

An inhibitor of *Staphylococcus aureus* exfoliative toxin

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منشط للذيفان المحدث للتقشر للعنقوديات الذهبية

أمين عبد الجبار السلمي وكاظم قاسم الربيعي وعلي مكس عفات

خلاصة: نُصِف في هذه المقالة منبسطاً للذيفان (التوكسين) المُحدث للتقشر من العنقوديات الذهبية. وقد تم استخلاص الذيفان من ذرية من العنقوديات الذهبية استُفردت من حالة مصابة بمتلازمة الجلد المسموط الناجمة عن العنقوديات. وتمت مقارنة نشاط الذيفان في مرق تربتيك الصويا ومرق نقيع القلب والدماغ. ولقد دعم كلا الوسطين نمو العنقوديات الذهبية، ولكن رشاحة مزرعة مرق نقيع القلب والدماغ خلت من أي نشاط للذيفان المُحدث للتقشر. ثم تبين أنها تحوي مادة عادت مفعول الذيفان المُحدث للتقشر. وتوحي هذه النتائج بإمكانية إيجاد علاج لمتلازمة الجلد المسموط الناجمة عن العنقوديات وللقوباء الفقاعية.

ABSTRACT We describe here an inhibitor of *Staphylococcus aureus* exfoliative toxin. The toxin was extracted from an *S. aureus* strain isolated from a case of staphylococcus scalded skin syndrome. The activity of the toxin was compared in tryptic soy broth and brain heart infusion broth. Both supported growth of *S. aureus* but the culture filtrate of brain heart infusion broth lacked exfoliative toxin activity. Furthermore it appeared to contain a substance that neutralized the action of exfoliative toxin. This suggests the possibility of a treatment for staphylococcal scalded skin syndrome and bullous impetigo.

Un inhibiteur de la toxine exfoliative de *Staphylococcus aureus*

RESUME Nous décrivons ici un inhibiteur de la toxine exfoliative de *Staphylococcus aureus*. La toxine était extraite d'une souche de *S. aureus* isolée sur un cas de syndrome de Lyell staphylococcique. L'activité de la toxine a été comparée dans le bouillon trypticase soja et le bouillon cœur-cerveille. Les deux bouillons favorisaient la croissance de *S. aureus* mais le filtrat de culture du bouillon cœur-cerveille manquait en activité en ce qui concerne la toxine exfoliative. En outre, il semblait contenir une substance qui neutralisait l'action de la toxine exfoliative. Ceci indique qu'il y a une possibilité de traitement du syndrome de Lyell staphylococcique et de l'impétigo bulleux.

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Introduction

The study of staphylococcal toxin in blistering diseases of the skin arose directly from Lyell's investigation of the Glasgow outbreak of staphylococcal impetigo and staphylococcal scalded skin syndrome (SSSS) in 1968-69 [1]. Impetigo is localized and scalding generalized [2]. An outbreak of bullous impetigo occurred in the United States, which included 34 cases of phage type II *Staphylococcus aureus* infection [3]. Arbutnott and Billcliffe recorded three cases of non-phage group II strain causing SSSS, and nine cases of phage group II [4]. Kaplan et al. reported a high incidence of bullous impetigo in the United States as a result of exfoliative toxin type A, phage type II *S. aureus* [5]. In France, nine cases of SSSS were found by Chan et al. [6]. Al-Ali found 10% of staphylococci from children with various skin lesions showed signs of exfoliative toxin (ET) effect [7]. Steve showed that the bullous impetigo and SSSS caused by *S. aureus*-producing ET led to clefting of skin in the upper epidermis [8].

It has been reported that a new type of staphylococcal exfoliative toxin elicited general exfoliation of the epidermis with Nikolsky signs when inoculated into 3-day-old mice and 1-day-old chicks [9]. Recent investigations have produced evidence suggesting that exfoliative toxins induce serine protease activity, although the specific substrate and mode of action are not known for certain [10,11]. Tanabe et al. found possible receptors for exfoliative toxins produced by *S. aureus* and *S. hyicus*, and that those toxins lost their toxicity by preincubation with ganglioside 4 (GM4)-like glycolipid from 1-day-old chicks and suckling mice [12]. In this study, we report the neutralizing effect of a phospholipid on

a powerful exfoliative toxin from a local clinical isolate of *S. aureus*.

Methods

Specimens and sampling

Skin swabs were collected from 716 patients with different skin diseases attending the outpatients clinic of the Dermatology Department of Basra General Hospital between January and September 1997. Specimens from patients with scalded skin syndrome and bullous impetigo were moistened with brain heart infusion broth (Difco) and transported to the laboratory for immediate processing and culturing on blood agar (Merck) and after 24 hours on mannitol salt agar (Oxoid) for isolation of *S. aureus*.

