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Antibacterial effect of β-thujaplicin on staphylococci isolated from atopic dermatitis: relationship between changes in the number of viable bacterial cells and clinical improvement in an eczematous lesion of atopic dermatitis

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β-Thujaplicin (hinokitiol) is a tropolone-related compound purified from the wood of *Chamaecyparis obtusa*, *Sieb. et Zucc.* and *Thuja plicata D. Don*. All *Staphylococcus aureus* isolates were inhibited by β-thujaplicin with MICs of 1.56–3.13 mg/L. However, a paradoxical zone phenomenon occurred, with each isolate producing regrowth at higher β-thujaplicin concentrations. Other antimicrobial agents showed a wide range of MICs. The combination of β-thujaplicin and zinc oxide inhibited the paradoxical zone phenomenon, and enhanced killing activity against clinically isolated staphylococci. Large numbers of viable bacterial cells, especially *S. aureus* cells, were detected in the skin surface of atopic dermatitis, in comparison with those in healthy volunteers. The number of cells increased as the severity of the skin condition worsened. Topical application of β-thujaplicin resulted in a reduction in the number of bacterial cells on the skin surface, and an improvement in skin condition after treatment. The results of this study suggest that the degree of reduction in the number of viable bacterial cells in an eczematous lesion of atopic dermatitis is related to the degree of improvement in skin condition.

Keywords: β-thujaplicin, staphylococci, MRSA, atopic dermatitis, zinc oxide

Introduction

β-Thujaplicin (hinokitiol) is a tropolone-related compound purified from the wood of *Chamaecyparis obtusa*, *Sieb. et Zucc*[1,2] and *Thuja plicata D. Don*.[3] Although the biological activities of this compound are not yet fully understood, it has been revealed to have antimicrobial activities.[4–7] β-Thujaplicin has been used as an antibacterial agent in foods and cosmetics due to its low toxicity in animals.[8]

Recently, we have found that (i) β-thujaplicin has antimicrobial activity against both bacteria and fungi; (ii) β-thujaplicin shows a paradoxical zone phenomenon (i.e. the phenomenon of inhibition of the growth of bacteria at a low concentration, followed by promotion of growth at a higher concentration and then complete inhibition of growth at an even higher concentration)[9] against staphylococci; and (iii) the simultaneous use of β-thujaplicin and Zn^{2+} inhibits the paradoxical zone phenomenon, and enhances antimicrobial activity towards various bacteria.[10]

Staphylococcus aureus has been shown to colonize in high density and at high frequency in eczematous lesions of atopic dermatitis (AD),[11–18] and is thought to be one of the factors exacerbating dermatitis.[19,20] It has also been reported that *S. aureus* colonies on the skin surface of patients with AD easily changed from methicillin-sensitive *S. aureus* (MSSA) to methicillin-resistant *S. aureus* (MRSA), when antibacterial agents were administered over a long period of time.[20] These facts suggest that if topical application of β-thujaplicin reduces the number of bacterial cells (especially *S. aureus* cells) on the skin surface of patients with AD, this reduction in the number of bacterial cells may lead to elimination of one of

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the factors exacerbating dermatitis, and to improvement in eczematous lesions of patients with AD. The purpose of this study was to determine whether β-thujaplicin alone, or in combination with zinc oxide (ZnO), acts on clinical isolates from patients with AD. Another purpose was to elucidate the relationship between changes in the number of viable bacterial cells, and clinical improvement in an eczematous lesion due to the topical application of β-thujaplicin.

Materials and methods

Materials

β-Thujaplicin (synthetic product, purity 100%) was obtained from Takasago International Corporation (Tokyo, Japan). Methicillin sodium, ofloxacin, gentamicin sulphate, erythromycin and minocycline were purchased from Banyu Seiyaku (Osaka, Japan), Daiichi Seiyaku (Osaka, Japan), Wako Pure Chemical Industries (Tokyo, Japan), Shionogi Seiyaku (Osaka, Japan) and Lederle Japan (Tokyo, Japan), respectively. Triton X-100 was obtained from Nacalai Tesque (Kyoto, Japan). Heart infusion broth (HIB), heart infusion agar (HIA) and mannitol salt agar (MSA), were purchased from Nissui Seiyaku (Kyoto, Japan). Two kinds of film stamp check (6×6 cm^2) were purchased from Bio Medical Laboratory (Kyoto, Japan); soy casein digest agar (SCDA) plates and MSA plates enabled the detection of viable bacterial cells and *S. aureus* cells. All other chemicals used in this study were commercially available products of analytical reagent grade. β-Thujaplicin was dissolved in ethanol and diluted with sterile water to various concentrations. The final concentration of ethanol was <4%. The addition of 4% ethanol showed no remarkable effect on these assay systems. Methicillin sodium, ofloxacin, gentamicin sulphate, erythromycin and minocycline were dissolved in sterile water, and ZnO was suspended in sterile water or dissolved in 0.1 M HCl solution.

