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A New Type of Staphylococcal Exfoliative Toxin from a *Staphylococcus aureus* Strain Isolated from a Horse with Phlegmon

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A new type of staphylococcal exfoliative toxin (sET) was isolated from the culture filtrate of a *Staphylococcus aureus* strain isolated from a horse with skin infection including phlegmon. The new sET was purified by precipitation with 80% saturated ammonium sulfate, column chromatography on DEAE–cellulofine A-500, gel filtration on a Sephadex G-75 column, and polyacrylamide gel electrophoresis (7.5% polyacrylamide). The new sET elicited general exfoliation of the epidermis with the so-called Nikolsky sign when inoculated into both 3-day-old mice and 1-day-old chicks, whereas sETA and sETB from human strains of *S. aureus* caused exfoliation in a 3-day-old mouse alone and shET from a porcine strain of *Staphylococcus hyicus* caused exfoliation in 1-day-old chicks alone. Intraepidermal splitting was observed at the granular layer of the epidermis of mice inoculated with the new sET as well as those inoculated with sETA. Exfoliation at the germinative layer of the epidermis was also observed in the chicks inoculated with the new sET as well as those inoculated with shET. The new sET was serologically different from sETA, sETB, and shET and showed the same molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was thermolabile and lost its toxicity after being heated at 60°C for 15 min. We propose that the new sET be designated as sETC.

Exfoliation in patients with staphylococcal scalded skin syndrome is caused by staphylococcal exfoliative toxin (sET) produced by some strain of *Staphylococcus aureus* (11). sET has been divided into two serotypes, sETA and sETB (8). sETA is a heat-stable toxin, whereas sETB is heat labile (6, 7). The production of sETA and sETB is controlled by the *eta* gene on chromosomal DNA and the *etb* gene on 42-kb plasmid DNA (9, 13–15), respectively. The molecular weights of sETA and sETB, as estimated by amino acid composition, are 26,950 and 27,274, respectively (9). Both humans and mice are susceptible to sETA and sETB; their target cells are epidermal cells in the granular layer and upper spinous layer of the epidermis (3, 12). In cultured cell lines, a rounding effect without cell death occurred after incubation with sET (5, 16).

In our previous studies (16, 17, 20), shET was isolated and purified from the culture filtrate of a *Staphylococcus hyicus* strain isolated from a pig with exudative epidermitis. shET induced exfoliation in piglets 8 to 12 h after subcutaneous injection. However, exfoliation was not seen for up to 24 h after injection in piglets inoculated with shET inactivated by heating at 60°C for 15 min. Histopathologically, an intraepidermal cleavage plane was observed between the corneal layer and the granular layer or at the granular layer of the skin of piglets injected with shET. The molecular weight of shET, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), is 27,000. Both piglets and young chicks are susceptible to shET (16). shET induced the Nikolsky sign in 1-day-old chicks 30 min after subcutaneous injection. In cultured cell lines, a rounding effect without cell death occurred after 6 to 24 h of exposure to shET (16).

The present paper describes the biological and serological characteristics of a new sET which was isolated and purified from a horse strain of *S. aureus*. We have named this new toxin sETC.

MATERIALS AND METHODS

Bacterial strains and mammalian cell lines. *S. aureus* Horse-1 (phage type 6/75) was isolated from a skin lesion (phlegmon) of a horse bred on a farm in Ibaraki prefecture (19). *S. aureus* ZM (an sETA-producing strain) and J-sETB-8 (an sETB-producing strain) were kindly supplied by S. Sakurai, Division of Molecular Genetics, School of Medicine, Jikeikai University. *S. hyicus* P-1 was isolated from a pig affected with erythema and incrustation of the body surface (18). These four strains were lyophilized and stored at 4°C. The lyophilized organisms were suspended in heart infusion broth (Difco Laboratories, Detroit, Mich.), inoculated onto heart infusion agar (Difco), and cultured at 37°C for 18 h before use as the inoculum for toxin production. Two established cell lines (HEp-2 and NCTC 2544) were used in the in vitro assay for sETs. HEp-2 cells were grown in Eagle's minimal essential medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (lot E-85506; Intergen Co., Purchase, N.Y.) and were maintained in Eagle's minimal essential medium without serum (maintenance medium). NCTC 2544 cells were grown in NCTC 135 medium (ICN Biomedicals Inc., Costa Mesa, Calif.) supplemented with 10% fetal calf serum and were maintained in NCTC 135 medium without serum (maintenance medium). These two cell lines

