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Lipids Including Cholesteryl Linoleate and Cholesteryl Arachidonate Contribute to the Inherent Antibacterial Activity of Human Nasal Fluid¹

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Mucosal surfaces provide first-line defense against microbial invasion through their complex secretions. The antimicrobial activities of proteins in these secretions have been well delineated, but the contributions of lipids to mucosal defense have not been defined. We found that normal human nasal fluid contains all major lipid classes (in micrograms per milliliter), as well as lipoproteins and apolipoprotein A-I. The predominant less polar lipids were myristic, palmitic, palmitoleic, stearic, oleic, and linoleic acid, cholesterol, and cholesteryl palmitate, cholesteryl linoleate, and cholesteryl arachidonate. Normal human bronchioepithelial cell secretions exhibited a similar lipid composition. Removal of less-polar lipids significantly decreased the inherent antibacterial activity of nasal fluid against *Pseudomonas aeruginosa*, which was in part restored after replenishing the lipids. Furthermore, lipids extracted from nasal fluid exerted direct antibacterial activity in synergism with the antimicrobial human neutrophil peptide HNP-2 and liposomal formulations of cholesteryl linoleate and cholesteryl arachidonate were active against *P. aeruginosa* at physiological concentrations as found in nasal fluid and exerted inhibitory activity against other Gram-negative and Gram-positive bacteria. These data suggest that host-derived lipids contribute to mucosal defense. The emerging concept of host-derived antimicrobial lipids unveils novel roads to a better understanding of the immunology of infectious diseases. *The Journal of Immunology*, 2008, 181: 4177–4187.

The respiratory tract is constantly challenged with airborne and aspirated microbes and its integrity depends on continuous removal of inhaled microbes by mucociliary clearance. Airway mucosal secretions have been intensely studied, and important defense functions have been attributed to antimicrobial polypeptides such as lysozyme, defensins, or LL37 (1). Other antimicrobial components include surfactant proteins (2) and mucins (3). Antimicrobial polypeptides are cationic and hydrophobic, and their functions in innate host defense are to kill microbes through membrane permeabilization and disruption of membrane-bound multienzyme complexes (4, 5) and to modulate

the immune response through chemotactic activity and sequestration of proinflammatory microbial products (6).

In addition, bodily secretions also contain lipids. Lipids form a heterogeneous group of hydrophobic substances ranging from simple fatty acids, linear chains of carbons carrying a carboxyl group, to more complex ring-structured molecules like cholesterol and cholesteryl esters, which can be further modified by the attachment of various side groups including alcohol, phosphate, and amino-functions. Major lipid classes comprise fatty acids (fatty acyls), glycerolipids, glycerophospholipids, sphingolipids, and sterols, including cholesterol and cholesteryl esters. Among these, glycerophospholipids and sphingolipids are the most polar and hydrophilic, and triglycerides and cholesteryl esters are the least polar and most hydrophobic (7). Although some fatty acids appear as free molecules, most of the lipids in bodily fluids are bound to carrier proteins, including albumin and apolipoproteins, the latter forming lipoproteins upon lipid-binding (8). Lipoproteins are classified based on their apolipoprotein and lipid composition, which affect their density. The five major lipoproteins described in plasma are chylomicrons, very-low-density lipoproteins, intermediate-density lipoproteins, low-density lipoproteins, and high-density lipoproteins (HDL),⁴ with increasing protein and decreasing lipid contents, respectively (9). Apolipoprotein A-I (apoA-I) is one of the nine major apolipoproteins. It is an integral component of HDL and is also found in chylomicrons and very-low-density lipoproteins. This 29-kDa protein is the main cholesterol acceptor

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⁴ Abbreviations used in this paper: HDL, high-density lipoprotein; HNP, human neutrophil peptide; SPE, solid phase extraction; rpHPLC, reversed phase HPLC; ELSD, evaporative light scattering detection; GC/MS, gas chromatography/mass spectrometry; AU, acid urea; apoA-I, apolipoprotein A-I.

