

Percutaneous Application of Peptidoglycan From *Staphylococcus aureus* Induces Infiltration of CCR4⁺ Cells Into Mouse Skin

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■ Abstract

Background: The lesional skin of patients with atopic dermatitis has an increased number of type 2 helper T (T_H2) cells in the dermis and is superficially colonized by *Staphylococcus aureus*. The purpose of this study was to determine the effects of peptidoglycan (PEG) from *S aureus* on T_H2 cell induction in murine skin.

Methods: Mice were sensitized with house dust mite antigen (MA) by topical application to barrier-disrupted abdominal skin. Seven days after sensitization, PEG was applied to the barrier-disrupted dorsal skin. After a further 3 days, C-C chemokine receptor type 4-positive (CCR4⁺) cells were counted in the PEG-treated skin. The production of chemokine (C-C) motif ligand 17 (CCL17) (thymus- and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine) in the skin was investigated using reverse transcriptase polymerase chain reaction and immunohistological analysis.

Results: Application of PEG to the dorsal skin of MA-sensitized mice led to a significant increase in the number of cells expressing CCR4 in the dermis. The skin of PEG-treated mice showed an increased level of CCL17 mRNA expression, which coincided with T_H2 cytokine mRNA expression. Immunohistological analysis demonstrated that levels of CCL17 transcripts corresponded to those of protein synthesis in the epidermis. CCL17 production was induced mainly by Langerhans cells stimulated with PEG. Furthermore, intraperitoneal injection of anti-CCL17 antibody abrogated the induction of CCR4⁺ cells in the skin.

Conclusion: These results suggest that PEG may induce T_H2 cells in the skin through the production of CCL17 by Langerhans cells and would explain the role of colonization by *S aureus* in patients with atopic dermatitis.

Key words: Atopic dermatitis. *Staphylococcus aureus*. Peptidoglycan. CCL17. CCR4.

■ Resumen

Antecedentes: La piel lesionada de pacientes con dermatitis atópica presenta un mayor número de linfocitos T cooperadores de tipo 2 (T_H2) en la dermis y está colonizada superficialmente por *Staphylococcus aureus*. El objetivo de este estudio fue determinar los efectos del peptidoglucano (PEG) de *S. aureus* en la inducción de T_H2 en la piel de ratones.

Métodos: Los ratones fueron sensibilizados con antígeno de ácaro del polvo doméstico mediante aplicación tópica en la piel abdominal con función de barrera alterada. Siete días después de la sensibilización, se aplicó PEG en la piel dorsal con función de barrera alterada. Transcurridos 3 días más, se efectuó un recuento de células positivas para el receptor de quimiocinas CC de tipo 4 (CCR4⁺) en la piel tratada con PEG. Se investigó la producción de ligando 17 de quimiocina (motivo CC) (CCL17) (quimiocina regulada por el timo y por activación) y de CCL22 (quimiocina derivada de macrófagos) en la piel utilizando la reacción en cadena de la polimerasa con transcriptasa inversa y análisis inmunohistológico.

Resultados: La aplicación de PEG en la piel dorsal de ratones sensibilizados con antígeno de ácaro del polvo doméstico dio lugar a un aumento significativo del número de células que expresaron CCR4 en la dermis. La piel de ratones tratados con PEG mostró un mayor nivel de expresión de ARNm de CCL17, lo que coincidió con la expresión de ARNm de citocinas T_H2. El análisis inmunohistológico demostró que los niveles de transcritos de CCL17 se correspondían con los niveles de síntesis de proteína en la epidermis. La producción de CCL17 estuvo inducida principalmente por células de Langerhans estimuladas por PEG. Asimismo, la inyección intraperitoneal de anticuerpos anti-CCL17 evitó la inducción de células CCR4⁺ en la piel.

Conclusión: Estos resultados indican que el PEG puede inducir linfocitos T_H2 en la piel por medio de la producción de CCL17 por parte de células de Langerhans. Asimismo, estos resultados ayudan a explicar el papel colonizador de *S. aureus* en pacientes con dermatitis atópica.

Palabras clave: Dermatitis atópica. *Staphylococcus aureus*. Peptidoglucano. CCL17. CCR4.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with immunopathologic features that vary depending on the duration of the lesion. Lesional skin of AD patients has an increased number of inflammatory cells (eg, type 2 helper T [T_H2] cells, mast cells, and eosinophils) in the dermis and is superficially colonized by *Staphylococcus aureus* [1]. *S aureus* is isolated from 75%-96% of skin lesions in AD patients, whereas the skin of only 5%-10% of healthy individuals is colonized by this organism [2,3]. We previously found that the rate of detection of *S aureus* on lesional skin in AD patients is higher than on nonlesional skin [3]. Furthermore, the bacterial cell count of *S aureus* on lesional skin is also significantly higher than on nonlesional skin. However, there appears to be no significant difference between lesional and nonlesional skin in the frequency of *S aureus* producing superantigenic exotoxin.

Gram-positive bacterial cell walls are composed of highly cross-linked peptidoglycan (PEG) decorated to different degrees with teichoic acid polymers, which are anchored to plasma membrane phospholipids, forming another major cell wall component, lipoteichoic acid (LTA) [4,5]. Gram-positive bacteria contain no lipopolysaccharide (LPS), and LTA and/or PEG are thought to be the major inflammatory products in their cell walls. Therefore, we hypothesized that LTA and/or PEG from *S aureus* might be more important as pathogenic factors than superantigenic exotoxins in AD patients [6,7]. In previous studies, we found that LTA and PEG were able to induce interleukin (IL) 5 production by peripheral blood mononuclear cells (PBMCs) from patients with AD [6] and that intradermal injection of LTA induced localized AD-like inflammation associated with significant increases in counts of eosinophils, mast cells, and mononuclear cells in the dermis of allergen-sensitized mice [7]. Furthermore, we demonstrated that percutaneous injection of another component of the gram-positive bacterial cell wall, PEG, induced infiltration of mast cells and eosinophils into the dermis [8,9]. However, it is unclear whether PEG has the ability to induce other inflammatory cells, such as T_H2 cells, in the dermis of patients with AD. In the present study, using percutaneous injection as a physiologically plausible antigen-invasion route, we investigated the effects of PEG on the infiltration of C-C chemokine receptor (CCR) 4–positive cells (CCR4⁺), as representatives of T_H2 cells, in mouse skin.