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were used for the detection of the rounding effect without cell death caused by sETs (16).

Isolation and purification of sETs. sETA and sETB were isolated and purified from the culture filtrates of *S. aureus* ZM and J-sETB-8 by the method described by Kondo et al. (6, 7). shET was isolated and purified from the culture filtrate of *S. hyicus* P-1 by the method of Sato et al. (17) and Tanabe et al. (20). sETC (the new type of sET) was isolated and purified from the culture filtrate of *S. aureus* Horse-1 as described for the shET isolated from *S. hyicus* P-1 in our previous paper (20). The final concentrated solutions of the above four sETs were designated as partially purified sETs. The protein concentration of each sample was determined as described in our previous paper (20).

PAGE. Native PAGE was performed as follows. Samples (3 ml) of partially purified sETs were mixed with 0.5 ml of 0.02% bromphenol blue in 80% glycerol–0.5 ml of 0.5 M Tris-hydrochloride buffer (pH 6.8). This sample was loaded on a polyacrylamide slab gel and was run at 60 mA per gel for 3 h with 0.005 M Tris–0.038 M glycine (pH 8.3) as the running buffer. After electrophoresis, the proteins in the gel slabs were transferred to polyvinylidene difluoride membranes (Atto Corp., Tokyo, Japan). A portion of the membrane was stained with 0.25% Coomassie brilliant blue R-250 (E. Merck AG, Darmstadt, Germany) and destained with 7% acetic acid. The corresponding portions of the gel slabs responsible for the protein band in the stained membranes were sliced out. sETs were extracted from these slices by electrophoresis with the Maxyield NP electroeluter (Atto). SDS-PAGE of the sET protein was performed as described for the shET isolated from *S. hyicus* P-1 (20). The protein in the gel slabs was transferred to polyvinylidene difluoride membranes. Half of each membrane was stained with 0.25% Coomassie brilliant blue R-250 and destained with 7% acetic acid. The other half was used for Western immunoblotting analysis.

In vivo assay for sETs. Eight 3-day-old inbred specific-pathogen-free mice (BALB/c; Japan SLC Co. Ltd., Hamamatsu, Japan) were used for the detection of exfoliative activity of each preparation by DEAE–cellulofine A-500 column chromatography, Sephadex G-75 gel filtration, and native PAGE. A total of 16 3-day-old SPF mice and 16 1-day-old specific-pathogen-free chicks (White Leghorn) bred on a Kitasato University farm were used for the detection of sETs (sETA, sETB, shET, and sETC). A 50-μg portion of each sample was injected subcutaneously into each of two chicks, and a 10-μg portion of each sample was injected subcutaneously into each of two mice. Exfoliative activity was regarded as positive when the Nikolsky sign (peeling off the skin surface easily caused by slight rubbing with the fingertip) was identified (6) within 3 h of injection.

Histopathological examination. Two mice and two chicks inoculated with sETC were sacrificed at 3 h after injection, and the skin lesions were collected for histological examination. Pieces of each skin lesion were fixed in 10% formalin solution and then embedded in paraffin. Sections were stained with hematoxylin and eosin.

In vitro testing for sETs. The rounding effect due to sETs was determined by using the cultured cells described above. A 100-μl portion of each toxin (50 μg/ml) diluted with maintenance medium was added to the cell monolayers in 96-well microculture plates (Corning Glass Works, Corning, N.Y.). After 24 h of incubation, the wells were examined microscopically for the rounding effect without cell death. Cell death was determined by trypan blue dye exclusion.

