日本財団 助成事業
The Nippon Foundation

２００１年度日中医学協会共同研究等助成事業報告書

－在留中国人研究者研究助成－

2002 年 2 月 27 日

財団法人 日中医学協会
理事長 殿

研究者氏名 范 文英 ㊞
所属機関名 浜松医科大学公衆衛生学
指導責任者氏名 竹内 宏一
職名 教授
所在地〒 431-3192 浜松市半田山 1 丁目 20-1
電話 053-435-2329 内線

1．研究テーマ

NC/Nga マウスにおけるヒトアトピー類似皮膚炎におよぼす食餌制限の影響

2．本年度の研究業績

(1) 学会・研究会等における発表 有 ・ (無) (学会名・演題)

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

雑誌名：Experimental Biology and Medicine (226: 1045-1050, 2001)

論文名：Effects of dietary restriction on spontaneous dermatitis in NC/Nga mice.

3．今後の研究計画

これまでに我々は、ヒトのアトピー性皮膚炎モデルである NC/Nga マウスに食餌制限（全栄養素制限）を行い、皮膚炎の発症、臨床症状、IgE、炎症細胞、炎症サイトカインが食餌制限により抑制されることを学術誌（Exp Biol Med 226: 1045-1050, 2001）に発表した。しかしながら、ヒトのアトピー性皮膚炎は、遺伝的要因、住居や食品等の環境要因、さらには心理的要因などが複雑に関連して発症、増悪するため、ヒトにおいて食事と症状の関係を明確に証明することは困難である。今後我々は、NC/Nga マウスを用い食餌制限や抗酸化食品の添加等による皮膚炎の発症抑制の有無や皮膚組織の酸化的障害、腸の絨毛細胞の変化、腸内細菌の変化等について検討する。腸の絨毛細胞、皮膚組織の酸化的障害、皮膚組織の病態評価は、computer-assisted image analysis を用いて行う。組織標本の光顕像をデジタル化し、視野内の目標物の hue、light、and saturation values（HLS）を自動設定し、目標物質を自動抽出する。この方法により細胞の数のみならず、免疫組織染色像における染色強度も連続変数として表現することが可能となる。腸内細菌のパターン（量的）変化について検討する。

4．指導責任者の意見

アレルギー疾患のなかでもアトピー性皮膚炎はわが国で急増している。以前は小児期に発症して成長するにつれて軽快する例が多かったが、最近は軽快しないばかりか成人期に発症してしかも難治性が多くなり深刻な状況を呈しているいわゆる現代文明病である。

中国の都市部でもこのようなアレルギー疾患が増加しているという。このような状況において、范氏はアトピー性皮膚炎の基礎的研究において、報告書に記載しているように極めて有意義な研究をした。この研究は長時間にわたって地道な努力を要する実験と観察であったが、それを持ち前の根気と冷静な判断で克服した。この成果は既に専門家によって評価されており、この領域の基礎的研究に大きく貢献した。さらに、上に記述しているように、これからの研究計画も興味深いものがある。

指導責任者氏名 竹内亮一 印

5．研 究 報 告 書

別紙報告書作成要領により、添付の用紙で研究報告書を作成して下さい。

研究発表中または研究中の本人のスナップ写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

※発表に当っては、*日中医学協会助成金による*旨を明記して下さい。

-日中医学協会助成事業-

-NC/Ngaマウスにおけるヒトアトピー類似皮膚炎におよぼす食餌制限の影響-

研究者氏名　范　文英
中国所属機関　中国北京化工医院　医師
日本研究機関　日本浜松医科大学公衆衛生学教室
指導責任者　教授　竹内　宏一
共同研究者名　甲田勝康，中村晴信，竹内宏一

ABSTRACT

In laboratory animals, dietary restriction prolongs lifespan, 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 group fed ad libitum or to 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-positive T 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.

