

Effects of TNCB Sensitization in DS-*Nh* Mice, Serving as a Model of Atopic Dermatitis, in Comparison with NC/Nga Mice

Setsuko Matsukura^a Michiko Aihara^a Tsutomu Hirasawa^b Zenro Ikezawa^a

^aDepartment of Environmental Immuno-Dermatology, Yokohama City University Graduate School of Medicine, Yokohama, and ^bDiscovery Research Laboratories, Shionogi and Co. Ltd., Osaka, Japan

Key Words

Atopic dermatitis · DS-*Nh* mouse · NC/Nga mouse · 2,4,6-Trinitrochlorobenzene · Splenocyte culture

Abstract

Background: It has been predicted that a type-1 and type-2 helper T cell (Th1/Th2) imbalance exists in atopic dermatitis (AD). In DS-*Nh* mice, an AD mouse model, *Staphylococcus aureus* increases on the skin surface. **Objective:** To investigate whether the Th1-dominant response has an influence on the development of AD, we induced chronic allergic hypersensitivity with 2,4,6-trinitrochlorobenzene (TNCB) in two AD mouse models: NC/Nga mice and DS-*Nh* mice. Th1 and Th2 cytokine production of splenocytes was assessed under stimulation with staphylococcal enterotoxin B (SEB) which induces a Th1 response in DS-*Nh* mice with or without TNCB sensitization. **Methods:** We examined clinical skin changes, trans-epidermal water loss (TEWL), the number of *S. aureus* on the skin and the serum IgE levels in these mice treated repeatedly with TNCB under conventional conditions (free of fur mites). The splenocytes of DS-*Nh* mice were cultured with SEB and the cytokine levels in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA). **Results:** Significant skin changes were observed on the skin even where TNCB was not applied in both mice treated with TNCB. Increases in *S. aureus*

on the skin and serum IgE levels were detected in DS-*Nh* mice, but not in NC/Nga mice. In DS-*Nh* mice, IFN- γ and IL-13 production of splenocytes increased in the mice treated with TNCB. **Conclusion:** These results suggest that there might be a different mechanism of dermatitis induction between NC/Nga and DS-*Nh* mice. Th1 responses might play an important role in the development of dermatitis and increase in serum IgE levels in DS-*Nh* mice through an increase in IL-13 production.

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Introduction

Atopic dermatitis (AD) in humans is a chronic pruritic inflammatory disease that fluctuates with remissions and progressions. The cause of AD is multifactorial involving immunological abnormalities and physiological and biochemical defects of the skin barrier structures [1–5]. As in immunological abnormalities, there are several reports suggesting that there are type-1 and type-2 helper T-cell (Th1/Th2) imbalances in AD [6, 7]. It has been reported that peripheral blood mononuclear cells (PBMC) from AD patients are Th2-predominant based on the analysis of cytokine production stimulated with specific antigens [6]. Former studies have demonstrated that in AD skin lesions, the IL-4/IFN- γ mRNA expression ratio significantly increases and mononuclear cells produce greater

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Correspondence to: Dr. Setsuko Matsukura
Department of Environmental Immuno-Dermatology
Yokohama City University Graduate School of Medicine
3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004 (Japan)
Tel. +81 45 787 2675, Fax +81 45 786 0243, E-Mail s.matsukura@aa.bb-east.ne.jp

amounts of IL-4 and IL-5 as compared to skin lesions of patients with psoriasis vulgaris or healthy skin [8]. These findings suggest that AD is a Th2-predominant disease that results in high levels of serum IgE.

On the other hand, the findings of several studies have suggested that Th1 cytokine production is important in AD. Grewe et al. [9] reported that IFN- γ mRNA in atopic skin lesions was augmented in the majority of patients, while IL-4 mRNA was augmented in the minority. They also reported that the chronological cytokine profiles on skin lesions induced by repeated patch testing with allergens showed augmentation of IL-4 mRNA in the early phase and those of IFN- γ mRNA in the late phase in AD patients. These data suggest that Th1 cytokines are predominant under certain conditions in AD [9].

On skin lesions in AD patients, an increase in the colonization of *Staphylococcus aureus*, which often produces staphylococcal enterotoxins, is frequently detected [10, 11]. It was reported that β -defensins and cathelicidins, antimicrobial peptides, are downregulated in the skin of AD patients [12]. These findings imply that both bacterial infections and reactions to the product of bacteria might be involved in the pathogenesis of AD.