Subjects

A total of 43 outpatients (15 males and 28 females, aged 22.0 ± 9.8 years) who fulfilled the criteria of the Japanese Dermatological Association[21] for AD, and a total of 30 healthy volunteers (14 males and 16 females, aged 22.5 ± 2.1 years), were selected for this study. Informed consent was obtained from all participants (or from parents in the case of patients <20 years old) prior to treatment. No patients with an apparent secondary infection were included in this study.

Antimicrobial treatment

Body shampoo containing 0.03% β-thujaplicin in the detergent, and with a main component of aminomethyl-β-alanine, and ZnO ointment containing 0.05% β-thujaplicin, were used for test preparations. In order to evaluate the effect of each β-thujaplicin preparation, the 43 patients were divided into two groups: a control group of 21 patients and a β-thujaplicin group of 22 patients. The control group was further divided into two groups: a non-soap-user group, in which patients who had not used soap before treatment continued not to use soap until the completion of treatment, and a soap-user group, in which patients who had been using soap continued to use the same soap. The patients in the soap-user group used various commercial soaps that did not contain antibacterial agents. In the β-thujaplicin group, the previously used face and body soaps were changed to β-thujaplicin body shampoo. β-Thujaplicin ointment was applied, if necessary, to the lesions in adequate amounts two or three times a day. The β-thujaplicin group (n = 22) was further divided into two groups: one group of 18 patients who used body shampoo alone (body shampoo group) and one group of four patients who used both body shampoo and ointment (ointment group). The period of application was from 4 to 12 weeks, unless symptoms deteriorated or serious side effects occurred. There were no differences in the therapeutic procedures, except for the use of β-thujaplicin preparations. The use of other oral or topical antimicrobial agents, antiseptics or oral corticosteroids that might affect the efficacy of β-thujaplicin was avoided, and topical corticosteroids were not used in involved areas where bacteriological stamp sampling had been performed. The lifestyle of the patients was otherwise the same as it had been before the study.

The severity of each symptom (dryness, desquamation and pityriasis, erythema, epidermal exfoliation and erosion) was classified into five grades (+++, severe; ++, moderate; +, mild; ±, light; –, none) and judged by the attending doctor before treatment and then at 2 or 3 week intervals. At the completion of treatment, the therapeutic efficacy was assessed from the results of objective judgement of skin condition and the patient's judgement of subjective symptoms and side effects. Overall improvement was classified into six grades: 'resolved', 'markedly improved', 'improved', 'slightly improved', 'poor' and 'worsened'.

The involved area was stamped before and during treatment by the contact-plate technique described above, and the colonies on each plate were counted.

Assay of viable bacterial cells on the skin surface

Assays of viable cells on the skin surface were carried out on 23 randomly selected patients (eight males and 15 females, aged 25.4 ± 7.1 years), and on all of the healthy volunteers using the scrub technique. Assays using the contact-plate technique were also carried out on all participants. The involved area of AD was classified as normal skin, or dry or wet lesions, as judged by the conditions of dryness, desquamation, pityriasis, erythema and exudation.

Scrub technique

The technique used in this experiment was a modified procedure of Akiyama *et al.*[20] A glass tube (22 mm in internal diameter) (Daiichi Kikai, Tokushima, Japan) was placed above the involved area and filled with 0.1% Triton X-100/0.01 M phosphate-buffered saline 1 mL, and mixed with a Teflon rod for 1 min. This procedure was then repeated. After mixing the 2 mL mixture, an 0.1 mL aliquot of the 10-fold diluted specimen was cultured at 37°C on HIA and MSA plates. After 24 h of culture at 37°C, viable cells on the HIA plates were counted for total colonies, and mannitol-degrading cells on the MSA plates were counted for *S. aureus* colonies. These counts were converted to the corresponding density of bacteria originally present in the area. The addition of 0.1% Triton X-100 showed no remarkable effect on this assay.

Contact-plate technique

MSA plates were stamped for 10 s on the severest site in the involved area, and SCDA plates were stamped for 10 s on a neighbouring site. After 48 h of culture at 37°C, viable cells on the SCDA plates were counted for total colonies, and mannitol-degrading cells on the MSA plates were counted for *S. aureus* colonies. When a very large number of colonies was observed, the viable cells per 1 cm^2 were counted, and these counts were converted to the corresponding density of bacteria originally present.

Organisms

Forty-six staphylococcal strains tested were isolated from eczematous lesions of 23 patients for MIC determination. A few predominant colonies were randomly selected from each culture plate. Organisms were identified by Gram's stain, coagulase-positivity and Staphaurex (rapid latex test kit for the identification of *S. aureus*; Murex Diagnostics Ltd, Dartford, UK) using standard procedures. To avoid duplication, only one isolate per patient in each group [*S. aureus* or coagulase-negative staphylococci (CNS)] was examined.

Susceptibility testing

The MIC of β-thujaplicin and related compounds for clinical isolates was determined by an agar dilution method.[22] All isolates were incubated overnight at 37°C in HIB and diluted to a final inoculum of 1×10^6 cfu/mL; the plates were incubated for 24 h at 37°C. MIC was defined as the lowest concentration of the compounds tested that completely prevented visible growth of the inoculum on the surface of HIA plates.