Materials and Methods

Mite Antigen and Peptidoglycan

Mite antigen (MA) was prepared from a commercially available house dust mite body extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan), which is a 50% glycerol saline extract of *Dermatophagoides farinae*. The extract was dialyzed against phosphate-buffered saline (PBS), pH 7.4, and concentrated to 1 mg/mL using a Centricon 10 (Millipore, Billerica, Massachusetts, USA) to produce the MA preparation. PEG derived from *S aureus* was obtained from Fluka (Buchs SG,

Switzerland). The PEG was reconstituted in PBS, pH 7.4, at a concentration of 1 mg/mL and sonicated for 1 hour before use.

Mice

Female specific pathogen-free BALB/c mice were obtained from (Japan SLC, Hamamatsu, Japan) and used when they were 6 to 8 weeks of age. They were housed in plastic cages in a clean, air-conditioned room at 24°C and allowed free access to a standard laboratory diet and water. All procedures were performed in accordance with the guidelines of the animal care and use committee of Meiji Pharmaceutical University, Tokyo.

Percutaneous Sensitization With Mite Antigen and Application of Peptidoglycan

Sensitization with MA was performed as described previously [7]. Briefly, mice were barrier-disrupted by repeated applications (8 times) of adhesive cellophane tape to shaved abdominal skin. Then, 100 µL of MA solution (1 mg/mL in PBS) was applied topically onto a 10-cm² area of the barrier-disrupted abdominal skin. PEG solution (10 µL at 1 mg/mL in PBS) was also administered percutaneously on a 1-cm² area of barrier-disrupted dorsal skin.

Purification of Epidermal Cells

Langerhans cells and keratinocytes in the epidermis were separated as described by Tada et al [10]. Briefly, mouse skin was treated with dispase (3000 U/mL, Godo Shusei, Tokyo, Japan) in RPMI 1640 medium with L-glutamine (Sigma, St. Louis, Missouri, USA) containing 10% fetal bovine serum (Sigma), 25 mM Hepes (Sigma), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco RBL, Grand Island, New York, USA) (RPMI 10) for 3 hours at 37°C. The epidermis was separated from the dermis and incubated in RPMI 10 containing 0.025% deoxyribonuclease I (Sigma) for 20 minutes at room temperature. An epidermal cell suspension was obtained by vigorous pipetting of epidermal sheets. The epidermal cell suspension was then treated with mouse anti-mouse I-A^d monoclonal antibody (clone 040-220, immunoglobulin [Ig] G2a) (1:600; Meiji Dairies Co., Tokyo, Japan) in RPMI 10 for 45 minutes on ice. The cells were then incubated in plates precoated with goat antimouse IgG polyclonal antibody (1:100, Sigma) for 45 minutes at 4°C. Floating cells were collected as keratinocytes, and adherent cells were used as Langerhans cells.

Reverse-Transcription Polymerase Chain Reaction

In order to determine the levels of expression of cytokine mRNAs in the skin, total RNA was extracted from the dorsal skin of mice by the single-step method using TRI-Reagent (Molecular Research Center, Cincinnati, Ohio, USA). In another experiment to compare levels of mRNA expression for toll-like receptor (TLR) in the epidermal cells, mRNA was extracted from Langerhans cells and keratinocytes using a Quick Prep Micro mRNA Purification Kit (GE Healthcare, Buckinghamshire, UK). Then, cDNA was synthesized from 8 µg of total RNA or 160

ng of mRNA using a First-strand cDNA Synthesis Kit (GE Healthcare). The polymerase chain reaction (PCR) was performed using the following primers: β -actin (540 bp) 5' primer, 5'-GTGGGCCGCTCTAGGCACCAA-3', and 3' primer, 5'-CTCTTTGATGTCACGCACGATTTC-3'; chemokine (C-C) motif ligand 17 (CCL17) (thymus- and activation-regulated chemokine) (301 bp) 5' primer, 5'-CAGGAAGTTGGTGAGCTGGTATA-3', and 3' primer, 5'-TTGTGTTTCGCCTGTAGTGCATA-3'; CCL22 (macrophage-derived chemokine) (206 bp) 5' primer, 5'-TCTGATGCAGGTCCTATGGT-3', and 3' primer, 5'-TTATGGAGTAGCTTCTTAC-3'; IL-4 (400 bp) 5' primer, 5'-AGTTGTCATCCTGCTCTTCTTTCTC-3', and 3' primer, 5'-CGAGTAATCCATTTGCATGATGCTC-3'; IL-5 (424 bp) 5' primer, 5'-ATGAGAAGGATGCTTCTGCACTTGA-3', and 3' primer, 5'-GTCACCATGGAGCAGCTCAGCC-3'; TLR2 (380 bp) 5' primer, 5'-TGGAGACGCCAGCTCTGGCTCA-3', and 3' primer, 5'-CAGCTTAAAGGGCGGGTCAGAG-3'; TLR6 (528 bp) 5' primer, 5'-AGTGCTGCCAAGTTCCGACA-3', and 3' primer, 5'-AGCAAACACCGAGTATAGCG-3'. Each PCR was performed using a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, Connecticut, USA) with 25 μ L of reaction mixture comprising 1.5 μ L of cDNA (corresponding to 800 ng total RNA or 16 ng mRNA starting material), 200 μ M of deoxynucleotide triphosphate mixture, 400 nM of each PCR primer, and 25 U/mL of Ex Taq DNA polymerase (Takara, Shiga, Japan). The reaction conditions were one 4-minute cycle at 94°C, 35 cycles of 45 seconds at 94°C, 45 seconds at 61°C, and 2 minutes at 72°C, followed by one 7-minute cycle at 72°C. The PCR products were separated on 2% agarose gel containing ethidium bromide.