Key Words dietary restriction, atopic eczema, allergy, inflammation

INTRODUCTION

Dietary restriction is acknowledged to prolong lifespan in laboratory animals (1, 2). Experimental studies have shown that such restriction can improve declining physiologic functions (3), and 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 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 to 25°C, relative humidity of 50 to 60%, and a light/dark cycle of 12 h/12 h. 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 and dietary restriction (DR) groups. All mice were given a standard rodent laboratory diet. Each AL mouse consumed 4.9 to 5.5 g of diet per day. Each DR mouse received the same diet, but the amount of food provided was adjusted daily to represent 60% 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 terminated at 15 weeks of age.

Evaluation of Dermatitis Severity. For objective evaluation of severity of dermatitis, we defined 3 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; 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.

Histochemical and Immunohistochemical Staining. All mice were killed 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 attached to a light microscope was used together with software run on a computer. To determine the number or area in regions of interests, MacSCOPE Image Analysis was used.

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 expressed as the mean number of cells per square millimeter. Mast cells were categorized into three types: granulated cells associated with less than 5 granules outside the cell, slightly degranulated cells with 5 to 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. 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.

RESULTS

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

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.

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.

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 DR female mice and tended to be smaller in DR male mice than in gender-matched AL mice. Mean area of IL-5 tended to be smaller in DR female mice than in AL female mice. And there were significant difference between total DR mice and total AL mice in both IL-4 and IL-5.

Serum IgE. The mean of serum IgE concentration in DR mice was lower than in AL mice at 15 weeks of age.

Relationships between Severity Indices for Dermatitis and Laboratory Findings. Scratching time showed a significant positive correlation with 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.

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 than other ad libitum fed mice. Further, mice with restriction showed only mild epidermal thickening, mild dermal inflammatory cells infiltration, and mild degranulation of mast cells. Dermal staining for inflammatory cytokines 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, while 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).

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 persists 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.

REFERENCES

1. Weindruch R, Walford RL. The retardation of aging and disease by dietary restriction. Springfield, IL: Charles C Thomas, 1988.
2. Yu BP. Aging and oxidative stress: modulation by dietary restriction. Free Radic Biol Med 21:651-668, 1996.
3. Frame LT, Hart RW, Leakey JEA. Caloric restriction as a mechanism mediating resistance to environmental disease. Environ Health Perspect 106:313-324, 1998.
4. Birt DF, Yaktine A, Duysen E. Glucocorticoid mediation of dietary energy restriction inhibition of mouse skin carcinogenesis. J Nutr 129:571-574, 1999.
5. Yoshida K, Inoue T, Nojima K, Hirabayashi Y, Sado T. Calorie restriction reduces the incidence of myeloid leukemia induced by a single whole-body radiation in C3H/He mice. Proc Natl Acad Sci U S A 94:2615-2619, 1997.
6. Kubo C, Gajjar A, Johnson BC, Good RA. The effects of dietary restriction on immune function and development of autoimmune disease in BXSB mice. Proc Natl Acad Sci U S A 89:3145-3149, 1992.
7. Klebanov S, Diais S, Stavinoha WB, Suh Y, Nelson JF. Hyperadrenocorticism, attenuated inflammation, and the life-prolonging action of food restriction in mice. J Gerontol Biol Sci Med Sci 50:B78-B82, 1995.
8. Kari F, Hatch G, Slade R, Crissman K, Simeonova PP, Luster M. Dietary restriction mitigates ozone-induced lung inflammation in rats: a role for endogenous antioxidants. Am J Respir Cell Mol Biol 17:740-747, 1997.
9. Perkins SN, Hursting SD, Phang JM, Haines DC. Calorie restriction reduces ulcerative dermatitis and infection-related mortality in p53-deficient and wild-type mice. J Invest Dermatol 111:292-296, 1998.
10. Leung DYM. Atopic dermatitis: new insights and opportunities for therapeutic intervention. J Allergy Clin Immunol 105:860-876, 2000.
11. Sicherer SH, Sampson HA. Food hypersensitivity and atopic dermatitis: pathophysiology, epidemiology, diagnosis, and management. J Allergy Clin Immunol 104:S114-S122, 1999.
12. Kouda K, Tanaka T, Kouda M, Takeuchi H, Takeuchi A, Nakamura H, Takigawa M. Low-energy diet in atopic dermatitis patients: clinical findings and DNA damage. J Physiol Anthropol 19:225-228, 2000.
13. Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, Matsumoto M, Ushio H, Saito S, Askenase PW, Ra C. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int Immunol 9:461-466, 1997.
14. Matsumoto M, Ra C, Kawamoto K, Sato H, Itakura A, Sawada J, Ushio H, Suto H, Mitsuishi K, Hikasa Y, Matsuda H. IgE hyperproduction through enhanced tyrosine phosphorylation of Janus kinase 3 in NC/Nga mice, a model for human atopic dermatitis. J Immunol 162:1056-1063, 1999.
15. Tsudzuki M, Watanabe N, Wada A, Nakane Y, Hiroi J, Matsuda H. Genetic analyses for dermatitis and IgE hyperproduction in the NC/Nga mouse. Immunogenetics 47:88-90, 1997.
16. European Task Force on Atopic Dermatitis. Severity scoring of atopic dermatitis: The SCORAD index.

