 7 to 14 days. One week after treatment was discontinued, the skin of the mouse with moderate AD-like eruption (Fig 2, B) was significantly improved, with new hair evident in almost all lesional areas (Fig 2, C). The mouse with severe AD-like lesions was also improved after 2 weeks, exhibiting a reduction of scratching accompanied by new hair growth (Fig 2, E). However, scaling and dryness did not appear to have improved significantly in this period. After treatment was discontinued, skin lesions recurred in the severely affected mouse but not in the mouse with moderate symptoms. Episodic dermatitis characterized by spontaneous remissions and relapses persisted in all untreated mice with AD-like lesions throughout the 8-month observation period.

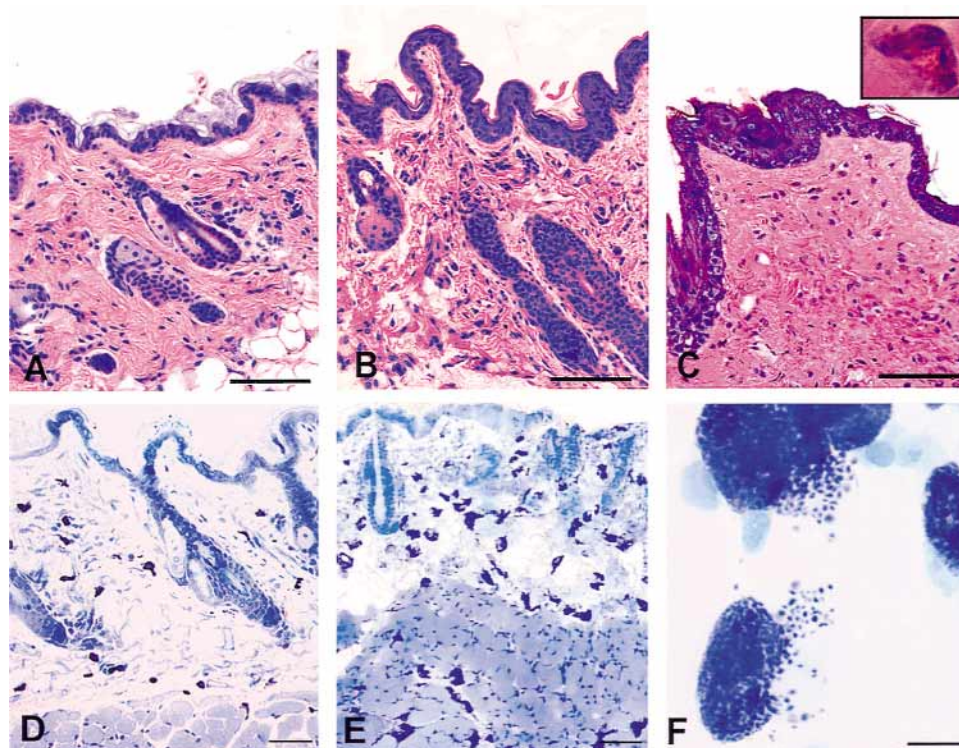


FIG 3. Histologic features of AD-like lesions. **A**, Normal inguinal skin from naive mouse (bar = 100 μ m). **B**, Biopsy specimens from inguinal area of AD-like lesion demonstrating mild spongiosis and epidermal thickening and marked mononuclear round cell infiltrate, especially about the hair follicles (bar = 50 μ m). **C**, Higher magnification of lesional skin depicted in **B**, showing spongiosis and epidermal thickening. *Insert* shows eosinophil (bar = 50 μ m). **D**, Frozen section of normal facial skin showing normal number of toluidine blue-stained mast cells (bar = 100 μ m). **E**, Frozen section of lesional facial skin showing markedly increased numbers of toluidine blue-stained mast cells (bar = 100 μ m). **F**, Degranulating mast cells in the dermis of lesional facial skin (bar = 10 μ m). **A**, **B**, and **C** are stained with hematoxylin and eosin stain, and **D**, **E**, and **F** are stained with toluidine blue.

