CMLS, Cell. Mol. Life Sci. 62 (2005) 2390–2399
1420-682X/05/202390-10
DOI 10.1007/s00018-005-5260-7

CMLS Cellular and Molecular Life Sciences

Research Article

Vernix caseosa as a multi-component defence system based on polypeptides, lipids and their interactions

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Received 14 June 2005; received after revision 1 August 2005; accepted 25 August 2005
Online First 22 September 2005

Abstract. *Vernix caseosa* is a white cream-like substance that covers the skin of the foetus and the newborn baby. Recently, we discovered antimicrobial peptides/proteins such as LL-37 in vernix, suggesting host defence functions of vernix. In a proteomic approach, we have continued to characterize proteins in vernix and have identified 20 proteins, plus additional variant forms. The novel proteins identified, considered to be involved in host defence, are cystatin A, UGRP-1, and calgranulin A, B and C. These proteins add protective functions to vernix such as antifungal activity, opsonizing capacity, protease inhibition and parasite inactivation. The composition of the lipids in vernix has also been characterized and among these compounds the free fatty acids were found to exhibit antimicrobial activity. Interestingly, the vernix lipids enhance the antimicrobial activity of LL-37 in vitro, indicating interactions between lipids and antimicrobial peptides in vernix. In conclusion, vernix is a balanced cream of compounds involved in host defence, protecting the foetus and newborn against infection.

Key words. Antimicrobial activity; innate immunity; neonates; protein fingerprinting; colonization; lipids.

Intra-amniotic infections are believed to be a trigger for miscarriage and preterm labour [1, 2]. The ability of the immunologically naïve foetus to avoid infections indicates strong innate defence mechanisms that offer physiological protection before adaptive immune responses are developed. During the last trimester of pregnancy, a protective biofilm, *vernix caseosa* (vernix), is formed on the foetal skin. Vernix is a creamy white deposit derived from the stratum corneum, sebaceous glands and remnants of the epitrichium. The composition of vernix is mainly water (80.5%), lipids (10.3%) and proteins (9.1%) [3]. There has been a debate for decades, whether vernix, together with the amniotic fluid, acts as a mechanical and chemical barrier that protects foetuses against infectious pathogens [4, 5]. However, the nature of the potentially active vernix compounds has been unknown.

Recently, a number of antimicrobial peptides and proteins have been identified in vernix [6–8]. Antimicrobial peptides and proteins are considered to be significant in

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the first line of host defence in organisms ranging from plants to mammals [9]. The main families of mammalian antimicrobial peptides, the cathelicidins and the defensins [10, 11], are represented in vernix together with the well-known defence proteins lysozyme, lactoferrin, secretory leukocyte protease inhibitor (SLPI) and psoriasin [6, 7]. Furthermore, vernix is rich in lipids derived from the sebaceous glands. The microbicidal effects of a variety of natural lipids have been extensively studied in recent years. A number of free fatty acids and their 1-monoacylglycerols have been found to kill enveloped viruses and various bacteria in vitro [12–15]. Like antimicrobial peptides, these active lipids affect cellular plasma membranes [12, 16] and are widespread as defence effectors in tissues exposed to microbes [9, 17–19]. In addition, breast milk has been shown to contain both antimicrobial lipids and peptides [18, 20–22].

The microenvironment, including ionic strength, ionic composition and pH, has considerable influence on the activity of different antimicrobial peptides [23, 24]. Furthermore, synergy has been demonstrated between antimicrobial peptides and proteins, e.g. between the human cathelicidin LL-37 and lactoferrin [25] and between specific antimicrobial peptides [26, 27]. On the other hand, surfactants partly block the microbicidal activity of LL-37 [28].

In this study, we identified a variety of protein and lipid components of vernix, including important components in host defence. In addition, the combined bactericidal activity of LL-37 and the lipids of vernix was examined, indicating a cooperative activity. Finally, we investigated the frequency of microbial colonization of babies at the time of birth, and analysed the individual variation of the antimicrobial activity found in vernix.

Material and methods

Collection of vernix caseosa. Vernix caseosa was collected from the skin of newborns shortly after delivery, before any washing of the neonates. The vernix samples were placed in sterile plastic containers and were immediately frozen and stored at –20 °C until analysed. All newborns were without prenatal or perinatal complications, and without clinical signs of infection.

Peptide/protein extraction. The vernix samples were homogenized in 60% acetonitrile containing 1% (v/v) trifluoroacetic acid (TFA) using a POLYTRON homogenizer (Kinematica, Littau, Switzerland), and were extracted during shaking overnight at 4 °C. After centrifugation of the extracts at 10,000 g, the supernatants were lyophilized. The lyophilized material was dissolved in 0.1% TFA and loaded onto OASIS HLB cartridges (Waters, Milford, Mass.), which had been activated with acetonitrile and equilibrated in 0.1% aqueous TFA. After the weakly bound material had been washed away with 0.1% aqueous TFA and 10% acetonitrile in 0.1% TFA, bound proteins were eluted with 80% acetonitrile in 0.1% TFA and the eluate was lyophilized.

