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Effects of topical application of a recombinant staphylococcal enterotoxin A on DNCB and dust mite extract-induced atopic dermatitis-like lesions in a murine model

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease with biphasic T cell-mediated abnormalities. Staphylococcal superantigens contribute to the exacerbation of inflammation in AD. The underlying immunopathological mechanisms are not fully understood. **Objective:** To determine whether epicutaneous application of recombinant staphylococcal enterotoxin A (rSEA) would exacerbate AD-like allergic inflammation induced by 2, 4-dinitrochlorobenzene (DNCB) and house dust mite extract (*Dermatophagoides farinae* extract, DFE) in a murine model. **Materials and Methods:** We first established an AD-like model using BALB/c mice exposed to DNCB/DFE on the ear. Next, *Staphylococcus (S.) aureus* or rSEA were topically applied to the mice. We evaluated the clinical and histopathological features of the animals. Serum immunoglobulin levels were also measured. In addition, real-time PCR analysis of cytokines produced by T cell subsets in the ears was conducted. **Results:** Mice treated with *S. aureus* and rSEA had more severe clinical symptoms, including increased mean dermatitis scores and ear thickness, compared to animals with only AD-like lesions. Total IgE, IgG2a and serum histamine levels were increased in all groups except the normal control group. The *S. aureus*- and rSEA-treated groups showed increased levels of cytokines such as IL-4, IL-13, INF- γ , IL-17, and IL-18. In particular, increased cytokine expression was more conspicuous in the rSEA-treated group than in mice exposed to *S. aureus*. **Conclusion:** The results of this study showed that topical exposure to rSEA as well as SEA-producing *S. aureus* aggravate atopic skin inflammation. This may be associated with the induction of a mixed Th1/Th2 type dermatitis.

Key words: Atopic dermatitis, Murine model, *Staphylococcus aureus*, Staphylococcal enterotoxin A

Article accepted on 11/29/2013

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with biphasic T cell-mediated abnormalities. T helper type (Th) 2-predominant dermatitis is observed during the acute phase and a mixed Th1/Th2 type dermatitis develops in the chronic phase [1, 2]. Patients with AD are very susceptible to bacterial, fungal and viral infections [3]. Bacterial colonization with *Staphylococcus (S.) aureus* is known to be the most common cause of skin infection in AD patients [4-6]. Furthermore, there appears to be a causative relationship between the numbers of bacteria present on the skin and disease severity in cases of AD [7, 8]. Exacerbation of the inflammatory immune response by *S. aureus* is largely mediated by the release of staphylococcal enterotoxins (SEs) such as SEA, SEB, and toxic shock syndrome toxin (TSST)-1; these compounds are also referred to as staphylococcal superantigens (SsAgs) [9]. SsAgs contribute to the persistence and exacerbation of inflammation associated with AD by penetrating the skin barrier. However, immunopathological mechanisms underlying the ability of these factors to aggravate AD are not fully understood.

In our previous study, we reported that application of SEA-producing *S. aureus* aggravates atopic skin inflammation in a mouse model [10]. In the present investigation, we determined whether epicutaneous application of recombinant SEA (rSEA) accelerates allergic skin inflammation in an AD-like murine model treated with 2, 4-dinitrochlorobenzene (DNCB) and house dust mite extract (*Dermatophagoides farinae* extract, DFE). We compared the inflammatory reactions induced by *S. aureus* and rSEA.

Materials and methods

Preparation *in vivo*

Mice

Six-week-old female BALB/c mice (n = 25) were obtained from SLC Inc. (Hamamatsu, Japan). The animals were housed 3-5 per cage in a laminar air flow room maintained at 22 $\pm$ 2 °C with 55 $\pm$ 5% relative humidity throughout

doi:10.1684/ejd.2014.2302

the study. Care and treatment of the mice were performed in accordance with the guidelines established by the Public Health Service Policy on the Human Care and Use of Laboratory Animals and the study was approved by the Institutional Animal Care and Use Committee at Kyungpook National University, Daegu, Republic of Korea.

***S. aureus* cultivation and rSEA production**

S. aureus strains containing the *sea* gene previously isolated from an AD patient were used for this study. Bacteria suspensions (10^{10} cfu/mL and 10^{11} cfu/mL) were produced by cultivating the cells in tryptic soy agar culture medium at 37 °C for 18 h. The genome of SEA was purified and PCR amplification using *sea*-specific primers (forward: 5'-CACCGTGATCCATGAAAAAACAGCA-TTTATACTAC-3' and reverse: 5'-CACCGTCTCGAGTTAACTTGTATATAATATATA-TCAA-3') with *Xho* I and *Bam*HI restriction sites was performed. All DNA analyses were performed according to standard procedures. The PCR product was ligated into a predigested pET28a vector. Positive recombinant clones were identified by restriction endonuclease digestion with *Xho* I and *Bam*HI, and were then used to transform *E. coli* DH5 α cells. PCR was performed using the following program: denaturation at 94 °C for 5 min and then 20 or 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec, and extension at 72 °C for 30 sec, followed by a final extension at 72 °C for 10 min and ending by holding at 4 °C. Nucleotide sequencing was performed by COSMO Genetech (Seoul, Republic of Korea). The constructed plasmid was used to transform *E. coli* BL21(DE3) strain using the electro-transformation method.

The pET28a-SEA construct was used to transform *E. coli* BL21(DE3) strain. This construct contained a chromosomally integrated T7 polymerase gene under the control of the lac promoter. Expression of the SEA protein was induced for 4 h by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside to the bacterial cultures (OD 1.0). The cells were then harvested by centrifugation and resuspended in a binding solution (5 mM imidazole, 250 mM NaCl, and 20 mM Tris-HCl, pH 7). The resuspended cells were then sonicated with a Sonifier 450 sonicator (Branson, Danbury, CT, USA). More than 90% of the rSEA was found in the soluble fraction. This fraction was loaded onto a Ni-NTA His-tag affinity column (Qiagen, Hilden, Germany) and the column was washed. The bound proteins were eluted with elution buffer (1 M imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9). Fractions containing the SEA protein were dialyzed against PBS. The purified protein was stored at -70 °C in storage buffer (50% glycerol, 125 mM NaCl, and 20 mM Tris-HCl, pH 7.9).