Microbiological examination

To exclude the possibility that the dermatitis was associated with fur mites³⁰ or bacterial infection,^{31,32} causes of dermatitis that have been previously reported, we performed parasitologic and bacteriologic examinations. No fur mites were found in the hair of mice with AD-like lesions or control mice. No bacterial organisms were isolated from the skin of mice with AD-like lesions or control mice.

Histologic features of AD-like lesions

Histologic examination of lesional biopsy specimens revealed mild epidermal spongiosis and epidermal thickening (Fig 3, *B* and *C*) and numerous inflammatory cells in the dermis (Fig 3, *B*), including eosinophils, lymphocyte-like cells (Fig 3, *C*), and mast cells (Fig 3, *E* and *F*). Quantification of inflammatory cells showed that the total number of cells in the dermis of mice with AD-like lesions reached 1828 ± 95 cells/mm², including 179 ± 42 cells/mm² (10%) eosinophils and 300 ± 7 cells/mm² (16%) mast cells, 68% of which were degranulated (Table II). In contrast, skin from naive mice contained fewer cells in the dermis (612 ± 107 cells/mm²) and no eosinophils. The number of mast cells was 85 ± 35 /mm²,

TABLE II. Quantification of cells in dermis (cells/mm²)

	Total cells	Eosinophils	Total mast cells	Degranulated mast cells
AD	$1828 \pm 95^*$	179 ± 42 (10%)	$300 \pm 7^*$	204 ± 24 (68%)*
Naive	612 ± 107	0 (0%)	85 ± 35	14 ± 5 (16%)

The total number of cells in the dermis, excluding the hair follicles, and the number of eosinophils and mast cells in 10 high-power fields of each of 3 to 5 sections of biopsy specimens from 3 mice were counted at 400 $\times$ magnification by using an Olympus BH2 microscope and micrometer eyepiece. Values are means $\pm$ SEM.

* $P < .001$ versus naive mice.

16% of which were degranulated. The differences in total number of cells, number of eosinophils, and number of mast cells between the 2 groups were statistically different ($P < .05$). In addition, the dermis and epidermis in mice with AD-like lesions appeared thicker than normal (Fig 2, *B*, vs Fig 2, *A*). These histologic features are similar to those seen in patients with AD,³³ as well as in cats and dogs with AD.^{13,14}

Increased numbers of blood eosinophils

Previous studies of human AD found that eosinophil numbers were marginally increased in involved skin but

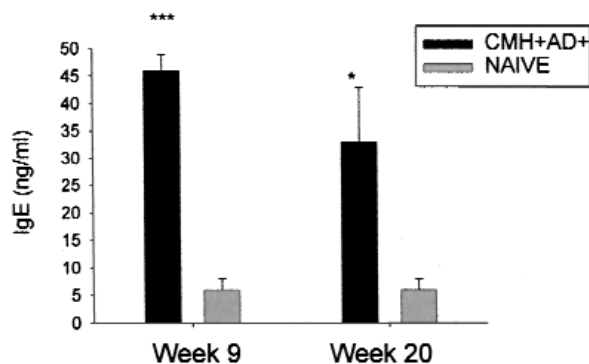


FIG 4. Increases in serum antigen-specific IgE. Blood was obtained from mice with AD-like lesions and naive mice ($n = 4-6$) at week 9 and week 20. CM-specific IgE levels were measured with an ELISA. CM-specific IgE levels were highest at week 9 and remained relatively constant between week 9 and week 20. *** $P < .001$ and * $P < .05$ versus naive mice.