Identification of proteins. Samples were separated and analysed by SDS-PAGE in 10–20% tricine gels (Invitrogen, Carlsbad, Calif.) at 125 V for 90 min. Protein bands were visualized by silver staining using Silver Xpress (Invitrogen) according to the manufacturer's instructions. In further SDS-PAGE runs, proteins/peptides were transferred from the tricine gel onto a PVDF membrane for 70 min at 25 V. Proteins on the membranes were visualized after staining for 4 min with 0.1% Coomassie brilliant blue R-250 in 45% methanol/0.35 M acetic acid, destaining for 5 min in 90% methanol/0.35 M acetic acid, and finally washed in water. The stained protein bands were cut out from the membrane, and N- and C-terminal sequences were determined chemically, utilizing PE-ABI Procise 494 cLC or 5HT 494 protein sequencers and an ABI 494C instrument (Applied Biosystems, Foster City, Calif.).

In-gel digestion and protein fingerprinting. After electrophoresis, the tricine gels were fixed for 3 x 30 min in 2% phosphoric acid in 30% ethanol, washed for 3 x 20 min in 2% phosphoric acid, and equilibrated for 30 min in equilibration buffer (2% phosphoric acid/5% aluminium sulphate/10% ethanol). The gels were then stained for 2 h in 0.01% Coomassie brilliant blue G-250 in equilibration buffer, and destained for 2 x 1 min in water. Protein bands were excised manually from the Coomassie-stained gels. The gel pieces were digested with trypsin using a MassPREP robotic protein handling system (Micromass/Waters), employing a protocol described previously [29]. Tryptic fragments were analysed by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS; Voyager DE-Pro, Applied Biosystems), using α-cyano-4-hydroxycinnamic acid as matrix (saturated in 70% acetonitrile) and mixed 1:1 (v/v) with the sample. Database searches were carried out utilizing the ProteinProspector MS-Fit program (http://prospector.ucsf.edu/). Peptides in chromatographic fractions were analysed for their molecular masses by MALDI MS in the same manner as above.

High-performance liquid chromatography of vernix extracts. Extracts were separated by reversed-phase (RP) chromatography on an ÄKTA purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden) with an RPC Source 4.6/150 (Amersham Pharmacia Biotech) column, equilibrated in 0.1% aqueous TFA. Elution was performed at a flow rate of 1 ml/min, and a gradient of acetonitrile from 0% to 15% in 5 min, 15% to 60% in 42

min, and 60% to 80% in 12 min was employed. The effluent was monitored at 214 and 280 nm. Fractions were lyophilized and redissolved in 30 µl 0.1% TFA before further analyses.

Microbial strains and culture conditions. *Bacillus megaterium* strain Bm11, *Escherichia coli* strain D21, group B *Streptococcus* (GBS), a clinical isolate verified by Phadebact Strep B test (Boule Diagnostic, Huddinge, Sweden) and *Candida albicans* (ATCC 14053) were utilized in a screening assay to monitor the antimicrobial activity of the vernix extracts. Prior to each experiment, microbial cells were seeded from frozen stocks (–70 °C) onto agar plates, in Luria Bertani (LB) medium (Gibco-BRL, Life Technologies, Paisley, U. K.) for the bacterial strains, and in YM medium (Difco Laboratories, Detroit, Mich.) for the yeast strain. The agar plates were incubated overnight at 37 °C. Bacterial colonies were then picked from the plates and suspended in LB broth, while yeast cells were suspended in YM broth, and both types of culture were incubated at 37 °C under shaking, until the desired cell density was reached.

Inhibition zone assay. Thin plates (1 mm) of 1% agarose containing 6 x 10^4 bacterial cells/ml were prepared in LB medium supplemented with medium E [30]. The assay for *C. albicans* was performed in the same manner except that YM medium was used. Wells, 3 mm in diameter, were punched in the agarose layer, and samples in 3 µl 0.1% TFA were loaded into each well. After overnight incubation at 37 °C for GBS, and 30 °C for all other microbes, inhibition zone diameters were recorded.

Lipid extraction. A lipid extract of vernix was obtained by the method described in Folch et al. [31], using 1.3 g vernix homogenized in a Potter-Elvehjem homogenizer (Kontes, Vineland, N. J.) with a chloroform:methanol mixture (2:1, v:v) containing butylated hydroxytoluene (BHT; 5 mg/100 ml) as antioxidant. After 1 h shaking at room temperature, and centrifugation at 3000 rpm for 10 min, methanol and saline were added to the liquid phase (final ratio, chloroform:methanol:saline, 1:1:0.8, v:v:v) and re-extraction was carried out followed by centrifugation. The chloroform phase was evaporated with N_2 and the total lipids were dissolved in chloroform and stored at –30 °C.

Thin-layer chromatography (TLC). Lipid extract (30 mg) was separated on thin-layer plates (Adsorbosil soft layer, Alltech, Deerfield, Ill.) using a mobile phase of petroleum ether/diethyl ether/acetic acid (80:20:1, v:v:v). The lipid components were visualized with 10% Rhodamine 6G in distilled water and then scraped from the plate and transferred into centrifuge tubes, in which they were extracted twice with hexane. The two hexane extracts were combined and the solvents were evaporated with N_2. Each lipid extract was then dissolved in 23 µl of chloroform and analysed for antimicrobial activity utilizing a modified inhibition zone assay (see below).

