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Palmitoleic Acid Isomer (C16:1Δ6) in Human Skin Sebum Is Effective against Gram-Positive Bacteria

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Key Words
Antimicrobial agents · Natural preservatives · Palmitoleic acid · Sebum · Skin lipids

Abstract
The percent lipid composition of pooled human sebum analyzed by thin-layer chromatography was: ceramides (13%), fatty acid (47%), cholesterol (7%), cholesterol esters (2%), squalene (11%), triglycerides (3%), and wax esters (17%). Total sebum lipids (2–4 mg/ml), sonicated into bacterial culture medium, caused 4- to 5-fold log reduction in growth of gram-positive bacteria, *Staphylococcus aureus, Streptococcus salivarius* and the anaerobe *Fusobacterium nucleatum*, but was ineffective against most gram-negative bacteria. Fractionation of the sebum lipids showed that both saturated and unsaturated fatty acids contained the bulk of the antimicrobial activity. Lauric acid (C12:0) was the most active saturated fatty acid. The unsaturated fatty acid, palmitoleic acid (C16:1Δ6, cPA) was both the most predominant monoene and the most active antimicrobial fatty acid. Purified cPA (>99%) yielded typical minimal inhibitory concentration (MIC) values of 10–20 μg/ml against gram-positive bacteria. Organically synthesized cPA isomer gave MIC values comparable to the natural material. Both natural and synthetic cPA were found to be the most active sebum lipid fraction in blocking the adherence of a pathogenic strain of *Candida albicans* to porcine stratum corneum. Ethanol in combination with cPA exerts a synergistic bactericidal activity against gram-negative pathogenic bacteria, including *Pseudomonas aeruginosa, Propionibacterium acnes, Escherichia coli*, and several methacillin-resistant strains of *S. aureus*. Palmitoleic acid may be useful in topical formulations for treatment of secondary gram-positive bacterial infections, as a gram-positive bacteria antimicrobial in wound dressings, and as a natural gram-positive antimicrobial preservative in skin and hair care products.

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Introduction

It has been know since the 1940s that sebum lipids impart a self-disinfecting activity to the skin surface [1, 2], and it has been accepted that free fatty acids are responsible for this property [2–4]. However, relatively little direct evidence has been published to support this claim or identify which fatty acids may be responsible.

The purpose of the present investigation was to compare different lipid classes from the skin surface and to identify the most potent antimicrobial components. In addition, an in vitro porcine stratum corneum model [5] was used to determine which of the sebum lipid classes was most active in inhibiting adherence of the yeast *Candida albicans* to delipidized stratum corneum. It was shown that the C16:1Δ6 isomer of palmitoleic acid (cPA) was the most active anti-bactericidal fatty acid component of human sebum. cPA was organically synthesized to confirm its activity compared to the native material. Additionally, studies were conducted to improve its spectrum of activity against gram-negative bacteria by formulating it with an alkyl alcohol as a co-solvent. A synergistic and broad spectrum bactericidal activity of cPA, when presented in combination with non-lethal amounts of ethanol (<15%), was demonstrated. By contrast, no such synergistic effect could be demonstrated for the gram-positive active topical antimicrobial agent, mupirocin.

Previous studies have used exfoliated epidermal or buccal keratinocytes in vitro for examination of the attachment of *C. albicans* to epithelial cells [6–9]. However, such cells may have been modified through the process of desquamation and this may have altered properties important for microbial attachment. For this reason, we have employed an in vitro model [5] to study the influence of surface lipids on the attachment of yeast cells to pig stratum corneum. Here we report our findings, showing that among the sebum fatty acids cPA was the most effective in preventing the adhesion of *C. albicans* to the surface layer of skin.

Materials and Methods

Chemicals

Synthetic palmitoleic acid (C16:1Δ6) and synthetic oleic acid (C18:1Δ8) were both obtained from Matreya, Inc., Pleasant Gap, Pa., USA. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., USA.

Lipid Fractionation

Lipids were collected by extraction of the skin surface or hair clippings by using chloroform:methanol (2:1) for 2 h. The lipids were fractionated by preparative silica gel thin-layer chromatography as previously described [5]. The fractions collected correspond to squalene, cholesterol esters plus wax esters, triglycerides, fatty acids, cholesterol, and a polar fraction consisting mainly of ceramides and cholesterol sulfate. The free fatty acids were subsequently fractionated into saturated and monosaturated fatty acids by argentation chromatography, and C16:1Δ6 and C18:1Δ8 were isolated from the monoene fraction by reverse phase thin-layer chromatography. Fatty acids used in the microbiological assays included commercially available saturated fatty acids of the type found in human sebum, including straight chain saturates and some methyl branched acids, C16:1Δ6, C18:1Δ8, C16:1Δ9, and C18:1Δ9.

