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# Effects of recombinant lysostaphin on cytotoxicity and interleukin-8 level in normal human epidermal keratinocytes cell line

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The normal human epidermal keratinocyte (NHEK) was used to evaluate the cytotoxicity of recombinant lysostaphin. As determined with the Neutral Red (NR) cytotoxicity assay, the midpoint toxicity value ($NR_{50}$) after 48 h exposure was 16 ± 0.4 g lysostaphin/l. Lysostaphin cytotoxicity effect is much less than the surface active agent, sodium laurate. However, the $NR_{50}$ value after 48-h exposure was 1.9 ± 0.02 g/l for *S. aureus* lysate derived from the bacterial lytic action of lysostaphin. A linear increase in interleukin-8 (IL-8) level in NHEK cells from resting levels of 65 ± 3 pg/ml to peak of 760 ± 15 pg/ml during the first 9 hours was noted for the cells treated with 800 mg lysostaphin/l. *S. aureus* lysate has the same effect on the induction of IL-8 levels. The induced rises in IL-8 were lysostaphin and *S. aureus* lysate concentration dependence.

**Keywords:** recombinant lysostaphin, cytotoxicity, interleukin-8

## Introduction

Infection by antibiotic resistant isolates of *Staphylococcus aureus* complicates the antibiotic treatment of staphylodermatitis, therefore, searching for alternative antimicrobial agents to combat resistant strains is urgent. Some proteinaceous antimicrobial agents used against *S. aureus* pathogens, such as defensins and lysostaphin, offer a potentially less hazardous approach to antibiotics currently available for use in antimicrobial treatment (Ganz *et al.*, 1990; Gennaro *et al.*, 1989; Zygmunt and Tavormina, 1972). Defensins have been used as antimicrobial agents for bovine mastitis therapy (Cullor *et al.*, 1990), however, the reduced activity outside the phagosome and potential cytotoxicity limit their application. Lysostaphin is an antimicrobial protein produced by *S. staphylolyticus* (Schindler *et al.*, 1964). It hydrolyses the pentaglycine links of the peptidoglycan of ***Staphylococcus aureus*** and has little activity against other microorganisms. In contrast to defensins, lysostaphin is active against antibiotic-resistant *S. aureus* isolates and has no oral toxicity at a wide range of doses (Schaffner *et al.*, 1967; Martin and White, 1967; Harris *et al.*, 1967; Quickel *et al.*, 1971). The lysostaphin gene has been sequenced and expressed in *Escherichia coli* and *Bacillus* species (Heinrich et al., 1987; Recsei et al., 1987). We previously expressed the gene encoding lysostaphin in *E. coli*, and its recombinant form was purified to homogeneity by using an one-step fractionation on bacterial cells of lysostaphin-resistant *S. aureus* mutant (Chan, 1996).

Using recombinant lysostaphin as an antimicrobial agent can potentially circumvent problems associated with current antibiotic therapy because of its targeted specificity and possible reduction of risk due to bacterial antibiotic resistance. In the present study, the cell toxicity test and inflammatory effects of recombinant lysostaphin on normal human epidermal keratinocyte (NHEK) are reported. Among potential proinflammatory cytokines, that are detectable in skin diseases in biologically active form, there are members of a family of small chemotactic peptides, of which interleukin-8 (IL-8) has been increasing studies (Nickoloff *et al.*, 1990; Gearing *et al.*, 1990). IL-8 is produced by various cell types including dermal fibroblasts and keratinocytes in response to inflammatory mediators such as IL-1, tumor necrosis factor-$\alpha$, interferon-$\gamma$, lipopolysaccharide or other insult substances (Matsushima and Oppenheim, 1989; Westick *et al.*, 1989). In view of the indigenous IL-8 producers and their different responsiveness to proinflammatory cytokines (Matsushima and Oppenheim, 1989; Westwick et al., 1989; Rozengurt,

1986), it is possible that IL-8 and its inducing mediators could be involved in the recruitment and proliferation of epithelial cells in cutaneous inflammation. In this study, we determined whether or not NHEK respond to the recombinant lysostaphin to produce IL-8. The obtained data should be valuable for evaluating whether lysostaphin can be a potential agent for staphylodermatitis therapy.

## Materials and methods

### Materials

All chemicals were purchased from Sigma Chemical Co. unless otherwise indicated. Lysostaphin was prepared by a recombinant DNA technique and purified according to the method described by Chan (1996).

