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The Antistaphylococcal Effect of Nisin in a Suitable Vehicle: A Potential Therapy for Atopic Dermatitis in Man

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Abstract

Staphylococcus aureus plays a central role in the pathogenesis of atopic dermatitis and is the predominant microorganism both in the lesions and in adjacent clinically normal skin. Chronic infection might aggravate the underlying lesion and serve as a source for further *S. aureus* infection. Nisin is a non-toxic and non-irritant peptide with no antibiotic-like side effects.

In this study the antistaphylococcal activity of nisin in six topical formulations was investigated in diffusion tests and is shown to depend both on the water content and on the technological system. Because topical products often adhere to the stratum corneum for only a short time, the kinetics of antimicrobial activity were examined using a membrane filter technique. Thirty minutes after nisin addition almost no living microorganisms were detectable in different aqueous samples.

The results demonstrate the potential of nisin preparations as an alternative to common antibiotics in the treatment of *S. aureus* infections in atopic dermatitis.

Members of the staphylococcal family of Gram-positive cocci are an essential component of the microbiological ecosystem of human skin (Williams & MacKie 1993; Morren et al 1994). In patients suffering from atopic dermatitis, however, the composition of the bacterial skin flora can be fundamentally altered where differences in sebum, sweat secretion and increased bacterial adhesion to epithelial cells in atopic eczema might lead to increased dermal *S. aureus* infections. It has been suggested that *S. aureus* might cause a direct inflammatory reaction in atopic dermatitis because protein A, a major *S. aureus* cell-wall component, causes an inflammatory response when applied to skin in which the stratum corneum has been removed or damaged. Lever et al (1988) demonstrated a significant reduction in staphylococcal colonization on use of topical mupirocin ointment, which is highly active against Gram-positive cocci, and correlated clinical atopic dermatitis improvement in apathogenesis after application of mupirocin in poly(ethylene glycol) base with a reduction in dermal staphylococcal colonization. The use of 2% mupirocin in white soft paraffin has also been reported to eradicate *S. aureus* in patients, and to result in slow recolonization rates (Williams & MacKie 1993).

Because prolonged use of antibiotics is complicated by the potential development of resistance and the possibility of systemic side-effects, the development and testing of other anti-microbial agents has grown in significance. Among the group of antibiotics (Jung 1991), nisin is known to have strong antibacterial activity against Gram-positive bacteria (Abee et al 1991). It has no allergic potency (Sears et al 1991) and has been demonstrated in a number of studies to be non-toxic (Molitor & Sahl 1991). In addition, the relatively high molecular mass of nisin (3400 Da) greatly reduces the risk of transdermal diffusion. Nisin, a lanthinonine-containing polypeptide from *Lactococcus lactis* is produced on a large scale and used mainly as a food preservative. Sahl (1991) suggested the energy-transducing cytoplasmic membrane as the primary target of *Staphlococcus stimulans* antimicrobicity, because nisin simultaneously blocks the biosynthesis of DNA, RNA, polysaccharides and proteins. Subsequent experiments with nisin and other lantibiotics demonstrated that all peptides induced a rapid efflux of ions and small molecules from the cytoplasma of various Gram-positive test bacteria, and that this coincided with a decrease in the trans-membrane potential. All the experimental evidence indicates that elongated cationic lantibiotics (type A lantibiotics) form potential-dependent pores in cytoplasma membranes, resulting in dissipation of vital ion gradients, loss of metabolites and eventually cell death.

The control of staphylococcal colonization is of considerable benefit in the treatment of atopic dermatitis. In this study the antistaphylococcal activity of nisin was investigated with regard to kinetics and the influence of different vehicles.

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Materials and Methods

Materials

Nisin (Ambicin N) was obtained from Aplin and Barrett (UK), unpreserved Ultrasicc was from Schering (Austria), hydroxyethylcellulose (Natrosol 250 MR) was from Aqualon (The Netherlands) and Unguentum Cordes was from Ichthyol (Germany). All other substances used were of analytical grade and were purchased from Sigma (St Louis, MO, USA).

Formulations

Only sterile formulations, checked by the standard sterility test of Pharmacopoea Europaea 1991, were used. Addition of nisin was performed aseptically under laminar air flow. Six topical

formulations containing various concentrations of nisin were prepared: 1. 4% hydroxyethylcellulose gel; 2. Ultrasicc; 3. Ung. Cordes-water (1 + 1); 4. Ung. Cordes-water (4 + 1); 5. Ung. Cordes; 6. 7% silicon dioxide-paraffin oil. Nisin was added to 1–5 from a 20 mg mL^{-1} stock solution in 0·1 M glycine-HCl buffer (pH 2·5) and undiluted to 6 because of the immiscibility of this vehicle with aqueous buffers.

