Effects of Dietary Restriction on Spontaneous Dermatitis in NC/Nga Mice

WENYING FAN,[1] KATSUYASU KOUDA, HARUNOBU NAKAMURA, AND HIROICHI TAKEUCHI

Department of Public Health, Hamamatsu University School of Medicine, 20-1, Handayama 1-Chome, Hamamatsu, Shizuoka 431-3192, Japan

In laboratory animals, dietary restriction prolongs life span, improves physiologic function, and prevents or lessens severity of several diseases including some experimental inflammatory states. We investigated the effect of dietary restriction on a spontaneously occurring mouse model of atopic dermatitis, an inflammatory skin disease. NC/Nga mice were assigned to a group fed *ad libitum* or to a restricted diet group receiving 60% of the amount of food consumed by the other group. Dermatitis was characterized according to extent, intensity, and scratching time. We then used computer-assisted image analysis to quantify immunologic findings in skin sections. Extent, intensity score, and scratching time in mice with restriction increased more gradually than in mice fed *ad libitum*. Infiltrating inflammatory cells (CD4-positive T cells, CD8-positiveT cells, eosinophils, and mast cells) as well as interleukin-4 and -5 secreted into tissue were reduced in mice with restriction. In conclusion, dietary restriction delayed onset and progression of spontaneous dermatitis in NC/Nga mice, an effect possibly involving inhibition of inflammatory infiltration cell and cytokine secretion. [Exp Biol Med Vol. 226(11):1045–1050, 2001]

Key words: dietary restriction; atopic eczema; allergy; inflammation

Dietary restriction is acknowledged to prolong life span in laboratory animals (1, 2). Experimental studies have shown that such restriction can improve declining physiologic functions (3), and can also prevent or lessen severity of spontaneously occurring (1), chemically induced (4), and radiation-induced neoplasia (5); and autoimmune diseases (6). Dietary restriction has also been reported to attenuate carrageenan-induced footpad inflammation (7), protect against ozone-induced lung inflammation (8), and alleviate chemically induced ulcerative dermatitis (9).

Atopic dermatitis (AD) is a human inflammatory skin disease triggered by interactions between genetic, immunologic, and environmental factors, including diet (10). Previous studies concerning dietary management of AD focused on elimination of allergenic proteins such as cow's milk or eggs (11). Recently, we reported that a calorically restricted diet was associated with remarkable improvement in AD patients in an open-label trial (12); a positive correlation was evident between improvement of dermatitis and decrease in body weight. Accordingly, we hypothesized that dietary restriction can suppress AD.

The NC/Nga mouse has recently been established as an animal model for human AD (13, 14). This strain of mouse spontaneously develops dermatitis associsted with excessive IgE production when animals are raised under conventional conditions. And the dermatitis in male mice is relatively more severe than that in female mice (13). This skin disorder was proposed to result from a combination of genetic propensity and environmental triggers (15).

In the present study, we investigated the effect of 40% dietary restriction involving calories, protein, vitamins, and minerals on AD-like dermatitis in NC/Nga mice.

Materials and Methods

Animals and Diets. Male and female NC/Nga mice purchased from Japan SLC (Hamamatsu, Japan) were bred and housed at the animal facility in the Institute for Experimental Animals at Hamamatsu University School of Medicine. Mice were maintained individually in plastic cages in a room with a conventionally regulated environment, including temperature of 23°–25°C, relative humidity of 50%–60%, and a 12:12-hr light:dark cycle. All mice received care in compliance with the *Guidelines for Animal Experimentation* of the Hamamatsu University School of Medicine. Mice were randomly divided into *ad libitum* (AL) groups (male, $n = 6$; female, $n = 13$) and dietary restriction (DR) groups (male, $n = 6$; female, $n = 13$). All mice were given a standard rodent laboratory diet (Lab Diet EQ 5L37; PMI Nutrition International, Brentwood, MO), containing 250 g of protein, 45 g of fat, 45 g of fiber, and 67 g of ash per kilogram of diet. Total digestible energy is

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[1] To whom requests for reprints should be addressed at Department of Public Health, Hamamatsu University School of Medicine, 20-1, Handayama 1-Chome, Hamamatsu, Shizuoka 431-3192, Japan. E-mail: fwy@hama-med.ac.jp

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13.1 MJ/kg of diet. Each AL mouse consumed 4.9–5.5 g of diet per day (energy consumed = 64.0–71.8 kJ/day). Each DR mouse received the same diet, but the amount of food provided was adjusted daily to represent 60% (3.0–3.3 g/day, 39.2–43.1 kJ/day) of the prior day's food consumption for a paired AL mouse. Both AL and DR mice had free access to water throughout the study. The regimen was initiated at 6 weeks of age and was terminated at 15 weeks of age.