Gas chromatography. The free fatty acids were converted into fatty acid methyl esters by incubation in 14% boron trifluoride/methanol (Sigma, St. Louis, MO.) for 45 min at 110 °C. The methyl esters were analysed by high-resolution gas liquid chromatography (Agilent 6890 N; Agilent Technologies, Palo Alto, Calif.) equipped with a flame ionization detector and a Chrompack CP-WAX 52 CB polyethylene glycol column (25 m x 0.32 mm i.d x 0.2 µm). Injector and detector temperatures were maintained at 235 °C and 250 °C, respectively. The oven was programmed to have an initial temperature of 90 °C for 2 min, then raised to 165 °C at 30 °C /min, and to 225 °C at 3 °C /min, and finally held isothermal for 6 min. The carrier gas was hydrogen at 31.8 kPa. Peaks obtained were identified and calibrated against those of standards (Nu-Chek-Prep; Sigma, Elysian, Minn.).

Modified inhibition zone assay. A modified inhibition zone assay was developed for screening lipid components separated by thin-layer chromatography (TLC), and to evaluate interaction between the total lipid fraction of vernix and the human cathelicidin LL-37. *B. megaterium* was used as the test organism. The samples were loaded on top of the agarose (without use of wells) at 4 °C. Lipid stock (~ 650 g/l) was made and diluted to the desired concentration in chloroform. A solution of the human cathelicidin LL-37 (2 g/l) was prepared in chloroform containing 0.7% TFA, giving a turbid solution. The LL-37 solution, mixed in a 1:1 ratio with different dilutions of the lipid stock, gave final lipid concentrations of ~ 300, 30, 7, 3 and 0.3 g/l, in chloroform containing 0.35% TFA. Each peptide-lipid sample (3 µl) was loaded onto the agarose plates without medium E and incubated overnight at 30 °C. The inhibition zones (mm) were analysed by two-tailed Mann-Whitney's U test. The differences in activity were regarded significant when the p values were less than or equal to 0.05.

Colonization analysis. Immediately after birth, samples were collected from the auditory canals, the axillae and the groins of newborn babies using a sterile cotton-tipped pin (Copan sterile transport swab, Brescia, Italy), and cultured for microbes by plating less than 12 h after collection according to standard procedures.

Results

Identification of proteins in vernix. Proteins/peptides were extracted from vernix (3 g) combined from eight neonates. Utilizing silver-stained SDS-PAGE, the pattern

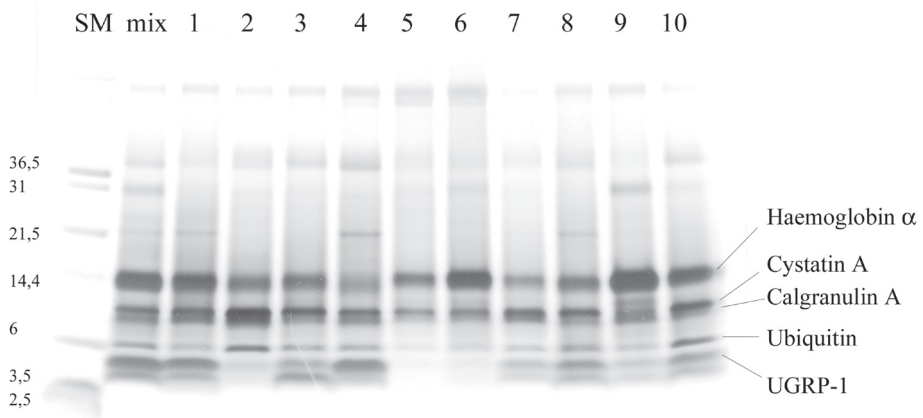

Figure 1. Gel electrophoresis of proteins in vernix caseosa. Peptide/protein extracts (5 μg) from ten individual babies (lanes 1–10), and from a vernix mixture of eight babies (mix), were separated by SDS-PAGE and visualized by silver staining. The proteins in the major bands were identified by N-terminal sequence analysis after transfer of the proteins from a preparative gel to a PVDF membrane, and are indicated to the right. SM, size marker.

Table 1. Proteins identified in vernix by MALDI MS, N- and C-terminal sequence analyses and peptide mass fingerprinting