Synthesis of 6-Z-Hexadecenoic Acid

Synthesis of cis-palmitoleic acid (6-Z-hexadecenoic acid) was performed by Matreya Corp., Pleasant Gap, Pa., USA. In *The Lipid Handbook* by Gunstone [10], C16:1Δ6 acid has been listed as isolated from natural oils, but no synthesis is listed. The C18:1Δ8 acid is listed as having been isolated from margarine, but never synthesized. Synthesis was accomplished in a five-step procedure, a detailed account of which is given elsewhere [11]. Briefly, in the first step, a chloro-compound is reacted with sodium salt of undecyne from Lancaster (Windham, N.H.). In the second step, the chloro-compound is reacted with cyanide in dimethylsulfoxide. In the third step, the nitrile is transformed to

Table 1. Sebum composition

	Gland	Surface	Hair
Polars (ceramides)	0	0	13
Cholesterol	1	1	7
Fatty acids	0	15	47
Triglycerides	57	42	3
Wax esters	25	25	17
Cholesterol esters	2	2	2
Squalene	15	15	11

All figures are weight percent and computed from the thin-layer chromatography data of Nicolaides [27] and Wertz (pers. commun.).

methyl ester by HCl in methanol, followed by saponification of the ester at low temperature. In the final step, reduction of the triple bond to the (Z) double bond is catalyzed by Palladium on barium sulfate (5%, from Aldrich, Milwaukee, Wisc.) using pyridine as solvent. The final product was purified by thin-layer chromatography.

Microbiology

The bacteria *Staphylococcus aureus, Streptococcus salivarius, Pseudomonas aeruginosa, Escherichia coli*, and *Propionibacterium acnes* were cultured in Brain Heart Infusion Broth (BHIB). *Fusobacterium nucleatum* was cultured in Schaedler's broth. *S. aureus* and *S. salivarius* were incubated statically in an aerobic incubator at 37°C. *P. acnes* and *F. nucelatum* were incubated anaerobically (5% CO_2, 10% H_2, 85% N_2) at 37°C. *P. aeruginosa* and *E. coli* were incubated in a gyratory water bath at 37°.

Bactericidal Assays

Cells were harvested by centrifugation and resuspended in their respective media. Suspensions were normalized to specific concentrations, based on the type of experiment conducted. Inhibitors were added to bacterial suspensions at concentrations indicated in the figure legends and text. Control suspensions received sterile distilled water and were processed in an identical manner. In some experiments, bacteria were preincubated with inhibitors prior to exposure to other compounds. Suspensions were incubated statically under appropriate atmospheric conditions for each test organism. At specific increments of time, samples were than plated on BHIB agar or Schaedler's agar using a spiral-plating system. Numbers of viable bacteria were determined following standard spiral-plating methodology [12]. Data obtained were transformed to $\log_{10}$ values prior to statistical analyses and graphing. For minimal inhibitory concentration (MIC) assays, lipids were suspended in culture medium and serial two-fold dilutions made in duplicate into test tubes, and inoculated with the test organism, incubated for 48 h and the optical density (OD 600) read in a spectrophotometer set up for turbidity measure. MIC is equal to the lowest concentration at which Δ OD 600 is <0.05.

Results

In a series of preliminary studies, we repeated and extended Burtenshaw's [1] original observation that human sebum sonicated into bacterial culture medium in milligram per milliliter amounts effectively reduced the viability of *S. aureus* and *S. salivarius* by 4–5 log CFUs, but was ineffective against most gram-negative bacteria, including *E. coli, P. aeruginosa* and *Echinococcus faecalis.* By contrast, as little as 0.1 mg/ml of sebaceous lipids was effective in killing the gram-negative oral anaerobe *F. nucleatum*. Lipids isolated from buccal epithelium, which lack free fatty acid, was entirely without effect on any of the above bacteria (data not shown).