DFE, DNCB and others

Freeze-dried crude DFE (Greer Laboratories, Lenoir, NC, USA) was used as an antigen. The DFE was dissolved in PBS containing 0.5% Tween 20. 2, 4-DNCB was dissolved in an acetone:olive oil (1:3, v:v) solution. All other reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

Sensitization

A total of 25 BALB/c mice were randomly assigned to the following six groups (figure 1A). Group 1 included normal

mice in which only tape stripping was performed (n = 3). Group 2 consisted of mice with AD-like lesions induced by treatment with DNCB and DFE after tape stripping (n = 4). Group 3 was composed of mice with AD-like lesions treated with 20 μ L of *S. aureus* on each ear (10^8 cfu/10 μ L) (n = 4). Group 4 included mice with AD-like lesions that were treated with 20 μ L of *S. aureus* on each ear (10^9 cfu/10 μ L) (n = 4). Group 5 included mice with AD-like lesions that were treated with 5 μ g rSEA (n = 5). Finally, group 6 was composed of mice with AD-like lesions that were treated with 10 μ g rSEA (n = 5).

AD-like lesions were induced with DNCB and DFE as previously described [11]. The experimental procedure schematic is presented in figure 1B. The surfaces of both ear lobes were stripped three times with surgical tape (Nichiban, Tokyo, Japan). After DNCB sensitization, 20 μ L of 1% DNCB was painted onto each ear lobe and 20 μ L of DFE (10 mg/mL) was applied 4 d later. Treatment with DFE/DNCB was repeated once every other week. The ears were also treated with *S. aureus* or rSEA twice a week after day 7. On days 14 and 28, blood samples were taken from the tails of the mice. On days 28, ear skin was obtained for histopathological analysis and tissue cytokine analysis.

Clinical assessment

Clinical symptoms of the mice were evaluated using the clinical dermatitis scoring system as previously described by Yamamoto *et al.* [2]. The development of 1) erythema/hemorrhage, 2) scarring/dryness, 3) edema, and 4) excoriation/erosion was scored on a scale of 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The sum of each score represented the dermatitis score. We also measured ear thickness 24 h after DNCB or DFE application with a dial thickness gauge (Kori Seiki MFG, Co., Tokyo, Japan).

Histopathological analysis

To analyze the effects of rSEA on epidermal thickening and inflammatory cell infiltration, sections of the mouse ear were stained with hematoxylin and eosin (H&E) and examined under a light microscope. Skin sections were stained with toluidine blue and the number of mast cells in five sites chosen at random was determined (200 $\times$ magnification) [12].

Laboratory analysis enzyme-linked immunosorbent assay (ELISA)

Blood samples (5 mL) were drawn from all mice. The blood samples were allowed to stand for 20 to 30 min before centrifugation at 3000 rpm for 15 min. The serum was removed and stored at -80 °C for subsequent analysis [13]. The levels of serum IgE, IgG2a, and DFE-specific IgE were measured using an ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's protocol. The protocol was performed according to the instructions provided with the ELISA kit.

Histamine assay

Blood samples (5 mL) were hemolyzed by the addition of 4.5 mL of water and 0.5 mL of concentrated perchloric acid. The tube was shaken for 10 min and then an 8 mL

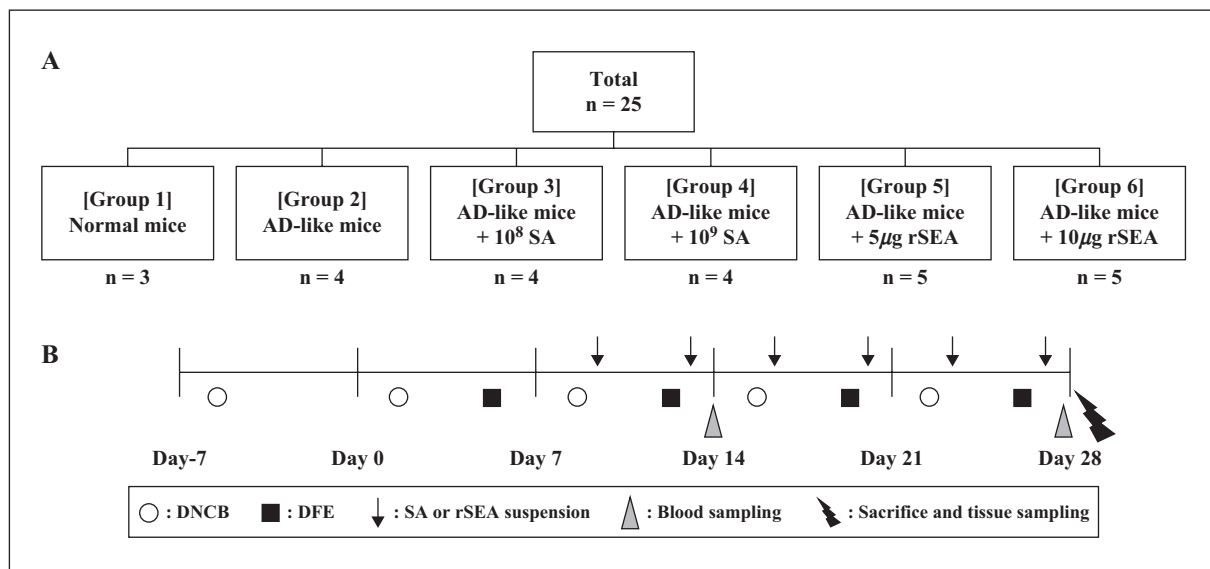


Figure 1. Overview of the animal experiments. **(A)** Grouping of 25 mice. **(B)** Induction of AD-like skin lesion on the ear. DNCB: 2, 4-dinitrochlorobenzene, DFE: *Dermatophagoides farinae* extract, SA: *S. aureus*, rSEA: recombinant staphylococcal enterotoxin A.

aliquot of butanol was transferred to a 40 mL glass-stopper shaking tube containing 2.5 mL of 0.1N HCl and 15 mL of n-heptane. After shaking for about 1 min, the tube was centrifuged at $400 \times g$ for 5 min at 4°C and histamine in the aqueous phase was fluorometrically analyzed [14]. The fluorescent intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 450 nm, using a Perkin-Elmer Fluorescence Spectrometer LS-50B (Perkin-Elmer, Norwalk, CT, USA).