Disc diffusion tests

The interaction between β-thujaplicin and ZnO was studied by an agar diffusion method[23] using an HIA plate. ZnO was suspended in sterile distilled water. Discs containing 60 μL of each solution obtained after dilution, were placed on a plate that had been inoculated at 10^6 cfu/mL, in the usual fashion, with the organism to be tested. After overnight incubation at 37°C, the results were interpreted according to the zone diameters (including the 6 mm disc), which were measured on the undersurface of the Petri dish with a ruler, or whether, according to the paradoxical zone phenomenon, they had disappeared.

Time–kill curve

The bactericidal activity of β-thujaplicin, and the effect of the combination of β-thujaplicin and ZnO, were determined, as described previously.[24] Briefly, overnight bacterial cultures in HIB were diluted with fresh HIB to ~10^7 cfu/mL, and incubated with the test compound at 37°C. The number of bacteria was counted after 2, 4 and 6 h incubation, by spreading 0.1 mL of the solution, obtained after diluting the culture 10-fold in sterile water, on to drug-free HIA plates. The colonies grown on the plates were counted after incubation for 24 h.

Statistical analysis

Statistical analysis was conducted as follows: the numbers of bacterial cells on skin surfaces were compared using the Kruskal–Wallis rank test, and Scheffe correction for multiple testing. Wilcoxon's signed rank test was used to compare numbers of viable bacterial cells in the same lesional site before and after treatment. Two-tailed tests were performed, and correlations with a level of $P < 0.05$ were considered to be significant.

Results

Assay of viable bacterial cells on the skin surface

The scrub and contact-plate techniques were used to evaluate the relationship between the number of viable bacterial cells on the skin surface and the skin conditions of AD. The results obtained by using the scrub technique (Figure 1) showed that the number of viable bacterial cells on the forehead was larger than that on the forearm of the healthy volunteers. *S. aureus* colonies were not detected in the involved areas of the healthy volunteers, but large numbers of colonies were detected on the skin of the patients with AD. In the AD group, the average number of total colonies or *S. aureus* on normal skin was less than that in dry lesions, which was less than that in wet lesions ($P < 0.001$, Kruskal–Wallis rank test). In both total and *S. aureus* colonies, there was a significantly larger number of

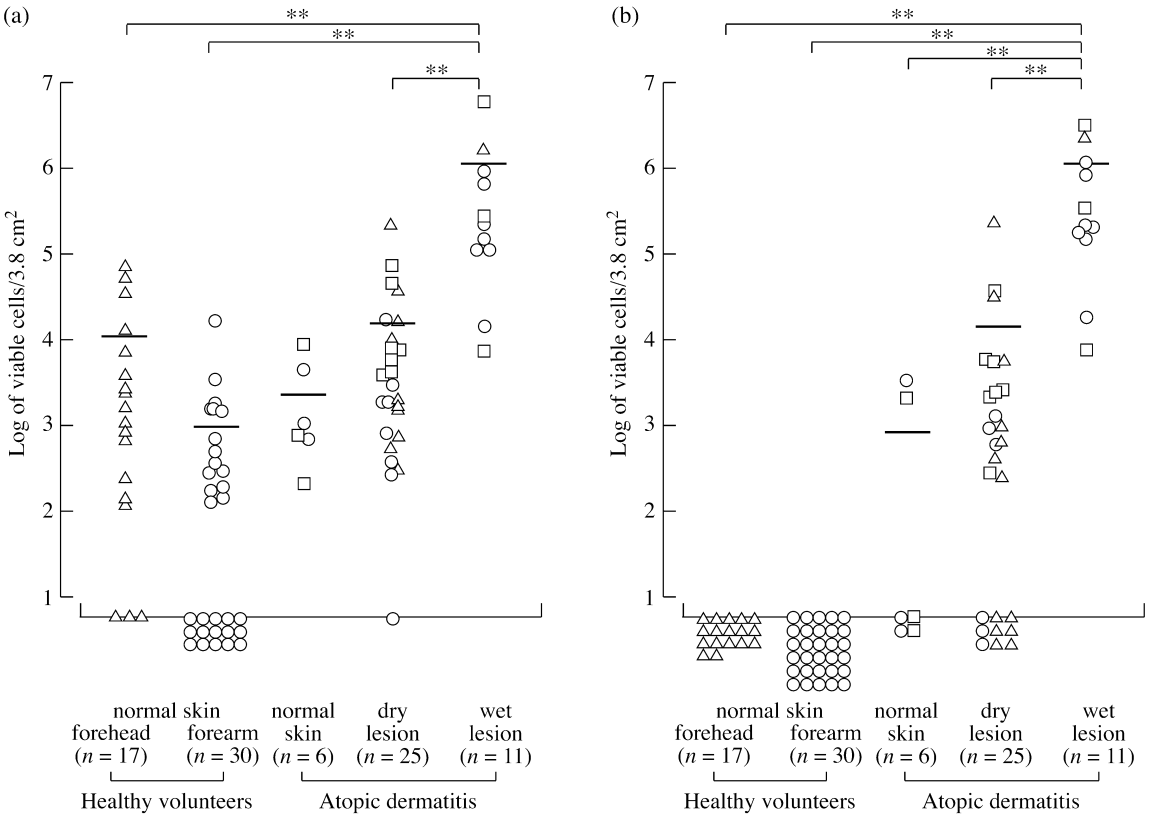

Figure 1. Relationship between the number of bacterial cells on the skin surface and the severity of the skin condition. The number of (a) total colonies and (b) *S. aureus* colonies on the skin surface, estimated by the scrub technique. $^{**}P < 0.01$ (Scheffe's test). Symbols: circles, limbs; squares, trunk; triangles, face; —, means.

viable cells in wet lesions than in other lesions ($P < 0.001$, Scheffe's test). This was also the tendency in more specific areas such as the forearm.