Table 1 presents results comparing the lipid composition of sebum from skin surface against hair clippings and lipid from sebaceous glands. The results show the high fatty acid content of hair clippings. This may be derived from bacterial hydrolysis of sebaceous triglycerides or represent free fatty acids produced directly by epithelial sebaceous gland cells. The percent lipid composition of stratum corneum lipids is quite different from sebum: ceramides (47%), cholesterol (25%), fatty acids (15%), cholesterol esters (10%) and cholesterol sulfate (3%).

The bactericidal activity of sebum-derived lipid fractions was evaluated. As shown in fig-

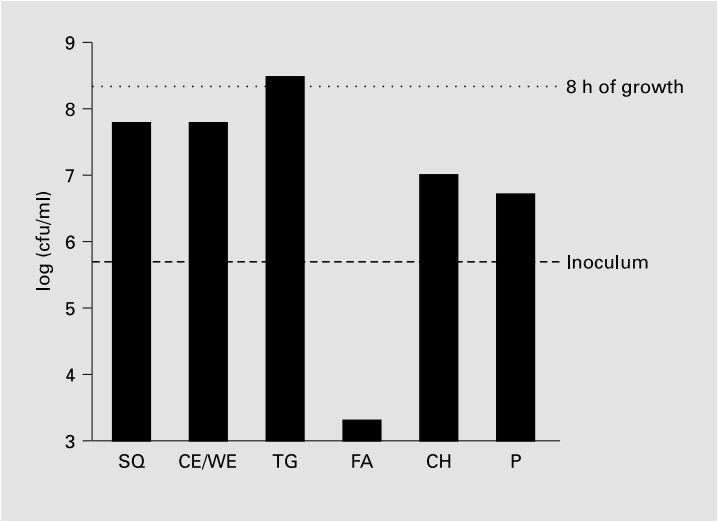


Fig. 1. Bactericidal activity of various lipid components fractionated from total human sebum. The bottom line displays the original inoculum size (log 5.8 cfu/ml), the top line the final growth size of the positive control (log 8.1 cfu/ml). SQ = squalene; CE/WE = cholesterol esters plus wax esters; TG = triglycerides; FA = fatty acid; CH = cholesterol; P = polar lipids. Each lipid was dispersed in culture medium at 4 mg/ml by sonication.

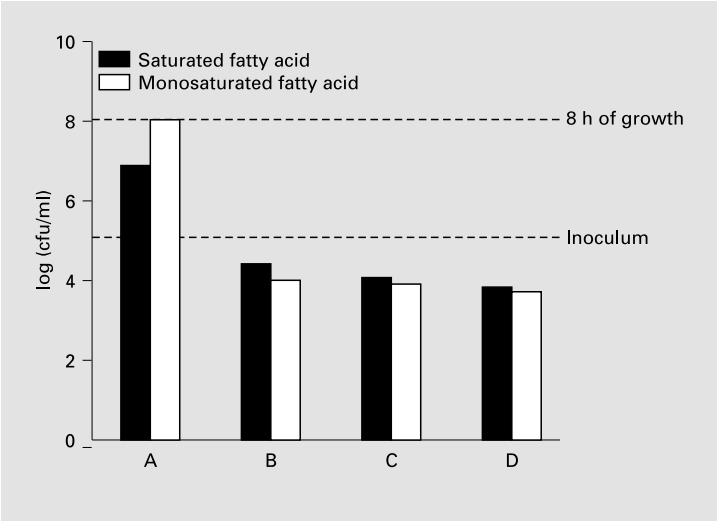


Fig. 2. Comparison of bactericidal activity of different amounts of saturated versus monosaturated fatty acids. A = 0.04 mg; B = 0.19 mg; C = 0.75 mg; D = 3.2 mg.

ure 1, only the fatty acid fraction displays bactericidal activity against *S. aureus*. The fatty acid fraction was further fractionated into saturates and monounsaturates. Figure 2 shows that both fractions were equally effective against *S. aureus*. Figure 3 shows that lauric acid (C12:0) was the most active of the saturated fatty acids, with decreasing bactericidal activity with either shorter or longer alkyl chain length. Figure 4a presents a typical gas-liquid chromatography (GLC) chromatograph of crude fatty acids derivatives (FAME) sample prepared from sebum fatty acids. The predominant saturated fatty acid was palmitic acid (C16:0) and the predominant monoene was palmitoleic acid (C16:1Δ6); the next most abundant monoene was an unusual oleic acid isomer (C18:1Δ8). Palmitoleic acid was iso-