The DS-*Nh* mouse strain originated from a mutant male mouse with deficient hair growth in an inbred DS strain colony at Aburabi Laboratories (Shionogi Co., Ltd., Shiga, Japan) in 1976. DS-*Nh* mice housed under conventional conditions, but not under specific pathogen-free (SPF) conditions, develop dermatitis spontaneously [13, 14]. The elevation in serum total IgE is detected from around 17 weeks of age after the development of skin lesions [13]. In these mice, a remarkable increase in *S. aureus* on the skin surface is observed under conventional conditions even without skin lesions [13]. In a previous report, applications of *S. aureus* on the skin accelerated the onset of skin eruption [14].

NC/Nga mice, in which skin eruptions appear spontaneously under conventional conditions, are well known as representative model mice for AD [15–21]. In NC/Nga mice, the fur mite is thought to be important in the induction of dermatitis and increase in serum IgE [21].

In the present study, we examined the effects of repeated application of 2,4,6-trinitrochlorobenzene (TNCB), which induces the Th1 response, on the development of AD-like dermatitis and production of IgE in DS-*Nh* and NC/Nga mice. We also investigated the cytokine production of splenocytes stimulated with staphylococcal endotoxin B (SEB), which is a bacterial super-antigen and induces Th1 response, in DS-*Nh* mice.

Materials and Methods

Animals

Five-week-old female DS-*Nh* mice raised under SPF conditions were provided by Aburabi Laboratories, Shionogi & Co., Ltd., Shiga, Japan. DS-*Nh* mice used for this study were obtained as F1(Nh/+) from male DS-*Nh*(Nh/Nh) $\times$ female DS(+/+). Five-week-old NC/Nga mice under SPF conditions were purchased from SLC (Shizuoka, Japan). They were acclimatized under conventional conditions (free of fur mites) before their use in the experiments at the Animal Experiment Center of Yokohama City University Graduate School of Medicine.

These mice were housed in micro-isolator cages, exposed to a 12-hour light/12-hour dark cycle, and standard feed and water ad libitum. This study was conducted according to the guidelines for animal experiments at Yokohama City University Graduate School of Medicine.

Reagents

TNCB, SEB, and concanavalin A (ConA) were purchased from Tokyo Kasei Kogyo Co., Ltd. Tokyo, Japan; Sigma Chemical Co., St Louis, Mo., USA, and Vector Laboratories, Inc, Burlingame, Calif., USA, respectively.

Sensitization and Repeated Application with TNCB

DS-*Nh* and NC/Nga mice were treated either with 1% TNCB dissolved in acetone:olive oil (4:1) or with control vehicle (acetone:olive oil 4:1), which was applied epicutaneously to the dorsal skin (50 μ l) at 8 weeks of age. The same reagents (either 50 μ l of 1%TNCB or control vehicle) were applied to 9- to 11-week-old mice 3 times a week on the dorsal skin. From 12 to 15 weeks of age, the mice were treated with the reagents once a week.

Evaluation of Skin Eruption Score

The skin eruptions defined as erythema, edema, excoriation/erosion, and dry skin were observed macroscopically. These symptoms were evaluated using a grading system: grade 0 = no visible skin change; grade 1 = slight skin change; grade 2 = moderate skin change, and grade 3 = severe skin change. The total scores of the skin eruptions are defined as the sum of the individual scores indicated above [13].

Measurement of Transepidermal Water Loss

Transepidermal water loss (TEWL) was measured using DERMALAB (Cortex Technology, Denmark). The room conditions were kept constant ($24 \pm 2^\circ\text{C}$ and $45 \pm 5\%$ relative humidity). Dorsal skin areas were selected for measurement of TEWL.

Isolation of *S. aureus* on the Skin Surface

DD checker Seiken, MSEY agar (Denka Seiken, Tokyo, Japan), which is a medium for the isolation and identification of *S. aureus*, was used to evaluate colonization of *S. aureus* on the skin surface [21]. Dorsal skin areas were selected for sampling. The examinations were performed at 7 weeks of age before application of TNCB, and at 15 weeks of age after the application of TNCB. The plates were applied for 10 s on the skin for sampling. Next, the medium plates were cultured in an incubator for 24 h at 37°C . After incubation, the numbers of colonies on the medium were counted to 1,000 cfu/10 cm^2 . Identification was determined according to the manufacturer's instructions.