S. aureus colonies were detected in the involved area in six (20%) of the healthy volunteers, by using the contact-plate technique: three colonies (per 6×6 cm^2) were detected in one volunteer, and one colony was detected in each of the other five volunteers. Large numbers of colonies were detected in the patients with AD. Other results were the same as those obtained with the scrub technique (data not shown). Some representative examples are shown in Figure 2.

Susceptibility testing

MICs of β-thujaplicin and related compounds were examined for 46 clinical isolates (23 *S. aureus* and 23 CNS), and the results are summarized in Table 1. Three methicillin-resistant staphylococci (methicillin MIC of ≥16 mg/L) were included in the 46 isolates. The paradoxical zone phenomenon, i.e. the phenomenon of inhibition of the growth of bacteria by an antimicrobial agent at a low concentration (1.56–3.13 mg/L), followed by promotion of growth by the agent at a higher concentration, and then complete inhibition of growth at an even higher concentration (50 mg/L), occurred in every isolate treated with β-thujaplicin. The MIC of β-thujaplicin was 1.56–3.13 mg/L, with regrowth at higher concentrations between 6.25 and 50 mg/L for all of the 46 clinical isolates (21 MSSA, two MRSA, 22 MSCNS and one MRCNS), while other tested antimicrobial agents showed a wide range of MICs. In terms of MIC_{50} value, the inhibitory effect of β-thujaplicin was lower than that of the five other test compounds, which are all well-known antimicrobial agents.

Disc diffusion tests

The results for clinically isolated *S. aureus* A-1, as representative examples, are shown in Figure 3. Prevention of the paradoxical zone phenomenon, and enhancement of the original inhibitory effect of β-thujaplicin against *S. aureus*, are observed at and near the junction of the two zones of inhibition. In other isolates, the same results were obtained.

Time–kill curves

The time–kill curves are shown in Figure 4. The combination of β-thujaplicin and ZnO enhanced the killing effect for both *S. aureus* A-5 and A-52, which were selected randomly. There was no significant decrease in the number of bacteria with the use of either β-thujaplicin (100 mg/L) or ZnO

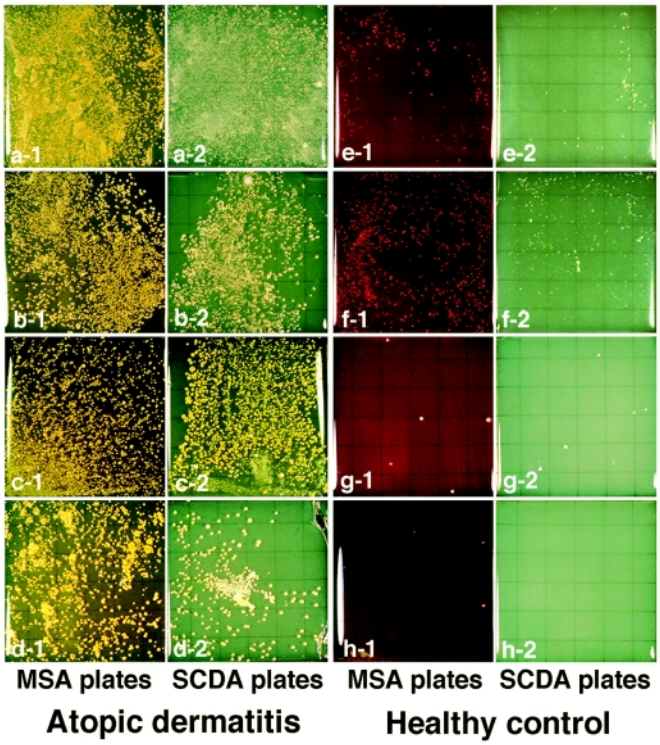

Figure 2. Viable bacterial cells in the involved area of atopic dermatitis and healthy control, obtained by the contact-plate technique. Large numbers of colonies, especially *S. aureus* (mannitol-degrading cells), were detected in the skin of the patients with atopic dermatitis. a-1, a-2, a 26-year-old woman, forehead; b-1, b-2, an 11-month-old boy, cheeks; c-1, c-2, a 28-year-old woman, forearm; d-1, d-2, a 24-year-old man, back of the hands; e-1, e-2, a 21-year-old woman, forehead; f-1, f-2, a 22-year-old man, cheeks; g-1, g-2, a 21-year-old woman, forearm; h-1, h-2, a 21-year-old man, back of the hands.