Quantitative real-time PCR (cytokine analysis)

Quantitative real-time PCR was carried out using a Thermal Cycler Dice TP850 (Takarabio Inc., Shiga, Japan), according to the manufacturer's protocol. Total RNA was isolated from the excised ear tissue. Conditions for the PCR assay were similar to ones previously described [15]. Briefly, 2 μL of cDNA (100 ng), 1 μL of a sense and antisense primer solution (0.4 μM), 12.5 μL of SYBR Premix Ex Taq (Takarabio Inc., Shiga, Japan), and 9.5 μL of dH_2O were mixed together in a final volume of 25 μL . The primers used for PCR were specific for interleukin (IL)-4 (F 5'-ACAGGAGAAGGGACGCCAT-3', R 5'-GAAGCCGTACAGACG-AGCTCA-3'), IL-13 (F 5'-GCAACATCAACAGGACCAGA-3', R 5'-GTCAGGGAATCCAG-GGCTAC-3'), IL-31 (F 5'-TCGGTCATCATAGCACATCTGGAG-3', R 5'-GCACAGTCCCT-TTG-GAGTTAAGTC-3'), INF- γ (F 5'-TCAAGTGGC ATAGATGTGGAAGAA-3', R 5'-TGG-CTCTGCAGG ATTTTCATG-3'), TNF- α (F 5'-CCTGTAGCCCCACGTCGTAGC-3', R 5'-TTG ACCTCAGCGCTGAGT TG-3'), IL-17 (F 5'-TCCCTCTGTGATCTGGGAAG-3', R 5'-CTCG-ACCCTGAA AGT GAA GG-3'), and IL-18 (F 5'-GGCCGACTTCACTGT ACA AC-3', R 5'-CCTTCACAGAGACCCTCACAG-3'). PCR was performed at 95°C for 10 s followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. mRNA expression was normalized and

quantified with TP850 software supplied by the manufacturer. β -actin served as an internal standard. Quantification was performed by online monitoring to identify the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point). The cycle numbers of the logarithmic linear phase were plotted against the logarithm of the template DNA concentration. The results were analyzed using TP850 software supplied with the machine. The software calculated each level of cytokine expression relative to that of the β -actin gene (delta CT) and then relative to that of the controls (delta delta CT), using the fluorescence threshold of the amplification reaction and the comparative CT method. Each quantitative PCR experiment was performed at least in duplicate [16].

Statistical analysis

Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were evaluated with an analysis of variance (ANOVA) followed by Dunnett's multiple range tests. P -values < 0.05 were considered statistically significant.

Results

Clinical assessment of the mice

Unlike in mice in group 1, eczematous skin lesions with hemorrhage, edema, excoriation or scaling were observed in groups 2-6 at the end of the experiment. On the 28th day, the mean dermatitis score of group 1 (0.7 ± 0.3) was the lowest compared to the scores for group 2 (6.3 ± 0.4), group 3 (6.8 ± 0.5), group 4 (8.0 ± 0.7), group 5 (6.2 ± 0.7), and group 6 (6.0 ± 0.8). Dermatitis scores were significantly increased for group 4 compared to group 2 ($p < 0.05$).

