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Original Article

Staphylococcus aureus colonization in contact hypersensitivity induces a shift in cutaneous cytokine milieu from a Th1- to a Th2-type profile

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Abstract

Background: Colonization of atopic dermatitis lesions with *Staphylococcus aureus* is a frequent phenomenon and may exacerbate inflammation of the skin. The aim of the present study was to determine the effect of *S. aureus* on T cell-mediated immune responses in contact hypersensitivity caused by a hapten or a protein with large molecule.

Methods: *Staphylococcus aureus* or phosphate-buffered saline was inoculated on experimental contact dermatitis induced by 2,4,6-trinitro-1-chlorobenzene (TNCB) or house dust mite antigen. At various times after inoculation, the experimental lesions were examined histologically and immunohistochemically. Furthermore, the kinetics of cytokine patterns (interferon (IFN)-γ, interleukin (IL)-2, IL-4 and IL-5) in each lesion were analyzed by reverse transcription–polymerase chain reaction.

Results: In TNCB-challenged lesions inoculated with *S. aureus*, the levels of IL-2 mRNA decreased; in contrast, mRNA levels of IL-4 and IL-5 were upregulated. In mite antigen-challenged lesions, IL-4 and IL-5 mRNA expression was detected throughout the period of the investigation, even without *S. aureus* inoculation. Interferon-γ mRNA expression in mite dermatitis without *S. aureus* inoculation was observed only in the later period and IL-2 mRNA expression in mite dermatitis with *S. aureus* was suppressed and observed much later than in the control group.

Conclusions: Infestation of *S. aureus* on skin lesions in experimental contact hypersensitivity induces a shift in the immune reaction from a Th1- to a Th2-dominant response.

Key words: atopic dermatitis, *Staphylococcus aureus*, Th1 cells, Th2 cells.

Introduction

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease characterized by increased IgE production and positive hypersensitivity to environmental allergens. Acute AD lesions are characterized by the presence of T cells producing classical type 2 cytokines, including interleukin (IL)-4 and IL-5.[1,2] T Cell activation is associated with an imbalance in cytokine production resulting in an increased production of IL-4 and a reduction of interferon (IFN)-γ,[3] although in the chronic phase of AD lesions the production of type 1 cytokines, such as IFN-γ, increases.[4] *Staphylococcus aureus* can be isolated from skin lesions in approximately 95% of all AD patients, but only in approximately 5% of non-atopic patients.[5] The presence of *S. aureus* on inflamed AD lesions without clinical superinfections can reach 10^6 colony forming units (c.f.u.)/cm^2,[6] and can cause severe exacerbation of the disease in many patients, although it is often responsive to antistaphylococcal therapy.[5,7]

The underlying mechanisms of this chronic bacterial colonization of the skin and the roles of *S. aureus* in allergic reactions in AD are unclear. To define the roles of *S. aureus* in AD, we examined the effect of inoculation with *S. aureus* on experimental contact dermatitis lesions developed with a hapten (2,4,6-trinitro-1-chlorobenzene;

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TNCB) or an aeroallergen (house dust mite antigen; *Dermatophagoides farinae*). Because hapten-induced contact dermatitis is considered to be a classic Th1-type immune response in the skin[8] and contact hypersensitivity induced by a high molecular weight protein, such as mite antigen, shows a Th2-type reaction,[9] in the present experimental model we are able to observe the effects of *S. aureus* on the two Th1- and Th2-type immune reactions, instead of original AD lesions showing complicated cytokine profiles.[4]

METHODS

Bacteria

We used an *S. aureus* strain isolated from a patient with atopic dermatitis to produce staphylococcal enterotoxin B (SEB). The *S. aureus* was grown overnight in Brain–Heart–Infusion broth (Nissui Pharmaceutical, Tokyo, Japan) at 37°C with shaking. The *S. aureus* was washed in sterile phosphate-buffered saline (PBS) and the *S. aureus* cells were resuspended in PBS. The cell suspension was then divided into individual inoculum sizes of 10^6 colony forming units (c.f.u.)/mL.

Sensitization and elicitation procedure

Female BALB/c mice were obtained from the Mouse Colony of Okayama University Medical School and were used at the age of 5–6 weeks.