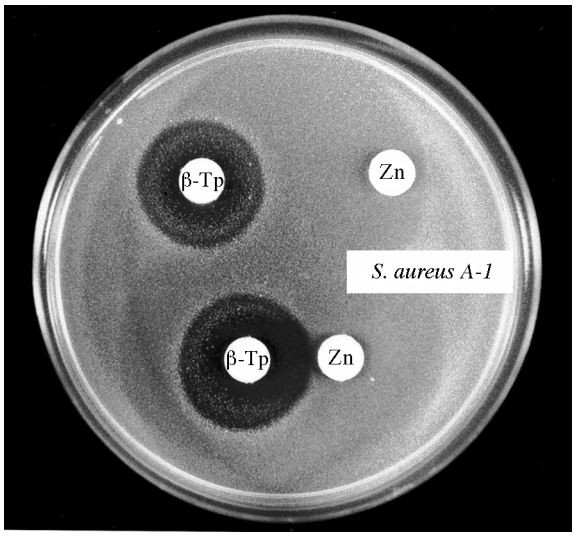

Figure 3. Synergic effects of β-thujaplicin (β-thujaplicin) (400 mg/L) and zinc oxide (Zn) (500 mg/L) on *S. aureus* A-1 isolated from a patient with atopic dermatitis. Inoculum size: 10 cfu/mL. Shaded areas indicate bacterial growth, and clear areas indicate zones of growth inhibition.

(400 mg/L) alone over a 6 h period, while their use combined resulted in a reduction in the number of viable cells for both strains at the end of the 6 h period (Figure 4a and d). The combination of β-thujaplicin (12.5 mg/L) and ZnO (100 or 400 mg/L) resulted in a dramatic decrease in the number of viable cells after 6 h, while exposure of both strains to β-thujaplicin (12.5 mg/L) or ZnO (100 or 400 mg/L) alone, did not result in a reduction in the number of bacteria during the 6 h period (Figure 4b, e, c and f).

Antimicrobial treatment

Background data of the patients, sites of involvement and treatment durations are shown in Table 2. No statistical differences between the β-thujaplicin group and control group were found for objective estimation. A comparison of the numbers of bacterial cells before and after treatment is shown in Figure 5. In the non-soap-user group, no relationship was found between treatment duration and number of total or *S. aureus* colonies. In the soap-user group, there was a slight decrease in the number of total colonies in the involved area ($P = 0.108$), but no significant change in the number of *S. aureus* colonies was observed. On the other hand, in the β-thujaplicin groups, both the number of total and *S. aureus* colonies decreased significantly ($P < 0.001$). In the ointment group, although no significant difference was found before or after treatment due to the small number of patients, the difference between the mean numbers of viable bacterial cells of total and *S. aureus* colonies was greater than that in the other groups. β-Thujaplicin preparations, including β-thujaplicin alone or in combination with ZnO, resulted in a significant reduction in the number of viable bacterial cells after treatment.

The degrees of overall improvement are shown in Table 3. The overall improvement rate, defined as the percentage of cases classified as 'improved' or better, in the β-thujaplicin-preparation use groups (90.9%) was higher than that in the control groups (61.9%). The overall improvement rates in the four subgroups were 100.0% in the ointment group, 88.9% in the body shampoo group, 66.7% in the soap-user group and 55.6% in the non-soap-user group. Side effects did not appear in any of the groups during the trial period.

Discussion

We investigated the numbers of viable bacterial cells on the skin surface of 23 patients with AD, and 30 healthy volunteers using the scrub technique and contact-plate technique. The same results were obtained by using the two techniques: (i) larger numbers of viable bacterial cells (especially *S. aureus* cells) were detected on the skin surface of the patients with AD than on the skin surface of the healthy volunteers; and (ii) in the patients with AD, the numbers of viable bacterial cells (both total and *S. aureus* cells) in each involved area of

Table 1. Antibacterial activity of β-thujaplicin against clinical isolates from patients with atopic dermatitis

Organism	Compound	MIC (mg/L)[a] range	50%	90%	Rate of paradoxical zone phenomenon (%)
S. aureus ($n = 23$)	β-thujaplicin	3.13[b] (50)[c]	3.13[b] (50)[c]	3.13[b] (50)[c]	100
	methicillin	1.56–>100	1.56	3.13	0
	gentamicin	0.20–>100	0.39	25	0
	erythromycin	0.20–>100	0.39	0.39	0
	minocycline	0.39–0.78	0.39	0.78	0
	ofloxacin	0.20–50	0.39	0.78	0
	zinc oxide[d]	400–800	400	400	0
	zinc oxide[e]	200	200	200	0
CNS ($n = 23$)	β-thujaplicin	1.56–3.13[b] (50)[c]	1.56[b] (50)[c]	3.13[b] (50)[c]	100
	methicillin	0.78–100	1.56	12.5	0
	gentamicin	≤0.1–50	0.2	25	0
	erythromycin	≤0.1–>100	0.2	>100	0
	minocycline	0.39–3.13	0.78	3.13	0
	ofloxacin	0.20–0.78	0.39	0.78	0
	zinc oxide[d]	100–200	200	200	0
	zinc oxide[e]	100–200	100	200	0

[a]50% and 90%, MIC_{50} and MIC_{90}.
[b]The value of lower MICs of β-thujaplicin is given.
[c]The value of higher MICs of β-thujaplicin is given in parentheses.
[d]Suspended in sterile distilled water.
[e]Dissolved in 0.1 M HCl solution.