Peak no.	Identified protein	Swissprot accession number	MALDI determined mass/ theoretical mass	N-terminal sequence	C-terminal sequence	MALDI sequence coverage (%)	MALDI matching fragments (n)
1	ubiquitin (1–76)	P62991	8568 / 8565	MQIFVKTLTGKTITL...	NA	64	5
1	ubiquitin (1–72)	P62991	8183 / 8181	MQIFVKTLTGKTITL...	NA	NA	NA
1	ubiquitin (1–70)	P62991	7916 / 7912	MQIFVKTLTGKTITL...	...HLV	NA	NA
3	psoriasin	P31151	11374 / 11367	DNTQAERSIIGMIDM...	NA	63	8
3	lymphocyte antigen Ly6E	Q16553	NA	LMXFSXLNQKSNLY...	NA	NA	NA
4	lysozyme C	P61626	NA	KVFERXELARTLKRL...	NA	59	8
4	transthyretin	P02766	NA	GPTGTGESKAPLMV...	NA	NA	NA
4	apolipoprotein C-1 (29–83)	P02654	6433 / 6432	DVSSALDKLKEFGNT...	NA	NA	NA
5	calgranulin C	P80511	NA	XXLEEHLEGIVNIFHQ...	NA	NA	NA
5	squamous cell carcinoma antigen 1 and/or 2(SCCA 1/2) (5-)*	P29508/ P48594	NA	SCANTKFMFDLFQQF...	NA	NA	NA
6	apolipoprotein A-II (38–101)	P02652	7145 / 7145	FQTVTDYGKDLMEKV...	NA	NA	NA
6	haemoglobin αchain	P69905	15135 / 15126	VLSPADKTNVKAAWG...	...KIR	74	6
6	haemoglobin βchain	P68871	15871 / 15867	VHLTPEEKSAVATLW...	NA	75	8
7	profilaggrin (1-)*	Q01720	NA	XTLLENIFAIINLFK...	NA	NA	NA
7	calgranulin A	P05109	NA	MLTELEKALNSIIDVYHKY...	NA	65	6
8	UGRP-2	Q96QR1	NA	FLVGSAKPVAQPVAA...	NA	NA	NA
9	hypothetical protein FLJ40379	Q8N7T4	NA	VTXEVXMNVVQKLDH...	NA	NA	NA
10	UGRP-1 (22–93)	Q96PL1	NA	FLINKVPLPVDKLAP...	...LSHLV	94	8
10	UGRP-1 (34–93)	Q96PL1	NA	LAPLPLDNILPFMD...	...LSHLV	93	6
10	UGRP-1 (35–93)	Q96PL1	NA	APXPLDNILPFMDPL...	...LSHLV	93	6
10	UGRP-1 (40–93)	Q96PL1	NA	DNILPFMDPLKLLLK...	...LSHLV	93	6
NA	cystatin A	P01040	NA	IPGGLSEAKPATPEIQEIVD...	NA	73	6
NA	calgranulin B	P06702	NA	NA	NA	80	9
NA	caspase 14 precursor/ C-terminal fragment*	P31944	NA	NA	NA	37/86	9/8
NA	haemoglobin γ1 / 2 chain	P69891/ P69892	NA	NA	NA	62	7

Peak numbers correspond to peaks in figure 2; *, fragment of unknown size; NA, not applicable.

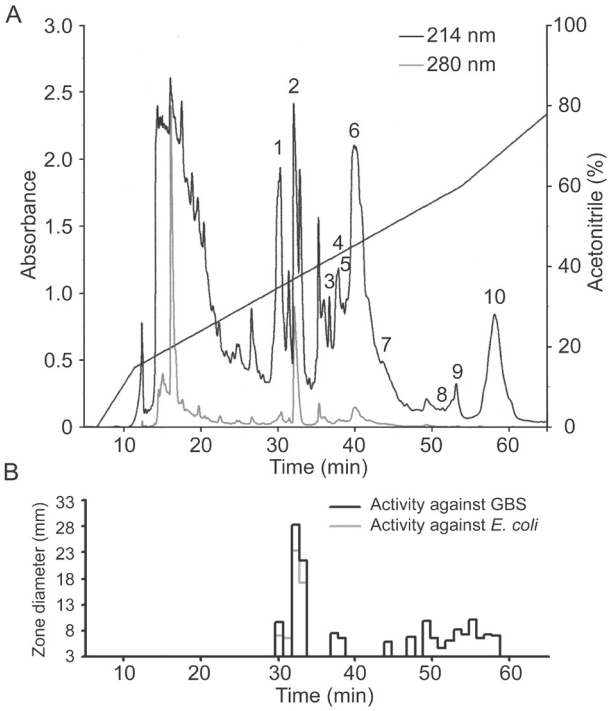

Figure 2. Protein separation and antimicrobial activity. HPLC separation of 4.5 mg protein/peptide extract originating from eight neonates is shown (A). After separation, fractions were dissolved in 30 µl 0.1% aqueous TFA and screened for activity against GBS and *E. coli* (B). The columns show the inhibition zone diameter (mm).

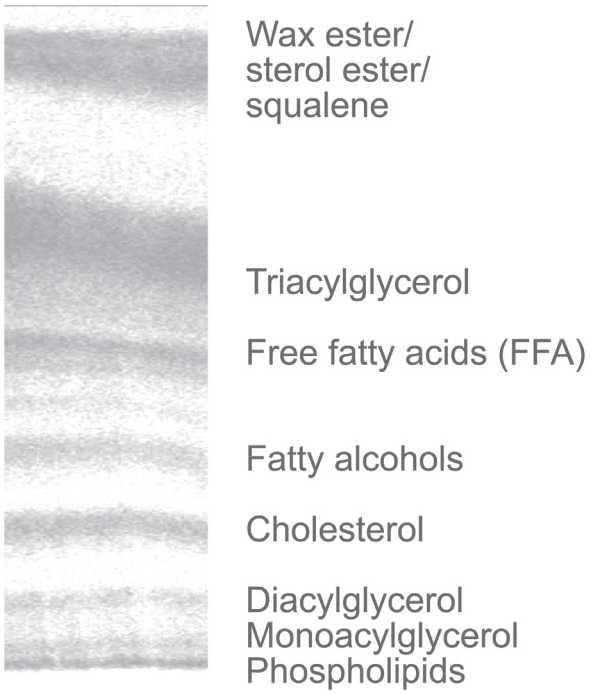

Figure 3. Separation of the lipid extract of vernix, using TLC. The separated lipid classes are indicated.