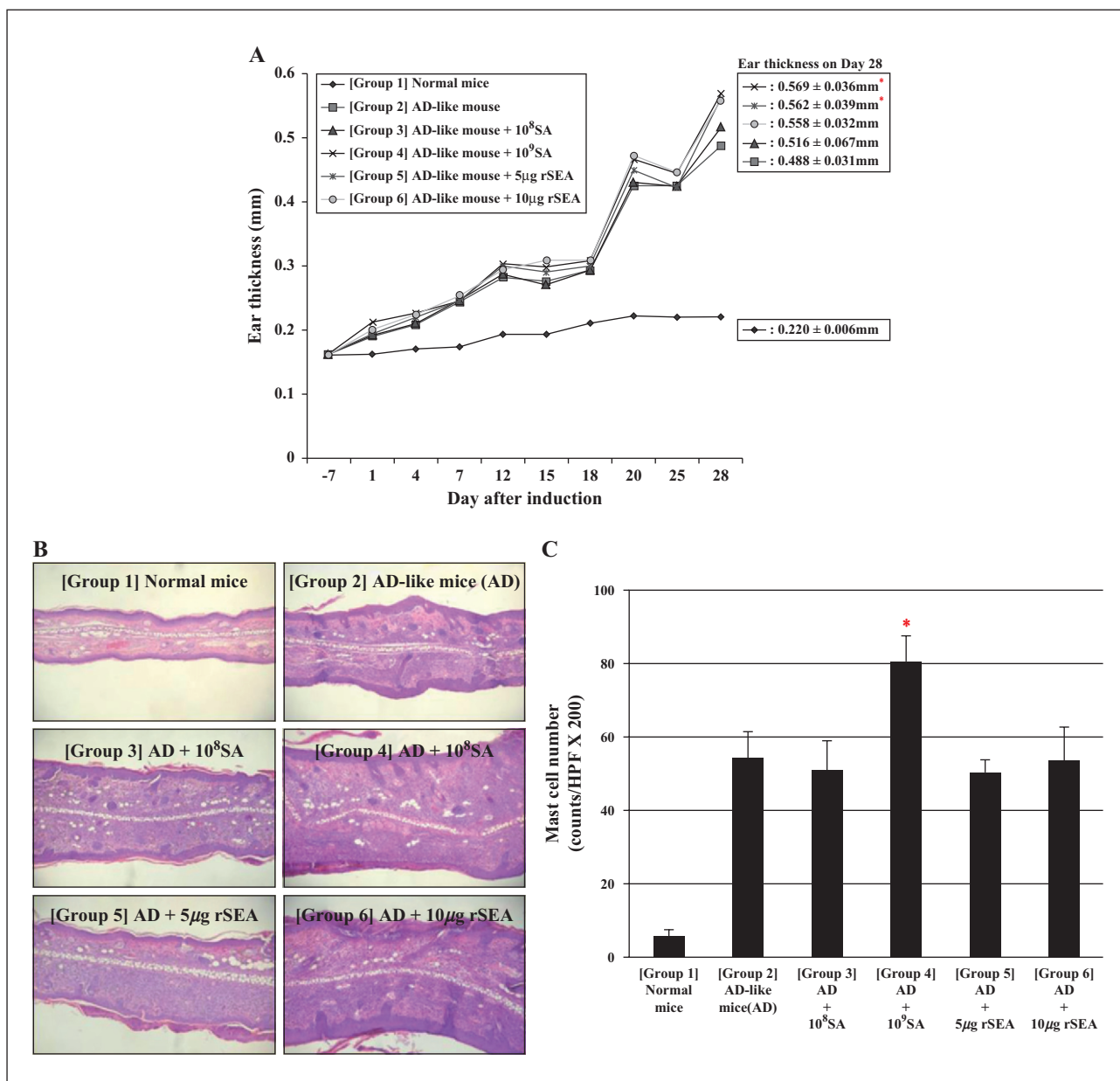


Figure 2. Clinical and histopathological changes of the ear. (A) Changes of ear thickness. (* $p < 0.05$ compared to group 2). (B) Histopathological findings. Significant inflammatory changes were observed such as thickening of the epidermis, fibrosis in the dermis, and accumulation of inflammatory cells such as lymphocytes, eosinophils and neutrophils in the ear tissue of the five groups with AD-like lesion (H&E, 100 × magnification). (C) Mast cell counts. The infiltration of mast cells induced by DFE/DNCB was promoted by *S. aureus* and rSEA. In particular, a high concentration of *S. aureus* resulted in significantly greater mast cell infiltration compared to group 2 (* $p < 0.05$ compared to the AD-like mice).

Otherwise, the scores for groups 3, 5 and 6 measured on the 28th day were not significantly different from those for group 2 ($p > 0.05$). As shown in figure 2A, repeated topical application of DFE/DNCB significantly increased ear thickness in the mice. Groups 2-6 showed thickened ears over time. On the 28th day, group 1 had an ear thickness of 0.220 ± 0.006 mm, which was not significantly different from the baseline value. Ear thickness was 0.488 ± 0.031 mm in group 2, 0.516 ± 0.067 mm in group 3, 0.569 ± 0.036 mm in group 4, 0.562 ± 0.039 mm in

group 5, and 0.558 ± 0.032 mm in group 6. Among the various groups, mice in groups 4 and 5 had significantly thicker ears when compared to group 2 ($p < 0.05$).

Histopathological findings

Repeated DFE/DNCB exposure caused significant inflammatory changes in the ear tissues of AD, such as thickening of the epidermis, dermal fibrosis and accumulation of inflammatory cells such as lymphocytes, eosinophils and

neutrophils (figure 2B). As shown in figure 2C, group 4 had significantly greater mast cell infiltration compared to that found in group 2 ($p < 0.05$).

Measurement of serum immunoglobulins and histamine release

Repeated application of DFE/DNCB elevated total serum levels of IgE, DFE mite-specific IgE and IgG2a (figures 3A-C). Although total IgE and IgG2a concentrations were increased in all five groups with AD-like lesions (groups 2-6), no statistically significant differences were found among the groups ($p > 0.05$). Although the mite-specific IgE levels also increased, no difference was observed among the five groups with AD-like lesions ($p > 0.05$). Serum histamine levels also increased in all five groups with AD-like lesions. Interestingly, mice in group 6 showed significantly enhanced levels of histamine compared to group 2 ($p < 0.05$; figure 3D).

Evaluation of cytokine expression

Repeated application of DFE/DNCB increased the expression of IL-4, IL-13, IL-31, INF- γ , TNF- α , IL-17, and IL-18 (figure 4). Groups 3-6 had higher levels of IL-4 compared to group 2. Among these groups, groups 4 and 6 had significantly increased expression of IL-4 ($p < 0.05$). Significantly increased IL-13 expression was observed in groups 5 and 6 compared to group 2 ($p < 0.05$). IL-31 expression was markedly increased in group 6 ($p < 0.05$). Compared to group 2, INF- γ was highly expressed in groups 4-6 ($p < 0.05$). TNF- α was highly expressed in group 5 alone ($p < 0.05$). Groups 3 and 6 had significantly high concentrations of IL-17 ($p < 0.05$). Additionally, significantly increased expression of IL-18 was found in groups 3, 4, and 6 ($p < 0.05$; figure 4).

Discussion

S. aureus are assumed to modulate AD skin inflammation via immunologic mechanisms rather than direct tissue destruction, since most AD patients do not show overt signs of infection even with skin colonization by *S. aureus* [17]. These findings raise the issue of the identification of various potent toxins, known as superantigens, produced by *S. aureus* in AD. The role of superantigens in AD development may be that of inducing skin inflammation by activating T cells, leading to the development of T cell skin-homing phenotypes [18].

In previous studies, SsAgs were found to reflect the genetic heterogeneity of the strains and the geographic distribution. Among *S. aureus* associated with AD, strains with the *sec* gene are the most frequently detected in Austria [19] while strains with the *sea* gene are most common in Germany [20]. Additionally, *seb* gene strains are reported to be the most frequently detected (54.3%) in AD patients in Japan [21] while the *sea* gene was found in most of the strains (97.2%) in Korea [18]. Since *S. aureus* with *sea*-expressing strains seem to be predominant among AD patients in South Korea [18, 22], *sea*-bearing *S. aureus* and rSEA were used in our experiment.