Immunohistological Analysis

Immunohistochemical staining for CCR4 and CCL17 was performed on sections of the PEG-treated dorsal skin. The skin specimens were embedded and frozen in OCT compound (Miles, Elkhart, Indiana, USA) at -80°C. Frozen sections cut at 6 μ m were fixed in cold acetone (-20°C) for 10 minutes, treated with blocking buffer (10% normal goat serum in PBS), and incubated with goat anti-mouse CCR4 polyclonal antibody (Abcam, Cambridge, Massachusetts, USA) or goat antimouse CCL17 polyclonal antibody (R & D Systems, Minneapolis, Minnesota, USA) at a concentration of 20 μ g/mL, followed by biotinylated rabbit anti-goat immunoglobulin polyclonal antibody (1:800; Dako, Carpinteria, California, USA). After washing with PBS, the skin sections were incubated with peroxidase-conjugated streptavidin (Dako). Endogenous peroxidase activity was blocked by incubation for 30 minutes with 0.3% hydrogen peroxide in PBS. Endogenous biotin was blocked by sequential incubations with avidin (Vector Laboratories, Burlingame, California, USA) and biotin (Sigma). The tissue sections were stained by incubation in a solution of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and treated with methyl green solution for visualization of nuclei. The number of CCR4⁺ cells was counted in 16 high power fields (\times 400) and expressed as cells/mm²; mean (SD) was calculated based on 6 independent experiments.

Quantification of CCL17 Production by Epidermal Cells

Keratinocytes and Langerhans cells were adjusted to 5×10^5 cells/mL in RPMI 10. The cultures (0.2 mL/well) were incubated in 96-well culture plates (Nunc, Roskilde, Denmark) in the presence of 10 μ g/mL of PEG at 37°C in a humidified atmosphere with 5% CO₂. In some experiments, the Langerhans cell cultures were incubated with 10 μ g/mL of rabbit antimouse IL-4 polyclonal antibody or rabbit antimouse tumor necrosis factor (TNF) α polyclonal antibody (Pepro Tech, London, UK) for 48 hours in the presence of 10 μ g/mL PEG. The culture supernatants were collected after incubation for 24-72 hours, and CCL17 concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits for quantification of murine CCL17 (R & D Systems).

Abrogation of CCR4⁺ Cell Infiltration With Anti-CCL17 Antibody

Sensitization with MA and subsequent percutaneous application of PEG to dorsal skin were performed as described above. After percutaneous application of PEG, 100 μ L of goat antimouse CCL17 polyclonal antibody (IgG) (1 mg/mL in PBS; R & D Systems) was injected intraperitoneally at 3, 24, and 48 hours. Goat IgG antibody (Sigma) was also used as a control antibody. Three days later, the PEG-treated skin was cut out, fixed, and stained in order to count the CCR4⁺ cells as described above.

Statistics

All experiments were performed using 4 or 6 mice. The data were expressed as means (SD) of 6 independent experiments, and differences between means were analyzed using a 2-tailed *t* test. Statistical significance was set at a *P* value of <.05.

Results

Effects of Peptidoglycan on Skin Infiltration by CCR4⁺ Cells

The first experiments were carried out to examine the induction of CCR4⁺ cell infiltration into mouse skin after percutaneous application of PEG. Mice were sensitized percutaneously with MA to obtain a T_H2-dominant cytokine response similar to that occurring in AD patients. Seven days later, the MA-sensitized mice were treated with 10 μ g of PEG applied to a 1-cm² area of barrier-disrupted dorsal skin; after a further 3 days the PEG-treated dorsal skin was cut out and processed for detection of CCR4⁺ cells. As shown in Figure 1A, CCR4⁺ cells were detected in the dermis of PEG-treated mice, and their number was significantly higher than in untreated mice and PBS-treated mice (Figure 1B). In the PEG-treated mice, all CCR4⁺ cells were observed in the dermis, and more than half of those were observed in the upper layer. These results indicated that percutaneous application of PEG is able to induce CCR4⁺ cell infiltration in the dermis of mice.