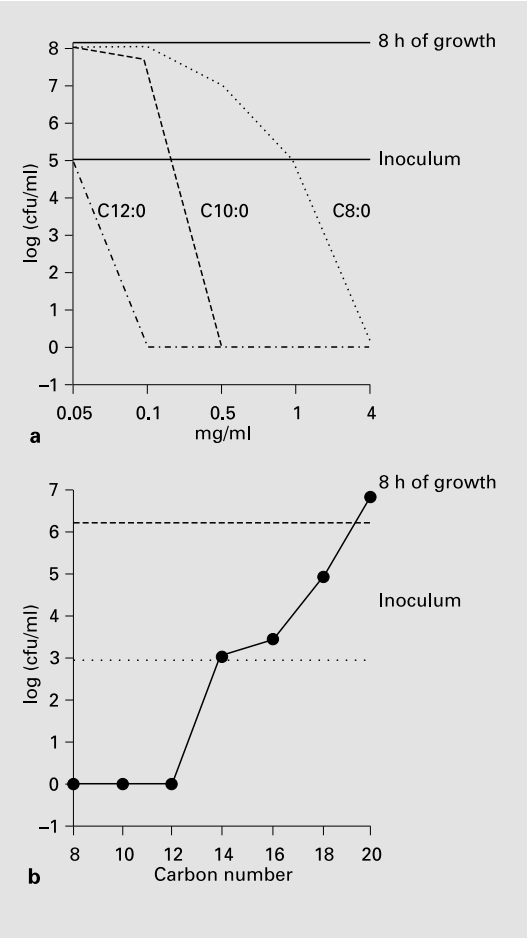


Fig. 3. a Comparison of bactericidal activity of lower- versus medium-chain length alkyl saturated fatty acid. Each fatty acid was dispersed in culture medium at 4 mg/ml by sonication. C12:0 = lauric acid; C10:0 = capric acid; C8:0 = caprylic acid. b Comparison of bactericidal activity of lower alkyl saturated fatty acids.

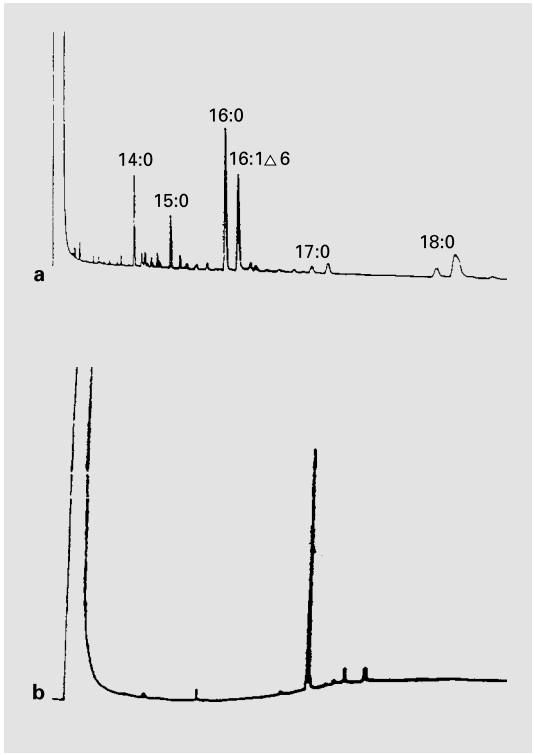


Fig. 4. a GLC of a crude mixture of methylated fatty acid fraction of human sebum. The labeled peaks above the baseline indicate the presence of both saturated and monounsaturated fatty acid. b GLC profile of FAME sample prepared from isolated and purified palmitoleic acid (C16:1Δ6).

lated from the monoene fraction by argentation TLC, followed by reverse phase TLC. Its purity was >95% as checked by GLC (fig. 4b).

Table 2 summarizes the results of a series of MIC assays. Both cPA isomers (C16:1Δ6 and C16:1Δ9) were equally effective against *S. aureus* (0.03 mg/ml) and *S. salivarius* (0.05 mg/ml). Neither isomer was effective against *E. coli* or *C. albicans*. In a separate study, a methyl-branched derivative, 15-methylpalmitoleic acid (2.54 mg/ml) was as effective as palmitoleic acid (2.64 mg/ml) in producing a 3–4 log reduction in the growth of *S. salivarius*, while 3 times as much cholesterol sulfate (6.34 mg/ml) was totally ineffective.