Many studies have revealed that cutaneous colonization of *S. aureus* significantly correlates with AD severity [7, 8, 23]. Williams *et al.* [23] reported that the density of *S. aureus* in AD lesions is associated with dermatitis severity. Our previous report supported such assertions because the prevalence of *S. aureus* is higher in AD patients than in normal children and eczematous lesions are more frequent in *S. aureus* retaining areas [22].

In addition to *S. aureus*, SsAgs may penetrate the skin barrier and promote the persistence and exacerbation of allergic skin inflammation in cases of AD [24]. However, dermatitis scores in the present study were significantly elevated only with high concentrations of *S. aureus*, while ear thickness was significantly increased only in mice treated with high concentrations of *S. aureus* and low concentrations of rSEA. Other groups also had increased dermatitis scores and ear thickness compared to the group with AD-like lesions induced by DNCB and DFE. In addition, even groups with insignificant results experienced aggravation of eczematous lesions by *S. aureus* or rSEA application that could be seen with the naked eye. These findings are consistent with the positive relationship between the clinical severity of AD and increased prevalence of *S. aureus* as well as bacterial toxins in AD-affected skin.

Similar to changes found in AD lesions [2], the *S. aureus*- or rSEA-treated groups also showed significant histopathological changes, such as epidermal hyperplasia, dermal fibrosis, and dermal infiltration of lymphocytes, eosinophils and neutrophils. Previous studies have reported that the number of mast cells infiltrating the dermis and mast cell activation are increased with AD [12, 25]. Another group used mast cell counts as a marker for monitoring AD improvement, to prove the efficacy of therapeutic agents [26]. In our investigation, the most highly elevated mast cell numbers were found in mice treated with a high concentration of *S. aureus*. These animals showed significantly increased dermatitis scores and ear thickness. Mice in the other groups had similar or less severe symptoms. These results indicate a positive correlation between clinical changes and histological changes. Therefore, we can speculate that there is a potential positive relationship between AD severity and the density of *S. aureus* as well as mast cell numbers. However, further studies are necessary to validate this assumption.

Among the reports on the role of *S. aureus* and immunopathogenesis in the aggravation of AD, some studies found that *S. aureus* induces a mixed Th1/Th2 response [27-29]. An animal experiment in which mice were treated with SEB showed that both Th1- and Th2-associated cytokines and chemokines were induced [27]. Vigorous production of specific IgE and IgG2a was also observed. Even though normal serum IgE levels do not imply that atopy does not exist, IgE concentrations are known to be increased in cases of AD [30]. A correlation between AD severity and the presence of SEA/SEB-specific antibodies with total serum IgE levels has also been reported [31, 32]. It is well known that Th1/Th2 imbalance plays an important role in the pathogenesis of AD and IgG2a immunoglobulin isotypes have been used as markers for Th1 lymphocytes [33]. Similarly, the results of the present study showed that IgE and IgG2a levels increased in all *S. aureus*- or rSEA-treated groups, although this change was not significant. Since inflammation induced by *S. aureus* or rSEA involves a mixed Th1/Th2- cytokine response, the ele-

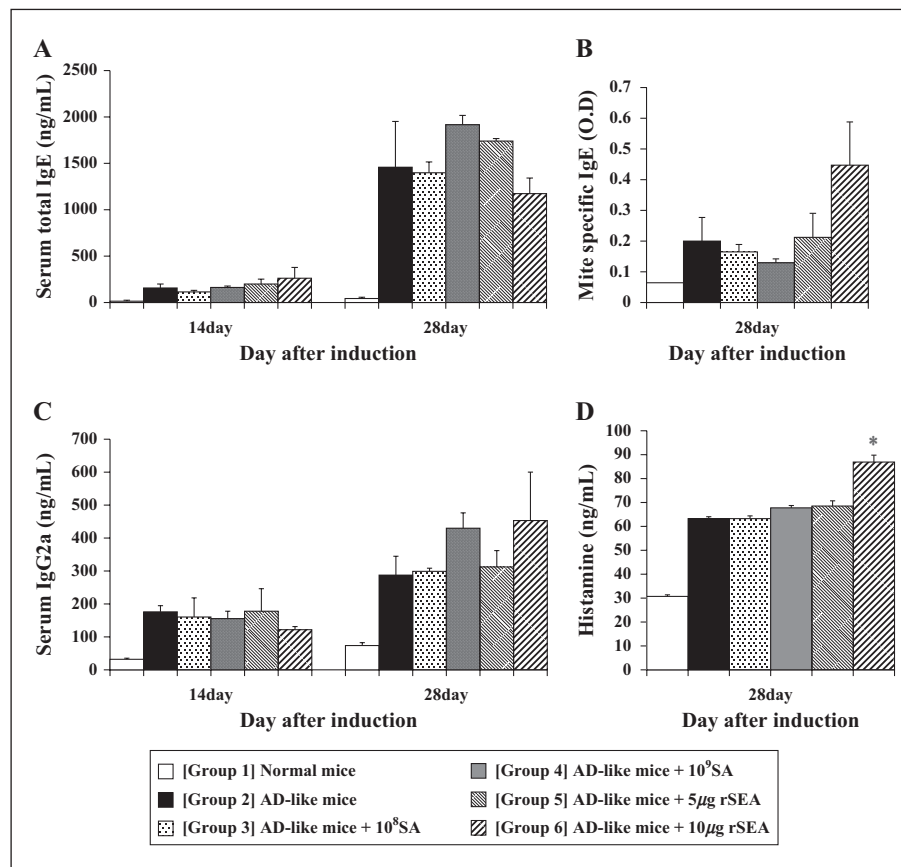


Figure 3. Effects of the application of *S. aureus* and rSEA on the serum levels of immunoglobulins measured by an ELISA and histamine evaluated by spectrofluometry (A-D). * $p < 0.05$ compared to group 2.

vation of IgE concentration through a Th2 response and IgG2a production via a Th1 response can influence each other. Furthermore, we postulate that the numerical values in experiments, the characteristics of the mice themselves and the small number of animals enrolled could be factors that affected our results. A previous study showed that histamine production, which is tightly regulated by IgE, is increased with AD [12]. In our investigation, elevated serum histamine levels were observed in all treated groups but these levels were significantly increased only with a high concentration rSEA.