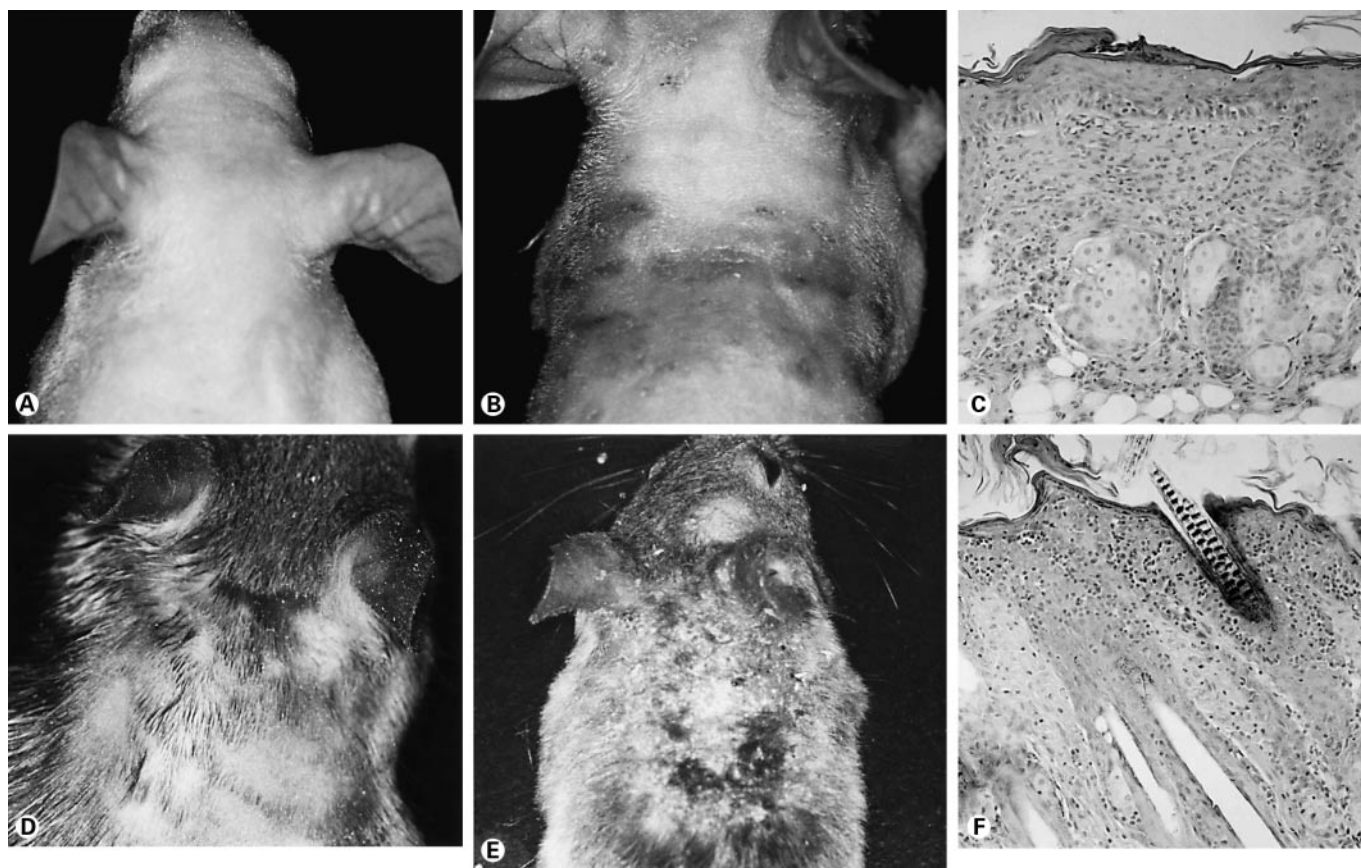


Fig. 1. Clinical and pathological features of DS-*Nh* and NC/Nga mice before and after treatment with TNCB. DS-*Nh* (A) and NC/Nga mice (D) before treatment with TNCB. DS-*Nh* (B) and NC/Nga (E) mice after treatment with TNCB. Erythema, erosion, crust and exco-riations were observed on the dorsal surface and face and neck skin

area in both mice. Pathological skin changes after treatment with TNCB in the DS-*Nh* (C) and NC/Nga mice (F). Hyperkeratosis, acan- thosis and intercellular edema in the epidermis and inflammatory cell infiltrations in the dermis were observed in the dorsal skin in HE samples from both mice.