Heat stability of sETs. The four sET solutions (sETA, sETB, shET, and sETC) were heated at 100°C for 20 and 40 min and at 60°C for 15 and 30 min. After heat treatment, 50 and 10 μg of each sET were injected subcutaneously into each of two chicks and two mice, respectively. As control, the same dose of nontreated sET was injected subcutaneously into chicks and mice.

Anti-sET antibodies. Sixteen inbred 7-week-old female specific-pathogen-free BALB/c mice (Japan SLC) were used for the production of anti-sETA, anti-sETB, anti-shET, and anti-sETC antibodies. Each of the four sETs was treated with 0.8% formalin at 37°C for 50 h to convert the toxin to toxoid. A 50-μg portion of each toxoid was mixed with incomplete Freund adjuvant (Difco) and injected intraperitoneally into a group of four mice four times within a 1-week interval. At 4 days after the fourth injection, 10^6 sarcoma cells were injected intraperitoneally into each mouse (21). At 3 days later, 50 μg of each toxoid was injected intravenously into each mouse. Most mice showed distended abdomens within 10 to 15 days after the sarcoma cell injection. At this time the ascitic fluid was withdrawn by paracentesis through an 18-gauge needle into a 10-ml syringe. Fluids from each group of mice were pooled and centrifuged at 10,000 × *g* for 15 min. The supernatant was then drawn off and stored at −20°C as anti-sET antibodies.

Immunological tests. The serotype of the sETs was determined by an immunodiffusion test by the method of Ouchterlony, with each purified sET as antigen and each anti-sET mouse ascitic fluid as antibody. Western blotting analysis was done by the method of Towbin et al. (22). Antibody to each sET was layered on the strip of the polyvinylidene difluoride membrane containing the corresponding sET, and the strip was incubated at 37°C for 30 min. After incubation, the strip was washed three times with 0.15 M phosphate-buffered saline supplemented with 0.05% Tween 20 (T-PBS). Then a 2,000-fold dilution of peroxidase-conjugated anti-mouse immunoglobulin G (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) was layered on the strip, which was incubated at 37°C for 30 min. After incubation, the strip was washed three times with T-PBS. Substrate solution (0.05% 3,3′-diaminobenzidine tetrachloride plus 0.01% H_2O_2 in 0.05 M Tris hydrochloride buffer [pH 7.6]) was layered on the strips, which were incubated at room temperature for 10 min and washed with tap water to stop the reaction.

RESULTS

Purification of new sET. Figure 1A shows a DEAE–cellulofine A-500 column chromatograph profile of the fraction precipitated by 80% saturated ammonium sulfate from the culture filtrate of *S. aureus* Horse-1. The elution profile showed three major peaks (D-1, D-2, and D-3). When 10 μg of each peak sample was injected subcutaneously into each of three suckling mice, exfoliative activity was found in the D-1 preparation alone. Figure 1B shows the Sephadex G-75 gel filtration profile of the D-1 preparation. The elution profile showed two major peaks, S-1 and S-2. When 10 μg of each peak sample was injected subcutaneously into each of two suckling mice, exfoliative activity was found in the S-2 preparation alone. Figure 2A shows the PAGE patterns of the S-2 preparation and sETA purified by the method of Kondo et al. (6). The S-2 preparation gave three major protein bands, B1, B2, and B3, whereas sETA gave one major protein band (B1). The mobility of the B2 band corresponded to that of the B1 band of sETA. When 10 μg of each protein extracted from gels corresponding to B1, B2, and B3 bands was injected subcutaneously into each of three suckling mice, exfoliative activity was found in the B2 extract alone. Figure 2B shows the SDS-PAGE patterns of the B2