Evaluation of Dermatitis Severity. For objective evaluation of severity of dermatitis, we defined three indices: extent (ratio of involved skin area to total body skin area); intensity score (sum of intensity scores of all skin regions surveyed), and scratching time (cumulative time spent scratching over a period of 10 min). Extent and intensity score were defined referring to the Scoring Atopic Dermatitis (SCORAD) system (16). For calculation of intensity score we assessed three items: erythema, edema or papulations, and oozing, crusts, or hemorrhage. Each of the three items was graded on a scale of 0 to 3 (0, absent; 1, mild; 2, moderate; and 3, severe) for the right ear, left ear, scalp, rostral back, caudal back, chest, and abdomen. The intensity score was the sum of individual item scores obtained for these seven areas. Severity of dermatitis was assessed once weekly in all mice.

Measurement of Serum IgE. Blood samples were collected at 15 weeks of age. Serum IgE concentrations were measured using a mouse IgE enzyme immunoassay kit (Yamasa Shoyu, Choshi, Japan).

Histochemical and Immunohistochemical Staining. All mice were sacrificed by cervical dislocation at 15 weeks of age, and skin samples from scalp located centrally between the ears were obtained as previously described (17). Samples were fixed in 10% formalin, embedded in paraffin, and sectioned perpendicular to the skin surface at a thickness of 3 μm. Sections were stained with hematoxylin and eosin (HE), acidic toluidine blue, or Congo red.

For immunohistochemistry, sections were stained with monoclonal antibody (mAb) against CD4, CD8, interleukin (IL)-4, or IL-5. Deparaffinized sections were treated for 15 min with 3% hydrogen peroxide (Wako, Osaka, Japan) in distilled water. After nonspecific binding of antibody was blocked with 10% normal goat serum (Immuno-biological Laboratories, Fujioka, Japan) diluted in Tris-buffered saline with 0.1% Tween 20 (TBST; Dako, Carpinteria, CA), sections were incubated overnight at 4°C with the primary mAb. The mAb used were rat anti-mouse CD4 mAb (RM4-5; PharMingen, San Diego, CA), rat anti-mouse CD8 mAb (53-6.7; Becton Dickinson, Mountain View, CA), rat anti-mouse IL-4 mAb (11B11; PharMingen), and rat anti-mouse IL-5 mAb (TRFK5; PharMingen). Subsequently, the sections were washed in TBST and then incubated with horseradish peroxidase-conjugated goat anti-rat IgG (PharMingen) at room temperature for 30 min. Reaction products were visualized with 3-3′-diaminobenzidine tetrahydrochloride (Wako) with hematoxylin counterstaining.

Computer-Assisted Histomorphometry. We used a computer-assisted image analysis system that permits automatic extraction and measurement.

Light microscopic images of skin sections were captured and then transformed into 32-bit color images with 945 × 738 resolution. For transformation, a digital camera (FUJIX HC-2000; Fuji Photo Film, Tokyo, Japan) attached to a light microscope (VANOX AHBS3; Olympus, Tokyo, Japan; with ×40 objective) was used together with software (Adobe Photoshop 5.5; Adobe Systems, San Jose, CA) run on a Macintosh computer (Apple Computer, Cupertino, CA). To determine the number or area in regions of interests, MacSCOPE Image Analysis was used (Version 2.5.6; Mitani, Maruoka, Japan).

The epidermal area was measured in five fields in each HE section. Results are expressed as the mean epidermal area in square micrometer for the five fields.

Densities of inflammatory cells in the dermis (toluidine blue-positive mast cells, Congo red-positive eosinophils, CD4-positive ($CD4^+$) T cells, and CD8-positive ($CD8^+$) T cells) were determined in five fields per section and were expressed as the mean number of cells per square millimeter. Mast cells were categorized into three types: granulated cells associated with less than five granules outside the cell, slightly degranulated cells with 5–15 granules outside the cell, and markedly degranulated cells with more than 15 granules outside the cell.

Portions stained with brown reaction product for IL-4 and IL-5 were extracted automatically based on hue, light, and saturation values, and were highlighted in green (Fig. 1, A and B). Areas of these extracted portions were measured in five fields per section, and the results are expressed as the mean stained area in square micrometers.