The results of the present study are consistent with those of previous reports, showing not only that Th2 cytokines (IL-4, IL-5, IL-13, and IL-31) but also Th1 cytokines (IL-12 and IFN- γ) were increased in mice treated with *S. aureus* and rSEA. In particular, greater augmentation of cytokine concentrations was found in animals treated with rSEA than in animals that received *S. aureus*, regardless of the concentration. Based on these findings, we assume that SEA is more closely related with cytokine-associated inflammation than other mechanisms that induce atopic skin inflammation aggravated by *S. aureus*.

IL-17 is known to be a pro-inflammatory cytokine that up-regulates the expression of inflammatory genes in keratinocytes, fibroblasts and epithelial cells. Levels of this factor are elevated in cases of AD [34, 35]. IL-17 also stimulates the production of TNF- α by keratinocytes [34, 35]. TNF- α is a pro-inflammatory cytokine important for the ini-

tial stage of AD because it finally recruits and proliferates leukocytes and it is produced by epidermal cells [12]. In our study, production of these two cytokines increased in both the *S. aureus*- and rSEA-treated groups. Furthermore, IL-17 levels were significantly elevated with high concentrations of both *S. aureus* and rSEA. In contrast, TNF- α production was significantly increased only with a low concentration of rSEA.

IL-18 is known to play an important role in the induction and aggravation of AD through the Th1 and Th2 cytokine production pathway [36]. In an AD mouse model study using *S. aureus*, mice showed increased serum levels of IL-18 but not IgE [28]. This previous study provided evidence that IL-18 contributes to intrinsic AD development by inducing and activating super Th1 cells that have the potential to produce both Th1 (IFN- γ) and Th2 cytokines (IL-13) as well as IL-3 [37]. Therefore, IL-18 has diverse abilities to cross-regulate Th1/Th2 derivation and function. Additionally, a previous study also reported augmented secretion of IL-18 with SEA stimuli in cases of AD [36]. In our investigation, we saw a significant increase of IL-18 in both the *S. aureus*- and rSEA-treated groups.

IL-31 is a cytokine produced by Th2 cell activation in cases of AD and induces pruritus. Many reports have been published showing that serum IL-31 levels significantly increase with AD [12, 38]. However, significantly elevated IL-31 levels were only observed in mice treated with a high concentration of rSEA in our study.

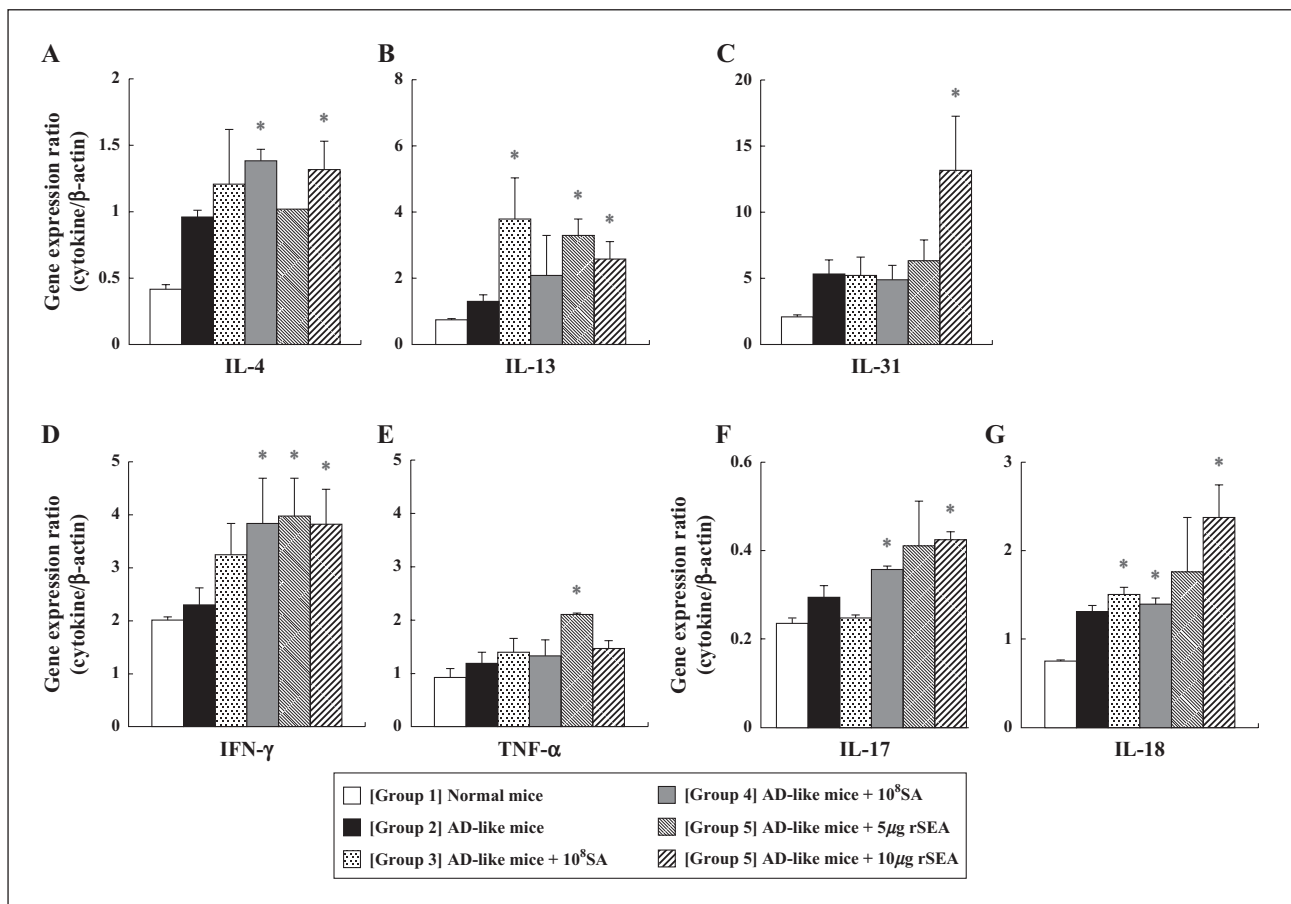


Figure 4. Effect of *S. aureus* and rSEA treatment on the expression of cytokines in ear tissues evaluated with real time-PCR. **p* < 0.05 compared to group 2. IL: interleukin, INF: interferon, TNF: tumor necrosis factor.