Consensus report of the European Task Force on Atopic Dermatitis. Dermatology 186:23-31, 1993.
17. Hiroi J, Sengoku T, Morita K, Kishi S, Sato S, Ogawa T, Tsudzuki M, Matsuda H, Wada A, Esaki K. Effect of tacrolimus hydrate (FK506) ointment on spontaneous dermatitis in NC/Nga mice. Jpn J Pharmacol 76:175-183, 1998.
18. Hishinuma K, Nishimura T, Konno A, Hashimoto Y, Kimura S. Augmentation of mouse immune functions by dietary restriction: an investigation up to 1 year of age. Ann Nutr Metab 34:76-84, 1990.
19. Matsuzaki J, Yamaji R, Kiyomiya K, Kurebe M, Inui H, Nakano Y. Implanted tumor growth is suppressed and survival is prolonged in sixty percent of food-restricted mice. J Nutr 130:111-115, 2000.
20. Kouda K, Ha-Kawa SK, Tanaka Y. Increased technetium-99m-GSA uptake per hepatocyte in rats with administration of dimethylnitrosamine or hepatocyte growth factor. J Nucl Med 39:1463-1467, 1998.
21. Nakamura H, Kouda K, Fan WY, Watanabe T, Takeuchi H. Suppressive effects on allergic contact dermatitis by short-term fasting. Toxicol Pathol 29:200-207, 2001.
22. Shi HN, Scott ME, Stevenson MM, Koski KG. Energy restriction and zinc deficiency impair the functions of murine T cells and antigen-presenting cells during gastrointestinal nematode infection. J Nutr 128:20-27, 1998.
23. Koski KG, Su Z, Scott ME. Energy deficits suppress both systemic and gut immunity during infection. Biochem Biophys Res Commun 264:796-801, 1999.
24. Leakey JEA, Chen S, Manjgaladze M, Turturro A, Duffy PH, Pipkin JL, Hart RW. Role of glucocorticoids and "caloric stress" in modulating the effects of caloric restriction in rodents. Ann NY Acad Sci 719:171-194, 1994.
25. Han ES, Evans TR, Nelson JF. Adrenocortical responsiveness to adrenocorticotropic hormone is enhanced in chronically food-restricted rats. J Nutr 128:1415-1420, 1998.
26. Sabatino F, Masoro EJ, McMahan CA, Kuhn RW. Assessment of the role of the glucocorticoid system in aging processes and in the action of food restriction. J Gerontol Biol Sci Med Sci 46:B171-B179, 1991.

注：本研究は Experimental Biology and Medicine (226:1045-1050, 2001)に掲載。

作成日:2002 年 2 月 28 日