revealed six abundant protein bands (fig. 1). Vernix extracts from ten newborn individuals analysed in the same manner showed similar patterns of abundant proteins (lanes 1–10 in fig. 1). However, the levels of the proteins varied between the extracts. After transfer of the proteins to a PVDF membrane, five of the six proteins were identified by N-terminal sequence analysis as haemoglobin α chain, cystatin A (stefin A), calgranulin A (S100A8, MRP-8, p8, L1 light chain), ubiquitin and uteroglobin related protein 1 (UGRP-1) (SCGB3A2, HIN-2) (fig. 1, table 1). The cystatin A identified was found to lack the N-terminal methionine, as has been shown to be characteristic of cystatin A of epidermal origin [32]. Further characterization involved C-terminal sequence analysis of haemoglobin α and UGRP-1 (table 1), as well as peptide mass fingerprinting after in-gel digestion (on sample number 8, fig. 1). By the latter method, three more variants of UGRP-1 were identified (table 1), in addition to haemoglobin β chain, lysozyme C, haemoglobin γ chain, psoriasin (S100A7), calgranulin B (S100A9, MRP-14, p14, L1 heavy chain), and caspase 14 precursor (table 1). The protein/peptide extract of the combined vernix sample from eight neonates (fig. 1) was separated by RP high-performance liquid chromatography (HPLC) (fig. 2) and proteins in fractions were further characterized by direct sequence analysis (table 1) and MALDI MS. In fractions corresponding to peak 10 (fig. 2), all UGRP-1 variants were identified. In addition, after separation on SDS-PAGE and blotting onto a PVDF membrane, several more proteins were identified by N-terminal sequence analysis (table 1). These were apolipoprotein C-1, apolipoprotein A-II, calgranulin C (S100A12), UGRP-2 (SCGB3A1 /HIN-1), profilaggrin, lymphocyte antigen Ly6E, squamous cell carcinoma antigen 1 and/or 2, transthyretin and the hypothetical protein FLJ40379. The mass-spectrometric analyses on the fraction corresponding to peak 1 in figure 2 revealed three different masses for ubiquitin corresponding to variant C-terminal forms (table 1).

Lipid composition of vernix, and antibacterial activity. The Folch extract of vernix was separated by TLC (fig. 3), detecting eight zones which were identified as wax esters/sterol esters/squalene, triacylglycerol, free fatty acids, fatty alcohols, cholesterol, diacylglycerol, monoacylglycerol and phospholipids. The hexane extract of the TLC zone containing free fatty acids was the only lipid fraction that exhibited antibacterial activity against *B. megaterium* (Bm11), giving a diameter of 1.7 mm in the modified antibacterial assay. The relative fatty acid composition of the free fatty acid zone was determined by gas chromatography (table 2) showing that 50.1% was saturated fatty acids, C16:0, C22:0 and C24:0 being the most abundant. The

Table 2. Free fatty acid composition of vernix

Free fatty acids	% (weight/weight)
SFA	**50.1**
C14:0	2.0
C15:0	1.0
C16:0	14.0
C17:0	0.4
C18:0	2.0
C20:0	0.8
C22:0	5.4
C24:0	24.5
MUFA	**11.4**
C16:1n-9	2.0
C16:1n-7	1.3
C18:1n-9	6.4
C18:1n-7	1.7
PUFA	**15.0**
C18:2n-6	9.6
C20:2n-6	1.3
C22:4n-6	4.1
Unidentified	**23.0**

SFA, saturated fatty acids (FA); MUFA, monounsaturated FA. PUFA, polyunsaturated FA; Data are expressed as weight % of total fatty acids in vernix. Values represent the average of two determinations.

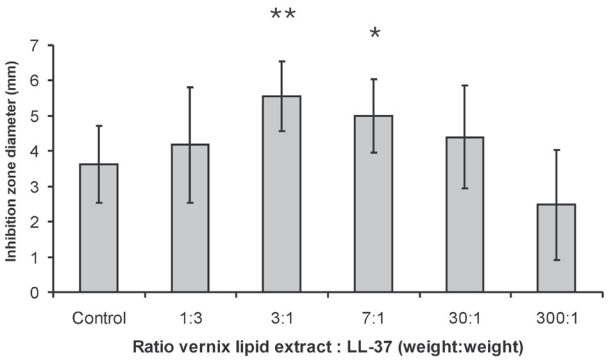

Figure 4. Bactericidal interactions of LL-37 and vernix lipids. LL-37 (3 μg) was mixed with different amounts of vernix lipid extract in chloroform with 0.35% TFA. The lipid amount in the samples is given as mass ratio (lipid:peptide). The positive control was the LL-37 peptide, and the negative control was chloroform with 0.35% TFA. The experiments were repeated four times in duplicate, and the Mann-Whitney U test used for the statistical analysis. ** $p < 0.01$, *$p < 0.05$.

major mono- and polyunsaturated fatty acids were C16:1, C18:1, C18:2 and C22:4 (table 2). No short- or medium-chain acids (4–12 carbons) were identified.