Our investigation demonstrated that the application of either SEA-producing *S. aureus* or rSEA may provoke an inflammatory response in a mouse model of AD. However, we could not find consistent differences between the effects of these two factors. SEA-producing *S. aureus* increased dermatitis scores and mast cell infiltration whereas rSEA increased the levels of IL-31 and TNF-α. Other parameters, including ear thickness and levels of IL-4, IL-13, IFN-γ, IL-17 and IL-18, were significantly elevated with both SEA-producing *S. aureus* and rSEA. We could not identify a possible reason for these differences; however, we assumed that IL-31 from CD4⁺ T cells may play an important role. Exposure to SsAgs rapidly induced IL-31 mRNA expression with AD both *in vivo* and *in vitro* and it has been previously shown that SsAgs play an important role in the regulation of IL-31 production [39]. The combination of IL-31 with rSEA may significantly increase the expression of TNF-α which has been found to serve as a pro-inflammatory cytokine in cases of AD [40]. In addition, a previous investigation demonstrated that IL-31 moderately up-regulates the secretion of IL-18 in stimulated macrophages [41]. As previously mentioned, IL-18 is a cytokine that influences the TH1 cell response. Therefore, our data suggest that IL-31 may have affected the difference between rSEA- and *S. aureus*-treated groups.

Interestingly, the degrees of clinical severity, including dermatitis scores and ear thickness, as well as the results of the

cytokine analysis, in our study were essentially independent of the *S. aureus* and rSEA amounts. Although these observations cannot be readily explained, we speculate that they may be attributed to a dose-independent immunologic response to a significant amount of *S. aureus* and/or rSEA. In some cases, this may have also been influenced by other factors, including tape stripping or the sensitizing process with DNCB and DFE. Dosage-independent responses may also be due to our study design, including the small number of mice, and subsequent research with a larger number of study subjects may therefore strengthen the speculations made in the current investigation.

In conclusion, we established an AD mouse model by the application of DNCB and DFE. The mice were subsequently treated with *S. aureus* and rSEA. Both the clinical and histopathological results showed that AD was aggravated in all treated groups. In particular, topical application of both SEA-producing *S. aureus* and rSEA promoted atopic skin inflammation. ■

Disclosure. Financial support: This research was supported by Kyungpook National University Research Fund, 2012 and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012-0007778). Conflict of interest: none.