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and activates lecithin-cholesterol ester transferase, which is involved in esterification of cholesterol and production of cholesterol esters. Recent studies suggest that apolipoproteins and their lipoprotein particles function also as immune modulators, regulating cytokine production and activity of immune, endothelial, and epithelial cells (10–12).

The functions of lipids are diverse. Lipids are essential components of all biological membranes, but the membrane lipid composition differs greatly between eukaryotes and prokaryotes (13, 14). Lipids are also precursors for hormones, are used for energy storage, and they have a prominent role as messengers and regulators of inflammation (15). Furthermore, lipids in lung surfactant, predominantly phospholipids, are essential for reducing the surface tension in the alveoli, thus preventing their collapse during exhalation (16).

Previous studies implicate that lipids also have antimicrobial functions. For example, the antimicrobial activity of fatty acids has been known for a long time and is used in food preservation (17), and certain chronic infectious diseases have been associated with an altered lipid profile (18–22). In cystic fibrosis, a hereditary disease with abnormal mucus production and chronic lung infection with *Pseudomonas aeruginosa*, reduced levels of docosahexaenoic and linoleic acid in saliva and serum have been documented and corroborated by a mouse model (23). A role in neonatal protection against infection has been also attributed to lipids in milk (24) and lipids in vernix caseosa, the newborn coating (25).

However, despite these reports on the antimicrobial actions of lipids and speculations on their use as therapeutic agents (26), the concept of a lipid-mediated component of innate host defense is new. We propose that host-derived lipids constitute the arsenal of antimicrobial agents in innate mucosal defense. We will show in this study that 1) selective removal of lipids from nasal fluid leads to diminished antibacterial activity, 2) this antibacterial activity is partially restored by resupplementing the fluid with lipids, and 3) selected cholesterol esters identified in nasal fluid exert antibacterial activity *in vitro*.

Materials and Methods

Nasal fluid collection

Under full institutional review board approval, nasal fluid was collected from healthy adult donors (D1–D11) without history of allergies or sinusitis into 50 ml of polypropylene centrifuge tubes by vacuum suction. Because nasal fluid may be contaminated with the mucosal microbiota, samples were incubated for 15 min at 60°C to heat-inactivate the normal flora. Trypticase soy broth (2 ml) inoculated with 20 μ l of heat-inactivated nasal fluid remained sterile after 72 h of incubation in contrast to nonheated nasal fluid. After heat inactivation, nasal fluid was homogenized with a tip sonicator (Fisher Dismembrator; Fisher Scientific), three times at level 2 for 20 s), and centrifuged for 5 min at 500 \times g. The resulting cell- and debris-free supernatant was further centrifuged for 30 min at 11,000 \times g and 4°C. Epithelial cells in nasal fluid were sparse and under these conditions, the contaminating cells were not disrupted and sedimented (data not shown). Cole et al. (27) reported a significant reduction of the cationic antibacterial protein fraction in nasal fluid upon boiling. To verify that heat inactivation at 60°C for 15 min does not significantly alter the biochemical and antibacterial properties of nasal fluid, we compared the overall protein profile, peptidoglycan hydrolyzing activity, antibacterial activity, and lipid profile of nasal fluid before and after heat inactivation. There was no major difference in the protein and lipid profile of nasal fluid demonstrable and there was no statistically significant difference in respect to the peptidoglycan hydrolyzing and antibacterial activity (data not shown).

Clarified nasal fluid was stored under nitrogen or argon at –20°C until further use. Nasal fluids were pooled for multiple analyses and functional assays to increase volume and to minimize interdonor variations in sample composition.

Epithelial cell culture

Normal human-derived bronchial epithelial cells grown at an air-liquid interface in 6-well plates (model no. AIR-606) were purchased from Mat-Tek Corporation. Upon receipt, cells had been grown for 6 days (in a serum-free DMEM-based medium with proprietary growth factors) and apical secretions had been allowed to accumulate. After overnight incubation (37°C, 5% CO₂), apical secretions from six wells were collected and pooled. Potentially contaminating cells and debris were removed by centrifugation (500 \times g, 4°C for 10 min), and the sample was subjected to lipid extraction, followed by reversed phase HPLC (rpHPLC) with evaporative light scattering detection (ELSD), as described below.