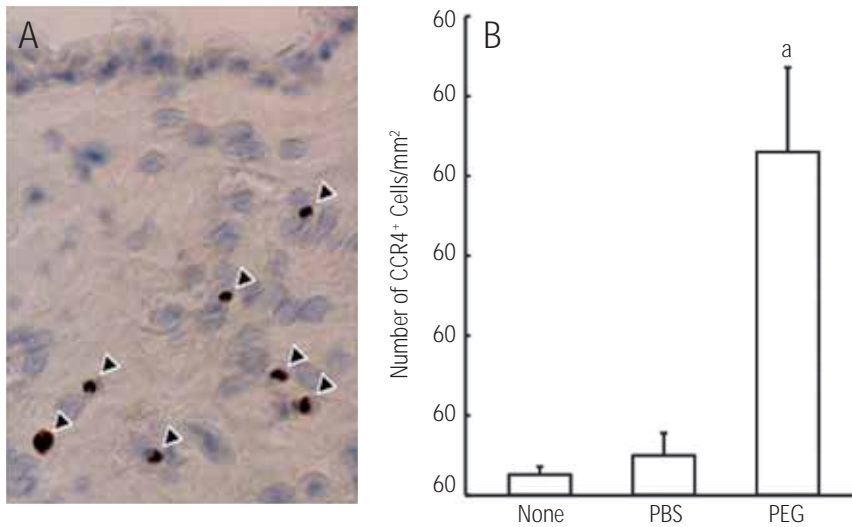


Figure 1. Quantification of CCR4⁺ cells in the dermis of mice treated with peptidoglycan. A, CCR4⁺ cells in the dermis (original magnification, ×400). B, CCR4⁺ cell counts. The results are expressed as means (SD) (n=6) of the number of CCR4⁺ cells per 1 mm² of skin section. Percutaneous application of PEG is able to induce CCR4⁺ cell infiltration in the dermis of mice. CCR4⁺ indicates C-C chemokine receptor type 4-positive; PBS, phosphate-buffered saline; PEG, peptidoglycan. ^aP<.001 vs PBS treatment.

Production of CCL17 in the Skin of Mice Treated With Peptidoglycan

Subsequently, in order to determine whether CCL17 and CCL22, which support the migration of CCR4⁺ cells, are produced in the skin of PEG-treated mice, expression of the mRNA for each chemokine in dorsal skin 3 days after administration of PEG was investigated using reverse transcriptase polymerase chain reaction (RT-PCR). Figure 2A shows that the skin from PEG-treated mice expressed CCL17 mRNA, but not CCL22 mRNA. Expression of mRNAs for T_H2 cytokines such as IL-4 and IL-5 was also observed (Figure 2B). We then carried out immunohistological analysis of dorsal skin biopsy samples 3 days after PEG application to clarify whether the expression of mRNA for CCL17 correlated with the synthesis of cytokine protein. Figure 3 shows that the skin of PEG-treated mice contained a large number of cells that were positive for CCL17 protein in the epidermis, whereas this was not the case in PBS-treated

mice. Production of CCL17 was restricted mainly to the epidermis; production of CCL17 in the dermis was scant.

Production of CCL17 by Epidermal Cells After Stimulation With Peptidoglycan

Since CCL17-positive staining was observed throughout the PEG-treated epidermis, it was thought that the source cells for CCL17 would be keratinocytes, Langerhans cells, or both. Therefore, keratinocytes and Langerhans cells were separated from the epidermal cells of a normal mouse and stimulated in vitro with PEG. As shown in Figure 4, although Langerhans cells produced CCL17 spontaneously, they responded to stimulation with PEG at 48-72 hours and showed significant increases in CCL17 production relative to untreated Langerhans cells. On the other hand, the levels of CCL17 produced by keratinocytes were lower than those produced by Langerhans cells, even at 72 hours after in vitro stimulation with PEG.

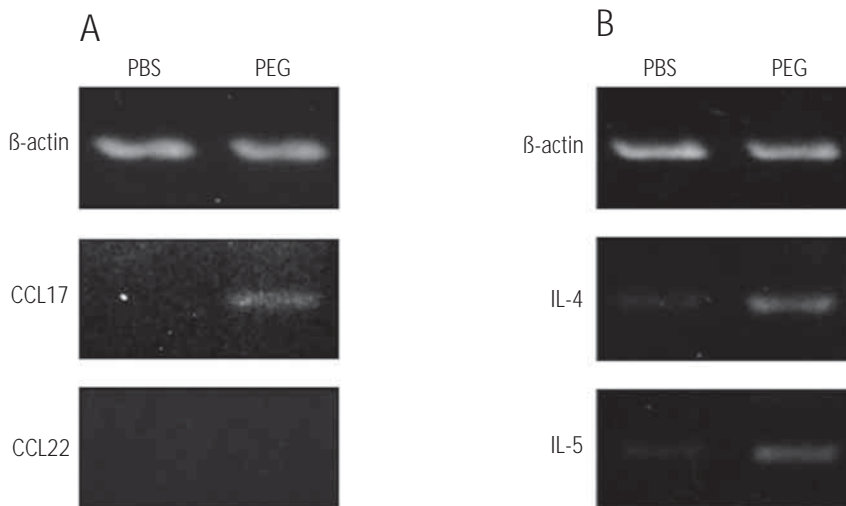


Figure 2. Effects of peptidoglycan on expression of cytokine mRNAs in the skin. A, T_H2 chemokines. B, T_H2 cytokines. The skin from PEG-treated mice expressed CCL17 mRNA, but not CCL22 mRNA. Expression of mRNAs for T_H2 cytokines such as IL-4 and IL-5 was also observed. CCL indicates chemokine (C-C) motif ligand; IL, interleukin; PBS, phosphate-buffered saline; PEG, peptidoglycan.