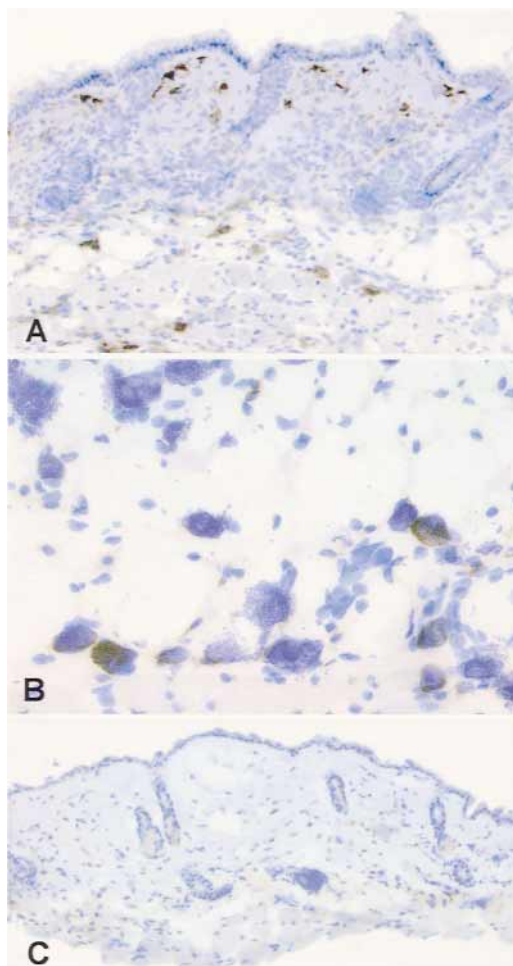


FIG 5. Increased numbers of IgE⁺ cells in AD-like lesions. **A**, Frozen section of facial lesion stained with anti-IgE mAb and counter stained with toluidine blue showing dark brown diaminobenzidine-stained IgE⁺ dendritic-shaped cells in the upper dermis, and IgE⁺ mast cells in the dermis and muscle (bar = 100 μ m). **B**, IgE⁺ mast cells with dark brown diaminobenzidine-stained cell membranes and toluidine blue-stained mast cell granules in a facial lesion at higher magnification (bar = 10 μ m). **C**, Frozen section of normal facial skin stained in the same manner as **A**.

markedly increased in the blood.^{24,34,35} We found in this study that mice with AD-like lesions also had higher peripheral blood eosinophil counts than control mice ($13.1\% \pm 0.9\%$ vs $2.6\% \pm 0.2\%$, $P < .001$).

Increased serum antigen-specific IgE, increased number of IgE-bearing mast cells, and positive immediate cutaneous hypersensitivity reactions to milk

To determine the relationship between IgE and CM-induced AD-like lesions, IgE levels in sera of AD-like and control mice were determined. Antigen-specific IgE levels were significantly increased at week 9 and remained elevated through at least week 20 (Fig 4). Antigen-specific IgE levels were also significantly increased in mice with peanut-induced AD-like lesions from week 9 to week 23 (data not shown). In addition, total serum IgE from both CM- and peanut-sensitized mice was significantly increased (data not shown).

Consistent with this finding, immediate cutaneous hypersensitivity reactions were induced by antigen injection of mice with AD-like lesions (100%). Mice with AD-like lesions receiving irrelevant antigen or naive mice injected with CM protein and Evans Blue dye did not exhibit positive reactions (Table II). These results, together with the observation of increased numbers of mast cells in lesional skin of mice with AD-like lesions, suggest that IgE-mediated activation of mast cells played a role in the pathogenesis of these AD-like lesions.

To confirm this hypothesis, we performed immunohistochemical staining on biopsy specimens from lesional and normal skin to assess the presence of IgE-bearing cells. Although no IgE-bearing cells were observed in naive mouse skin, numerous IgE⁺ cells ($123 \pm 8/\text{mm}^2$) were observed in eczematous lesions of mice with AD-like lesions (Fig 5). To identify IgE-bearing mast cells, IgE-stained sections were counter stained with toluidine blue. Increased numbers of mast cells were present in lesional biopsy specimens, and these cells comprised 69.9% of the IgE⁺ cells. No IgE⁺ cells were detected in the skin of naive mice. A small number of toluidine blue-negative IgE⁺ cells with dendritic morphology were also observed in lesional skin (data not shown).