Lipid standards

Lipid standards, selected according to the literature (28) and guided by our studies, were purchased from Matreya or Sigma-Aldrich. These were dissolved in organic solvents (chloroform or dichloromethane) in glass vials, overlaid with nitrogen or argon gas, and stored at –20°C. Free fatty acids used were as follows: myristic acid (C14:0, defining the number of C atoms to the number of double bonds), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0, plant fatty acid used as internal standard), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and docosahexaenoic acid (C22:6). Glycerolipids used include dipalmitic acid and tripalmitic acid. Glycerophospholipid used was phosphatidylserine. Sphingolipid used was sphingomyelin. Sterols used include cholesterol, cholesterol stearate, cholesterol palmitate, cholesterol oleate, cholesterol linoleate, and cholesterol arachidonate.

Lipid extraction

Lipid extractions were based on the Bligh and Dyer method (29). Briefly, per 0.4 ml of sample, 1.5 ml of methanol:chloroform (2:1, v/v) were added, the sample flushed with nitrogen (N₂ gas), and vigorously vortexed for 1 min. Then, 0.5 ml of chloroform was added, the sample flushed with N₂, and vortexed for 1 min. The procedure was repeated using 0.5 ml of dH₂O. Samples were then incubated on a rotary shaker for 10 min and centrifuged (500 \times g, 25°C, 10 min). The lower phase was collected and an equal volume of chloroform was added to the upper phase to repeat the lipid extraction. The chloroform layers from both steps were pooled and the organic solvent was removed under a gentle stream of N₂ gas at 37°C. The extracted lipids were resuspended in a small volume of appropriate organic solvent for further studies and analysis. For quantitative experiments, to control for lipid extraction efficiency, heptadecanoic acid was added (20 μ g unless stated otherwise) to all samples before lipid extraction. Extraction efficiencies were in average 40–80%, and test extractions showed that the various lipid classes were recovered with similar relative efficiencies (data not shown).

Thin layer chromatography

The TLC system used two separation systems run serially (30). Glass-backed silica gel TLC plates (250- μ m layer thickness, 60 Å, 20 \times 20 cm; EMD Chemicals) prewashed in methanol and heat-activated for 10 min at 130°C in an oven were spotted with concentrated lipid extracts and standards and first developed in chloroform:methanol:water (65:25:4 v/v/v) to 13 cm from the origin, then in hexane:diethyl ether (36:9 v/v) to near the top. Plates were dried and lipid components visualized in iodine vapor. The darkness of the resulting spots is influenced by the quantity of each lipid and the number of unsaturated bonds, limiting the suitability of TLC for comparative lipid quantification of samples with different lipid composition.

rpHPLC

Separation and quantification of lipids was performed with a low-pressure quaternary gradient system (Summit HPLC System; Dionex) with Dionex PCS1 Chromeleon software on a reverse phase column (C18, 5 μ m, 250 \times 3.0 mm; Higgins Analytical), pre-equilibrated in acetonitrile (solvent A)/dichloromethane (solvent B) (87.5/12.5) with ELSD 800 (Alltech Associates) operated at 40°C in 1 or 2 bar nitrogen (N₂). The 5- μ l samples were injected and eluted (0.6 ml/min) with an increasing concentration of solvent B (time (minutes) per percentage of solvent B: 4/12.5, 7/47.5, 28/50.0, 32/12.5, 35/12.5). Response curves were established for authentic standards (data not shown) and used to quantify lipids in test samples, whereby the amount detected in the HPLC chromatogram was corrected for the actual extraction efficiency evident in the recovery of the internal standard heptadecanoic acid. In this system linoleic acid (C18:2) coelutes with myristic acid (C14:0) and palmitoleic acid (C16:1), oleic acid (C18:1) with palmitic acid (C16:0), and cholesterol palmitate with cholesterol

oleate (data not shown). In some cases, peak fractions were collected between 2 and 10 min or 11.5 and 13 min, and further analyzed by gas chromatography/mass spectrometry (GC/MS) for free fatty acid analysis, or a commercial enzymatic cholesterol kit according to the manufacturer's instruction (BioVision), respectively.