Bactericidal interactions of LL-37 and vernix lipids. To investigate whether LL-37 and the vernix lipids interact with each other, and hence affect the antibacterial activity, we developed a modified inhibition zone assay. Different concentrations of the vernix lipid fraction were prepared and mixed with 3 μg LL-37 in choloroform with 0.35% TFA. An inhibition zone of 3.6 mm in diameter was recorded for 3 μg of LL-37 alone (control in fig. 4), while a zone diameter of 1.5 mm was observed for the highest concentration of lipids alone. In contrast, neither the other lipid concentrations, nor the negative control (chloroform with 0.35% TFA) exhibited any activity. Notably, the activity of 3 μg LL-37 was enhanced when mixed with lipids in certain proportions (fig. 4). A maximum of 5.6 mm in zone diameter was recorded at a lipid:peptide ratio of 3:1. The antibacterial activity of samples with a ratio of 3:1 and 7:1 was significantly increased versus that with LL-37 alone ($p = 0.005$ and $p = 0.031$, respectively).

Identification of chlorhexidine in vernix. The highest activity against both *E. coli* and GBS was recorded in fractions corresponding to peak 2 (fig. 2). The component giving rise to this activity was isolated by two consecutive RP chromatographic steps, utilizing Vydac C18 (4.6 x 250) mm and C8 (2.1 x 150) mm columns (The Separations Group, Hesperia, Calif.), respectively. The active component eluted at 38.6% acetonitrile in the first run using 0.1% heptafluorobutyric acid as a counter ion, and at 32.4% acetonitrile in the second run using 0.1% TFA as a counter ion. This component gave rise to symmetric absorbancy peaks at both 214 and 280 nm. Utilizing nanoelectrospray mass spectrometry in the positive-ion mode, the isotopic composition of a cluster of protonated molecules at m/z 505 indicated the presence of two chlorines in the compound. The accurate molecular mass, 504.203, obtained at a working resolution of ~15,500 (full-width at half maximum) was used to calculate possible elemental compositions and to search the database registry created by the Chemical Abstract Service (CAS), hosted by the Scientific & Technical Information Network (STN) in Karlsruhe, Germany. One compound ($C_{22}H_{30}Cl_2N_{10}$) corresponded to the antimicrobial substance chlorhexidine. Comparison of the collision-induced dissociation spectrum with that of authentic chlorhexidine showed that the compound was chlorhexidine.

Antimicrobial activity in protein/peptide extracts of vernix. Vernix samples were collected from 88 neonates, and peptides and proteins extracted from each sample. The extracts were screened for antimicrobial activity against four different microbial species, i.e. *B. megaterium* (71 samples), *E. coli* (86 samples), GBS (88 samples) and *C. albicans* (81 samples). All samples analysed exhibited activity against *C. albicans* and *B. megaterium,* while 78% were active against GBS and 31% against *E.*

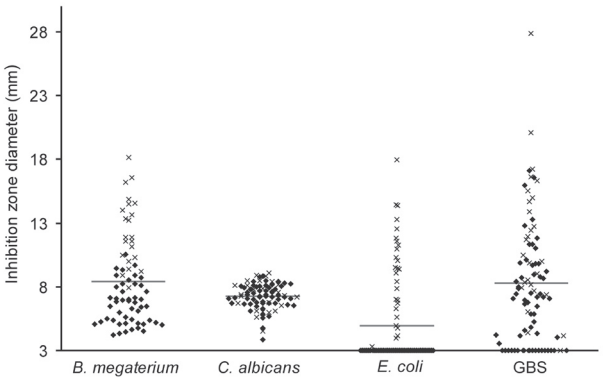

Figure 5. Antimicrobial activity of crude protein/peptide extracts of vernix against *B. megaterium*, *E. coli*, GBS, and *C. albicans*. Extracts exhibiting activity against *E. coli* are marked with ×.

coli (fig. 5). The highest activity was recorded against *B. megaterium* and GBS, an average of 8.4- and 8.3-mm zone diameters, respectively. Intermediate activity was obtained against *C. albicans* (7.3 mm), and the lowest activity was recorded against *E. coli* (5.6 mm). High variation in antibacterial activity was observed against all bacteria. In contrast, low variation in antifungal activity was noted against *C. albicans.* The samples exhibiting activity against *E. coli,* (× in fig. 5), are clustered in the higher-activity range against both GBS and *B. megaterium*. However, samples active against *E. coli* (×) have similar activity distribution against *C. albicans* as the samples that exhibit no activity against *E. coli* (fig. 5), demonstrating a different origin of the antibacterial and antifungal activities in vernix.