Statistical Analysis. Data are expressed as the mean ± SEM. We used the Mann-Whitney *U* test to evaluate statistical difference between groups. Pearson's correlation coefficient was employed to assess relationships between dermatitis severity indices and laboratory values. A *P* value less than 0.05 was considered to indicate statistical significance. Statistical analysis was performed using StatView for Macintosh (Version J-4.11; Abacus Concepts, Berkeley, CA).

Results

Body Weight. Body weights in DR mice decreased between 6 and 9 weeks of age, after which the animals maintained stable body weight (Fig. 2).

Occurrence. Median time of occurrence of dermatitis in AL mice was 7 weeks of age, with 100% prevalence noted at 10 weeks. In contrast, dermatitis appeared at a median of 11 weeks of age in DR mice, whereas four DR mice did not show evidence of dermatitis even at the end of the study.

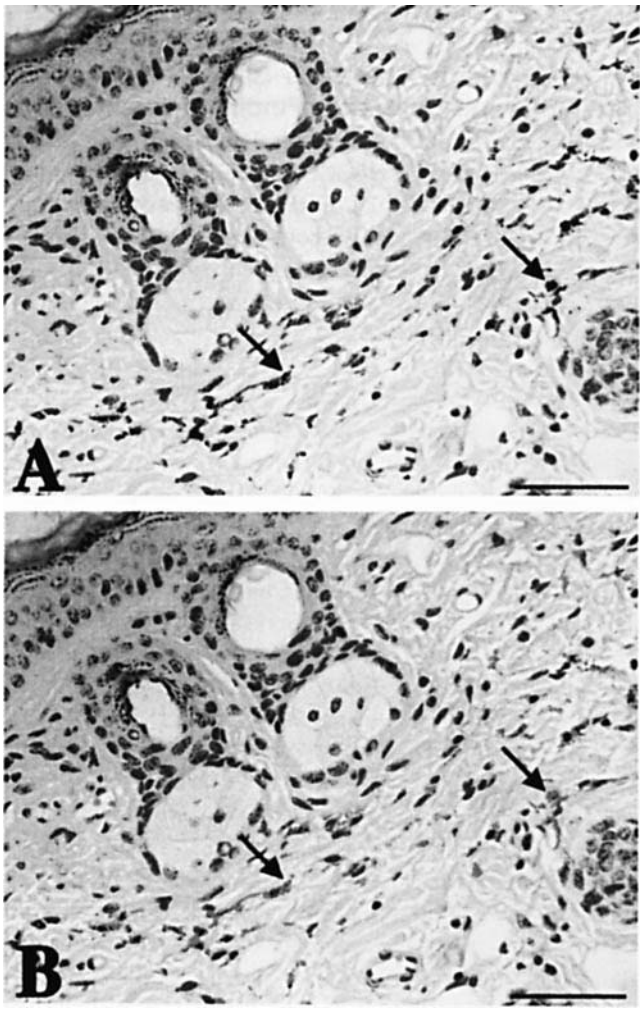

Figure 1. Extraction of the interleukin-4 portion in an immunostained skin section. (A) Original image; interleukin-4 is stained brown (arrows). (B) Extracted image; stained portion is highlighted in green (arrows). Bar = 50 μm.

Appearance. AL mice spontaneously developed dermatitis characterized by erythema, papulation, hemorrhage, erosion, and alopecia. With time, dermatitis worsened and spread over most of the body surface. Involvement was most intense in the scalp and dorsal skin. In contrast, dermatitis in DR mice appeared to be relatively mild.

Severity of Dermatitis. During the study AL mice showed increases in the severity indices of dermatitis, including extent, intensity score, and scratching time. In contrast, DR mice showed more gradual increases in these severity indices. At the end of the study, the extent, intensity score, and scratching time in DR mice were significantly suppressed compared with corresponding values in gender-matched AL mice (Figs. 3, 4, and 5).

Histologic Examination. In AL mice, the epidermis showed remarkable hyperkeratosis and marked acanthosis; severe inflammatory cell infiltration was seen in the dermis. In contrast, these histologic findings were mild in DR mice.