Exfoliative toxin-producing *S. aureus*

Isolates of *S. aureus* were characterized and identified according to Gram stain, morphological characteristics and conventional biochemical tests [13,14]. Identified isolates were grown on Difco tryptic soy broth (TSB) and assayed for ET activity in newborn mice [2]. Mice (*Mus musculus*), breed BALB/c, aged 1-7 days, weight 2-5 g, were divided into two groups, each containing three mice. TSB containing 10^7 , 10^8 , and 10^9 cells/mL (McFarland standard) were prepared and one group of mice injected subcutaneously with 0.1 mL each. The control group of mice was injected with normal saline. Injected mice were observed for 24 hours for the appearance of Nikolsky signs at the site of injection, i.e. erythema, cyanosis, bullous formation and skin sloughing [15].

Subsequent experiments concentrated on the culture filtrate of *S. aureus* BS1

strain isolated from a case of SSSS. The activity of ET of *S. aureus* BS1 was compared both in TSB and brain heart infusion broth (BHIB).

Steps that led to the discovery of an inhibitor of ET

- Filtrate from *S. aureus* BS1 grown on TSB in shake culture at 140 rpm for 24 hours was injected subcutaneously (0.1 mL) in rabbits at different sites.
- Filtrate of the same strain grown for 24 hours on BHIB in shake culture was injected (0.1 mL) into rabbits subcutaneously in different sites.
- Aliquots of BHIB (0.1 mL each) were injected into rabbits either immediately after their injection with 0.1 ml TSB filtrate, or after 1 hour.
- Brain heart infusion medium (2g) was dissolved in 10 mL of absolute alcohol. Two fractions appeared, a supernatant and a precipitate. After evaporating alcohol of the supernatant at room tem-

perature, a pasty brownish substance remained. This substance and the precipitate were each dissolved in sterile normal saline. Then each fraction was mixed in 1:1 volume with TSB filtrate, i.e. the toxin-containing filtrate, and injected (0.1 mL) into rabbits subcutaneously.

- In addition, 0.5 g of the brownish substance was mixed with 2.5 g of Vaseline to make an ointment. Rabbits were injected each with 0.1 mL TSB filtrate subcutaneously, and the skin site on each rabbit was rubbed with the ointment, either:
 - immediately after the injection; or
 - 1 hour later; or
 - 24 hours after the injection.

Lecithin

Different concentrations of lecithin were used as injection or ointment for rabbits receiving ET subcutaneously.

Table 1 Activity of exfoliative toxin produced by *Staphylococcus aureus* BS1 grown on TSB and BHIB at 37 °C and 140 rpm

Age of culture (hours)	Growth OD 540 nm	Filtrate of BHI	Filtrate of TSB	Skin reaction
4	0.3	-	+	Erythema only
6	1.0	-	++	Cyanosis, dry skin,
10	1.5	-	+++	eruption, vesicles,
12	1.9	-	+++	bullae, skin sloughing
16	2.4	-	++++	and separation from the
20	3.0	-	+++++	body, some cases
24	3.0	-	+++++	showed abscess

Control: TSB (-), only erythema.
Filtrate of BHIB: (-), only erythema.
TSB = tryptic soy broth.

Control: BHIB (-), only erythema.
OD = optical density.
BHIB = brain heart infusion broth.

Table 2 Inhibitory effect of injected BHIB on the exfoliative toxin after injecting rabbits with TSB filtrate

TSB filtrate (mL)	Saline (mL)	BHIB (mL)	No. rabbits tested	Skin reaction
0.1	0.1	Nil	3	Erythema, cyanosis, nodule then skin dried
0.1	Nil	0.1 ^a	3	Erythema, healing after 1 hour
0.1	Nil	0.1 ^b	3	Erythema, healing after 6 hours

^aGiven immediately after injection of TSB filtrate.

^bGiven 1 hour after injection of TSB filtrate.

BHIB = brain heart infusion broth.

TSB = tryptic soy broth.

Results

Filtrates from cultures of different cell densities showed no activity of ET when *S. aureus* BS1 was grown on BHIB, but parallel growth on TSB clearly revealed the activity of the toxin on injection into young mice (0.1 mL each density). Animals showed erythema and cyanosis, skin eruption, vesicles, bullae, sloughing of the skin and separation from the body, and in some cases, abscess (Table 1). Skin sections of animals injected with saline showed no pathological change, whereas sections of those injected

with *S. aureus* BS1 or its culture filtrate, TSB filtrate, showed typical intraepidermal cleavage caused by acantholysis of superficial epidermal cells.