Table 2. Clinical features of patients

	Control group			β-Thujaplicin group		
	non-soap-user group	soap-user group	total	body shampoo group	ointment group	total
Patients (male/female)	9 (2/7)	12 (4/8)	21 (6/15)	18 (6/12)	4 (3/1)	22 (9/13)
Age (years)	26.9 ± 8.4[a]	23.4 ± 4.1	24.9 ± 6.4	21.8 ± 10.9	9.0 ± 10.6	19.5 ± 11.8
Region:						
wet	1	4	5	5	1	6
dry	8	8	16	13	3	16
Overall severity[b]						
severe	1	1	2	4	1	5
moderate	4	9	13	14	3	17
mild	4	2	6	0	1	0
Site of involvement						
face	5	4	9	7	2	9
trunk	1	4	5	5	1	6
limbs	3	4	7	6	1	7
Treatment duration (weeks)	7.3 ± 1.9[a]	6.8 ± 1.4	7.0 ± 1.6	6.6 ± 1.9	6.0 ± 1.6	6.5 ± 1.8

[a]Values are mean ± S.D.
[b]Overall severity according to the criteria of the Japanese Dermatological Association.

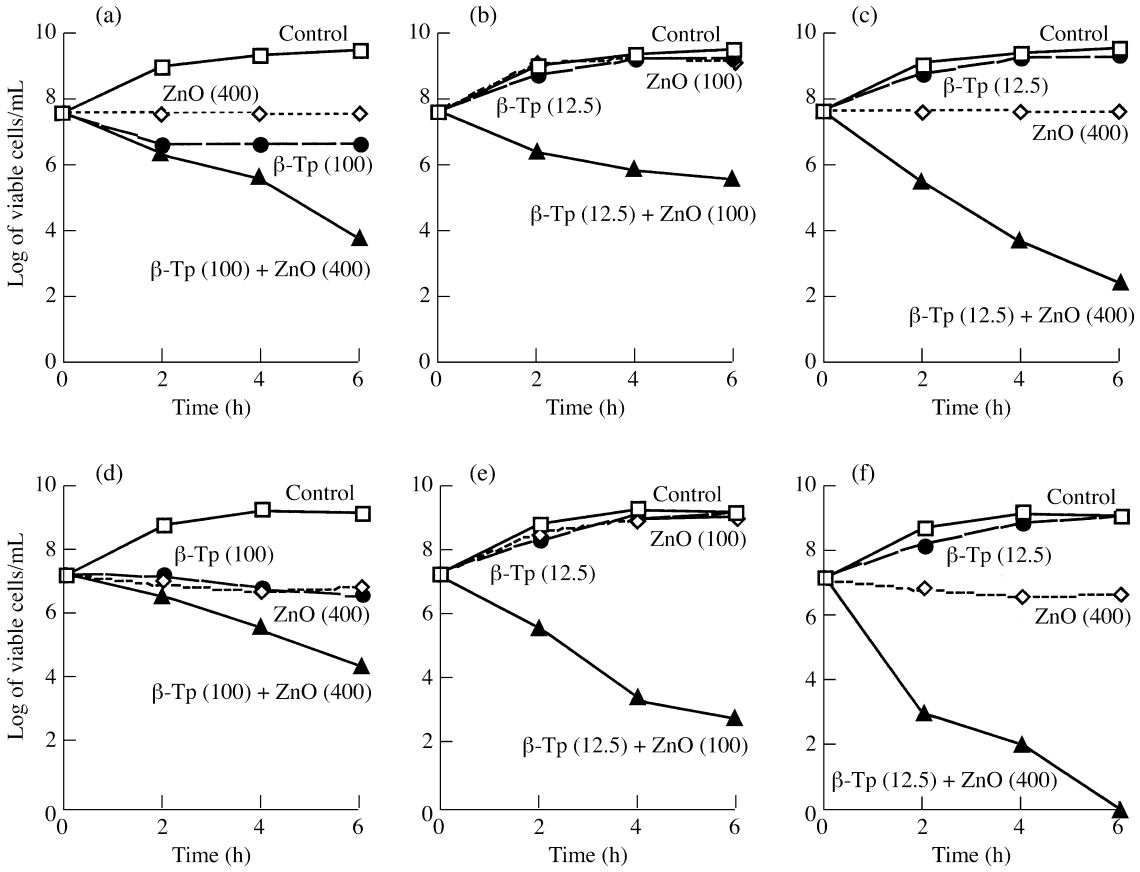

Figure 4. Effect of the combination of β-thujaplicin (β-Tp) and zinc oxide (ZnO) on *S. aureus* A-5 (a–c) or A-52 (d–f) isolated from patients with atopic dermatitis. Symbols: (a and d) squares, control; circles, β-thujaplicin (100 mg/L); diamonds, zinc oxide (400 mg/L); triangles, β-thujaplicin + zinc oxide; (b and e) squares, control; circles, β-thujaplicin (12.5 mg/L); diamonds, zinc oxide (100 mg/L); triangles, β-thujaplicin + zinc oxide; (c and f) squares, control; circles, β-thujaplicin (12.5 mg/L); diamonds, zinc oxide (400 mg/L); triangles, β-thujaplicin + zinc oxide.