The *n*-octanol-water and *n*-octanol-0·1 M glycine-HCl buffer (pH 2·5) partition coefficient (K_{oct}) was determined by adding 0·7–1·2 mg nisin to 4 mL of the biphasic system and equilibrating by shaking for 24 h. The concentration of nisin in the aqueous phase was measured photometrically at 240 nm (Perkin-Elmer Lambda 16) against reference standards comprising the biphasic system with no added nisin.

Diffusion test

A modified agar diffusion test (United States Pharmacopeia, 1990) was employed. Briefly, overnight cultures (OD_{600}: 0·2–0·3; 1 mL) of *Staph. aureus* (Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany; DSM 1104) were inoculated on to Petri dishes containing bacterial media B (Pharmacopoea Europaea, 1991). After 30-min pre-diffusion at room temperature, repeat samples (1 g) of each formulation (1–6) containing various nisin concentrations (5–600 $\mu g\ mL^{-1}$) were added as spots to each plate. After incubation at 37°C for 48 h antimicrobial activity was estimated by measuring the zone of reactivity around each specimen. All formulations containing nisin were stored at room temperature for 5 weeks after which the diffusion tests were repeated. For formulation 1 the diffusion test was also performed after 6 months storage.

Membrane filter technique

A test was established to investigate both the onset time of antimicrobial activity and the incubation time required to eliminate *S. aureus* activity completely. Overnight cultures were diluted in four steps to 1 : 10 000 with 0·1 M $MgSO_4$ at 4°C and then further diluted 1 : 10 with 0·1 M $MgSO_4$-glycerol (1 + 1). One-hundred-microlitre volumes of this bacterial suspension were vortex-mixed with a sterile solution containing 8·9 mL water and 1 mL Tween 80 (= 10 mL). Tween 80 was necessary to aid distribution on the membrane filter surface.

This solution (10 mL) was incubated for 1 min with nisin (0 and 10 $\mu g\ mL^{-1}$) added from the stock solution and the suspension was passed through a sterile membrane filter (nominal porosity 0·22 μm, diameter 50 mm). The filter was then transferred directly to test medium B and incubated at 37°C for 48 h after which the number of macroscopic colonies on the filter were counted. The effect of incubation time (1, 5, 10 and 30 min) on *S. aureus* survival was determined using 10 ng mL^{-1} nisin. All assays were performed in triplicate and compared with controls containing no nisin. Nisin adsorption on the filter was tested: 10 mL nisin solution (10 ng mL^{-1}) was filtered, the filter was washed with aqueous sodium chloride solution (0·9%; 100 mL), the organism was added, the filter washed with sterile water (100 mL) and incubated, and the colony-forming units counted. If solely the bacterial suspension was filtered, the counts were identical.

Results and Discussion

The antimicrobial action of nisin is pH-dependent and shows the highest inhibitory activities at pH values less than 5 (Liu & Hansen 1990). Although remarkably stable in acidic solutions, nisin is also considerably inactivated at pH > 7 owing to the formation of multimeric biologically inactive products (Liu & Hansen 1990), possibly representing aggregates of nisin (Garcera et al 1993). All formulations under test had pH values between 5 and 6·5, values which are also more compatible with the acidic environment of the stratum corneum. In the equilibrated biphasic *n*-octanol-water (*n*-octanol-glycine HCl buffer) system, nisin was only detected in the aqueous phase. ($K_{oct} < 1$). In atopic dermatitis patients, however, application of nisin in hydrophobic fat vehicles would be preferred because the dry lichenous eruptions characteristic of atopic dermatitis also contain a high density of *S. aureus*. The horny layer in atopic dermatitis patients is also affected by a significant impairment of sebaceous gland secretion (Wirth et al 1981) and often accompanying ichthyosis (Borelli & Schnyder 1962).

Transepidermal water loss is, furthermore, increased and, paradoxically, the water content of dry skin is increased as expected in areas of eczema (Finley et al 1980).

Because water-content and inhibition of transepidermal water loss are both important, six formulations with decreasing water content were tested. Antimicrobial activity was determined in the diffusion assay by measuring the zone around each specimen in which visible bacterial growth was inhibited (Fig. 1). The lowest concentration of nisin required to inhibit bacterial growth in each formulation is shown in Tables 1 and 2; with the exception of formulation 2, antistaphylococcal activity correlated with increasing water content: 1 > 3 > 2 > 4 > 5 > 6. The highest activity was obtained with formulations 1 and 3, 24 h after application of nisin (Table 1). No significant changes in antimicrobial activity were seen after 5-weeks storage of each nisin formulation (Table 2). A long-term stability test of preparation 1 for six months indicated no decrease in the antimicrobial activity of nisin. The dermatological treatment of atopic dermatitis necessitates the use of bases, such as formulation 5 and 6, that do not cause drying of the horny layer. Although no antimicrobial activity was observed for formulation 6, a concentration of 600 $\mu g\ g^{-1}$