The sites injected with TSB filtrate followed immediately, or after 1 hour, by injection with BHIB, remained normal — i.e. no signs on the skin, except erythema that disappeared within 1–6 hours (Table 2). There was also no effect of ET on the sites injected with TSB filtrate, as long as the extracted pasty brownish substance was mixed with TSB filtrate before injection, or used as an ointment (Table 3).

Table 3 Inhibitory effect of the BHIB-alcohol-extracted brownish substance on the exfoliative toxin when applied as an ointment, after injecting rabbits with TSB filtrate

TSB filtrate (mL)	Saline (mL)	BHIB extract (0.5 g/2.5 g Vaseline)	No. rabbits tested	Skin reaction
0.1	0.1	Nil	3	Cyanosis, nodule then skin dried
0.1	Nil	Yes ^a	3	Erythema only, healing after 1 hour
0.1	Nil	Yes ^b	3	Erythema, nodule then healing

^aApplied immediately after injection of TSB filtrate.

^bApplied 1 hour after injection of TSB filtrate.

BHIB = brain heart infusion broth.

TSB = tryptic soy broth.

Table 4 Inhibitory effect of injected lecithin (50 mg/mL) on the activity of exfoliative toxin after injection of rabbits with TSB filtrate

TSB filtrate (mL)	Saline (mL)	Lecithin (mL)	No. rabbits tested	Skin reaction
0.1	0.1	Nil	3	Cyanosis, nodule and bullous eruption
0.1	Nil	0.1 ^a	3	Erythema only, healing after 1 hour
0.1	Nil	0.1 ^b	3	Erythema, peeling of affected skin then healing

^aGiven immediately after injection of TSB filtrate.

^bGiven 1 hour after injection of TSB filtrate.

TSB = tryptic soy broth.

Application of lecithin by injection or as an ointment immediately or 1 hour after injecting the rabbits with TSB filtrate, showed a neutralizing effect on ET similar to what was seen with BHIB or the pasty brownish substance from BHIB (Tables 4 and 5). Meanwhile, preincubation of the toxin either with lecithin or BHIB, as well as the follow-up injections, were more effective than the ointment. However, increasing the concentration of lecithin in the ointment up to 200 mg/g of Vaseline was more effective in neutralizing the action of ET. Aliquots of 0.1 mL TSB filtrate represent a sub-lethal dose. Injection of rabbits with a dose of 0.2 mL TSB filtrate and then immediately rubbing the injected area with 100 mg lecithin/g Vaseline was not sufficient to save the animal from death. Post-mortem examination indicated that viscera

congested, cyanosis took place, there was liver enlargement with cyanosis, ecchymosis in the intestine, gall bladder enlargement and non-absorbed food material. Clinically, the animal showed depression, congested eye and anorexia.

ET purified by combination with ammonium sulfate, gel filtration and ion-exchange gave one band on gel electrophoresis. It showed the same signs of action on suckling mice and was also neutralized by lecithin.

Discussion

During this study, a search was conducted to find an inhibitor of ET. Early signals came from the fact that although BHIB supported the growth of *S. aureus*, its

Table 5 Inhibitory effect of lecithin ointment on the activity of exfoliative toxin after injection of rabbits with TSB filtrate

TSB filtrate (mL)	Lecithin ointment (mg/g Vaseline) ^a	No. rabbits tested	Skin reaction
0.1	100	3	Erythema, one small vesicle each, no cyanosis
0.1	200	3	No erythema, normal skin

^aThe ointment was applied immediately after injection of the TSB filtrate.

TSB = tryptic soy broth.

culture filtrate lacked ET activity. In addition, preparation of BHIB used as injection into rabbits inhibited exfoliation and appearance of Nikolsky signs. Therefore, it was thought that BHIB, in contrast to TSB, contained a substance that somehow neutralized the action of ET.

It was shown that the pasty brownish substance from the alcohol fraction of BHI possessed the neutralizing effect on ET, which implies that a lipid component is more likely to be the neutralizer or inhibitor of ET. The introduction of lecithin as an inhibitory substance of ET was inferred from these results and from other findings [12,16], coupled with the fact that lecithin inhibits another toxin of *S. aureus*, the delta haemolysin [17].

Kondo described the partial characterization of an inhibitor of ET extracted from mouse skin with sodium dodecyl sulfate [16]. Also, a possible receptor has been found for ET, designated GM4-like glycolipid; it has been reported that *S. hyicus* and *S. aureus* exfoliative toxins lost their toxicity on preincubation with GM4-like glycolipid from 1-day-old chicks and suckling mice respectively [12]. Injecting or rubbing the experimental animal with lecithin showed effective inhibition of ET. It seems that BHI does not inhibit the synthesis of ET, but rather ET loses its activity due to the presence of a lipid component. Similarly, lecithin renders ET ineffective by an, as yet, unknown mechanism.

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