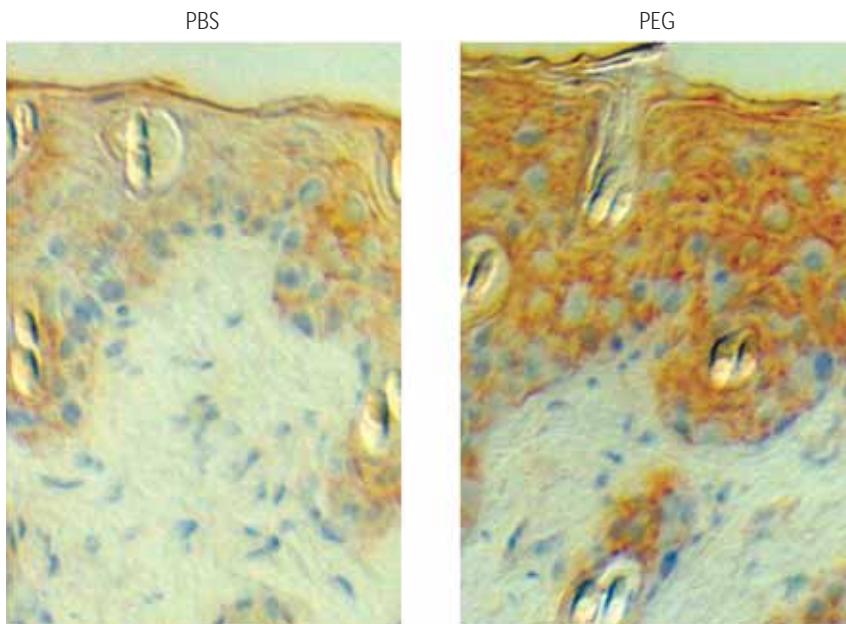


Figure 3. Immunohistochemical staining of the skin after percutaneous application of peptidoglycan (original magnification, $\times 400$). The skin of PEG-treated mice contained a large number of cells positive for CCL17 protein in the epidermis, whereas this was not the case in PBS-treated mice. PBS indicates phosphate-buffered saline; PEG, peptidoglycan.

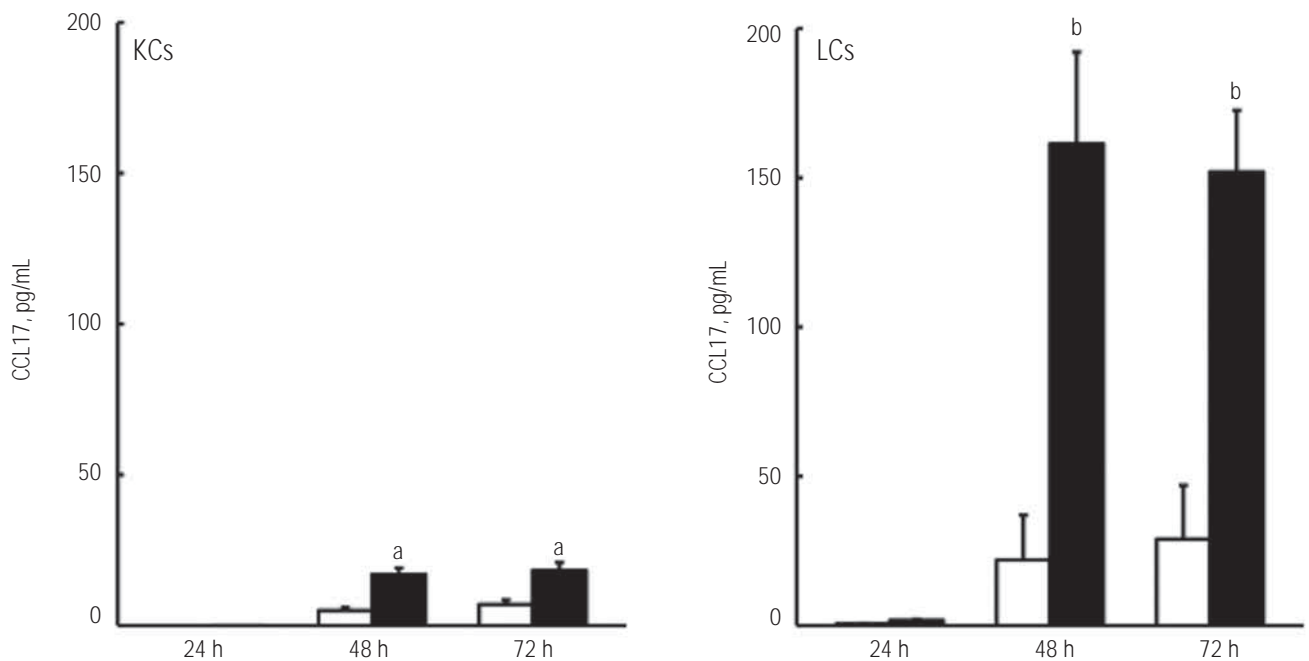


Figure 4. Effects of peptidoglycan on production of CCL17 by epidermal cells. Shaded bars represent cells treated with peptidoglycan for 24-72 hours; white bars indicate cells not treated with peptidoglycan. LCs treated with peptidoglycan induced significant increases in CCL17 production. However, the levels of CCL17 produced by KCs were lower than those produced by LCs. CCL indicates chemokine (C-C) motif ligand; KC, keratinocyte; LC, Langerhans cell.

^a $P < .05$.

^b $P < .01$ vs nontreatment.

TLR2 has been shown to recognize PEG from *S aureus* in combination with TLR6 [11-13]. Therefore, using RT-PCR analysis, we investigated whether the difference in the levels of CCL17 production between keratinocytes and Langerhans cells was related to levels of TLR2 and TLR6 expression. mRNAs for TLR2 and TLR6

were expressed spontaneously in both keratinocytes and Langerhans cells (Figure 5). Langerhans cells stimulated with PEG showed increased expression of mRNAs for both TLR2 and TLR6, peaking at 24 hours and declining thereafter. However, PEG-stimulated keratinocytes showed no increase in mRNA expression.

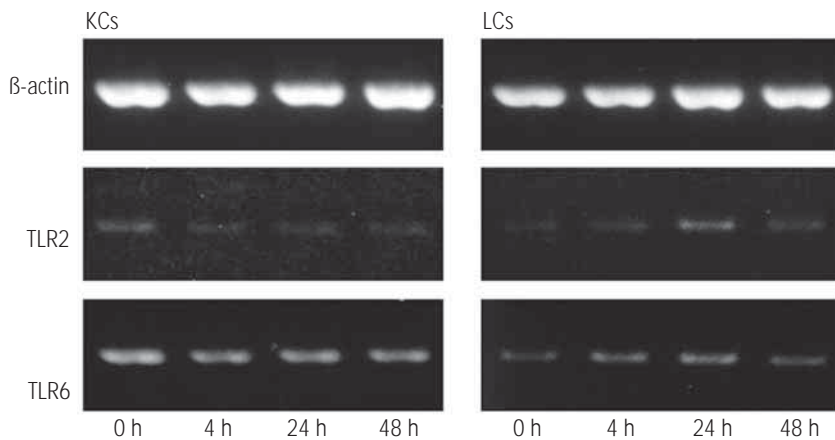


Figure 5. Effects of peptidoglycan on expression of TLR2 and TLR6 mRNAs in KCs and LCs. LCs stimulated with peptidoglycan showed increased expression of mRNAs for both TLR2 and TLR6, peaking at 24 hours. However, peptidoglycan-stimulated KCs showed no increase in mRNA expression. KC indicates keratinocyte; LC, Langerhans cell; TLR, toll-like receptor.