Measurement of Serum IgE

Sera were collected and stored at -80°C until IgE measurement. Serum total IgE levels were measured using a sandwich ELISA kit (Yamasa Shouyu Co., Ltd., Chiba, Japan). The ELISA procedures were conducted according to the manufacturer's instructions.

Isolation of Splenocytes and Culture of Cells

At the ages of 7 and 15 weeks, DS-*Nh* mice were sacrificed under anesthesia by exsanguination from the subclavian arteries and veins. A single cell suspension (1.25×10^6 cells/0.5ml) prepared from the spleen was cultured with SEB (10 $\mu\text{g}/\text{ml}$) or ConA (5 $\mu\text{g}/\text{ml}$) as a posi- tive control, in RPMI1640 (Sigma Chemical Co.) with 10% fetal bovine serum (FCS) at 37°C in 5% CO_2 for 48 h. The supernatants stored at -80°C until the measurement of cytokines.

Measurement of Cytokines in Supernatants of Splenocyte Cultures

The levels of IFN- γ , IL-4, IL-12, and IL-13 in the supernatants were measured using sandwich ELISA kits; Cytoscreen Mouse IFN- γ , IL-4, IL-12 (Biosource International, Camerillo, Calif., USA) and ANALYZA Mouse IL-13 (Genzyme Techné, Minneapolis,

Minn., USA). The procedures were conducted according to the man- ufacturer's instructions.

Statistics

Statistical significance was determined by the Mann-Whitney's t test for unpaired 2-group comparison using the Stat View System, if not otherwise mentioned.

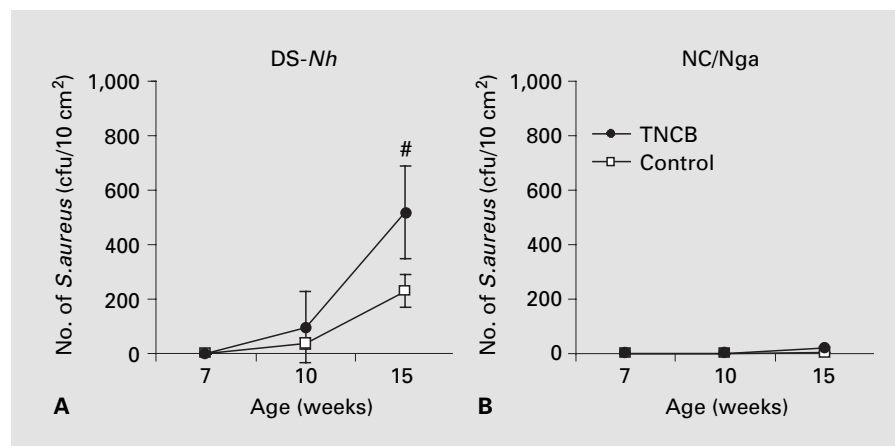
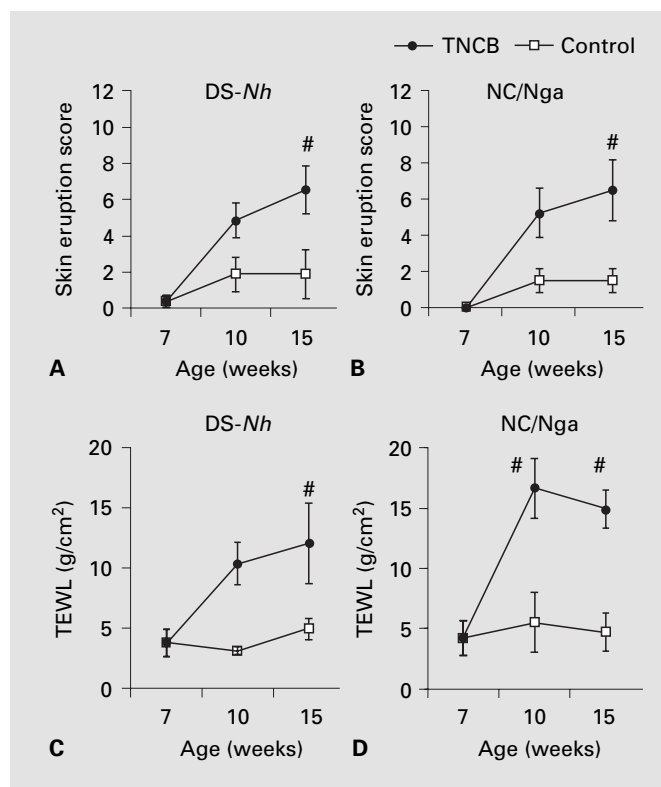
Results