### *Staphylococcus aureus* cultivation

*S. aureus* strain purchased from ATCC. was grown in tryptic soy broth supplemented with glucose (20 g/l). Culture was incubated at 37°C and aerated by shaking at 250 rpm. *S. aureus* lysate was prepared by treating the 10 ml suspension of bacterial cells ($10^9$ cells/ml) with 20 units of recombinant lysostaphin for 30 min at 37°C, and centrifugation was performed to remove the debris from lysate. The unit definition of lysostaphin was defined as the amount of preparation causing 50 % reduction of turbidity of *S. aureus* cells suspension by $A_{600}$ = 0.5 within 10 min at 37°C.

### Cultivation of normal human epidermal keratinocyte (NHEK)

NHEK cells were purchased from Clonetics Corp. (Walkersville, MD). NHEK cells were grown in Clonetics Corporation's Keratinocyte Growth Medium ($KGM^{TM}$) at 37°C under 5% $CO_2$ (v/v), and disaggregated by trypsinzation by 0.25% trypsin overnight at 4°C. NHEK cells used in this study were derived from the second passage grown as a monolayer to 80% confluence.

### Neutral Red (NR) cell viability assay

NHEK cells were seeded into 96 well microplates with 2500 cells/well. The cells were grown in KGM containing 10 mM $Ca^{2+}$ and incubated at 37°C and 5% $CO_2$ for 3 days. At the fourth day, lysostaphin at various concentrations (range from 500 to $10^5$ mg/l) was added into each well to a final volume of 200 µl, and the cells were incubated for another 2 days. Six to eight replicate wells were used per concentration of lysostaphin. After 48 h of exposure, cytotoxicity was assessed with the Neutral Red (NR), which is based on the uptake and lysosomal accumulation of the supravital dye (Borenfreund et al., 1990). Neutral Red, as a 4 mg/ml aqueous stock solution, protected from light with foil, was added into each well to a final concentration of 5 ppm and incubated for 3 h. Cells were then rapidly washed and fixed with 0.5% formalin/1% $CaCl_2$ (w/v) and the NR incorporated into viable cells was released into the supernatant with 0.2 ml 95% ethanol. Absorbance was measured at 540 nm with a microtiter plate spectrophotometer. Data were expressed as percent of untreated controls ± the standard errors of the mean, and were used to construct concentration-response cytotoxicity graphs. A midpoint toxicity value was reported as $NR_{50}$.

### Measurement of IL-8

IL-8 was determined by a commercial specific enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN, USA), using a monoclonal antibody as the capturing and polyclonal rabbit anti-IL-8 antibody as the secondary antibody, both of which were raised against human recombinant IL-8. The sensitivity of assay was 31 pg of IL-8 per ml. Three-day incubating broth of pretreated and control NHEK cells were used for IL-8 determinations.

## Results

The cytotoxicity of a 48-h exposure to lysostaphin was compared to that of surface active agent, sodium laurate. Fig. 1A shows the concentration-response cytotoxicity curves of these test agents for the NHEK and Table 1 lists their midpoint cytotoxicity, $NR_{50}$ values (i.e. the concentration of test agent that reduced the uptake of NR by 50% as compared to untreated control cells). Lysostaphin toxicity is much milder to NHEK cells than the surface active agents. We next determined the cytotoxicity of *S. aureus* lysate which was derived from the bacterial lytic action of lysostaphin. As shown in Fig. 1B, 48-h exposure of *S. aureus* lysate shows more potent to NHEK cells than lysostaphin itself, and 6-h exposure of *S. aureus* lysate is not harmful. The concentrations of recombinant lysostaphin and *S. aureus* were determined by their protein contents measured by the Bradford dye binding assay using bovine serum albumin as a standard.

The influence of lysostaphin and *S. aureus* lysate on IL-8 induction was studied in NHEK cells. As shown in Fig. 2, within 9 hours following treatment, the induction of IL-8

**Table 1** Midpoint cytotoxicity ($NR_{50}$) values of the test agents towards NHEK cells after 48-h exposure.

| *Test agent* | *$NR_{50}$ (mg/l)* |
|---|---|
| Lysostaphin | 16000 ± 407 |
| S. aureus lysate | 1887 ± 20 |
| Na laurate | 25 ± 3 |

Data are presented as the arithmetic means ± SEM.
The concentration of lysostaphin and *S. aureus* lysate were determined as their portein contents measured by the Bradford dye binding assay.