T cells in AD-like skin lesions

Previous studies have demonstrated that T cells, particularly CD4⁺ T cells, are prominent in lesional skin of human patients with AD.³⁶ Histologic analysis of lesional skin of mice with AD-like lesions revealed many lymphoid cells in the dermis (Fig 3, *B*), and immunohistochemical staining revealed numerous CD4⁺ T cells ($282 \pm 72/\text{mm}^2$; Fig 6, *A*) and few CD8⁺ T cells ($18 \pm 8.8/\text{mm}^2$; Fig 6, *B*). The ratio of CD4⁺/CD8⁺ T cells was 15.6:1. In contrast, rare CD8⁺ T cells (Fig 6, *D*) and no CD4⁺ T cells (Fig 6, *C*) were observed in the skin samples of naive mice.

To characterize the cytokine profiles in mice with AD-like lesions, we determined the expression of IL-4, IL-5, IL-13, and IFN- γ mRNA in lesional skin biopsy specimen

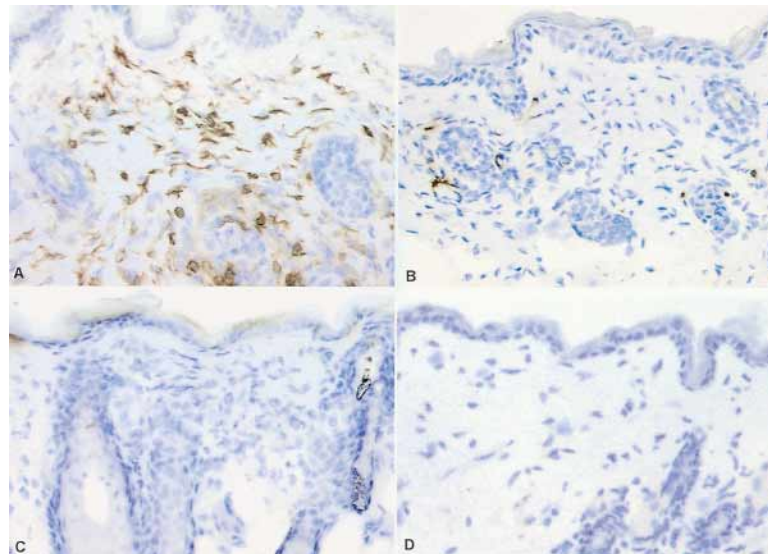


FIG 6. Increased numbers of CD4⁺ T cells in AD-like lesions. **A** and **B** are frozen section of the same lesion shown in Fig 5. **A**, Numerous dark brown diaminobenzidine-stained CD4⁺ cells, some of which have infiltrated the perifollicular area (bar = 50 μ m). **B**, Few dark brown diaminobenzidine-stained CD8⁺ cells, most of which are near hair follicles (bar = 50 μ m). **C** and **D**, Normal facial skin stained in the same manner as in **A** and **B**, showing the absence of CD4⁺ T cells in **C** and CD8⁺ T cells in **D** (bar = 50 μ m).