Fractions from the RP chromatography (fig. 2) were screened for antibacterial activity against GBS and *E. coli* (fig. 2). Anti-streptococcal activity was detected in fractions distributed over more than half of the chromatogram, indicating that many different components in vernix are responsible for this activity. On the other hand, the activity against *E. coli* was found in a much narrower range, only in fractions corresponding to peak 1 and 2 (fig. 2). As explained above, the most active component in these fractions is chlorhexidine. This indicates that chlorhexidine is the major contributor of total anti-*E. coli*-activity, and that vernix samples found active against *E. coli* were possibly contaminated by higher levels of chlorhexidine than samples with no *E. coli* activity. In samples active against *E. coli*, part of the high activity against *B. megaterium* and GBS is concluded to originate from chlorhexidine. Notably, if we exclude the anti-*E. coli*-active samples (marked x in fig. 5), the activity range against *B. megaterium* decreases remarkably, while the activity range against GBS is still high.

Table 3. Frequency and type of bacterial colonization of newborns at birth.

Microbe	Frequency of colonization among 87 neonates
Gram positive:	
Staphylococcus (coagulase negative)	75 (86%)
Staphylococcus aureus	4 (5%)
Lactobacillus sp.	1 (1%)
Corynebacterium	9 (11%)
Streptococci, viridans group	10 (12%)
Streptococci, haemolytic group G	1 (1%)
Streptococci, haemolytic group B	3 (4%)
Streptococci, non-haemolytic	5 (6%)
Bacillus sp.	23 (27%)
Bacillus subtilis	1 (1%)
Gram negative:	
Coliform bacilli	10 (12%)
E. coli	18 (21%)
Enterococcus	2 (2%)
Yeast:	
Candida albicans	2 (2%)

Microbial colonization of newborns. Immediately after birth, samples were collected from the skin surface of 87 newborn babies for detection of microbial colonization of the skin. Fourteen groups of bacteria were found to colonize the surface of the babies. Only one baby was not colonized with any bacteria, 26 (31%) babies were colonized with one group of bacteria, 34 (40%) were colonized with two groups, 20 (24%) were colonized with three groups, and two (2%) were colonized with four groups. *Staphylococcus* (coagulase negative) colonized 86% of the babies, *Bacillus* sp. 27%, and *E. coli* 21%. Only 4% of the babies were colonized with GBS, and 2% with *C. albicans* (table 3).

Discussion

In previous studies, we have characterized antimicrobial peptides and polypeptides in vernix [7, 8]. In the present study, we demonstrate the presence of many more proteins of immunological importance in vernix. Among the most abundant proteins now characterized are cystatin A, calgranulin A, ubiquitin and UGRP-1, which are all implicated in innate immunity of humans. Vernix lipids were also characterized, and antimicrobial activity was detected, in particular for free fatty acids. In addition, our results indicate that lipids may contribute to a favourable microenvironment in vernix by interacting with antimicrobial components such as LL-37. Our characterization

of proteins, lipids and their interactions suggests that vernix is a complex innate defence barrier, protecting the foetus and the newborn from infectious microbes, in an apparently crucial manner, since the adaptive immunity of newborns is immature. The antimicrobial property of vernix may also act to facilitate colonization by the normal flora following birth and to block the colonization of unwanted microbes or pathogens. For example psoriasin, which is identified in vernix, directly kills *E. coli* but not *Staphylococcus aureus* [33, 34]. The shedding of the vernix in late pregnancy may suggest that the level of protection has to be adjusted to allow proper colonization of the normal flora.

Several now identified proteins are expressed in skin such as cystatin A, profilaggrin, psoriasin and calgranulin C. Due to the close contact of vernix and amniotic fluid, they share some of the same components. This is now shown regarding calgranulin A and B, proteins previously known to be present in amniotic fluid [35]. The origin of UGRP-1 may be the lungs, and a transfer of this protein to vernix may occur via the amniotic fluid. Blood may be another source of the identified proteins in vernix. Using mass fingerprinting, we identified not only α- and β-haemoglobin but also γ-haemoglobin. During the last two trimesters of pregnancy, the foetus produces γ-haemoglobin, which is replaced by β-haemoglobin after birth, enabling an efficient transfer of oxygen from the blood of the mother to the foetus. Thus, the γ-haemoglobin detected in vernix originates from the foetus.

Calgranulin A, B and C, and psoriasin all belong to the S100 family of calcium binding proteins. The S100 family of proteins has two calcium-binding motifs of the EF-hand type [36]. These proteins have been shown to exhibit chemotactic properties and may play a role in the pathogenesis of epidermal diseases [36]. Notably, an N-terminal fragment of profilaggrin, with sequence similarity to the two EF-hands [37], was also identified in vernix.

Calprotectin is an antifungal and antibacterial complex consisting of a heterodimer of calgranulin A and calgranulin B [38]. Both subunits were identified, revealing that the active holoprotein is present in vernix. Accordingly, the crude peptide/protein extract of vernix exhibited good antifungal activity. However, after separation of the protein extract by RP-HPLC, we could not detect any antifungal activity in the collected fractions (data not shown). Our interpretation of this difference is that the two subunits of calprotectin have been separated upon HPLC, leading to loss of activity. Calprotectin is suggested to kill microbes by chelating zinc, thereby depriving microbes of an essential metal ion [39]. This mode of action has also been described for lactoferrin and psoriasin, the latter being a major *E. coli*-killing compound in human skin [33].