House dust mite antigen (Df), *Dermatophagoides farinae* body extract, was obtained from Torii Pharmaceutical (Tokyo, Japan). We dissolved TNCB (Tokyo Kasei, Tokyo, Japan) in a solvent (acetone and olive oil 1 : 4) and used a 0.5% solution for sensitization and a 0.005% solution for elicitation. The Df antigen dissolved in PBS at 5 mg/mL was prepared for sensitization and 1% Df antigen in white petrolatum containing 0.1% sodium dodecyl sulfate (SDS) was prepared for elicitation.

Mice were barrier disrupted by repeated applications and detachments of adhesive cellophane tape on shaved abdominal skin. Percutaneous sensitization with the antigens was performed by topical application of 20 μL of 0.1% TNCB or 50 μL Df (5 mg/mL in PBS) onto the barrier-disrupted abdominal skin. Seven days after sensitization, 10 μL of 0.005% TNCB (TNCB contact dermatitis) or 0.1 g of 1% Df ointment (Df contact dermatitis) was applied to the shaved dorsal skin of the mice.

Inoculation on mice

The *S. aureus* cells were inoculated on the skin according to the method of Abe *et al*.[10] Briefly, 12 h after elicitation, 50 μL of each of the bacterial suspensions was applied onto the back skin lesions of the mice. The inoculated sites were occluded with sterile plastic film followed by a tight dressing with vinyl adhesive tape. As a control, PBS was applied instead of *S. aureus* suspensions in some cases.

Histologic examinations

Biopsy specimens were taken at 0, 6, 12, 18 and 60 h after inoculation of the bacterial suspension. Half the specimens were fixed in 10% formalin solution and embedded in paraffin, whereas the other half were snap-frozen in liquid nitrogen and stored at –70°C until use. Paraffin sections were stained using hematoxylin and eosin.

To confirm IL-4 and IFN-γ expression in the lesions, frozen sections (5 μm) were prepared with a cryostat and stained by immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated anti-IL-4 monoclonal antibody (mAb) and rhodamine-conjugated anti-IFN-γ mAb (PharMingen, San Diego, CA, USA).

Count of viable bacteria in lesions

Circular biopsy specimens (1.5 cm in diameter) were taken from the experimental lesions at 6, 12, 18, 36 and 60 h after inoculation with *S. aureus*. Specimens were minced with a pair of surgical scissors and homogenized in 1 mL sterile PBS. A sample of the homogenized tissue was added to 9 mL sterile PBS and 0.1 mL of this mixture was cultured on staphylococcal medium no. 110 'Nissui' (Nissui Pharmaceutical) using the 10-fold dilution method. After 48 h incubation at 37°C, the number of colonies was counted and the c.f.u. measurement was made.

RNA extraction and reverse transcription

Biopsy specimens were taken from dorsal skin at various times after inoculation with *S. aureus*. Samples were homogenized in a solution containing phenol and guanidium isothiocyanate (TRIzol™ Reagent; Life Technologies, Gaitherburg, MD, USA). After the addition of chloroform, the solution was mixed and centrifuged. Then, the aqueous phase was removed and RNA was precipitated with ethanol. Reverse transcription was

accomplished with Ready-to-go™ You prime First-Strand Beads (Pharmacia Biotech, Grand Island, NY, USA), according to the manufacturer's instructions. Briefly, total RNA (5 μg) from each sample was incubated with 1 U DNase (Gibco, Grand Island, NY, USA) and was used as a template. This was transferred to each tube in reaction mix beads and 0.5 μg oligo dT primer was added. Reverse transcription was performed at 37°C for 60 min in a total volume of 33 μL.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using a thermal cycler (MJ Research, Watertown, MS, USA). The cDNA was amplified in a 25 μL reaction volume containing 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L (each) dNTPs, 0.4 mmol/L primers and 0.2 U Taq polymerase (Gibco) overlaid with mineral oil. The PCR primers for IL-2, IL-4, IL-5, IFN-γ and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were purchased from Clontech Laboratories (Palo Alto, CA, USA). Cycle conditions were 94°C for 30 s, 58°C for 30 s and 72°C for 1 min and 30 s. This process was repeated for 28 cycles with G3PDH primer and for 33 cycles with IL-2, IL-4, IL-5 and IFN-γ primers. After the final cycle, the temperature was maintained at 72°C for 6 min. The PCR products were electrophoresed through 2% agarose gels in Tris–acetate–EDTA (TAE) buffer. After staining with ethidium bromide, gels were photographed under ultraviolet light. Both positive and negative controls were included in each assay to confirm that only cDNA PCR was detected and that none of the reagents was contaminated.