Development of Skin Changes by Sensitization and Repeated Applications with TNCB in DS-*Nh* and NC/Nga Mice

Both DS-*Nh* and NC/Nga mice, which received TNCB treatment, developed more severe AD-like skin eruptions compared with the mice receiving only the vehicle control. Erythema, erosion and discharge and crust by exco- riation were observed not only on the dorsal areas applied

Fig. 2. Effects of sensitization and repeated applications of TNCB on skin eruption score (**A, B**) and transepidermal water loss (TEWL), (**C, D**) in DS-*Nh* and NC/Nga mice. Data are shown for TNCB- and vehicle- (acetone:olive oil, 4:1, control) treated mice. Mice were housed under conventional conditions (free of fur mites). Sensitization and repeated applications with TNCB induced a significant increase in skin eruption score (**A, B**) and TEWL (**C, D**) in DS-*Nh* and NC/Nga mice at the age of 15 weeks. Each value represents the mean $\pm$ SE of 6–8 DS-*Nh* or NC/Nga mice. # $p < 0.05$ vs. control at the same age.

Fig. 3. Effects of sensitization and repeated applications of TNCB on the increase in the number of isolates of *S. aureus* on the skin surface of DS-*Nh* (**A**) and NC/Nga mice (**B**). Mice were housed under conventional conditions (free of fur mites). Medium plates were applied for 10 s on the skin for sampling and were cultured in an incubator for 24 h at 37°C. After incubation, the number of colonies on the medium was counted to 1,000 cfu/10 cm². Sensitization and repeated applications of TNCB induced an increase in *S. aureus* on the skin surface of DS-*Nh* mice but not NC/Nga mice. Each value represents the mean $\pm$ SE of each group. Each group consists of 6–8 DS-*Nh* mice or NC/Nga mice. # $p < 0.01$ vs. 15-week-old control mice.



with TNCB, but also on the face and neck areas without systemic application (fig. 1). Histopathological changes in the skin of the 15-week-old mice treated with TNCB were hyperkeratosis, acanthosis, and intercellular edema in the epidermis and inflammatory cell infiltrations in the dermis in hematoxylin-eosin (HE)-stained samples (fig. 1). Inflammatory cells consisted mostly of monocytes and eosinophils. Necrotic epidermis with neutrophils or ballooning cells which are observed in irritable contact dermatitis, were not recognized. The skin eruption score

increased in the 15-week-old DS-*Nh* and NC/Nga mice treated with TNCB as compared to the mice treated with vehicle control (fig. 2A, B). The values of TEWL on the skin lesions also increased in the 15-week-old DS-*Nh* and NC/Nga mice treated with TNCB (fig. 2C, D).

Increase in *S. aureus* on the Skin Surface

Under the conventional conditions (free of fur mites), *S. aureus* increased significantly in the group of 15-week-old DS-*Nh* mice treated with TNCB ($p < 0.01$ vs. 15-

week-old control mice; fig. 3A). An increase in *S. aureus* on the skin was even observed from 10 weeks of age in the group of DS-*Nh* mice treated with vehicle control. In contrast, in NC/Nga mice no bacterial proliferation was observed in any of the groups of mice treated with TNCB or vehicle control until 15 weeks.

Serum Levels of Total IgE

In DS-*Nh* mice after treatment with TNCB, the levels of serum IgE significantly increased at the age of 15 weeks (fig. 4A). However, in NC/Nga mice under conventional conditions (free of fur mites), the increase in serum IgE was not significant even after treatment with TNCB at 15 weeks of age (fig. 4B).