Table 3 presents the results of experiments comparing the MIC values of palmitoleic acid isomers (C16:1Δ6 and C16:1Δ9), the ammo-

nium salt of C16:1Δ6, and mupirocin, a topical antibiotic against a number of gram-positive and gram-negative bacteria. As expected, mupirocin was 30 times more effective than any of the lipids tested against either *S. aureus* or *Streptococcus pyogenes*. By contrast, the palmitoleic acid isomers were more effective against an isolate of *Corynebacterium* sp., isolated from a human wound. Neither the palmitoleic acid isomers nor the ammonium salt, nor mupirocin were effective against several different gram-negative bacteria, including *Enterobacter aerogenes, Klebsiella pneumoniae* and *P. acnes*.

It was expected that palmitoleic acid would be just as effective against clinical isolates of *S. aureus* on the hypothesis that it would have a surfactant effect on bacterial membranes. Figure 5 shows that palmitoleic acid (100 μg/ml) and mupirocin (100 μg/ml) were without effect. By contrast, exposure of this strain of *S. aureus* to the combination of palmitoleic acid plus 15% ethanol for a minimum of 15 min was highly effective (7 log kill). Similar exposure of cells to 15% ethanol had no effect.

The synergistic effect of palmitoleic acid with ethanol was further confirmed in additional experiments in which a 5-min exposure of another independent strain of MRSA to the combination resulted in a 8-fold log kill (fig. 6). By contrast, mupirocin had little activity, and ethanol and cPA alone required at least a 30-min exposure to achieve a two-fold

Table 2. MIC values for the C16:1Δ6 and C16:1Δ9 isomers of palmitoleic acid against several microorganisms

Microorganism	MICs, mg/ml	
	C16:1Δ6	C16:1Δ9
Staphylococcus aureus	0.03	0.03
Streptococcus salivarius	0.05	0.05
Escherichia coli	>0.5	>0.5
Candida albicans	>0.5	>0.5

Table 3. Microbiological evaluation of the ammonium salt and isomers of palmitoleic acid versus mupirocin

Bacteria	Palmitoleic acid isomers		Mupirocin
	acid C16:1Δ6/C16:1Δ9	ammonium salt (C16:1Δ6)NH_4	
Gram-positive bacteria			
Staphylococcus aureus	31.3/7.8	31.3	0.5
Streptococcus pyogenes	7.7/7.8	7.8	0.25
Corynebacterium sp. (wound isolate)	15.6/15.6	15.6	>250
Gram-negative bacteria			
Enterobacter aerogenes	>250/>250	>250	>125
Klebsiella pneumoniae	>250/>250	>125	>125
Propiobacterium acnes	>125	>125	>125

The results are MICS (μg/ml).

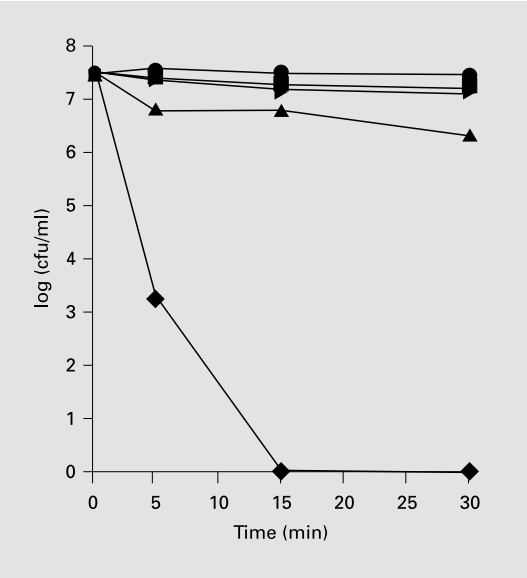


Fig. 5. Kinetic of bactericidal action of a clinical isolate of *S. aureus* of palmitoleic acid (100 µg/ml) in combination with 15% ethanol (♦), mupirocin alone (100 µg/ml, ■), palmitoleic acid alone (100 µg/ml, ▲), ethanol alone (15%, ▼) and control (●).