Free fatty acid analysis

Lipid extracts from nasal fluid (800 μ l) were subjected to rpHPLC, and fractions collected between 3.5 and 10 min of the gradient were dried under a stream of nitrogen and treated with 250 μ l of boron trifluoride in methanol (14% w/v; Alltech Associates) (31) for 20 min at 60°C. Fractions were extracted after addition of 2 ml water twice with 2 ml CH_2Cl_2 , dried under nitrogen, dissolved in chloroform, and then injected (1 μ l, splitless mode, Agilent 7638B autoinjector, 280°C) onto a bonded-phase nonpolar fused silica capillary column (HP1, 50 m \times 0.2 mm, 0.11- μ m film thickness) housed within an Agilent 6890N gas chromatograph. The column was eluted (constant flow, 1 ml/min) with ultra-high purity helium, the oven was held at 50°C for 1 min following injection, then ramped (10°C/min) to a plateau at 320°C. The end of the column (GC/MS transfer line at 280°C) was directly inserted into the electron-impact (230°C, 70 eV) source of a quadrupole mass spectrometer (Agilent 5975 MSD) scanning from m/z 40–1050 (0.67 s/scan). Data were analyzed with instrument-supplied software, and compound identification was achieved with the National Institute of Standards and Technology Mass Spectral Search algorithm (version 2.0). Identification of fatty acyl methyl ester derivatives in nasal fluid was based on elution times and by comparing the peak mass spectra with library spectra and the spectra from authentic standards.

Cholesteryl ester analysis

Identification of cholesteryl esters was performed according to Duffin et al. (32). Total lipid extracts from 100 μ l of individual or pooled nasal fluid were resuspended in 100 μ l of chloroform:methanol (4:1, v/v) containing 10 mM ammonium formate. Standards (cholesteryl palmitate, cholesteryl arachidonate, cholesteryl linoleate, and cholesteryl oleate) were dissolved similarly at 1 pmol/ μ l. Mass spectra were recorded by direct injection (20 μ l) of samples into a stream of the same solvent (20 μ l/min), entering an Ionspray source attached to a triple-quadrupole mass spectrometer (Sciex API III⁺). Mass spectra (m/z 100–1000, 0.3-Da step size, 6.16 s/scan, orifice 65 V) and fragment and parent ion MS/MS spectra (m/z 350–1000, orifice 50 V, 0.3-Da step size, 6.67 s/scan, instrumental setting for collision gas (argon) thickness of 100) were recorded using hydrocarbon- and particulate-depleted air (so-called zero air) for spray nebulization, and the vapors of liquid nitrogen for the curtain gas, both at a flow rate of 0.6 L/min. Data were collected (version 2.5 (FPU) software; Tune) and displayed (version 3.3; MacSpec) with instrument manufacturer-supplied software. The prominent cholesteryl esters in nasal fluid were identified after comparison of the spectra from samples and standards, and with reference to spectra reported in the literature (32).

Lipoprotein analysis

To assess the presence of lipid-binding proteins, Titan Gel electrophoresis was performed with 20- μ l aliquots of individual nasal fluid, lyophilized and dissolved in 2 μ l of electrophoresis buffer, according to the manufacturer's instructions (Lipoprotein kit; Helena Laboratories). Immunoblotting was used to specifically identify apolipoprotein A-I (apoA-I). Aliquots of nasal fluid were subjected to 12% SDS-PAGE, followed by immunoblotting without Ag fixation (32). The primary Abs were mouse mAbs against apoA-I, apoB100, and apoE (Chemicon International). Specific bands were visualized with alkaline phosphatase-conjugated polyclonal goat anti-mouse Abs (Pierce) and BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium substrate; Research Products International), and quantified using Versadoc imaging documentation and QuantityOne software (Bio-Rad).