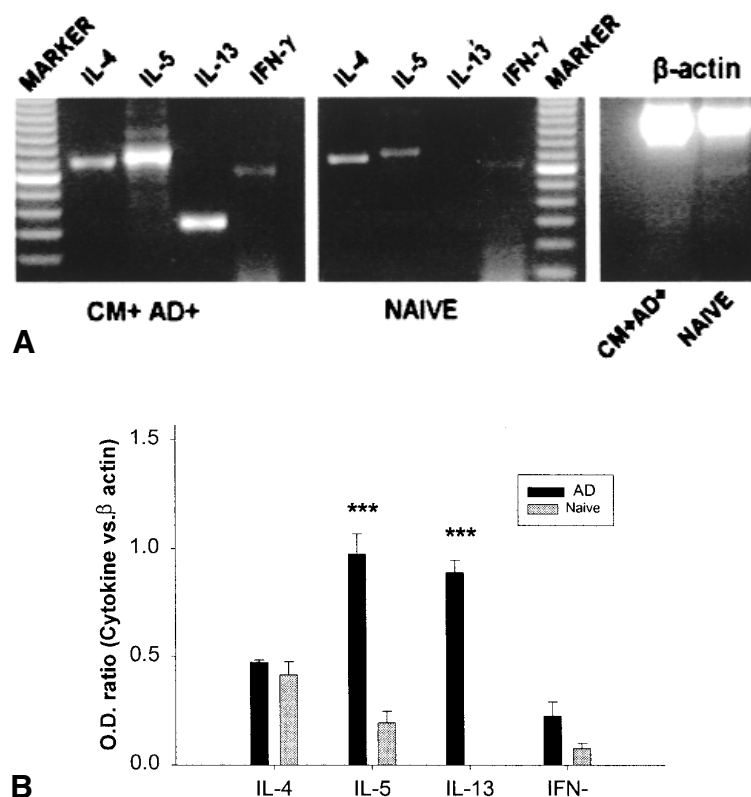


FIG 7. Increased IL-5 and IL-13 mRNA expression in AD lesions. Total RNA was extracted from biopsy specimens of lesional skin of mice with AD-like lesions and normal skin of naive mice (n = 4). Semiquantitative RT-PCR was performed in triplicate, as described in the "Methods" section. **A**, Gel illustrating cytokine (IL-4, IL-5, IL-13, and IFN- γ) mRNA from lesional skin of mice with AD or normal skin from naive mice. β -Actin mRNA expression is shown for comparison. **B**, OD ratios of cytokine (IL-4, IL-5, IL-13, and IFN- γ) versus β -actin. The results are expressed as means $\pm$ SEM of OD ratios of cytokine (IL-4, IL-5, IL-13, and IFN- γ) versus β -actin. *** P < .001 versus naive.

samples collected 8 to 10 weeks after the first appearance of dermatitis. IL-4 expression and IFN- γ expression were slightly but not significantly increased compared with expression in naive mice. IL-5 expression was markedly increased in mice with AD-like lesions compared with naive mice ($P < .001$), and IL-13 mRNA expression was only detected in the lesional skin of mice with AD-like lesions (Fig 7). These results suggest that CD4 $^{+}$ T $_{H}$ 2-like cells are the predominant infiltrating lymphocyte and that IL-13 and IL-5 play an important role in the maintenance of chronic AD-like lesions in this model.

Proliferative responses of skin mononuclear cells of mice with AD-like lesions

To assess the T cell-specific proliferative responses to CM antigen, dermal cells were isolated from biopsy specimens of lesional skin of mice with CM-induced AD-like lesions. Cells were stimulated with CM antigen, and the proliferative responses were compared. Dermal cells from AD-like lesions showed significantly increased proliferative responses to CM proteins (3666 ± 143 cpm) compared with unstimulated cells (1375 ± 325 cpm, $P < .05$).

DISCUSSION

The cause of AD in human subjects remains enigmatic, but a recent study demonstrated that approximately 40% of children with moderate-to-severe AD attending a university dermatology clinic had food hypersensitivity.⁷ Double-blind placebo-controlled food challenges in children with food allergy and AD typically elicit a pruritic, erythematous, morbilliform eruption within 15 to 120 minutes after the challenge.³⁷ The eruption is accompanied by a rise in plasma histamine levels³⁸ and generally clears within a few hours. However, it is the subsequent activation of CLA $^{+}$ lymphocytes that is likely responsible for the development of the eczematous lesion.³⁹ In an earlier study it was demonstrated that elimination of specific foods from the diet of patients with food allergy and AD resulted in significant improvement in their eczematous skin symptoms.⁴⁰ In addition, patients with food allergy and AD were found to have increased numbers of circulating hypodense (activated) eosinophils, increased spontaneous basophil histamine release (ie, primed basophils), and increased numbers of T cells expressing IL-5 and IL-13 mRNA.⁶ Despite a substantial body of clinical and laboratory evidence, it has been difficult to establish the exact immunopathogenic role of food hypersensitivity in AD.

This study demonstrates that oral sensitization to food proteins in mice may elicit a systemic immune response and inflammatory skin lesions that resemble human AD. After oral sensitization with milk protein and cholera toxin,²⁰ a group of mice was noted to have a dry, apparently pruritic eruption, resulting in scratching and hair loss. A review of potential causes revealed that new mouse chow was contaminated with low levels of milk protein. Subsequent attempts to reproduce the dermatitis resulted in approximately one third of mice similarly

sensitized with CM or peanut proteins having a dry, erythematous, scaly, pruritic eruption involving 15% to 100% of their body surface within 9 to 14 weeks of initiating the sensitization protocol. As with human AD, treatment of the skin lesions with topical corticosteroids led to decreased pruritus and erythema, with return of hair growth (Fig 1). In addition, episodic dermatitis was noted in untreated mice, with recurrences and remissions occurring over an 8-month observation period.