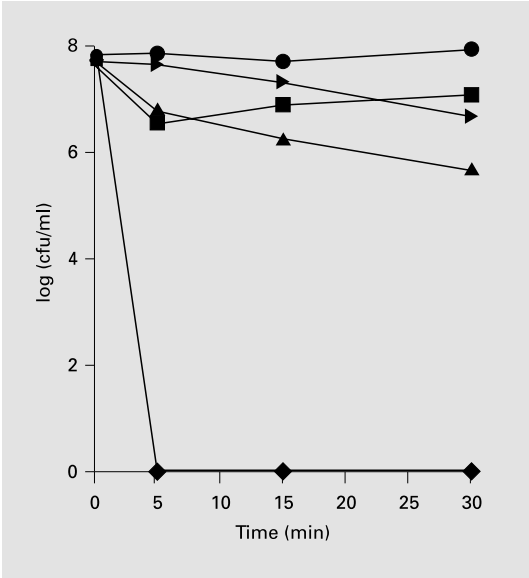


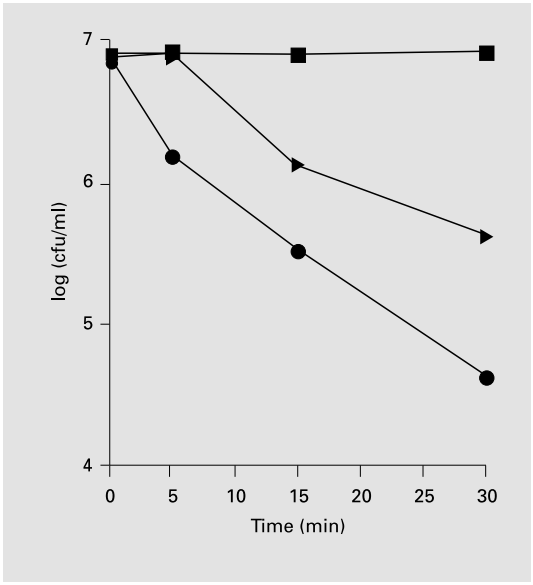
Fig. 6. Kinetics of bactericidal action on a methacillin-resistant strain of *S. aureus* of a combination of the ammonium salt of palmitoleic acid (100 µg/ml) and ethanol (15%) (♦), mupirocin alone (100 µg/ml, ■), ammonium salt of palmitoleic acid (100 µg/ml, ▲), 15% ethanol alone (▼) and control (●).

log kill. A similar result to this was obtained with another independent MRSA strain (data not shown).

Of greater significance was the finding that the combination of palmitoleic acid with ethanol conferred a synergistic killing activity versus a number of pathogenic gram-negative bacteria. Figure 7 shows that a 30-min exposure to the combination of cPA (100 µg/ml) plus ethanol (15%) was effective against *P. aeruginosa*. Neither cPA nor mupirocin alone were effective, while 15% ethanol alone was only partially effective. Figure 8 presents the results of a kinetic experiment showing that the combination of cPA (100 µg/ml) plus ethanol (15%) yielded a 3 log reduction in viable *P. acnes.* Again, neither mupirocin nor cPA, nor ethanol by itself was effective.

The next question addressed was whether a short pre-exposure of cells to ethanol would enhance the bactericidal activity of cPA. Figure 9 shows that as little as either 2 or 5 min of pre-exposure of an MRSA strain was sufficient to greatly enhance the bactericidal activity of cPA. By contrast, such short exposures had no effect on the ability of mupirocin to kill this MRSA strain.

The sebaceous lipid fatty acid fraction was further fractionated into saturated and unsaturated fatty acid. As shown above, GC analysis of FAME demonstrated the presence predominately of C14–C18 alkyl chain length saturated fatty acids, as well as two unusual and predominate monoene unsaturated fatty acids, palmitoleic acid (C16:1Δ6) and oleic acid (C18:1Δ8). Preliminary adhesion experi-



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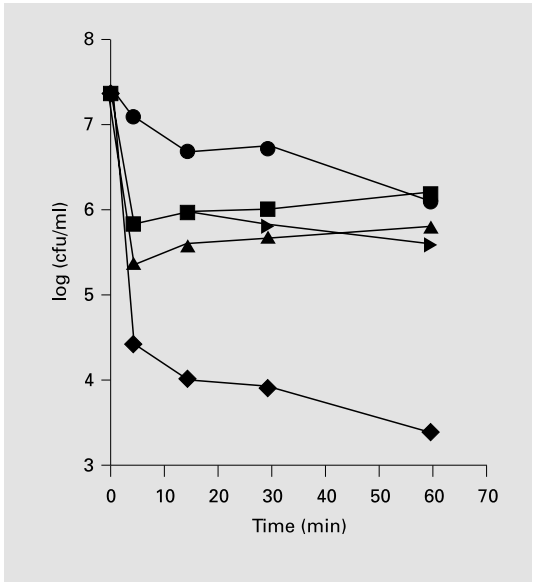