Cytokine Levels in the Supernatants of Splenocyte Cultures with SEB

In DS-*Nh* mice, IFN- γ , IL-4, IL-12 and IL-13 levels were measured in the supernatants of splenocyte cultures stimulated with SEB. The levels of IFN- γ stimulated with SEB significantly increased as compared to those of the medium controls in both groups at 15 weeks of age ($p < 0.01$; fig. 5A). The increase in IFN- γ levels in the group of 15-week-old mice treated with TNCB was significantly higher than those in 15-week-old mice treated with vehicle control ($p < 0.05$; fig. 5A).

The levels of IL-13 stimulated with SEB increased significantly in the 10- and 15-week-old mice treated with both vehicle control and TNCB as compared to those of the medium controls ($p < 0.01$; fig. 5B). With SEB stimulation, the IL-13 levels in the groups treated with TNCB were higher than those in vehicle control mice although they were not significant (fig. 5B).

On the contrary, the levels of IL-4 did not increase significantly as compared to those of the medium controls from 7 to 15 weeks of age in both groups of mice (fig. 5C). The levels of IL-12 stimulated with SEB did not increase at 10 and 15 weeks of age as compared to those of medium controls of the same age (fig. 5D).

In contrast, the splenocytes of NC/Nga mice did not respond to SEB (data not shown).

Discussion

DS-*Nh* mice housed under conventional conditions spontaneously develop dermatitis mainly on the face and the neck from 7 weeks of age [13]. Dermatological skin eruptions exacerbated with aging and serum IgE levels increase in association with the development of dermati-

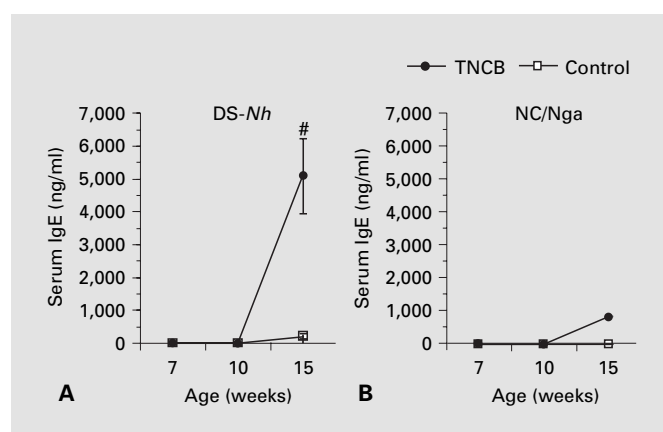


Fig. 4. Effects of sensitization and repeated applications of TNCB on the serum IgE level in DS-*Nh* and NC/Nga mice under conventional conditions (free of fur mites). Sensitization and repeated applications induced a significant increase in serum IgE in DS-*Nh* mice (**A**) but not in NC/Nga mice (**B**). Each value represents the mean $\pm$ SE of 6–8 DS-*Nh* or NC/Nga mice. [#] $p < 0.01$ vs. control mice at the same age.

tis. Increases in TEWL on the skin lesions were also detected suggesting that the skin barrier system was impaired [11]. Furthermore, an increase in *S. aureus* on the skin surface was observed. These clinical features in DS-*Nh* mice are in accordance with those observed in human AD patients. Pathological changes, such as hyperkeratosis, acanthosis, intercellular edema in the epidermis and inflammatory cell infiltration, were also compatible with human AD [14]. Hikita et al. [14] reported fluctuating data on mast cells and eosinophils in the skin of DS-*Nh* mice from sections stained with acidic toluidine blue. The number of total mast cells increased in 17-week-old and older mice under conventional conditions. Moreover, infiltrating eosinophils increased at the age of 13–29 weeks [14]. They also reported that degranulated mast cells increased under conventional conditions. Interestingly, it has been reported that an increase in *S. aureus* on the skin would play an important role in the induction of dermatitis in DS-*Nh* mice because applying *S. aureus* to their skin exacerbates their skin symptoms [14]. Previous studies in humans have also shown that *S. aureus* and staphylococcal enterotoxin play an important role in the pathogenesis of AD [10, 11].