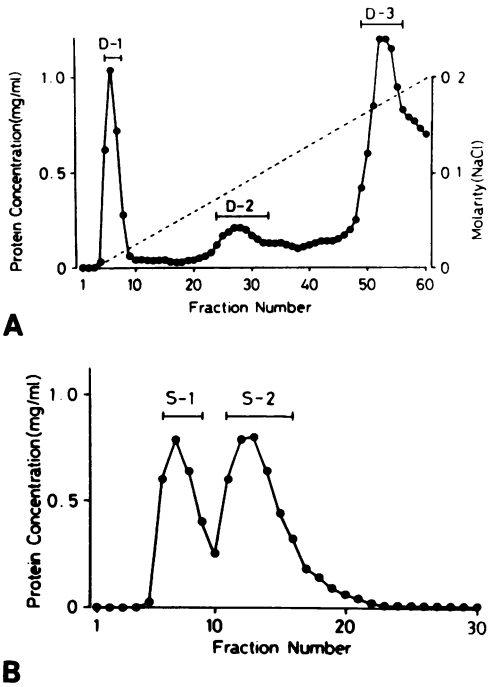

FIG. 1. (A) DEAE-cellulofine A-500 chromatography of the preparation of *S. aureus* Horse-1 precipitated by 80% saturated ammonium sulfate. The sample was placed on a column (1 by 20 cm) equilibrated with 0.01 M Tris-HCl (pH 7.5) and was eluted with a linear gradient from 0 to 0.2 M NaCl at a flow rate of 15 ml/h. (B) Sephadex G-75 gel filtration of the D-1 preparation obtained by DEAE-cellulofine chromatography. The sample was applied to a Sephadex G-75 gel column (2.2 by 35 cm) and was eluted with 0.01 M Tris-HCl (pH 7.5) at a flow rate of 24 ml/h.

extract and three sETs (sETA, sETB, and shET) purified by the method of Kondo et al. (6, 7) and Tanabe et al. (20). The B2 extract gave a single protein band, and its molecular weight was approximately 27,000. The molecular weights of sETA, sETB, and shET were also 27,000. From these results, the B2 extract was considered to be purified exfoliative toxin. We temporarily named the B2 extract sETC.

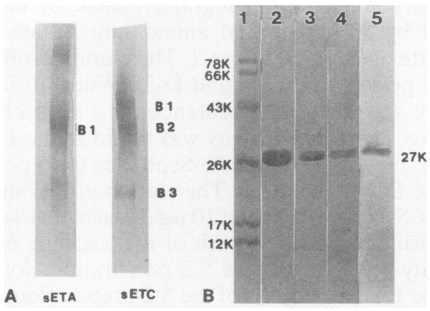

FIG. 2. (A) PAGE patterns of sETs. The left lane shows the result for purified sETA, and the right lane shows the result for the S-2 preparation obtained by Sephadex G-75 gel filtration. A protein band in the left lane was designated band 1 (B1). Three protein bands in the right lane were designated bands 1, 2, and 3 (B1, B2, and B3), respectively. (B) SDS-PAGE patterns of sETs. Lanes 1 to 5 show the marker proteins, purified sETA, purified sETB, purified shET, and the protein extracted from the B2 band (sETC), respectively.

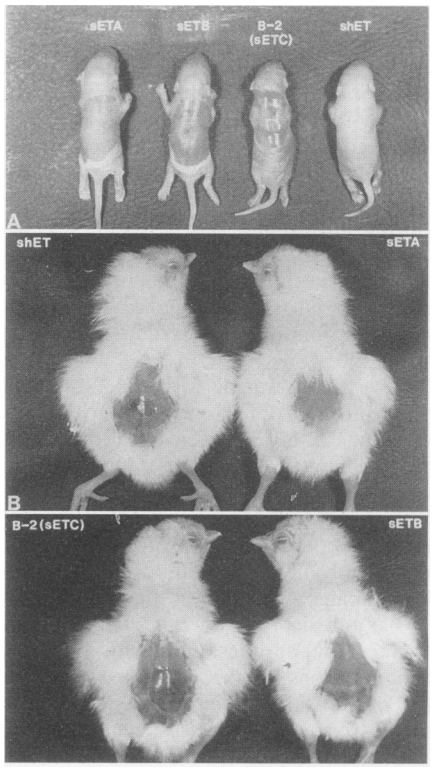

FIG. 3. (A) Exfoliation of the epidermis in suckling mice inoculated with each sET. (B) Exfoliation of the epidermis in 1-day-old chicks inoculated with each sET. sETA, sETB, shET, and B2 (sETC) show the mice and chicks inoculated with sETA, sETB, shET, and sETC.