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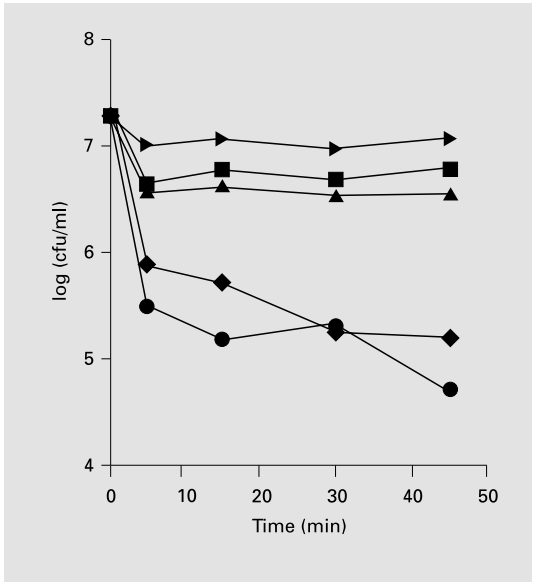
Fig. 7. Kinetics of bactericidal action on *P. aeruginosa* of palmitoleic acid (100 µg/ml) plus 15% ethanol (●), mupirocin alone (100 µg/ml, ◆, same as control values), palmitoelic acid alone (100 µg/ml, ▲, same as control values), 15% ethanol alone (▼) and control (■).

Fig. 8. Kinetics of bactericidal action on *P. acnes* of palmitoleic acid (100 µg/ml) plus 15% ethanol (◆), mupirocin alone (100 µg/ml, ■), palmitoleic acid alone (100 µg/ml, ▲), 15% ethanol alone (▼) and control (●).

Fig. 9. Susceptibility of MRSA pre-exposed to mupirocin versus palmitoleic acid. Mupirocin (100 µg/ml, 5 min ethanol exposure, ■), mupirocin (100 µg/ml, 2 min ethanol exposure, ▲), palmitoleic acid (100 µg/ml, 5 min ethanol exposure, ●), palmitoleic acid (100 µg/ml, 2 min ethanol exposure, ◆) and control (5 min distilled water exposure, ▼).



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ments indicated that both the saturated and unsaturated fatty acid fractions gave approximately equivalent inhibitory activity on the adherence of *Candida* to the stratum corneum. In order to determine the most active monoene species, the unsaturated fatty acid fraction was further subfractionated by argentation TLC, followed by reverse phase TLC. The C16:1Δ6 fraction was judged to be pure by GLC. Insufficient amounts of the fractionated oleic acid isomer were available for further testing.

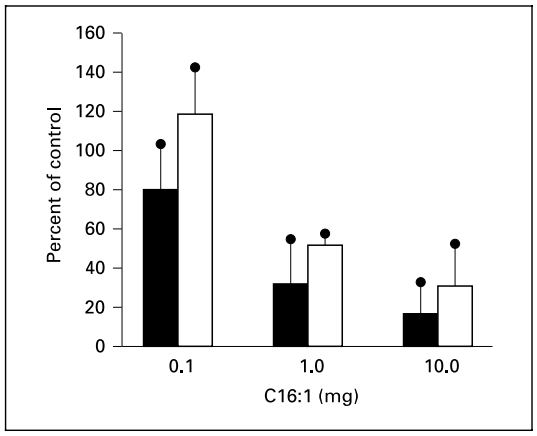

Fig. 10. Effect of C16:1 isomers on adherence of *C. albicans* to stratum corneum. ■ = C16:1Δ6; □ = C16:1Δ9.

Figure 10 compares the effect of increasing amounts of two C16:1 isomers on the inhibition of *Candida* adhesion to the stratum corneum. Both the C16:1Δ6 and C16:1Δ9 isomers were strongly inhibitory at concentrations equal to or above 1.0 mg of applied lipid per 1-cm-diameter stratum corneum disc. Both synthetic palmitoleic acid (C16:1Δ6) and synthetic oleic acid isomer (C18:1Δ8) gave similar results.

Discussion

The present results demonstrate that free fatty acids in human sebum do account for the self-disinfecting activity at the skin surface, and that cPA isomer accounts for most of this activity. The notion that free fatty acid of the stratum corneum provide a protective 'acid mantle' has received support from studies showing that inhibition of fatty acid synthesis from skin phospholipids interferes with barrier homeostasis and stratum corneum integrity [13].