The PCR products were quantitated by analyzing Polaroid negatives using National Institutes of Health image (v1.61; Wayne Rasbaund, US National Institutes of Health, Bethesda, MD, USA) and the results integrated and normalized to the value of G3PDH. In semiquantitative experiments, the PCR was set up as described above and the amplifications were run for 24 cycles with G3PDH primers and for 30 cycles with IL-4 and IFN-γ primers. In the initial experiments, we established standard curves for G3PDH and these cytokines by titration of cDNA samples prepared from samples that had the highest signals for G3PDH and these cytokines. The cycle numbers were selected because they represented midpoints of their respective linear ranges for amplification of cDNA (1 μL) and because there was a linear correlation between the input cDNA and the yields of PCR products. Quantities of cDNA were normalized to yield equivalent amounts of PCR products for G3PDH and they were compared with the positive controls.

RESULTS

Number of viable bacteria in lesions

After inoculation with *S. aureus*, the mean values of viable bacteria on the surface per 1 cm^2 were plotted and are shown in Fig. 1. The bacterial counts were maintained at 10^3–10^4 c.f.u./cm^2 over time in both the TNCB and Df contact dermatitis groups.

Histological findings

At the time of inoculation (12 h after elicitation), the dermis in both groups showed a similar perivascular lymphocytic infiltration with scattered eosinophils. The epidermis also showed signs of spongiosis. After inoculation with *S. aureus*, the dermis showed a marked lymphocytic infiltrate with neutrophils and eosinophils (Fig. 2a).

Immunohistochemical analysis was also performed with anti-IL-4 mAb and anti-IFN-γ mAb using frozen sections of the TNCB contact dermatitis with *S. aureus*, when increased expression of IL-4 mRNA was detected (see below). The IL-4 immunoreactivity was localized to small mononuclear cells in the lower dermis and partly in the subcutaneous tissue (Fig. 2b). The distribution of IFN-γ

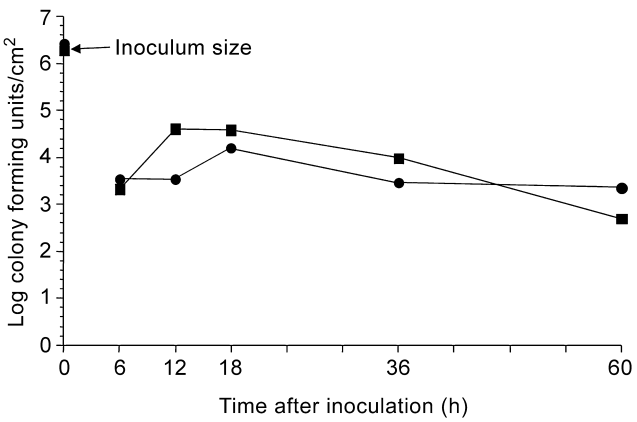

Fig. 1 Number of viable bacteria in contact dermatitis lesions at various time points after inoculation with *Staphylococcus aureus*. The bacterial counts are maintained at 10^3–10^4 c.f.u./cm^2 over time in both the 2,4,6-trinitro-1-chlorobenzene (■) and Df (●) contact dermatitis groups.