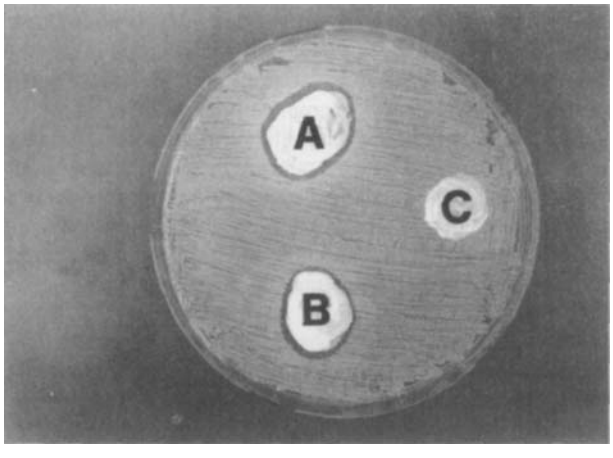

FIG. 1. Diffusion test of formulation 3: A: 400 $\mu g\ g^{-1}$ nisin; B: 200 $\mu g\ g^{-1}$ nisin; C: negative control.

Table 1. Results of diffusion tests one day after addition of nisin to the preparation: 0, zero reactivity, no detectable zone around specimen; 1, slight activity, thin zone around specimen; 2, mild activity, zone extends less than 0·5 cm; 3, high activity, zone extends more than 0·5 cm.

Formulation	Concentration of nisin ($\mu g\ g^{-1}$)	Effectiveness			
		3	2	1	0
1	5				+
	25			+	
	50		+		
	100	+			
	200	+			
	300	+			
2	100				+
	200			+	
	250		+		
	300		+		
	600	+			
3	50				+
	75		+		
	100	+			
	200	+			
	400	+			
4	200				+
	400			+	
	500			+	
	600		+		
5	400				+
	500				+
	600		+		
6	400				+
	500				+
	600				+

Table 2. Results of diffusion tests five weeks after addition of nisin to the preparation; 0, zero reactivity, no detectable zone around specimen; 1, slight activity, thin zone around specimen; 2, mild activity, zone extends less than 0·5 cm; 3, high activity; zone extends more than 0·5 cm.

Formulation	Concentration of nisin ($\mu g\ g^{-1}$)	Effectiveness			
		3	2	1	0
1	25				+
	50			+	
	100		+		
	200		+		
	300	+			
2	150				+
	300		+		
	600	+			
3	75				+
	100		+		
	200	+			
	400	+			
4	400				+
	500			+	
	600		+		
5	400				+
	500				+
	600		+		
6	400				+
	500				+
	600				+

nisin in 5 shows mild antistaphylococcal activity which can be increased by adding water: with 20% water (formulation 4) the effect against *S. aureus* is similar; if 50 % water is included, however (formulation 3), the antimicrobial effect is significantly increased. Efficient anti-staphylococcal activity is seen at 100 $\mu g\ g^{-1}$ nisin, which increases to 200 $\mu g\ g^{-1}$ nisin after 5 weeks storage.

Because topical preparations adhere for only a short time, the rapidity of onset of antimicrobial activity was determined. In a modification of a standard Pharmacopoea europaea membrane filter technique, aqueous test solutions were inoculated with 10^4–10^6 microorganisms (*S. aureus*) mL^{-1} and diluted so that in the final dilution 8–10 colony-forming units could be detected. As shown in Fig. 2, almost all microbial activity was eliminated by 30-min incubation at a nisin concentration of 10 ng mL^{-1}. Only aqueous solutions could be tested by the membrane filter technique, however, because rapid filtration of ointments was not possible.

In conclusion, formulations 1 (hydrogel) and 3 (Ung. Cordes-water) proved to be pharmaceutically the most effective vehicles for administration of nisin as an antibacterial agent. Formulation 3 has the added advantage that systems might be prepared with variable water content for the treatment of atopic dermatitis in which prevention of desiccation of the horny layer is important. In this respect, the antistaphylococcal activity of the non-irritant and non-toxic drug nisin might provide the basis for a novel treatment of atopic dermatitis.

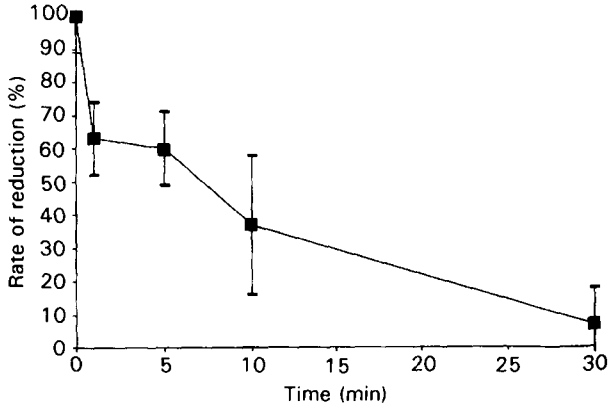

FIG. 2. Reduction in *Staph. aureus* as a function of time; nisin concentration 10 ng mL^{-1}; data incorporate the standard deviations of three experiments.

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