Calgranulin C was first identified on the surface of onchoceral worms in human subcutaneous nodules [40]. It is proposed to be released by activated neutrophils, thereby attacking and killing nematodes [40]. Thus, the presence of calgranulin C in vernix contributes to the protective role of vernix.

Cystatin A is a protease inhibitor that is mainly expressed by epithelial and polymorphonuclear cells [41, 42]. Cystatin A is also a minor cross-linking component of the cornified cell envelope [43] and a part of the mechanical barrier of the skin. Unlike cystatin C, cystatin A has not been shown to possess any direct antimicrobial effect. However, cystatin A has been suggested to be a first-line protector against cysteine proteases released from infectious micro-organisms and parasites [44]. Thus, cystatin A could have a dual role in the innate defence of the foetus.

Our results reveal that UGRP-1 (HIN-2/SCGBA2) is one of the major proteins in vernix, whereas UGRP-2 (HIN-1/SCGBA1) is not as abundant. These proteins are both expressed at high levels in neonatal lungs by different subsets of secretory cells within the surface and glandular epithelia [45]. UGRP-1 has been shown to bind bacteria and to the macrophage scavenger receptor MARCO [46], indicating opsonizing properties. In the lungs of mice, the expression of UGRP-1 is upregulated by interleukin (IL)-10 [47], while it is downregulated by IL-5 [48], suggesting that UGRP-1 is a target of anti-inflammatory pathways. In vernix, we characterized three novel forms of UGRP-1, which are differently processed at the N-terminus. These forms may have altered binding affinities to bacteria, leading to enhancement of the opsonizing spectra.

Vitamin A has been detected at high levels in vernix [49] and is proposed to serve as a nutritional depot of vitamin A. Vitamin A is secreted from the amniotic epithelium into the amniotic fluid, and is taken up by vernix [49]. Our results show that transthyretin is present in vernix, a protein that binds to the retinol binding protein, which in turn binds vitamin A.

Like lipids previously isolated from human stratum corneum and sebum [50, 51], our results demonstrate inhibitory effects of the free fatty acids in vernix against the Gram-positive bacterium *B. megaterium*. We also demonstrate that palmitoleic acid (C16:1) and linoleic acid (C18:2), known to exhibit potent antimicrobial activity [14, 52], are a considerable part of the total free fatty acids. The long-chain unsaturated fatty acids found in vernix (C20–C22 in table 2) are also antimicrobial, and the activity is enhanced with an increase in the number of double bonds [15]. Like antimicrobial peptides [53], fatty acids and monoacylglycerols disintegrate the lipid envelope of viruses [15] and bacterial plasma membranes [12, 16].

Considering the high lipid content of vernix (10%) [3], lipids influencing the function of other components of

vernix seems possible. Other factors such as salts and pH have been demonstrated to influence the conformation of the human cathelicidin LL-37 [23]. Therefore, we speculate that the lipid fraction of vernix can exhibit similar functions. Under our experimental conditions, lipids isolated from vernix enhanced the antimicrobial potency of LL-37. Thus, LL-37 can be active in a lipid-rich environment.

When studying the antimicrobial activity in peptide/protein extracts of vernix, we found a high antimicrobial activity against bacteria and fungi. The most active antibacterial compound against *E. coli* and GBS in these samples was isolated and identified as chlorhexidine. Chlorhexidine is a microbicidal substance of vaginal cream used as a lubricant during vaginal examination prior to delivery. For this reason, some of the vernix samples were found to contain chlorhexidine. We noted that the samples with anti-*E. coli*-activity (labelled x in fig. 5) also exhibited activity against *B. megaterium* and GBS. Since we demonstrated that the main component active against *E. coli* was chlorhexidine, we speculate that high *E. coli* activity indicates a higher degree of chlorhexidine in these samples. To minimize the contribution of chlorhexidine, we excluded the samples active against *E. coli* from the analyses. This showed that the interindividual variation of activity is high against GBS and lower against *B. megaterium* and *C. albicans*. Similarly, the colonization data could also be affected by chlorhexidine. However, when the *E. coli*-positive samples were removed, only small differences in colonization were observed (data not shown).

In conclusion, we have now characterized proteins and lipids that add protective functions to vernix, such as antifungal properties, opsonizing features, protease inhibiton and parasite inactivation. In addition, the antimicrobial action of LL-37 can be potentiated by the lipids in vernix in vitro, stressing the importance of the microenvironment for the function of antimicrobial components.

Acknowledgements. This work was supported by grants from The Icelandic Research Fund for Graduate Students, The Swedish Foundation for International Cooperation in Research and Higher Education (STINT), and The Swedish Research Council (no. 11217, 13X-3532). We thank E. Cederlund, C. Palmberg, M. Ståhlberg, G. Alvelius and M. Lindh for excellent assistance. We also thank M. Chromek and A. Brauner for the GBS strain.

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