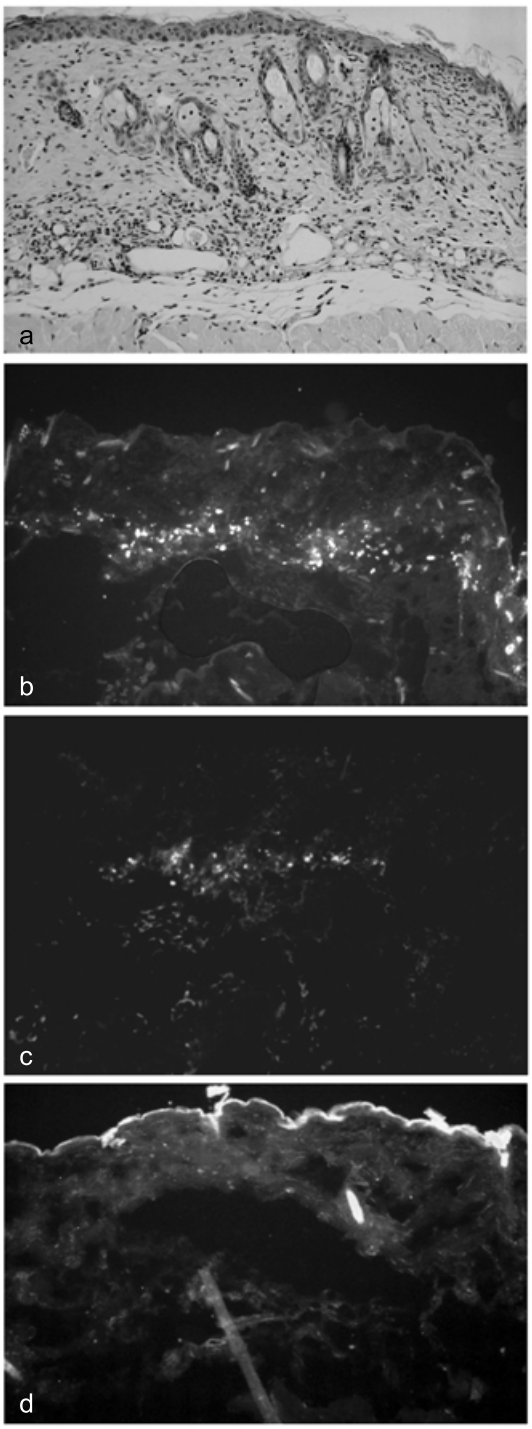

Fig. 2 Light micrographs showing histopathological (a) and immunohistochemical findings for interleukin (IL)-4 (b) and interferon (IFN)-γ (c) expression in 2,4,6-trinitro-1-chlorobenzene (TNCB) contact dermatitis 12 h after inoculation with *Staphylococcus aureus* (24 h after elicitation with TNCB). (a) The TNCB contact dermatitis with *S. aureus* is showing a marked lymphocytic infiltrate with neutrophils and eosinophils in the dermis. The overlying epidermis shows some spongiotic changes (hematoxilin–eosin). (b) The IL-4$^+$ cells are clearly detectable in the deep dermis and partly in the subcutaneous tissues of TNCB contact dermatitis with *S. aureus* 12 h after inoculation. (c) The distribution of IFN-γ in TNCB contact dermatitis with *S. aureus* 12 h after inoculation is similar to that of IL-4, although it is not always consistent with that of IL-4. (d) No immunoreactivity is present in the TNCB control.

was similar to that of IL-4, but not always consistent with that of IL-4 (Fig. 2c). In contrast, no immunoreactivity for IL-4 was detected in the TNCB controls (Fig. 2d).

Kinetics of cytokine mRNA expression

As shown in Fig. 3, different patterns of cytokine gene expression were apparent when comparing the time-course in the TNCB and Df contact dermatitis groups. Of the four cytokines tested, the mRNA level for IL-2 and IFN-γ was higher in the TNCB control group. The expression of IL-2 mRNA was detected throughout the investigation period in both control groups, although in the control group for Df dermatitis mRNA expression for IFN-γ was markedly suppressed early after inoculation. The mRNA for the Th2 cytokines IL-4 and IL-5 was not expressed in the TNCB contact dermatitis control group. However, the expression of the cytokines was markedly upregulated in the TNCB contact dermatitis with *S. aureus* group. In contrast, the expression of mRNA for IL-4 and IL-5 was clearly detected in the Df control group and was moderately enhanced in the Df contact dermatitis with *S. aureus* group. Among the four contact dermatitis groups, the TNCB control group showed a distinctly suppressed pattern of mRNA expression for IL-4 and IL-5 compared with the other groups.