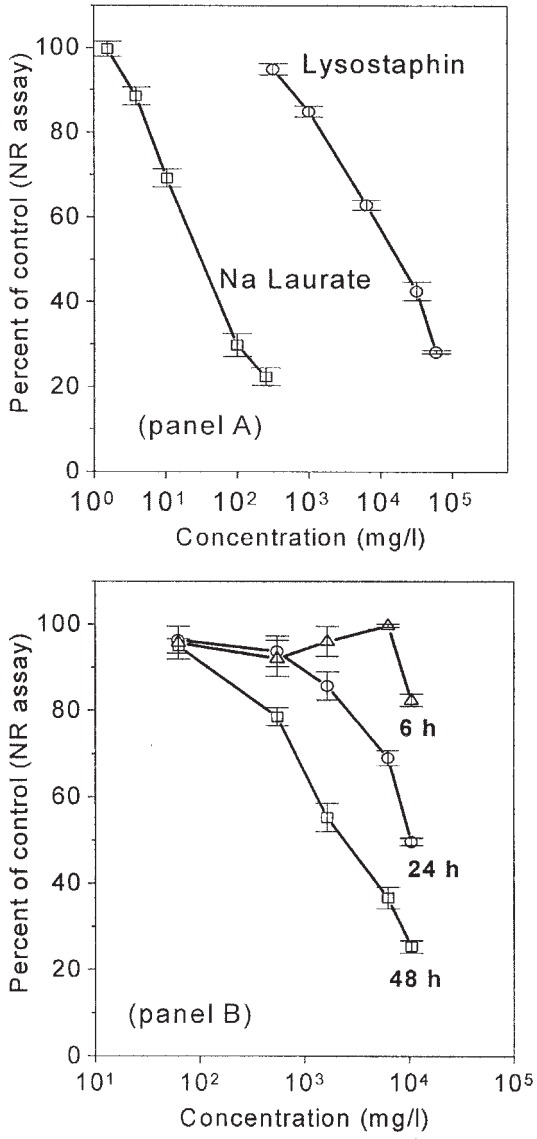


**Figure 1** (A) Comparative 48-h cytotoxicities of lysostaphin and sodium laurate towards NHEK cells with the NR assay. The data are presented as the arithmetic mean in percent of the controls ± SEM. (B) Response of the NHEK cells to the various period exposure to *S. aureus* lysate by NR assay. Individual data points are presented as the arithmetic mean in percent of the controls ± SEM.

in lysostaphin-treated (300 mg/l) and *S. aureus* lysate-treated (800 mg/l) NHEK cells were approximately 8-fold and 11-fold, respectively, higher than in cells that had not received treatment. The IL-8 concentration in NHEK cells decreased after 9 hours exposure.

Measurements of IL-8 levels in lysostaphin-treated NHEK cells are presented in Fig. 3. NHEK cells had detectable basal IL-8 concentrations, and lysostaphin-treated cells

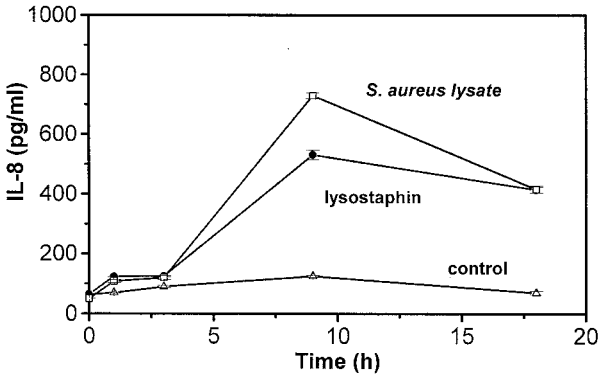


**Figure 2** Kinetics of IL-8 production in lysostaphin-treated and *S. aureus* lysate-treated NHEK cells. NHEK were treated with 300 mg/l recombinant lysostaphin and 800 mg/l *S. aureus* lysate, respectively. Data present mean ± SEM of IL-8 levels.

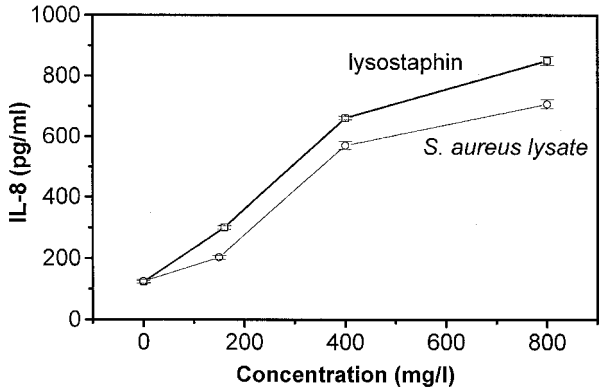


**Figure 3** Comparative response of IL-8 in NHEK cells to a 9-h exposure to recombinant lysostaphin and *S. aureus* lysate. Results are the mean ± SEM of more than six experiments.

showed levels that appeared significantly increased when compared to control group ($P < 0.015$). The induced levels of IL-8 were dose-dependent and peak values (850 pg/ml) were observed at purified recombinant lysostaphin concentrations of about 800 mg/l (Fig. 3). Under similar experimental conditions, *S. aureus* lysate (800 mg/l) stimulated rise in IL-8 level to peak values of about 700 pg/ml.