Histologic examination of the skin lesions in the sensitized mice revealed areas of mild thickening and spongiosis of the epidermis and a marked inflammatory infiltrate and thickening of the dermis compared with that of naive mice. The infiltrate consisted of large numbers of lymphocytes, predominantly CD4 $^{+}$ T cells, and few CD8 $^{+}$ cells, eosinophils, and mast cells. RT-PCR of lesional skin in mice with AD-like lesions and normal skin in naive mice revealed similar expression of IL-4 and IFN- γ , markedly elevated IL-5 expression in mice with AD-like lesions compared with naive mice, and uniquely elevated IL-13 expression in the skin of mice with AD-like lesions. This is similar to findings reported in human subjects with AD.^{9,41} These findings differ from the histopathologic findings in patients with chronic urticaria, in whom mast cell numbers (predominantly perivascular) are reportedly increased 10-fold, and the increased numbers of infiltrating lymphocytes show no predominance of CD4 $^{+}$ or CD8 $^{+}$ lymphocytes.⁴² The presence of increased numbers of eosinophils in the skin and peripheral blood, increased cutaneous mast cells, and elevated levels of serum IgE antibodies are consistent with the predominantly T $_{H}$ 2 response seen in AD in human subjects.

Although having some similarity in appearance to that of alopecia areata described in aging C3H/HeJ mice,⁴³ the clinical and histologic findings in our model of AD-like lesions differ significantly from those seen in mice with spontaneous alopecia. The hair loss in spontaneous alopecia typically develops diffusely or in circular areas on the dorsal surface of mice beginning at 6 to 8 months of age. In the AD model alopecia developed on the snout, head, and trunk about 9 to 14 weeks after the initial sensitizing dose. In mice with spontaneous alopecia, histologic analysis of the skin demonstrated a mononuclear cell infiltrate around the hair follicles. The infiltrate was composed primarily of CD8 $^{+}$ cytotoxic T cells and few CD4 $^{+}$ T cells (CD4 $^{+}$ /CD8 $^{+}$ = 1:3). No obvious changes were noted in the dermis. Eosinophilia was not found in the blood or affected skin. In our AD model there was spongiosis of the epidermis and dermis, with increased numbers of eosinophils, mast cells, and CD4 $^{+}$ T cells (CD4 $^{+}$ /CD8 $^{+}$ = 15.6:1). Although there are no reports of cytokine profiles in mouse alopecia, human studies have demonstrated that alopecia is an autoimmune disease with predominantly T $_{H}$ 1 cytokines.^{44,45} About one third of sensitized mice had the eczematous eruption and alopecia, whereas the incidence of spontaneous alopecia areata in female C3H/HeJ mice is reportedly 0.25%. Recently, spontaneous alopecia in aging mice was shown to be an autoimmune disorder.⁴⁶

Another potential cause of an eruption and alopecia in mice is the presence of ectoparasites or infection. Microscopic examination of plucked hair from both mice with AD-like lesions and naive mice showed no evidence of parasitic infestation. In addition, skin cultures for aerobic and anaerobic bacteria⁴³ showed no evidence of pathogenic organisms.