The PCR products were applied to the analysis of IL-4 and IFN-γ mRNA expression (Fig. 4) in order to better quantify the comparisons. In TNCB control lesions, the production of IFN-γ mRNA was increased from the time of inoculation (12 h after elicitation) and reached a peak at 12 h. In the TNCB contact dermatitis with *S. aureus* lesions, the levels of IFN-γ mRNA decreased at 6–12 h without reaching the peak levels observed in the TNCB control lesions. Interleukin-4 mRNA was never detected in TNCB control lesions. In contrast, the levels of IL-4 mRNA in the TNCB contact dermatitis with *S. aureus*

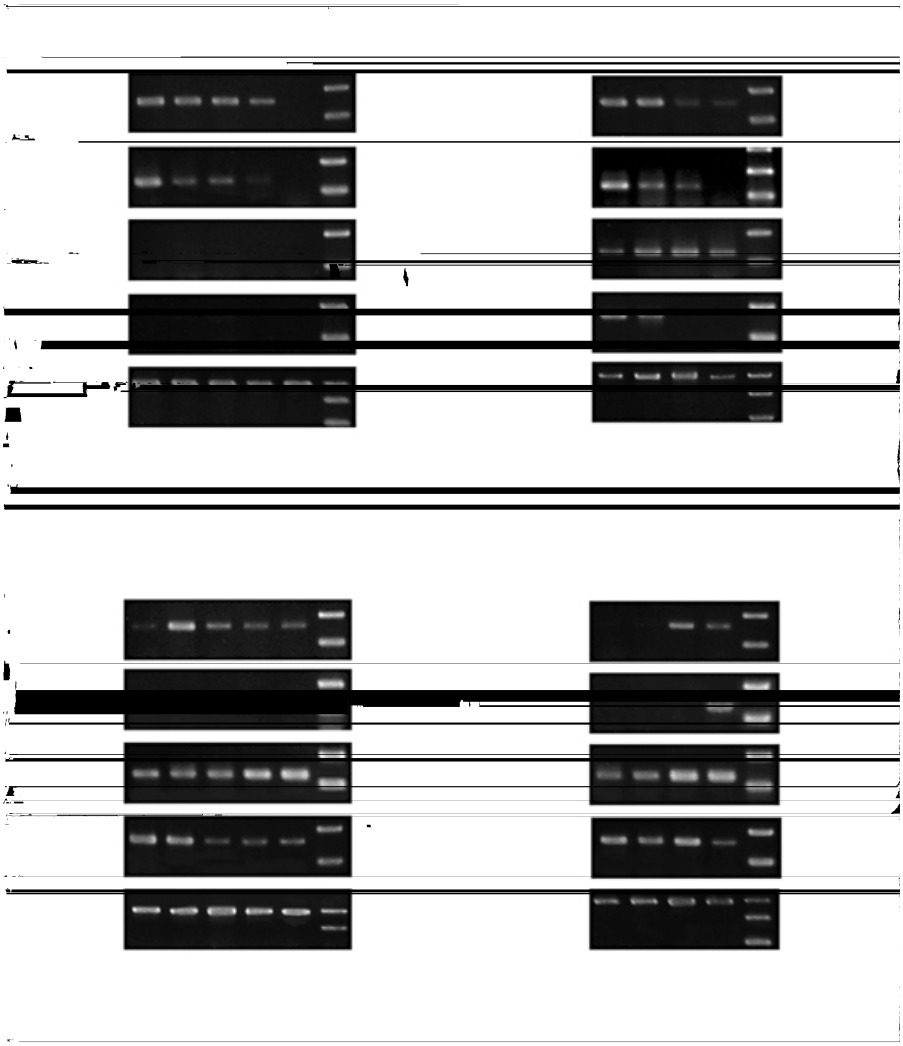

Fig. 3 Reverse transcription–polymerase chain reaction analysis of the kinetics of cytokine mRNA expression at various time points in the 2,4,6-trinitro-1-chlorobenzene (TNCB) and Df contact dermatitis groups with or without *Staphylococcus aureus* inoculation. The TNCB control group (a) shows a distinctly suppressed pattern in mRNA expression for the Th2 cytokines compared with the other groups. However, the expression of these cytokines is heavily upregulated in the TNCB contact dermatitis with *S. aureus* group (b). IL, interleukin; IFN-γ, interferon-γ; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