Lysostaphin was tested clinically at a wide range of doses and different routes of administration, and no significant adverse responses were reported (Schaffner *et al.*, 1967; Dixon *et al.*, 1968). However, if lysostaphin is used as an antimicrobial agent for staphylodermatitis infection, its cytotoxic and inflammatory effects on skin have to be closely examined.

In the present study, recombinant lysostaphin showed about 640 times less cytotoxic potential to the NHEK cells than was surfactant sodium laurate, as determined with the NR assay after a 48-h exposure. This result indicates that recombinant lysostaphin alone, concentraiton range from 100 to $10^5$ mg/l, shows almost no cytotoxicity evaluated with NHEK cells. However, *S. aureus* lysate, which was derived from the bacterial lytic action of lysostaphin on $10^7$ *S. aureus* cells/ml, was found about 8 times more cytotoxic potential to the NHEK cells than was lysostaphin itself. Even though the calculated $NR_{50}$ value of 1887 $\pm$ 20 mg/l for *S. aureus* lysate is considered as a less-cytotoxic agent, the side-effect of recombinant lysostaphin action should be noted, if it is used as an antimicrobial agent for staphylodermatitis infection.

In this study, results also indicated that NHEK cells were characterized by increased concentrations of IL-8 when treated with a similar amount of either lysostaphin or *S. aureus* lysate. The generation and secretion of IL-8 may provide a useful biomarker for determining skin irritants (Luster *et al.*, 1995). However, further determining the source, kinetics of production, and the regulation of inflammatory mediators in the skin will be of value in predicting various toxicities arising from exposure to recombinant lysostaphin or *S. aureus* lysate. IL-8 is thought to play an important role as an autocrine or paracrine growth factor regulating the characteristic keratinocyte hyperproliferation seen in skin disease such as psoriasis, although its mechanism is yet unclear. Overall, the finding reported here suggest that although lysostaphin alone or *S. aureus* lysate is not toxic to NHEK cells, they stimulate cellular L-8 production which may cause skin diseases.

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## References

Borenfreund, E, Babich, H and Martin-Alguacil, N (1990) *In Vitro Cell DevBiol* 26 :1930–1034.

Chan, EC (1996) *Biotechnol Lett* 18 :833–838.

Cullor, JS, Smith, WL and Selsted, ME (1990) *Proc Intl Symp on Bovine Mastitis* pp55.

Dixon, X, Goodman JS, and Koenig, MG (1968) *Yale J Biol Med* 41:62–67.

Ganz, T, Selsted, ME and Lehrer, RI (1990) *Eur K Haematol* 44 :1–5.

Gearing, AJH, Fincham, NJ, Bird, CR, Wadhwa, M, Meager, A, Cartwright, JE and Camp, RDR *Cytokine* 2 :68–75.

Gennaro, R, Skerlavaj, B and Romeo, D (1989) *Infect Immun* 57 :3142–3147.

Harris, RL, Nunnery, AW and Riley, HD (1967) *Antimicrob Agents Chemother* 7 :110–116.

Heinrich, P, Rowenstein, R, Bohmer, M and Sonner, P (1987) *Mol Gen Genet* 209 :563–569.

Luster, MI, Wilmer, JL, Germolec, DR, Spalding, J, Yoshida, T, Gaido, K, Simeonoma,PP, Burleson, FG and Bruccoleri, A (1995) *Toxicol Lett* 82 :471–476.

Martin, R and White, A (1967) *J Lab Clin Med* 70 :1–5.

Martin, R and White, A (1967) *J Lab Clin Med* 70 :1–8.

Martin, R and White, A (1968) *J Lab Clin Med* 71 :791–799.

Matsushima, K and Oppenheim, JJ (1989) *Cytokines* 1 :2–13.

Nickoloff, BJ, Griffiths, CEM and Barker, JNWN (1990) *J Invest Dermatol* 94: 151S–157S.

Quickel, KE, Selden, R, Caldwell, JR, Nora, NF and Schaffner, W (1971) *New Eng J Med* 291 :239–246.

Recsei, PA, Gruss, AD and Novick, RP (1987) *Proc Natl Acad Sci USA* 84 :1127–1131.

Rozengurt, E (1989) *Science* 234 :161–166.

Schindler, CA and Schuhardt, VT (1964) *Proc Natl Acac Sci USA* 51 :1127–1131.

Schaffner, W, Melly, MA and Koening, MG (1967) *Yale J Biol Med* 39 :230–336.

Westwick, J, Li, SWand Camp, RDR (1989) *Immunol Today* 10 :146–147.

Zygmunt, WA and Tavormina, PA (1972) *Prog Drug Res* 16 :309–314.

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