Biological activity of the new sET. Figure 3A shows the results of the mouse inoculation test. When the four types of sETs were inoculated into the suckling mice, the Nikolsky sign was observed in the mice inoculated with sETA, sETB, and sETC within 3 h but was not seen in the mice inoculated with shET. Figure 3B shows the results of the chick inoculation test. When the four types of sETs were inoculated into the 1-day-old chicks, the Nikolsky sign was observed in the chicks inoculated with shET and sETC within 30 min but was not seen in the chicks inoculated with sETA and sETB. Table 1 shows the toxic activity of each sET in the cultured cells. shET caused the rounding effect in both HEp-2 and NCTC 2544 cells, whereas sET derived from *S. aureus*, such as sETA, sETB, and sETC, induced the effect in NCTC 2544 cells alone. After the appearance of the rounding effect of the cells inoculated with sETC, sETA, sETB, and shET, these cells were stained with

TABLE 1. Rounding effect in cultured cells after 24 h of exposure to the four sETs

sET	Presence of rounding effect in:	
	NCTC 2544 cells[a]	HEp-2 cells[b]
sETA	+	−
sETB	+	−
sETC	+	−
shET	+	+

[a] Cell line derived from the human epidermis.
[b] Cell line derived from the human larynx.

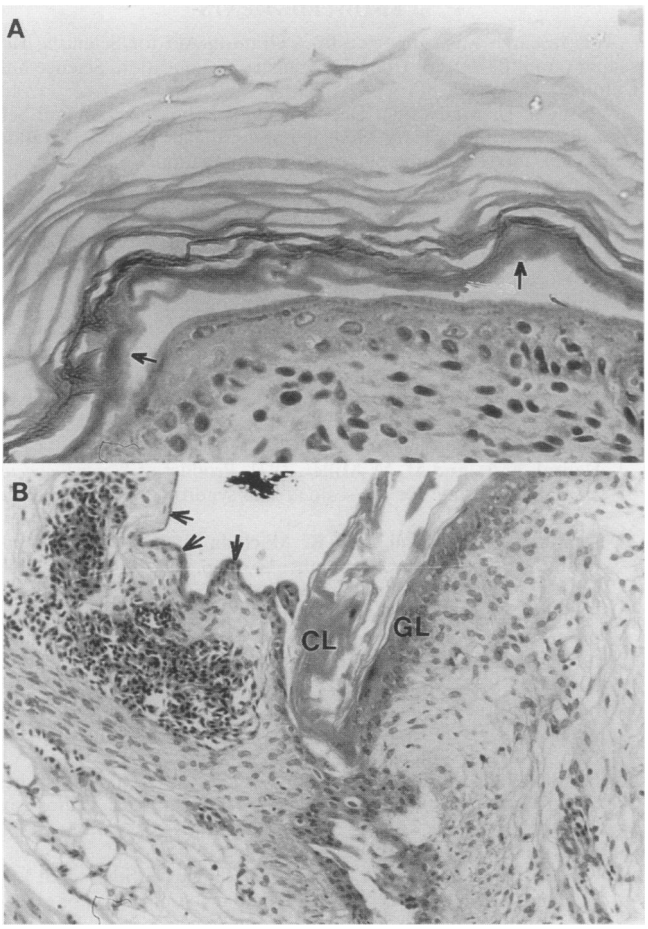

FIG. 4. (A) Intraepidermal splitting of the epidermis of a mouse inoculated with sETC. The arrows show the granular layer. (B) Exfoliation of the epidermis of a chick inoculated with sETC. The arrows show the disappearance of the upper germinative layer. CL and GL, corneal layer and germinative layer, respectively.

trypan blue. However, trypan blue dye exclusion could not be seen in all cells inoculated with the sETs. Therefore these cells were considered to be viable.