AD were found to be in the following order of magnitude: normal skin < dry lesion < wet lesion (i.e. the number of viable bacterial cells increased with the severity of the skin condition). Although no *S. aureus* colonies were detected in the involved area in the healthy volunteers by using the scrub technique, a few colonies (one to three colonies per 6×6 cm^2) were detected in the involved area of six (20%) of the 30 healthy volunteers by using the contact-plate technique. This result is similar to rates of detection of *S. aureus* colonies reported previously in healthy subjects.[15] The difference between the results obtained by using the scrub technique and those obtained by using the contact-plate technique are attributed to the following factors: (i) the sampling area using the scrub technique (3.8 cm^2) is only about one-tenth of that using the contact-plate technique (36 cm^2); and (ii) although the scrub technique is suitable for counting the number of bacteria in a small area, the sample solution taken from the measurement site is diluted >100 times, making a bacterial count of <100 in the solution undetectable.

The effects of β-thujaplicin alone, and combinations of β-thujaplicin and ZnO on 46 strains of staphylococci isolated from eczematous lesions of AD, were investigated. In terms of MIC_{50}, the antimicrobial activity of β-thujaplicin was lower than that of the other five antimicrobial agents tested (Table 1). However, susceptibilities to the other five antimicrobial agents showed a wide range of MICs, while the MIC of β-thujaplicin was 1.56–3.13 mg/L, with regrowth producing the paradoxical phenomenon for all of the 46 clinical isolates, including three methicillin-resistant staphylococci (two MRSA and one MRCNS). In other words, β-thujaplicin acts on MRSA, which was used in the present study, at the same concentration as it does on MSSA. There have been no reports of β-thujaplicin-resistant strains, and no resistant strains were found in the present study. The paradoxical zone phenomenon, the mechanisms of which are still not clear, occurred in all of the strains of staphylococci treated with β-thujaplicin in the present study. For example, *S. aureus* A-46 cells stopped proliferating when treated with 3.13 mg/L of β-thujaplicin, then began to regrow when the concentration of β-thujaplicin was increased to 12.5 mg/L, and was finally inhibited once more when the concentration of β-thujaplicin was further increased to 50 mg/L (Figure 3a). Since this