Next, we examined whether PEG-induced CCL17 production by Langerhans cells was inhibited by anti-IL-4 antibody and/or anti-TNF- α antibody. Anti-TNF- α antibody, but not anti-IL-4 antibody, significantly inhibited the PEG-induced production of CCL17 by Langerhans cells (Figure 6).

Abrogation of PEG-Induced CCR4⁺ Cell Infiltration by Anti-CCL17 Antibody

The appearance of CCR4⁺ cells after application of PEG to the skin and subsequent treatment with anti-CCL17 antibody was investigated to assess the role of CCL17 in the infiltration

of CCR4⁺ cells into the dermis. Anti-CCL17 antibody or control IgG antibody was injected intraperitoneally 3, 24, and 48 hours after application of PEG. At 3 days after application, the skin was removed and processed for detection of CCR4⁺ cells by immunohistochemical staining. As shown in Figure 7, significant inhibition of PEG-induced CCR4⁺ cell infiltration was seen in the skin of mice treated with anti-CCL17 antibody, but not in mice treated with the control IgG antibody. These results indicated that CCR4⁺ cells migrate into the dermis upon stimulation with CCL17 produced by epidermal Langerhans cells.

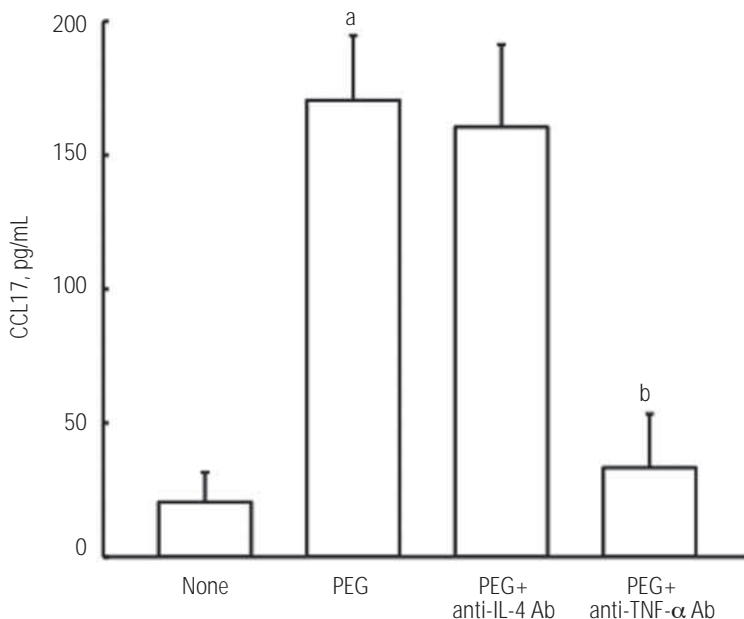


Figure 6. Abrogation of peptidoglycan-induced CCL17 production by Langerhans cells. Anti-TNF- α antibody, but not anti-IL-4 antibody, significantly inhibited the PEG-induced production of CCL17 by LCs. CCL indicates chemokine (C-C) motif ligand; IL, interleukin; PEG, peptidoglycan; TNF, tumor necrosis factor.

^a $P < .01$ vs no treatment.

^b $P < .05$ vs treatment with PEG.

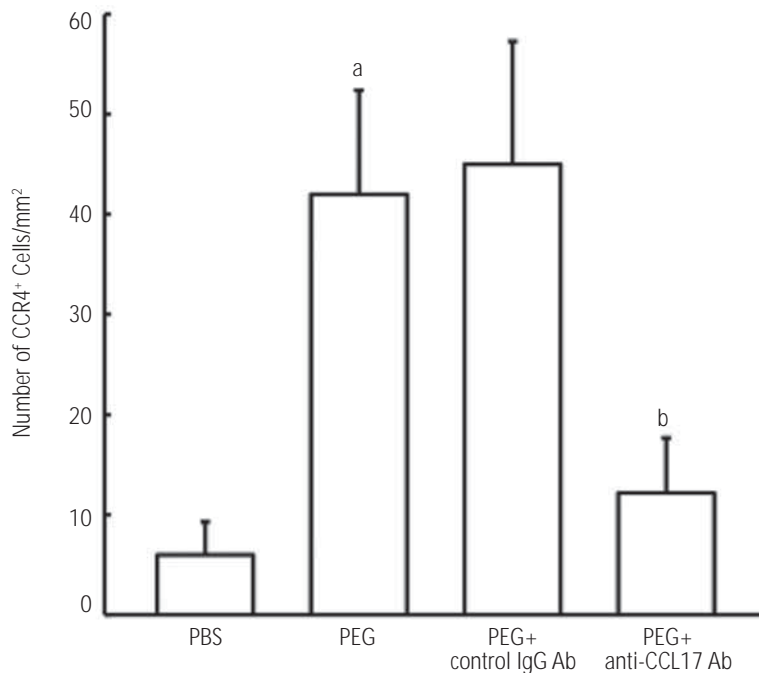


Figure 7. Abrogation of CCR4⁺ cell migration in peptidoglycan-treated mouse skin. Significant inhibition of PEG-induced CCR4⁺ cell infiltration was seen in the skin of mice treated with anti-CCL17 antibody, but not in that of mice treated with the control IgG antibody. CCL indicates chemokine (C-C) motif ligand; CCR, C-C chemokine receptor; Ig, immunoglobulin; PBS, phosphate-buffered saline; PEG, peptidoglycan. ^a $P < .005$ vs PBS treatment. ^b $P < .01$ vs PEG treatment.