Protein analysis

Protein composition and concentrations were assessed by Coomassie- and silver-stained 12% acid urea (AU)-PAGE (33), analyzed with a gel imaging system (Versadoc; Bio-Rad) and bicinchoninic acid assay using BSA standard protein according to the manufacturer's instructions (Pierce), respectively.

Lipid depletion of nasal fluid by solid phase extraction (SPE)

To deplete nasal fluid of nonpolar lipids, SPE was performed on pooled nasal fluids using tC18 1cc Sep-Pak cartridges (Waters Corporation) with a 12-port vacuum manifold and a bench-top vacuum station (Alltech Associates). The method had been first developed using mock nasal fluid (data

not shown). Cartridges were sequentially conditioned and washed with 2 ml of acetonitrile and 2 ml of dH_2O , respectively. The pooled nasal fluid (50 μ l) diluted 1/2 in dH_2O was loaded per cartridge and the flow-through was collected. The cartridges were washed with 2×1 ml of dH_2O and then eluted with 2 ml of 45% acetonitrile containing 0.05% trifluoroacetic acid to recover hydrophobic proteins, including antimicrobial peptides like human neutrophil peptides (HNP) that may have bound to the tC18 matrix. The latter two fractions were separately collected, lyophilized, dissolved in a small amount of 0.01% acetic acid, and pooled, with the flow-through yielding lipid-depleted nasal fluid. The protein concentrations of nasal and lipid-depleted nasal fluid were determined and nasal fluid was further diluted in dH_2O to match the protein concentration of lipid-depleted nasal fluid (typically 1/2.5). Samples of both fluid, matched for total protein concentrations, were then compared with assess the extent of lipid depletion and their similarities in protein composition. Between 100– and 300- μ l aliquots of nasal fluid and lipid-depleted nasal fluid were subjected to lipid extraction and rpHPLC/ELSD. To calculate the overall lipid depletion, nasal fluid and lipid-depleted nasal fluid were first normalized to the internal standard heptadecanoic acid and their peak areas were calculated for peaks eluting after 3.75 min (to exclude interfering peaks associated with nonretained material). Aliquots of total protein-matched nasal and lipid-depleted nasal fluid were subjected to Coomassie-stained AU-PAGE to verify similar protein composition (2 μ l) and to immunoblotting (20 μ l) (33) for HNP-1–3, to ascertain that antimicrobial peptides had not been removed through our SPE procedure. In addition, 6 μ l of each sample were subjected to immunoblotting to quantify apoA-I.

Antimicrobial peptides and polypeptides

Lysozyme was purified in the E. Porter laboratory (California State University, Los Angeles, CA). Purified HNP-2 and lactoferrin had been provided by Dr. T. Ganz (University of California, Los Angeles, CA).

Antibacterial assays

For CFU assays, mid-logarithmic cultures of *P. aeruginosa* (a cystic fibrosis strain originating from the Dr. Welsh's laboratory (University of Iowa, Iowa City, IA) were adjusted to McFarland 0.5 ($\sim 1 \times 10^8$ CFU/ml) in saline and nutrient-supplemented phosphate buffer (assay buffer; 100 mM NaCl, 10 mM NaPi (pH 7.4), 4% trypticase soy broth). Silicized microfuge tubes were used, and assays were performed in duplicates. The 6 μ l of bacterial stock suspension were added to 54 μ l of complete nasal fluid or lipid-depleted nasal fluid. After incubation at 37°C and 150 rpm for 30 and 60 min, samples were serially diluted in assay buffer and plated in duplicates or triplicates on trypticase soy agar plates. Colonies were counted after 24 h of incubation at 37°C, and the original bacterial concentrations (in CFU per milliliter) were calculated. As control, bacteria were added to assay buffer and plated immediately (T0) and after incubation as described.