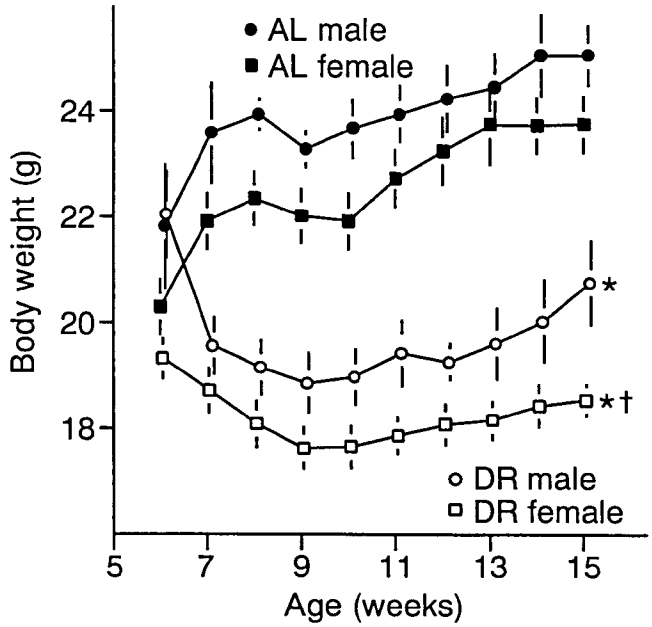

Figure 2. Effect of dietary restriction on body weight of NC/Nga mice. AL male, $n = 6$; AL female, $n = 13$; DR male, $n = 6$; DR female, $n = 13$. Values represent the mean ± SEM. $^*P < 0.01$ compared with gender-matched AL mice. $\dagger P < 0.05$ compared with male DR mice.

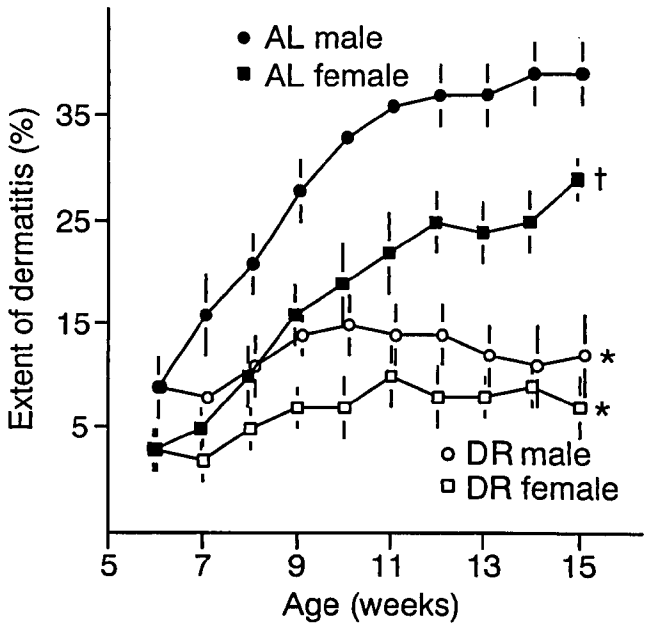

Figure 3. Effect of dietary restriction on extent of dermatitis in NC/Nga mice. AL male, $n = 6$; AL female, $n = 13$; DR male, $n = 6$; DR female, $n = 13$. Values represent mean ± SEM. $^*P < 0.01$ compared with gender-matched AL mice. $\dagger P < 0.05$ compared with male AL mice.

Image Analysis. Mean epidermal area in DR mice was significantly smaller than in gender-matched AL mice. Mean densities of mast cells, eosinophils, $CD4^+$ T cells, and $CD8^+$ T cells in DR mice were significantly lower than in gender-matched AL mice. Moreover, mean area of IL-4 was significantly smaller in female DR mice and tended to be smaller in male DR mice than in gender-matched AL mice.