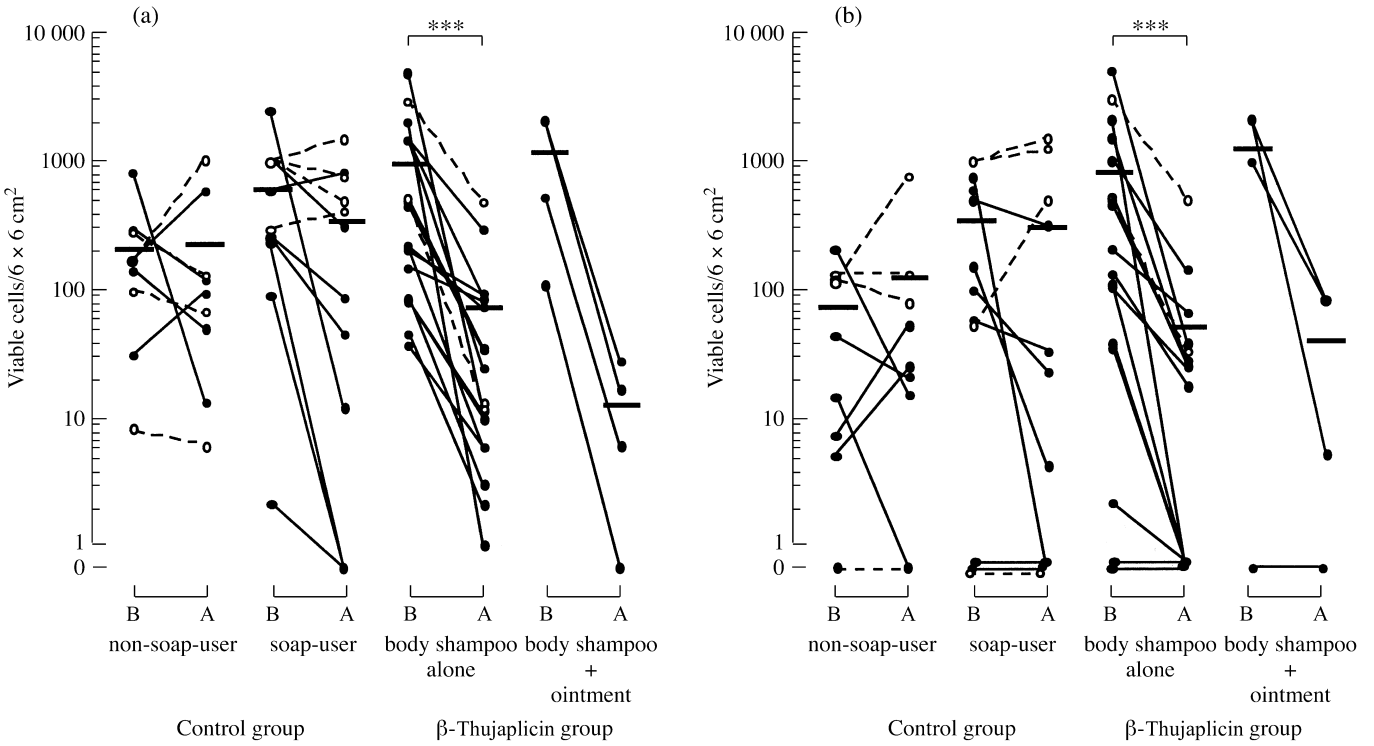

Figure 5. Comparison of the number of bacterial cells in the involved area before (B) and after (A) treatment. (a) Total colonies; (b) *S. aureus* colonies, ***$P < 0.001$ (Wilcoxon's signed rank test). Symbols: filled circles, cases achieved 'improved' or better response; open circles, cases achieved 'slightly improved' or less response; —, means.

Table 3. Overall improvement

	Control group		β-Thujaplicin group	
	non-soap-user group	soap-user group	body shampoo group	ointment group
Resolved	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)
Markedly improved	2 (22.2)	3 (25.0)	7 (38.9)	2 (50.0)
Improved	3 (33.3)	5 (41.7)	9 (50.0)	1 (25.0)
Slightly improved	3 (33.3)	3 (33.3)	2 (11.1)	0 (0.0)
Unchanged	1 (11.1)	1 (8.3)	0 (0.0)	0 (0.0)
Aggravated	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	9 (100.0)	12 (100.0)	18 (100.0)	4 (100.0)
≥Improved	5 (55.6)	8 (66.7)	16 (88.9)	4 (100.0)

No. of patients (%).
Non-soap-user group: not used soap before treatment; continued not to use soap until completion of treatment.
Soap-user group: used various conventional soaps without antibacterial agents.
Body shampoo group: used body shampoo that contained 0.03% of β-thujaplicin without ointment.
Ointment groups: used body shampoo that contained 0.03% β-thujaplicin, and ointment that contained 0.05% β-thujaplicin.

phenomenon has been shown previously to disappear when ZnO is added,[10] the combined effect of β-thujaplicin and ZnO on the paradoxical zone phenomenon was investigated with the clinical isolates. It was found that a combination of β-thujaplicin and ZnO inhibited the paradoxical zone phenomenon, and reduced the number of clinically isolated staphylococci, even at the concentrations that had no inhibitory effect when the agents were used alone. Their action on the clinical isolates was the same as that on the standard strain, *S. aureus* 209 JP (data not shown).

Since the bactericidal effect of β-thujaplicin on clinical isolates was confirmed by tests *in vitro*, use-tests were carried

out in order to determine whether the number of bacterial cells, especially that of *S. aureus*, on the skin surface of patients with AD is reduced by topical application of β-thujaplicin, and whether topical application of β-thujaplicin is clinically effective for eczematous lesions of AD. Because the contact-plate technique is sufficiently reliable, accurate and simple, it was used to count the numbers of viable bacterial cells on the skin surface before and after β-thujaplicin treatment, and the results were compared with those in the control groups (Figure 5). The use of β-thujaplicin preparation significantly reduced the number of bacterial cells, especially *S. aureus* cells, on the skin surface. The degrees of reduction in the number of viable bacterial cells in the four subgroups were in the following order: non-soap-user group < soap-user group < β-thujaplicin body shampoo use and ointment non-use group < β-thujaplicin body shampoo and ointment use group.

The degree of reduction in the number of viable bacterial cells on the skin was found to be related to the degree of improvement in skin condition; the improvement rate was higher in groups with greater reductions in the number of viable bacterial cells on the skin. Moreover, in cases that did not show a noticeable improvement in skin condition (i.e. cases classified as 'slightly improved' or worse), the number of viable bacterial cells (especially *S. aureus* cells) on the skin at the end of the study period had not significantly decreased compared with the number before treatment was commenced.

S. aureus has been shown to colonize in high density and at high frequency in eczematous lesions of AD, and is thought to be one of the factors exacerbating dermatitis. With regard to patients with AD colonized with *S. aureus*, Akiyama *et al*.[25] reported that the attachment of *S. aureus* cells to coverslips was suppressed in the presence of 5% ZnO. Leyden & Kligman[26] reported that patients with AD colonized with *S. aureus* responded better to a combination of topical corticosteroid and antibiotic therapy than to topical steroid therapy alone. The efficacy of β-thujaplicin ointment may be due not only to the antimicrobial action of β-thujaplicin and ZnO, but also to ZnO's inhibitory action[27] on the attachment of *S. aureus* bacteria.

The results of this study indicate that the use of an antibacterial agent, such as β-thujaplicin, which has a mild action, alone or in a preparation combined with ZnO, can reduce the number of viable bacterial cells on the skin surface of AD, and this reduction in the number of bacterial cells may be clinically effective for treating eczematous lesions of AD.

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