lesions were upregulated 6 h after inoculation and this persisted throughout the investigation period. In Df control lesions, production of IL-4 mRNA gradually increased from just after the time of inoculation and persisted for 60 h. In the Df with *S. aureus* lesions, IL-4 mRNA was upregulated and reached a peak at 12 h, where it remained for 60 h. The expression of IFN-γ mRNA in Df control lesions was first detected at 24 h, whereas that in the Df with *S. aureus* lesions was first detected at 60 h, although the expression was very low in comparison with the IL-4 mRNA level. These findings from PCR analysis indicate that patterns of cytokine expression in contact hypersensitivity induced by TNCB shift from a Th1- to a Th2-type cytokine profile in the presence of *S. aureus*.

Discussion

In experimental contact hypersensitivity by application of haptens, $CD4^+$ T cells, especially Th1 cells producing cytokines such as IL-2 and IFN-γ, play an important role in the pathophysiological mechanism[8] of this condition. However, although some haptens tend to induce Th1 cells in skin lesions of contact hypersensitivity, others tend to induce Th2 cells. Kitagaki *et al*. reported that Th1 cells predominantly infiltrated in the acute elicitation phase in mouse contact sensitivity induced by TNCB, although, in the chronic lesions after repeated applications of the same hapten, Th2 cells became inversely more predominant.[11] Asada *et al*. also demonstrated that Th2 cells expressing mRNA for IL-4 appeared late in

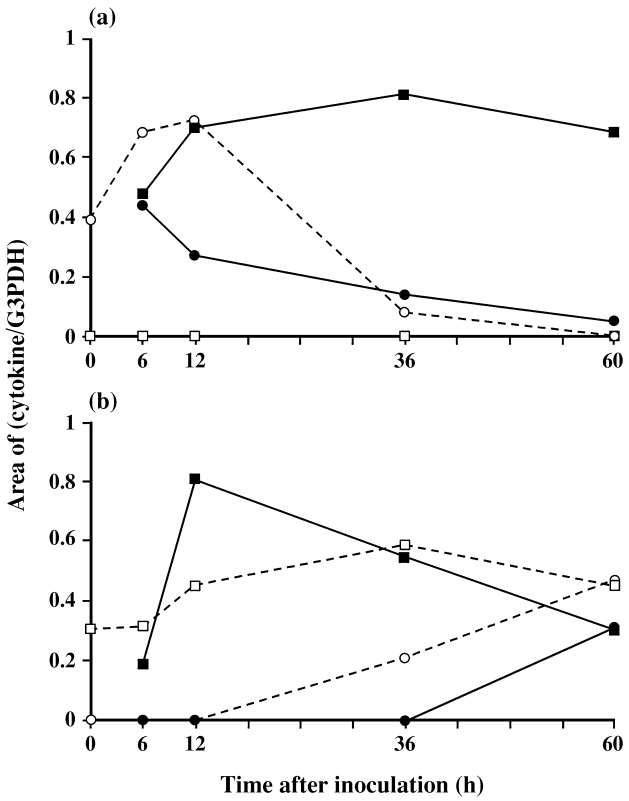

Fig. 4 Relative levels of cytokine mRNA expression at various time points after inoculation with *Staphylococcus aureus*. (a) Interleukin (IL)-4 mRNA is never detected in the 2,4,6-trinitro-1-chlorobenzene (TNCB) control lesions; in contrast, in the TNCB contact dermatitis with *S.aureus* lesions, the levels of IL-4 mRNA are upregulated after inoculation. (□), TNCB control (IL-4); (○), TNCB control (interferon (IFN)-γ); (■), TNCB + *S. aureus* (IL-4); (●), TNCB + *S. aureus* (IFN-γ). (b) In the Df with *S. aureus* lesions, IL-4 mRNA is upregulated and the expression of IFN-γ mRNA is clearly suppressed for a long time. (□), Df control (IL-4); (○), Df control (interferon (IFN)-γ); (■), Df + *S. aureus* (IL-4); (●), Df + *S. aureus* (IFN-γ).

the elicitation phase of contact sensitivity developed by TNCB, but not during the sensitization phase, and proposed that the hapten TNCB induced Th2 cells in the developing stage of contact dermatitis.[12] Furthermore, when contact sensitivity is induced by some haptens in BALB/c mice, a predominant infiltration of Th2 cells is seen compared with other mouse strains.[13] In the present study, we demonstrated that regular contact dermatitis due to the hapten TNCB, showing a Th1 cytokine pattern, can change following inoculation with *S. aureus* to the Th2 pattern.