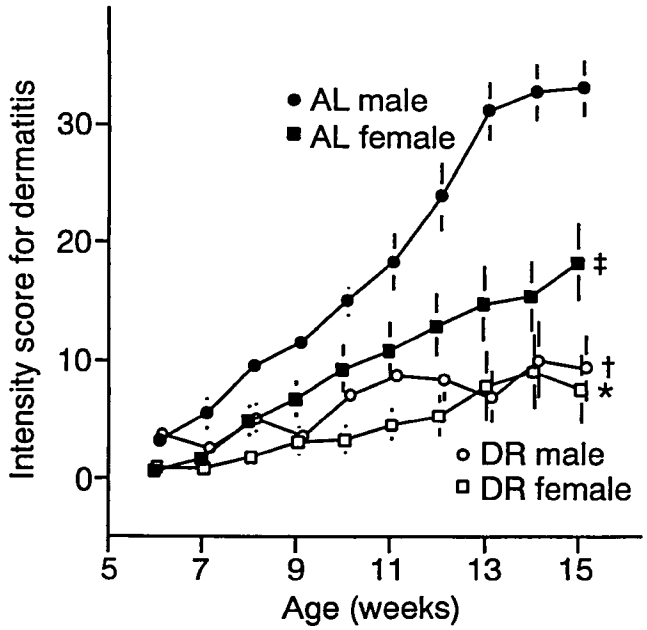

Figure 4. Effect of dietary restriction on intensity score for dermatitis in NC/Nga mice. AL male, n = 6; AL female, n = 13; DR male, n = 6; DR female, n = 13. Values represent mean ± SEM. $*P < 0.05$, $\dagger P < 0.01$ compared with gender-matched AL mice. $\ddagger P < 0.05$ compared with male AL mice.

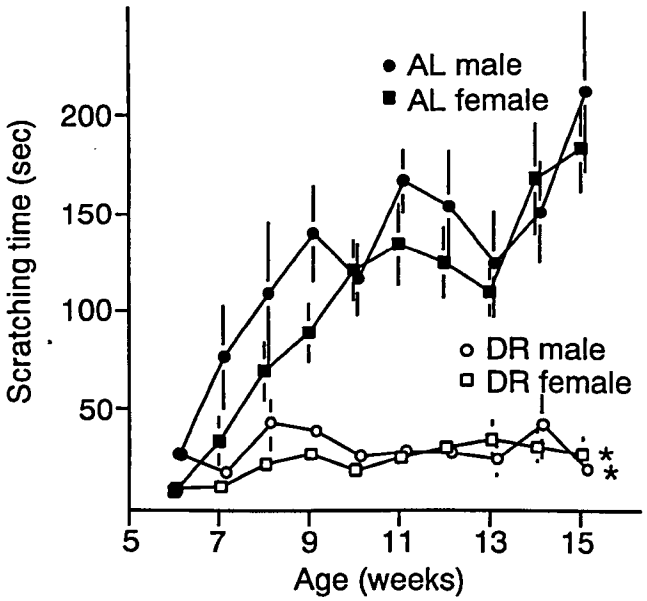

Figure 5. Effect of dietary restriction on scratching time in NC/Nga mice. AL male, n = 6; AL female, n = 13; DR male, n = 6; DR female, n = 13. Values represent mean ± SEM. $*P < 0.01$ compared with gender-matched AL mice.

Mean area of IL-5 tended to be smaller in female DR mice than in female AL mice (Table I). And there were significant differences between total DR mice and total AL mice in both IL-4 and IL-5 ($P < 0.05$ and $P < 0.05$, respectively; $n = 19$ in each group).

Serum IgE. The mean of serum IgE concentration in DR mice was lower than in AL mice at 15 weeks of age (AL mice, 32,500 ± 3,100 ng/ml, $n = 19$; DR mice, 22,400 ± 3,300 ng/ml, $n = 19$; $P = 0.06$).

Relationships between Severity Indices for Dermatitis and Laboratory Findings. Scratching time showed a significant positive correlation with each laboratory variable ($CD4^+$ T cells, $CD8^+$ T cells, eosinophils, mast cells, IL-4, IL-5, and IgE). Extent and intensity score also showed a significant positive correlation with all laboratory variables. Of all laboratory data, density of markedly degranulated mast cells was most closely correlated with scratching time (Table II).

Discussion

Dietary restriction, which is synonymous with such terms as calorie restriction and food restriction, has been well studied in laboratory animals. Dietary intake restriction can be accomplished with avoidance of malnutrition by a 40% reduction from average unrestricted food intake, including a balanced decrease in calories, protein, vitamins, and minerals (1–3). This regimen results in a limited period of weight loss, after which the animals maintain stable body weight or gradually regain some of the weight originally lost despite continued dietary restriction (1, 18, 19). Our results as to changes in body weight were consistent with previous observations.

Our mice with dietary restriction showed lower serum IgE concentrations and less severe dermatitis (as evaluated by the extent, intensity score, and scratching time) than other *ad libitum*-fed mice. Further, mice with restriction showed only mild epidermal thickening, mild dermal inflammatory cells (lymphocyte and eosinophil) infiltration, and mild degranulation of mast cells. Dermal staining for inflammatory cytokines (IL-4 and IL-5) was also suppressed.