This is the first murine model of AD-like lesions induced by oral sensitization with a food protein. Other murine models of AD have been reported. Wang et al⁴⁷ initially reported that the repeated epicutaneous exposure of protein in the absence of adjuvant could induce a predominantly T_H2 inflammatory response in the skin of BALB/c mice. More recently, Spergel et al^{16,17} demonstrated that epicutaneous sensitization with OVA in 4- to 6-week-old BALB/c mice could provoke an eczematous skin eruption and airway hyperreactivity. The mice had high levels of OVA-specific IgE antibodies and an eczematous eruption characterized by an infiltration of predominantly CD4⁺ T cells, eosinophils, and neutrophils and local expression of IL-4, IL-5, and IFN- γ mRNA. After aerosolized OVA, these mice were found to have airway hyperresponsiveness and increased eosinophils in their bronchoalveolar lavage fluid. This constellation of symptoms is similar to that seen in human subjects, where virtually all children with AD demonstrate hyperresponsiveness to inhaled histamine. Although not tested in this study, it is likely that our model also involves airway hyperresponsiveness because we use a very similar oral sensitization protocol to generate mice with food-induced anaphylaxis.²⁰ Other murine models of AD have also been proposed. One model uses an inbred strain of NC/Nga mice that are raised in a conventional, non-pathogen-free environment.¹⁵ These mice have chronic relapsing inflammation of the skin with infiltration of CD4⁺ T cells, early infiltration of eosinophils, and increased expression of IL-4 mRNA. The trigger of the cutaneous inflammation in this model is unknown, although similar mice raised in a pathogen-free environment failed to have the eczematous eruption, suggesting some environmental trigger.⁴⁸ In another model recombinant Sj26 protein, a glutathione S-transferase of *Schistosoma japonicum*, was injected with alum into the peritoneum of 6- to 8-week-old BALB/c mice.²⁴ Within 2 to 3 weeks of immunization, the mice had Sj26-specific IgE antibodies and a progressive eczematous skin eruption. Biopsy specimens of the skin revealed mild spongiosis in the epidermis and infiltration of mononuclear round cells and eosinophils in the dermis. Peripheral blood eosinophilia was also noted. The mouse models of AD reported by us and others mimic human AD in many aspects. However, mouse models of AD, like any animal models, are not exactly the same as the human condition. They have some undesirable characteristics, including dense hair covering the skin, high hair follicle density, and very thin epidermis. Nevertheless, these models make it possible to dissect immunopathogenic responses in ways that are not possible in human subjects.

A clinical picture similar to AD has been reported in both dogs and cats. Butler et al¹⁴ reported recurrent non-

seasonal pruritic dermatitis in basenji-greyhound dogs. Histologic examination of a papular eruption on the chin of the dogs revealed hyperkeratosis and parakeratosis of the epidermis and a dense inflammatory infiltrate of the dermis consisting of lymphocytes, histiocytes, plasma cells, and neutrophils. These animals demonstrated non-specific and *Ascaris* species-specific airway hyperreactivity and blunted cyclic adenosine monophosphate responsiveness to β -adrenergic agents, which is similar to that of atopic patients. Allergic dermatitis has been reported in cats as a result of food hypersensitivity.⁴⁹ Affected cats have a pruritic papular eruption and alopecia, primarily on the head, on the neck, and about the ears, that resolves when the allergenic food is removed from the diet (most commonly fish and milk). Histologic evaluation of the dermatitis revealed an infiltration of primarily CD4⁺ cells.¹³ Although the dermatitis is similar to that found in human subjects, these animals have not proven to be practical models for the study of immunopathologic mechanisms related to AD in human subjects.

In summary, we have been able to induce an eczematous eruption in food-sensitized mice that resembles AD in human subjects. Like AD in children with food allergy, these mice have elevated levels of food-specific IgE antibodies, peripheral blood eosinophilia, and an inflammatory skin eruption characterized by an infiltration of CD4⁺ T_H2 lymphocytes and increased numbers of eosinophils and mast cells. Repeated ingestion of small amounts of the food allergen appears responsible for the induction of the eczematous eruption and hair loss, which improves with topical corticosteroid therapy. Further studies are necessary to determine why the dermatitis develops in only about one third of mice with food allergy and what immunopathogenic mechanisms are responsible for the skin eruption. It will also be of interest to determine whether these mice have gastrointestinal symptoms and malabsorption, as reported in children with food allergy,⁵⁰⁻⁵² and whether they have airway hyperreactivity, as noted in human subjects⁵³ and dogs.¹⁴ With a better understanding of the relationship between food hypersensitivity and AD, more effective forms of therapy may be developed.

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