Dermatophagoides-derived antigens, which have a much greater molecular weight than TNCB, usually induce different immune reactions than those induced by conventional haptens. Comoy *et al*. showed that protease activity in *Dermatophagoides pteronyssinus* 1 antigen played an important role in deviating the immune response.[9] The results of the study of Comoy *et al.*,[9] combined with the present findings, indicate that infestation of *S. aureus* on skin lesions in mite antigen-induced experimental contact sensitivity showing a Th2 cytokine pattern early plays an important role in further promoting an imbalance to the Th2 pattern. Colonization by *S. aureus* not only switches the immune responses in inflammatory skin lesions from a Th1 cytokine pattern to a Th2 pattern, but also increases the duration of the Th2 immunoreaction pattern, leading to chronicity of the inflammation. Atopic dermatitis can appear to be chronic contact dermatitis due to Df and the experimental contact dermatitis in the present study presents an ideal model of the inflammatory skin lesions with *S. aureus* colonization.

The number of *S. aureus* cells of the skin involved in AD is usually less than 10^7 c.f.u./cm^2, which represents a colonization rather than an infection, meaning that there would be no biological defense response such as infiltration of polymorphonuclear leukocytes phagocytozing the microorganisms. Nevertheless, colonization of *S. aureus* on AD skin lesions is considered to be one of the exacerbating factors in AD, even in the absence of clear infectious signs.[7,14,15] The number of *S. aureus* cells that were inoculated in the present study is presumably consistent with that seen in patients with AD. Even several hours after inoculation, the number of *S. aureus* cells remained at approximately 10^4 c.f.u./cm^2 and, during this period, the cytokine profile in the experimental lesions is still kept in the Th2 pattern. Because the number of *S. aureus* obtained from moderate AD lesions, but not from severe lesions, is approximately 10^3 c.f.u./cm^2, our present findings are considered to be appropriate as a model of AD lesions with colonization of *S. aureus*. Colonizing *S. aureus* can induce various cytokines, including tumor necrosis factor-α from epidermal keratinocytes.[15–17] Tumor necrosis factor-α stimulates the eosinophils that have infiltrated into the dermis to produce type 2 cytokines, such as IL-4,[18] and further stimulates dermal fibroblasts to produce eotaxin,[19] which promotes further infiltration of Th2 cells[20] into the lesion. Furthermore, IL-4 produced by Th2 cells may activate Th2 cells. This activation series may lead to the predominance of Th2 cells in inflammatory lesions.

Staphylococcal enterotoxin B is the most investigated of the exotoxins produced by *S. aureus* and *S. aureus* cell components in terms of its effects on human T lymphocytes. Campbell *et al*. reported that the production of IFN-γ by peripheral blood mononuclear leukocytes (PBML) from children with AD is significantly suppressed after incubation with SEB, compared with that by PBML from controls.[21,22] Strickland *et al*. reported that superantigens increased the frequency of memory T cells capable of migrating to activated AD lesions.[23] The cell wall elements of *S. aureus*, teicoic acid and peptide glycan are also known to activate human T lymphocytes to produce some cytokines.[24,25] In the present study, we used viable cells of *S. aureus* producing only SEB to experimentally simulate human AD lesions colonized with abundant *S. aureus*. Therefore, it is not clear which component of *S. aureus* or SEB was responsible for modifying the profile of the immune responses. However, considering recent *in vitro* and *in situ* studies together,[21–25] as shown in the present study, colonization with *S. aureus* and its exotoxins can lead to imbalanced cytokine production by allergen-specific T cells in the skin involved in AD, never the preferential binding of *S. aureus* to skin sites of established Th2-mediated inflammation.[26]

Further investigation is required to clarify the effect of *S. aureus* on AD, especially in terms of the exotoxin level produced by *S. aureus* to block the binding of *S. aureus* to inflamed skin, leading to a new treatment for common and refractory AD.

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