In previous studies, NC/Nga mice have been noted to be a Th2-predominant AD model because their serum IgE levels increase from 5 weeks under conventional conditions with fur mites and keep increasing until about 12 weeks of age [15–19]. As mentioned, the start of elevation in serum IgE level is earlier than that in DS-*Nh* mice. The

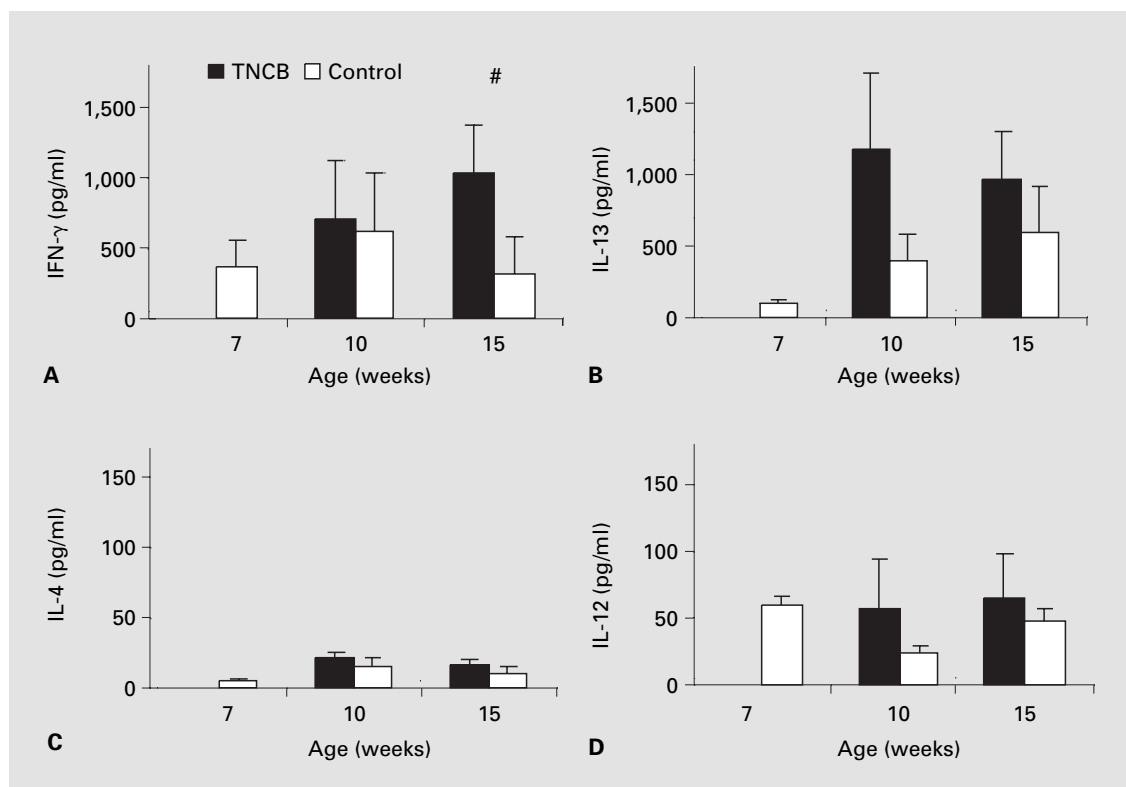


Fig. 5. Cytokine production (**A** IFN- γ , **B** IL-13, **C** IL-4, **D** IL-12) from spleen MNC of DS-*Nh* mice. Mice were sensitized at 8 weeks of age and repeatedly treated with TNCB under conventional conditions. Control mice were treated with vehicle (acetone:olive oil, 4:1). 1.25×10^6 spleen MNC in 500 μ l of 10% FBS RPMI 1640 medium were cultured with SEB (10 μ g/ml) in 24-well flat-bottom plates in 5% CO₂ at 37°C for 48 h and the culture supernatants were stocked at -80°C and thereafter subjected to ELISA. Mean values of IFN- γ (**A**) and IL-13 (**B**) in TNCB-treated mice were higher than in control mice. Mean values of IL-4 (**C**) and IL-12 (**D**) were low as in controls. Mean values represent the mean $\pm$ SE from 4–6 DS-*Nh* mice. # $p < 0.05$ vs. controls at the same age. In medium (10% FBS RPMI 1640) control, IFN- γ and IL-13 were not produced from spleen MNC, and 0–3 pg/ml of IL-4 and 0–10 pg/ml of IL-12 were produced from spleen MNC.