Discussion

A high incidence of chronic skin colonization with *S aureus* is a prominent feature of AD. Although many attempts have been made to characterize the role of *S aureus* in the skin of AD patients, most studies have focused on the role of staphylococcal exotoxins [14]. However, half of the *S aureus* strains isolated from AD patients are not capable of producing superantigens [3,15,16], and, therefore, the roles of these strains in the skin lesions of AD patients are not fully understood. In a previous study, we demonstrated that intradermal injection of a cell wall component of *S aureus*, LTA, was able to induce localized AD-like inflammation, which was characterized by infiltration of eosinophils, mast cells, and mononuclear cells into the dermis [7]. Therefore, *S aureus* strains that do not produce toxins acting as superantigens would also be capable of causing inflammation in AD lesions.

Our aim in the present study was to determine the effects of PEG from *S aureus* on the migration of CCR4⁺ cells into murine skin. PEG was applied percutaneously, a physiologically plausible route for antigen invasion. CCR4 is selectively expressed on T_H2 cells, and chemokines such as CCL17 and CCL22 have been thought to elicit migration of T_H2 cells to sites of inflammation [17-19]. In fact, expression of these chemokines and the number of CCR4⁺ cells are increased not only in the serum but also in the lesional skin of AD patients, suggesting that at least these 2 chemokines may be associated with the infiltration of CCR4⁺ T_H2 cells into lesional skin [20-22]. T_H2 cell counts are increased in the acute skin lesions of AD patients and release T_H2 cytokines, such as IL-4 and IL-5 [1]. However, the cytokine profile in the skin changes during the course of the disease towards a T_H1-T_H2 mixed cytokine profile. Therefore, it is generally believed that infiltration of

T_H2 cells into the skin plays an important role, at least in the early phases of AD [23].

Our data showed that application of PEG to the skin of MA-sensitized mice induced a significant increase in the number of CCR4⁺ cells in the dermis relative to that observed in mice treated with PBS. In addition, application of PEG to the skin induced expression of mRNA for CCL17, but not for CCL22. Furthermore, expression of mRNA for CCL17 coincided with that of mRNAs for IL-4 and IL-5 in the skin. Immunohistological analysis demonstrated that the levels of CCL17 transcripts corresponded to those of synthesized protein. CCL17 was produced mostly in the epidermis, showing that epidermal cells such as keratinocytes and/or Langerhans cells did, in fact, produce CCL17. Uchida et al [20] reported that keratinocytes in the basal layer of the skin lesions of AD patients produce CCL17. However, our in vitro experiments using keratinocytes and Langerhans cells suggested that PEG would stimulate mainly Langerhans cells and increase production of CCL17. Since PEG is a well-known TLR2 agonist [24], signaling through TLR2 on Langerhans cells would be associated with the production of CCL17, but not with that of CCL22. These results suggested that production of CCL17 and CCL22 would be regulated by the different signal transduction pathway in Langerhans cells. The results of RT-PCR using a TLR2 primer set predicted the presence of TLR2 in Langerhans cells and suggested that an increase in CCL17 production by means of stimulation with PEG might be associated with an increase in the level of TLR2 expression on Langerhans cells. The inhibitory effect of anti-TNF- α antibody on CCL17 production suggested that the latter was associated with PEG-induced TNF- α production and that TNF- α acted on Langerhans cells through an autocrine mechanism. Since normal human keratinocytes do not produce CCL17 in vitro

[25], production of CCL17 by keratinocytes in the epidermis of AD patients may be initiated through inflammatory cytokines such as TNF- α secreted from Langerhans cells. Xiao et al [26] demonstrated that production of CCL17 by murine Langerhans cells is upregulated by IL-4 and TNF- α and suggested that the T_H2 cytokine microenvironment in skin may increase production of CCL17 by mature Langerhans cells, thus attracting T_H2 cells to the skin. Accordingly, it appears that Langerhans cells play a critical role in the development of AD-like inflammation characterized by T_H2-prone dermatitis [27]. Since CCR4⁺ cells were mostly observed in the upper dermis of PEG-treated skin, CCL17 produced predominantly in the epidermis would play an important role in inducing the chemotaxis of CCR4⁺ T_H2 cells into the upper dermis and the creation of a T_H2 cytokine microenvironment in the skin. Finally, IL-4 derived from T_H2 cells would induce further CCL17 production. Our previous study also demonstrated that repeated application of PEG to the skin induces mast cells in the dermis [8]. Mast cells are the most important source of TNF- α [28], and the TNF- α they secrete might be capable of upregulating production of CCL17 by Langerhans cells.

Previous reports have not explained the role of *S aureus* in the recruitment of T_H2 cells into the lesional skin of AD patients. Our results suggest that colonization by *S aureus* may play a critical role in perpetuating inflammation of skin tissue by stimulating infiltration of T_H2 cells through a bacterial cell wall component such as PEG. As the skin of most AD patients shows superficial *S aureus* colonization and barrier disruption due to reduced ceramide levels [29], PEG would be expected to penetrate the skin. Therefore, antimicrobial treatment in at least a subgroup of AD patients, irrespective of whether they show clinical signs of superinfection, may be considered a new therapeutic strategy for AD.