For experiments in which lipid-depleted nasal fluid was resupplemented with lipids, total lipid extract (without heptadecanoic acid) from the corresponding nasal fluid was subjected to rpHPLC. Peak fractions were pooled, dried, and dissolved in chloroform to yield a 9-fold concentrated stock (relative to the nasal fluid volume used for lipid extraction). Of this, 6 μ l were added to the respective microfuge tubes and the chloroform was allowed to evaporate. Control received 6 μ l of chloroform only. The 54 μ l of nasal fluid, lipid-depleted nasal fluid, or assay buffer (for controls) were added and lipids were allowed to solubilize at 37°C for 30 min before bacteria were added.

To determine the antibacterial activity of nasal fluid lipids in the presence and absence of HNP-2, native liposomes were prepared freshly for each experiment from crude lipid extracts according to Sadzuka and colleagues (34). Lipid extracts or chloroform as solvent control were dried in round-bottom glass tubes that had been prewashed with 5% acetic acid, incubated in assay buffer (1.2-fold concentrated compared with original nasal fluid volume) at 65°C for 10 min, and subsequently sonicated at 75°C for 30 min. After cooling to room temperature, liposomes were placed on ice. Per sample tube the following was pipetted: 2.5 μ l of 10-fold concentrated HNP-2 dissolved in 0.01% acetic acid or solvent only, 42.5 μ l of native liposomes, solvent control or assay buffer (the latter for T0 only), and 5 μ l of mid-logarithmic growth phase bacteria (*P. aeruginosa* and *Staphylococcus aureus*; American Type Culture Collection (ATCC) 29213), cultured in trypticase soy broth, adjusted to McFarland 0.5 in assay buffer, and further diluted in assay buffer, 1/10). At T0 and after 3 h of incubation, 200 μ l of assay buffer were added to each tube and the number of CFUs was determined after serial dilution and plating on trypticase soy agar plates. Samples were prepared in duplicates and plated twice.