The origin of free fatty acid on the skin surface is a subject of recent studies. In the past it was ascribed to the action of resident skin bacteria. In agreement with this idea, we report a low value for gland-derived lipids [27] versus our relatively high value (47% of total lipids) reported here for hair clippings. Recent studies employing cyanoacrylate follicular cast, derived from patients treated with a variety of topical antimicrobial agents, showed a relative decrease in free fatty acids combined with an increase in triglycerides [14], supporting the hypothesis of lipolysis due to microbial colonization. On the other hand, isolated human sebocytes synthesize their own fatty acid without the influence of bacteria [15–17]. It is likely that some proportion of sebum fatty acids is gland derived and some formed by bacterial degradation of sebaceous triglycerides.

It is pertinent to this report that C16:1Δ6 isomer of palmitoleic acid was reported by Nicolaides [27] to be an exclusive lipid of sebum. This makes it likely that the antibacterial activity of sebum is an intrinsic property of sebaceous glands, to be added to the several other detected sebaceous gland properties, including the storage/provider compartment of the antioxidant, vitamin E [18], and the site for storage/production of skin androgens [19].

Our results showed that all of the gram-positive bacteria tested were exceedingly susceptible to the total sebum samples with significant (>4 orders of magnitude) decrease in numbers of viable cells. However, none of the gram-negative bacteria tested were affected by these concentrations of total sebum samples.

Fractionation of skin surface lipids revealed that both the saturates and monounsaturates had about equal antimicrobial activity. The literature is replete with evidence that the lower alkyl straight chain fatty acids are

active in killing dermatophytes and gram-positive bacteria [20–24]. We confirm here that lauric acid is the most potent of these saturated fatty acids. It should be noted in passing that lauric acid is a minor component, as seen in the GLC chromatograph of the methylated fatty acid from sebum. The predominant saturated fatty acid recovered from human hair clippings is palmitic acid (C16:0). In fact, lauric acid at concentrations above 1% irritates skin [25] and, as a consequence, is not useful as a topical antimicrobial, except in short-term acting germicidal soaps [26]. It was surprising to find that palmitoleic acid was the most predominant monoene fatty acid. Nicholaides [27] reported the presence of the C16:1 family of fatty acids in chromatographic analyses of human skin surface lipids, but he did not report that they were responsible for the antimicrobial activity of sebum. Although Burtenshaw [1] was the first to report the self-disinfecting activity of sebum fatty acids, he failed to identify the active components.

In previous microbiological growth and bactericidal assays, palmitoleic acid (C16:1Δ9) was reported to be a potent inhibitor of gram-positive bacteria [28], but no connection was shown between these findings and the antimicrobial activity of human sebum. Recently, there has been renewed interest in the antimicrobial activity of lipid components of skin lipids [29, 30]. In particular, sphingosine, a building block of stratum corneum ceramides, has been identified as a potent and broad spectrum antimicrobial [31]. Free sphingosine is present in human stratum corneum lipids [32], but it is not likely to be freely available on the surface of skin because it combines readily with cholesterol esters [33].

Other studies have shown that various short- and medium-chain free fatty acids and their corresponding monoacyl glycerol esters have antibacterial activity in vitro against primarily gram-positive bacteria [34–41]. Recently, it was reported that certain medium-chain monoacyl glycerol esters and FFAs were bactericidal for *Helicobacter pylori* [42], and older literature reported that oleic acid had virucidal activity for membrane-enveloped mammalian viruses [43]. In addition, n-docosanol, a 22-carbon saturated fatty alcohol, has been reported to have antiviral activity against lipid-enveloped viruses, including herpes simplex viruses [44].

While we found that cPA acid at concentrations below 0.5 mg/ml had little effect on the growth of *C. albicans,* at concentrations above 0.1 mg/ml it is effective in preventing the attachment of pathogenic yeast cells to isolated sheets of mammalian stratum corneum. In this regard, cPA may be a potential prophylactic treatment to prevent some types of yeast infections. cPA or its zinc salt share similar therapeutic activities with the monoene fatty acid, undecylenic acid (C11:1Δ10; found in sweat) and the active agent in monographed OTC topical anti-fungal formulations [45, 46]. Among the potential applications for cPA inhibition of *Candida* adhesion to skin are inclusion in a medical adhesive to prevent *Candida* infection of wounds, and catheter coatings. An important advantage of cPA may be that it is a natural gram-positive bacteria antimicrobial of the skin.

Acknowledgments

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