References

- Pastar Z, Lipozencic J, Ljubojevic S. Etiopathogenesis of atopic dermatitis-an overview. *Acta Dermatovenereol Croat* 2005; 13: 54-62.
- Yamamoto M, Haruna T, Yasui K, et al. A novel atopic dermatitis model induced by topical application with dermatophagoides farinae extract in NC/Nga mice. *Allergol Int* 2007; 56: 139-48.
- Lubbe J. Secondary infections in patients with atopic dermatitis. *Am J Clin Dermatol* 2003; 4: 641-54.
- Leyden JJ, Marples RR, Kligman AM. *Staphylococcus aureus* in the lesions of atopic dermatitis. *Br J Dermatol* 1974; 90: 525-30.
- Ring J, Abeck D, Neuber K. Atopic eczema: role of microorganisms on the skin surface. *Allergy* 1992; 47: 265-9.
- Lin YT, Wang CT, Chiang BL. Role of bacterial pathogens in atopic dermatitis. *Clin Rev Allergy Immunol* 2007; 33: 167-77.
- Guzik TJ, Bzowska M, Kaspruwicz A, et al. Persistent skin colonization with *Staphylococcus aureus* in atopic dermatitis: relationship to clinical and immunological parameters. *Clin Exp Allergy* 2005; 35: 448-55.
- Nilsson EJ, Henning CG, Magnusson J. Topical corticosteroids and *Staphylococcus aureus* in atopic dermatitis. *J Am Acad Dermatol* 1992; 27: 29-34.
- Proft T, Fraser JD. Bacterial superantigens. *Clin Exp Immunol* 2003; 133: 299-306.
- Jung HJ, Jun JH, Cha HW, et al. Effect of topical application of *staphylococcus aureus* in the DNCB and dust mite extract-induced murine model with atopic dermatitis-like lesions. *Korean J Dermatol* 2011; 49(Suppl. 2): 124.
- Kwon HK, Lee CG, So JS, et al. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. *Proc Natl Acad Sci USA* 2010; 107: 2159-64.
- Choi EJ, Lee S, Hwang JS, et al. DA-9601 suppresses 2, 4-dinitrochlorobenzene and dust mite extract-induced atopic dermatitis-like skin lesions. *Int Immunopharmacol* 2011; 11: 1260-4.
- Liu F, Lin LR, Zhang HL, et al. Laboratorial characteristics of patients with diarrhea suffering from egg white allergy. *Allergol Immunopathol (Madr)* 2013 Jul 11 [Epub ahead of print].
- Shore PA, Burkhalter A, Cohn VH Jr.. A method for the fluorometric assay of histamine in tissues. *J Pharmacol Exp Ther* 1959; 127: 182-6.
- Kim SH, Lee S, Suk K, et al. Discoidin domain receptor 1 mediates collagen-induced nitric oxide production in J774A.1 murine macrophages. *Free Radic Biol Med* 2007; 42: 343-52.
- Karrer S, Bosserhoff AK, Weiderer P, Landthaler M, Szeimies RM. Influence of 5-aminolevulinic acid and red light on collagen metabolism of human dermal fibroblasts. *J Invest Dermatol* 2003; 120: 325-31.
- Cardona ID, Cho SH, Leung DY. Role of bacterial superantigens in atopic dermatitis: implications for future therapeutic strategies. *Am J Clin Dermatol* 2006; 7: 273-9.
- Kim DW, Park JY, Park KD, et al. Are there predominant strains and toxins of *Staphylococcus aureus* in atopic dermatitis patients? Genotypic characterization and toxin determination of *S. aureus* isolated in adolescent and adult patients with atopic dermatitis. *J Dermatol* 2009; 36: 75-81.
- Tomi NS, Kränke B, Aberer E. Staphylococcal toxins in patients with psoriasis, atopic dermatitis, and erythroderma, and in healthy control subjects. *J Am Acad Dermatol* 2005; 53: 67-72.
- Bunikowski R, Mielke ME, Skarabis H, et al. Evidence for a disease-promoting effect of *Staphylococcus aureus*-derived exotoxins in atopic dermatitis. *J Allergy Clin Immunol* 2000; 105: 814-9.
- Yagi S, Wakaki N, Ikeda N, et al. Presence of staphylococcal exfoliative toxin A in sera of patients with atopic dermatitis. *Clin Exp Allergy* 2004; 34: 984-93.
- Kim BS, Kim JY, Lim HJ, et al. Colonizing features of *Staphylococcus aureus* in early childhood atopic dermatitis and in mothers: a cross-sectional comparative study done at four kindergartens in Daegu, South Korea. *Ann Allergy Asthma Immunol* 2011; 106: 323-9.
- Williams RE, Gibson AG, Aitchison TC, Lever R, Mackie RM. Assessment of a contact-plate sampling technique and subsequent quantitative bacterial studies in atopic dermatitis. *Br J Dermatol* 1990; 123: 493-501.
- Gong JQ, Lin L, Lin T, et al. Skin colonization by *Staphylococcus aureus* in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial. *Br J Dermatol* 2006; 155: 680-7.
- Guttman-Yassky E, Nograles KE, Krueger JG. Contrasting pathogenesis of atopic dermatitis and psoriasis-part II: immune cell subsets and therapeutic concepts. *J Allergy Clin Immunol*. 2011; 127: 1420-32.
- Liu FT, Goodarzi H, Chen HY. IgE, mast cells, and eosinophils in atopic dermatitis. *Clin Rev Allergy Immunol*. 2011; 41: 298-310.
- Savinko T, Lauerma A, Lehtimäki S, et al. Topical superantigen exposure induces epidermal accumulation of CD8+ T cells, a mixed Th1/Th2-type dermatitis and vigorous production of IgE antibodies in the murine model of atopic dermatitis. *J Immunol* 2005; 175: 8320-6.
- Terada M, Tsutsui H, Imai Y, et al. Contribution of IL-18 to atopic-dermatitis-like skin inflammation induced by *Staphylococcus aureus* product in mice. *Proc Natl Acad Sci USA* 2006; 103: 8816-21.
- Hong SW, Kim MR, Lee EY, et al. Extracellular vesicles derived from *Staphylococcus aureus* induce atopic dermatitis-like skin inflammation. *Allergy* 2011; 66: 351-9.
- Aral M, Arican O, Gul M, et al. The relationship between serum levels of total IgE, IL-18, IL-12, IFN-gamma and disease severity in children with atopic dermatitis. *Mediators Inflamm* 2006; 2006: 73098.
- Bunikowski R, Mielke M, Skarabis H, et al. Prevalence and role of serum IgE antibodies to the *Staphylococcus aureus*-derived superantigens SEA and SEB in children with atopic dermatitis. *J Allergy Clin Immunol* 1999; 103: 119-24.
- Ong PY, Patel M, Ferdman RM, Dunaway T, Church JA. Association of staphylococcal superantigen-specific immunoglobulin e with mild and moderate atopic dermatitis. *J Pediatr* 2008; 153: 803-6.
- Kim MJ, Choung SY. Mixture of Polyphenols and Anthocyanins from *Vaccinium uliginosum* L. Alleviates DNCB-Induced Atopic Dermatitis in NC/Nga Mice. *Evid Based Complement Alternat Med* 2012 Oct 23. doi: 10.1155/2012/461989 [Epub ahead of print].
- Matsushima Y, Kikkawa Y, Takada T, et al. An atopic dermatitis-like skin disease with hyper-IgE-emia develops in mice carrying a spontaneous recessive point mutation in the Traf3ip2 (Act1/CIKS) gene. *J Immunol* 2010; 185: 2340-9.
- Koga C, Kabashima K, Shiraishi N, Kobayashi M, Tokura Y. Possible pathogenic role of Th17 cells for atopic dermatitis. *J Invest Dermatol* 2008; 128: 2625-30.
- Orfali RL, Sato MN, Takaoka R, et al. Atopic dermatitis in adults: evaluation of peripheral blood mononuclear cells proliferation response to *Staphylococcus aureus* enterotoxins A and B and analysis of interleukin-18 secretion. *Exp Dermatol* 2009; 18: 628-33.
- Inoue Y, Aihara M, Kirino M, et al. Interleukin-18 is elevated in the horny layer in patients with atopic dermatitis and is associated with *Staphylococcus aureus* colonization. *Br J Dermatol* 2011; 164: 560-7.
- Raap U, Weißmantel S, Gehring M, Eisenberg AM, Kapp A, Fölster-Holst R. IL-31 significantly correlates with disease activity and Th2 cytokine levels in children with atopic dermatitis. *Pediatr Allergy Immunol* 2012; 23: 285-8.
- Sonkoly E, Muller A, Lauerma AI, et al. IL-31: a new link between T cells and pruritus in atopic skin inflammation. *J Allergy Clin Immunol* 2006; 117: 411-7.
- Akdis M, Trautmann A, Klunker S, Blaser K, Akdis CA. Cytokine and anti-cytokine therapies for psoriasis and atopic dermatitis. *Acta Odontol Scand* 2001; 59: 178-82.
- Kasraie S, Niebuhr M, Werfel T. Interleukin (IL)-31 induces pro-inflammatory cytokines in human monocytes and macrophages following stimulation with staphylococcal exotoxins. *Allergy* 2010; 65: 712-21.