In NC/Nga mice, dermatitis has been reported to be closely associated with excessive IL-4 and IL-5 production, as well as inflammatory cell infiltration in the dermis (13, 14). IL-4 induces IgE synthesis, whereas IL-5 promotes IL-4-dependent IgE synthesis and stimulates eosinophils (10). In most previous studies, intensity of cytokine immunostaining in local skin lesions was evaluated by subjective observation or a morphologic scoring system based upon microscopic examination (13, 17). Observer bias cannot be completely avoided with those methods. Recently, computer-assisted image analysis has been used increasingly for quantitative histopathologic examination (20, 21). We used such a method to quantify both histologic findings and cytokine secretion as continuous variables, and could demonstrate that dietary restriction suppresses production of inflammatory cytokines in NC/Nga mice.

We also obtained good correlations of microscopic and blood assay results with severity of dermatitis. Interestingly, the number of degranulated mast cells was the laboratory finding best correlated with scratching time. Degranulation of mast cells releases histamine and other mediators associated with itching (pruritus).

Table I. Computer-Assisted Imaging Analysis of Skin Sections in NC/Nga Mice at 15 Weeks of Age[a]

	Male AL[b] (n = 6)	Male DR[b] (n = 6)	Female AL (n = 13)	Female DR (n = 13)
Epidermal area (μm^2)	5000 ± 1000	2000 ± 200‡	4000 ± 400	2000 ± 200‡
Mast cells (cells/mm^2)				
Granulated	359.6 ± 15.6	208.5 ± 9.5‡	373.7 ± 21.0	214.8 ± 10.9‡
Slightly degranulated	34.6 ± 10.4	2.1 ± 1.5‡	41.0 ± 16.5	6.7 ± 4.5†
Markedly degranulated	45.9 ± 10.6	11.4 ± 4.9†	45.0 ± 7.0	21.4 ± 3.7‡
Eosinophils (cells/mm^2)	329.2 ± 40.8	86.2 ± 12.2‡	164.3 ± 21.3¶	98.1 ± 7.4‡
$CD4^+$ T cells (cells/mm^2)[b]	422.6 ± 43.8	124.4 ± 22.3‡	251.5 ± 33.4§	57.6 ± 15.0‡§
$CD8^+$ T cells (cells/mm^2)[b]	151.7 ± 32.8	36.1 ± 8.3†	72.1 ± 9.9§	21.7 ± 5.3‡
Interleukin-4 (μm^2)	143 ± 31	70 ± 16*	143 ± 51	46 ± 12†
Interleukin-5 (μm^2)	212 ± 74	71 ± 15	83 ± 18	44 ± 10*

[a] Values are expressed as the means ± SEM.
[b] AL, ad libitum; DR, dietary restriction; $CD4^+$, CD4 positive; $CD8^+$, CD8 positive.
* $P < 0.1$, † $P < 0.05$, and ‡ $P < 0.01$ compared with AL mice of the same gender. § $P < 0.05$ and ¶ $P < 0.01$ compared with male mice of the same diet group.

The precise mechanism responsible for suppression of dermatitis by dietary restriction is unclear. In previous studies, dietary restriction has been reported to reduce T cell function (such as IL-4 and IL-5 production), and to suppress lymphocyte proliferation and serum IgE response in infected mice (22, 23). Also, dietary restriction causes elevation of circulating cortisol in normal rodents (24–26). Thus, interrelationships are likely among immunologic, endocrinologic, and other responses to allergic conditions in combination with dietary restriction. It is also unclear if dietary restriction can improve AD once the disease has begun in this model, and if the effect of dietary restriction persist after the termination of this regimen. Further studies are needed to more fully explain these questions.

In conclusion, dietary restriction delayed onset and suppressed progression of AD-like dermatitis in NC/Nga mice, an effect possibly involving inhibition of inflammatory infiltration cell and cytokine secretion.

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Table II. Correlations between Dermatitis Severity Indices and Laboratory Variables[a]

	Scratching time	Extent	Intensity score
$CD4^+$ T cells[b]	0.65†	0.78†	0.70†
$CD8^+$ T cells[b]	0.59†	0.66†	0.59†
Eosinophils	0.48†	0.50†	0.42†
Slightly degranulated mast cells	0.43†	0.50†	0.51†
Markedly degranulated mast cells	0.72†	0.68†	0.65†
Interleukin-4	0.39*	0.48†	0.47†
Interleukin-5	0.38*	0.51†	0.52†
Immunoglobulin E	0.56†	0.75†	0.82†

[a] n = 38. Values are correlation coefficients according to Pearson's correlation test.
[b] $CD4^+$, CD4 positive; $CD8^+$, CD8 positive.
* $P < 0.05$; † $P < 0.01$.

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