References

- Leung DYM. Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic diseases. *J Allergy Clin Immunol*. 1995;96:302-19.
- Gloor M, Peters G, Stoika D. On the resident aerobic bacterial skin flora in unaffected skin of patients with atopic dermatitis and in normal controls. *Dermatologia*. 1992;164:258-65.
- Matsui K, Nishikawa A, Suto H, Tsuboi R, Ogawa H. Comparative study of *Staphylococcus aureus* isolated from lesional and non-lesional skin of atopic dermatitis patients. *Microbiol Immunol*. 2000;44:945-7.
- Lambert PA, Hancock IC, Baddiley J. Occurrence and function of membrane teichoic acids. *Biochim Biophys Acta*. 1977;472:1-12.
- Fischer W. Bacterial phosphoglycolipids and lipoteichoic acids. In: Hannah DJ, editor. *Handbook of lipid research*. New York: Plenum; 1989. p. 123-234.
- Matsui K, Motohashi R, Nishikawa A. Cell wall components of *Staphylococcus aureus* induce interleukin-5 production in patients with atopic dermatitis. *J Interferon Cytokine Res*. 2000;20:321-4.
- Matsui K, Nishikawa A. Lipoteichoic acid from *Staphylococcus aureus* induces Th2-prone dermatitis in mice sensitized percutaneously with an allergen. *Clin Exp Allergy*. 2002;32:783-8.
- Matsui K, Nishikawa A. Percutaneous application of peptidoglycan from *Staphylococcus aureus* induces an increase in mast cell numbers in the dermis of mice. *Clin Exp Allergy*. 2005;35:382-7.
- Matsui K, Wirotasangthong M, Nishikawa A. Percutaneous application of peptidoglycan from *Staphylococcus aureus* induces eosinophil infiltration in mouse skin. *Clin Exp Allergy*. 2007;37:615-22.
- Tada Y, Asahina A, Nakamura K, Tomura M, Fujiwara H, Tamaki K. Granulocyte/macrophage colony-stimulating factor inhibits IL-12 production of mouse Langerhans cells. *J Immunol*. 2000;164:5113-9.
- Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem*. 1999;274:17406-9.
- Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of gram-positive bacterial cell wall components by the innate immune system occurs via toll-like receptor 2. *J Immunol*. 1999;163:1-5.
- Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci USA*. 2000;97:13766-71.
- Taskapan MO, Kumar P. Role of staphylococcal superantigens in atopic dermatitis: from colonization to inflammation. *Ann Allergy Asthma Immunol*. 2000;84:3-12.
- Jappe U, Heuck D, Witte W, Gollnick H. Superantigen production by *Staphylococcus aureus* in atopic dermatitis: No more than a coincidence? *J Invest Dermatol*. 1998;110:844-6.
- Zollner TM, Wichelhaus TA, Hartung A, Von Mallinckrodt C, Wagner TOF, Brade V, Kaufmann R. Colonization with superantigen-producing *Staphylococcus aureus* is associated with increased severity of atopic dermatitis. *Clin Exp Allergy*. 2000;30:994-1000.
- Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. *J Biol Chem*. 1997;272:15036-42.
- Imai T, Chantry D, Raport C, Wood C, Nishimura M, Godiska R, Yosie O, Gray P. Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4. *J Biol Chem*. 1998;273:1764-8.
- Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol*. 1999;11:81-8.
- Uchida T, Suto H, Ra C, Ogawa H, Kobata T, Okumura K. Preferential expression of Th2-type chemokine and its receptor in atopic dermatitis. *Int Immunol*. 2002;14:1431-8.
- Horikawa T, Nakayama T, Hikita I, Yamada H, Fujisawa R, Bito T, Harada S, Fukunaga A, Chantry D, Gray PW, Morita A, Suzuki R, Tezuka T, Ichihashi M, Yoshie O. IFN- γ -inducible expression of thymus and activation-regulated chemokine/CCL17 and macrophage-derived chemokine/CCL22 in epidermal keratinocytes and their roles in atopic dermatitis. *Int Immunol*. 2002;14:767-73.

22. Nakazato J, Kishida M, Kuroiwa R, Fujiwara J, Shimoda M, Shinomiya N. Serum levels of Th2 chemokines, CCL17, CCL22, and CCL27, were the important markers of severity in infantile atopic dermatitis. *Pediatr Allergy Immunol*. 2008;19:605-13.
23. Leung DYM, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. *J Clin Invest*. 2004;113:651-7.
24. Re F, Strominger JL. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *J Biol Chem*. 2001;276:37692-9.
25. Tsuda T, Tohyama M, Yamasaki K, Shirakata Y, Yahata Y, Tokumaru S, Sayama K, Hashimoto K. Lack of evidence for TARC/CCL17 production by normal human keratinocytes in vitro. *J Dermatol Sci*. 2003;31:37-42.
26. Xiao T, Fujita H, Saeki H, Mitsui H, Sugaya M, Tada Y, Kakinuma T, Torii H, Nakamura K, Asahina A, Tamaki K. Thymus and activation-regulated chemokine (TARC/CCL17) produced by mouse epidermal Langerhans cells is upregulated by TNF- α and IL-4 and downregulated by IFN- γ . *Cytokine*. 2003;23:126-32.
27. Elentner A, Finke D, Schmuth M, Chappaz S, Ebner S, Malissen B, Kissenpfennig A, Romani N, Dubrac S. Langerhans cells are critical in the development of atopic dermatitis-like inflammation and symptoms in mice. *J Cell Mol Med*. 2009;13:2658-72.
28. Chen H, Centola M, Altschul S, Metzger H. Characterization of gene expression in resting and activated mast cells. *J Exp Med*. 1998;188:1657-68.
29. Imokawa G, Abe A, Jin K, Higaki Y, Kawashima M, Hidano A. Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J Invest Dermatol*. 1991;96:523-6.

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