IgE levels are substantially higher (10^4 – 10^5 ng/ml) in these mice [17] than in DS-*Nh* mice (10^2 – 10^3 ng/ml). A defective IFN- γ response to SEB in NC/Nga mice was reported by Habu et al. [16]. They stated that the low IFN- γ response to SEB or endotoxin (lipopolysaccharides, LPS) was due to the lack of regular V β 8+T and V β 8+ NK T cells in the mice. They concluded that this systemic deficiency in the Th1 response to bacterial antigens leads to a Th2-dominant state in NC/Nga mice [16].

It is said that human AD patients are polarized to Th2-predominant states in view of the high levels of serum IgE and cytokine expressions of their peripheral blood and infiltrated cells in skin lesions [15]. However, Th2 predominance cannot always explain the pathogenesis of AD because there are some patients whose serum IgE levels are within the normal range or are not at such high levels

[22, 23]. The subgroup of AD patients with normal serum IgE and without specific IgE sensitization has been termed the ‘non-allergic form of AD’, or ‘non-atopic eczema’ or ‘intrinsic type AD’ [22, 23].

Scheerens et al. [24] suggested that long-term exposure to contact-sensitizing agents in mice induced a significant increase in both the total IgE and IgG levels in the sera when compared with non-sensitized control mice. Antigen-specific IgE and IgG were also elevated in the sera when compared with the control mice [24]. In this study, repeated application of TNCB on the dorsal skin of DS-*Nh* and NC/Nga mice induced systemic inflammatory skin eruptions not only on the dorsal skin, but also on the face and neck, which is severer than those of control mice. This might mean that repeated exposure to TNCB, which causes Th1-predominant contact hypersensitivity, facili-

tates the natural occurrence of AD-like skin eruptions. These skin lesions mimicked AD clinically and pathologically. In view of pathological changes, hyperkeratosis, acanthosis, and spongiosis were observed in the epidermis. Infiltration of inflammatory cells, including eosinophils, was detected in the dermis. There were no apparently significant differences between DS-*Nh* and NC/Nga mice both clinically and pathologically. In company with the development of skin eruptions induced by TNCB application, an increase in serum IgE levels and also an increase in *S. aureus* colonization on the skin surface were detected in DS-*Nh* mice. Total serum IgE levels in DS-*Nh* mice treated with TNCB was much higher than those in the control mice at the age of 15 weeks. On the contrary, there was no significant increase in the total IgE levels and no significant increase in *S. aureus* on the skin surface in NC/Nga mice treated with TNCB. Therefore it seems that repeated allergic contact dermatitis induced an increase in *S. aureus* on the skin surface and AD-like lesions with IgE hyperproduction in DS-*Nh* mice, while the same treatment induced only skin lesions without an increase in *S. aureus* and IgE production in NC/Nga mice.

In order to investigate the mechanism of these clinical and serological changes in DS-*Nh* mice, we examined splenocyte cytokine response to SEB in the mice before and after the development of skin eruptions. As a result, an increase in IFN- γ induced by SEB was shown in accordance with the exacerbation of skin eruptions and an increase in *S. aureus* on the skin surface in DS-*Nh* mice

treated with TNCB. This suggests that the Th1 response to SEB is important in DS-*Nh* mice in the development of dermatitis.

In addition, IL-13 production stimulated by SEB increased in DS-*Nh* mice treated with TNCB. Previous research has indicated that IL-4 plays an important role in the overproduction of IgE in AD. It has also been shown that IL-13, which is secreted not only by CD4-positive Th2 cells but also by CD8-positive T cells and dendritic cells, shares many biological activities with IL-4 and induces IgE synthesis [25–27]. However, IL-13 does not have direct effects on T cells, while IL-4 enhances the differentiation of Th2 cells by inhibiting the production of IFN- γ . Without IL-4, IL-13 facilitates IgE synthesis by acting directly on B cells [28, 29].

These data suggest the possibility that IL-13 might play an important role in the production of IgE, and IFN- γ might facilitate IL-13 production in the DS-*Nh* mice.

Taken together our results suggest that the pathogenesis of AD-like eruptions in DS-*Nh* mice is different from that in NC/Nga mice. It is possible to state that Th1 cells might play a more important role in DS-*Nh* mice than in NC/Nga mice to develop dermatitis. Further investigation should be performed to establish an involvement of the Th1-predominant response in the development of AD-like eruptions in DS-*Nh* mice. Currently, an analysis of the spleen cells and draining lymph node cells by flow cytometry in DS-*Nh* mice before and after the development of skin eruptions is in progress.

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