Histopathological examination. In the section of the epidermis of a mouse inoculated with sETC (Fig. 4A), the intraepidermal splitting was observed at the granular layer or between the corneal layer and the granular layer (arrows). Figure 4B shows the exfoliation of the epidermis of chicks inoculated with sETC. In the normal area, the germinative layer consisted of two or three cell layers under the corneal layer. On the other hand, in the exfoliated area (arrows), the corneal layer and most of the cells in the germinative layer disappeared.

Heat stability of sETC. The effect of heat treatment on sETC is shown in Table 2. The toxic activity of sETA was stable when the toxin was heated at 100°C for 20 min but was lost after heating at 100°C for 40 min. The toxic activities of shET and sETB were lost after heating at 60°C for 15 and 30 min, respectively. When sETC heated at 60°C for 15 min was inoculated into both mice and chicks, the Nikolsky sign was not observed in either animal. These results show that sETC is a heat-labile toxin.

Serological nature of sETC. Figure 5 shows the Western blotting analysis of the sETs. Anti-sETC antibody reacted with the 27-kDa protein band of sETC alone. Similarly, anti-sETA, anti-sETB, and anti-shET antibodies reacted with the 27-kDa protein bands of their homologous sETs alone. In the immunodiffusion test of sETs, sETC formed a precipitin line with anti-sETC antibody but did not form any precipitin lines among anti-sETA, anti-sETB, and anti-shET antibodies. These results suggest that sETC is serologically different from the other three sETs. Therefore, we designated the new exfoliative toxin produced by *S. aureus* Horse-1 as staphylococcal exfoliative toxin C (sETC).

TABLE 2. Effect of heat treatment on exfoliative activity of the four sETs

Heat treatment	Presence of Nikolsky sign in animals[a] given:			
	sETA	sETB	sETC	shET
None	+	+	+	+
60°C for 15 min	+	+	−	−
60°C for 30 min	+	−	−	−
100°C for 20 min	+	−	−	−
100°C for 40 min	−	−	−	−

[a] The Nikolsky sign was tested in mice for sETA, sETB, and sETC and in chicks for sETC and shET.

DISCUSSION

Several investigators have isolated sETs from *S. aureus* strains isolated from patients affected with staphylococcal scalded skin syndrome (4, 6, 7, 12). However, it was not fully understood whether sET-producing strains are limited to human strains or whether sET could be isolated from both human and animal strains. In our previous studies (16, 17, 20), we isolated a new sET from a porcine strain of *S. hyicus*. This new sET was different from both sETA and sETB in its antigenicity and susceptible animal species. We designated the new sET shET. Recently, Adesiyun et al. (1) reported that 3.9% of the animal strains of *S. aureus* examined produced sET and that 91.1% of the sET-producing strains produced sETA alone. In our previous study (19), only one (strain Horse-1) of the 76 horse strains of *S. aureus* produced an sET other than sETA and sETB. We showed in the present study that sET produced by *S. aureus* Horse-1 is a new serotype of sET. Previously, Kondo et al. (8) reported that 3 of 43 human strains of *S. aureus* produced a nontypeable

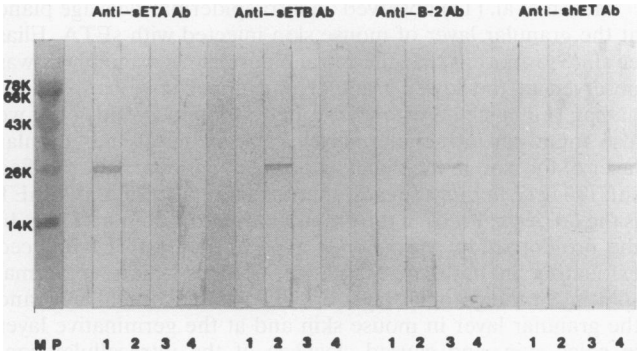

FIG. 5. Western blotting analysis of sETs. MP, Marker proteins stained with Coomassie brilliant blue R-250; molecular weights are given in thousands. Lanes: 1, purified sETA; 2, purified sETB; 3, B2 extract; 4, purified shET. Ab, antibody.

sET. These findings suggested that sET-producing strains exist among not only human strains but also animal strains and that the new serotype of sET exists in both animal and human strains.

The new sET isolated from *S. aureus* Horse-1 is a heat-labile toxin, since its toxicity was lost after heating at 60°C for 15 min. Its molecular weight is approximately 27,000, like that of sETA, sETB, and shET. Exfoliation occurred in both mice and chicks inoculated with the purified new sET from *S. aureus* Horse-1. Susceptibility to sETA and sETB is limited to humans and mice (2, 10, 11), and susceptibility to shET is limited to piglets and chicks (16). The toxic activity of sETB was lost after heating at 60°C for 30 min, and that of shET was lost after heating at 60°C for 15 min. From these findings, two possibilities were proposed. The first is that *S. aureus* Horse-1 produces both sETB and shET. The second possibility is that *S. aureus* Horse-1 produces a new serotype of sET. In Western blotting analysis and immunodiffusion tests, antiserum against sET from strain Horse-1 reacted with the homologous sET but not with antibodies against sETA, sETB, or shET. These results suggest that the sET obtained from *S. aureus* Horse-1 is a new serotype of sET. Therefore, we propose to designate the new serotype of sET staphylococcal exfoliative toxin C (sETC).

In our previous study (16), sETA isolated from a human strain of *S. aureus* caused a rounding effect without cell death in cultured NCTC 2544 cells but not in HEp-2 cells, whereas shET isolated from a porcine strain of *S. hyicus* caused a rounding effect in both NCTC 2544 and HEp-2 cells. Kondo et al. (5) also reported a rounding effect in JTC-17 cells after exposure to 25 μg of sETA solution per ml. Both NCTC 2544 and JTC-17 cells are human epithelial cell lines. Kondo et al. (5) suggested that the rounding effect of cultured epidermal cells was caused by the cleavage of intracellular contacts in JTC-17 cells. Therefore, the rounding effect in NCTC 2544 cells inoculated with sETs was also thought to be caused by the cleavage of intracellular contacts. When sETC was inoculated onto monolayers of NCTC 2544 and HEp-2 cells, the rounding effect was observed in NCTC 2544 cells alone. sETA and sETB, which caused exfoliation in mice, induced the rounding effect in NCTC 2544 cells alone, whereas shET, which caused exfoliation in chicks, induced the rounding effect in both NCTC 2544 cells and HEp-2 cells. sETC caused exfoliation in both mouse and chicks, but it did not induce the rounding effect in HEp-2 cells. The above results suggest that the receptor substance of sETC was different from sETA, sETB, and shET.

Melish et al. (11) observed an intraepidermal cleavage plane at the granular layer of mouse skin injected with sETA. Elias et al. (3) reported that the intraepidermal cleavage plane was observed at the lower granular and upper spinous layers of human skin injected with sETA. In our previous study (17), we also found an intraepidermal cleavage plane at the granular layer of the skin in the piglets inoculated with partially purified shET. These findings suggest that the target of sETA and shET is the epidermal cells in the granular layer and spinous layer. In the present study, the new serotype of sET (sETC) induced exfoliation in both mice and chicks, caused intraepidermal splitting at the granular layer or between the corneal layer and the granular layer in mouse skin and at the germinative layer in chick skin, and caused cleavage of the intracellular contacts in NCTC 2544 cells. These results suggest that the target of the new serotype of sET (sETC) is epidermal cells in the granular or spinous layer of the skin, as with sETA, sETB, and shET.

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