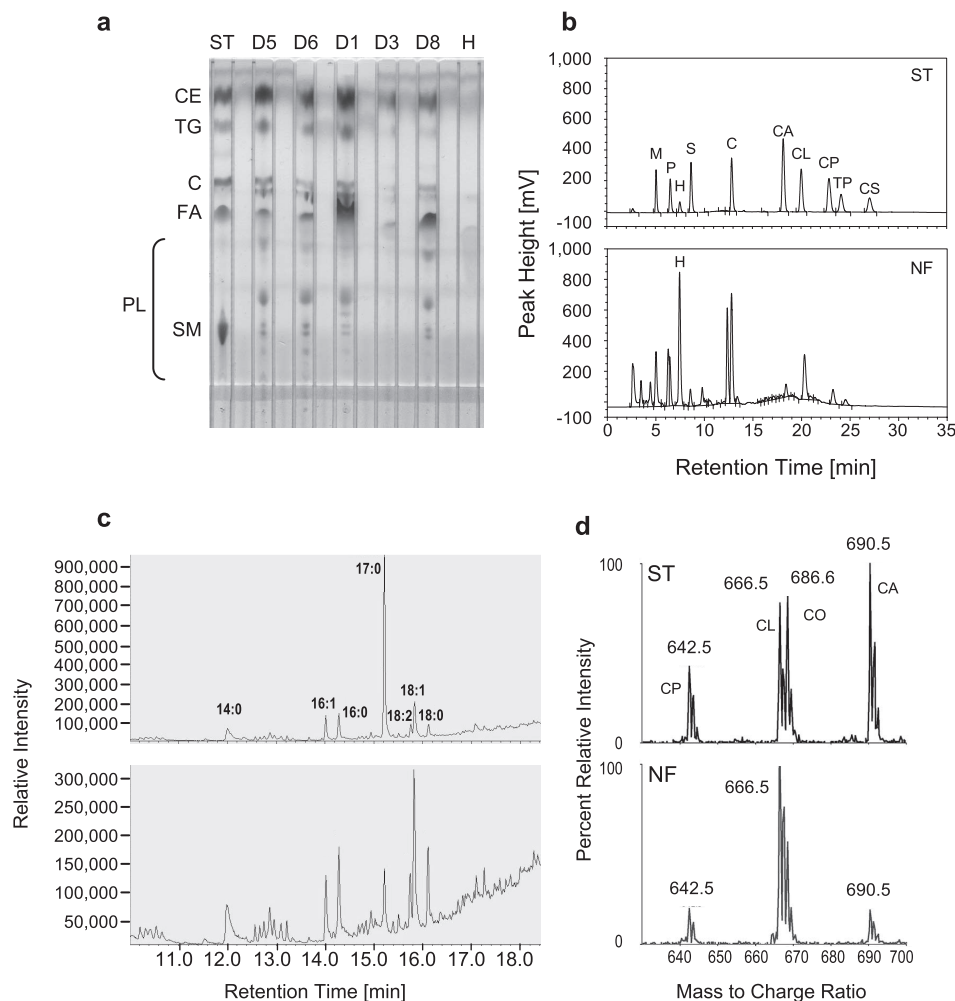


FIGURE 1. Nasal fluid is rich in lipids and contains all major lipid groups. Lipid extracts from individual or pooled nasal fluid samples were subjected to TLC (a), rpHPLC/ELSD (b), gas chromatography followed by mass spectral analysis (c), and tandem mass spectrometry (d). Standard lipids (ST, 25 μg each), cholesteryl ester (CE, cholesteryl palmitate), triglyceride (TG, tripalmitin), cholesterol (C), fatty acid (FA, palmitic acid, C16:0), and sphingomyelin (SM) were detected. Polar lipids (PL) migrate where indicated; lipid extracts from individual nasal fluid samples (350 μl) were collected from respective donors (D1–D8), and all samples had been spiked with heptadecanoic acid (H) before extraction (25 μg). b. Standard lipids (5 μg each) and lipid extract from 150 μl of pooled nasal fluid (NF) were collected from donors D1–D3 and spiked with 50 μg of heptadecanoic acid (H, C17:0), myristic acid (M, C14:0), palmitic acid (P, C16:0), stearic acid (S, C18:0), cholesterol (C), cholesteryl-arachidonate, -linoleate, -palmitate, and -stearate (CA, CL, CP, CS), and tripalmitin (TP). c. Total ion current profiles of methylated, rpHPLC-purified fatty acids from 400 μl of nasal fluid samples (top) pooled from donors D1–D3 and 800 μl of nasal fluid samples (bottom) from donor D6 are shown. Peaks represent the methyl esters of myristate (14:0), palmitoleate (16:1), palmitate (16:0), heptadecanoate (17:0), linoleate (18:2), oleate (18:1), and stearate (18:0). d. Representative electrospray ionization parent ion mass spectra (m/z 369.4, cholesterol) of standard cholesteryl (ST) esters (each 60 nmol) (top) and the lipid extract of 100 μl of nasal fluid (NF) samples (bottom), pooled from donors D1–D3. Cholesteryl-palmitate, -linoleate, -oleate, and -arachidonate (CP, CL, CO, and CA) are shown. Calculated molecular mass for the NH_4^+ adducts is 642.62, 666.62, 668.63, and 690.62 Da, respectively.

To assess their antibacterial activity, cholesterol and cholesteryl esters (cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate, and cholesteryl palmitate; Sigma-Aldrich) were incorporated in liposomes to ensure consistent and homogenous suspension in an aqueous medium. Briefly, thin lipid films consisting of phosphatidylcholines, phosphatidylglycerols, and cholesterol with and without test lipid (test liposomes and vehicle liposomes, respectively) were prepared by pipetting aliquots of lipid stock solutions (in methanol:chloroform) into glass tubes and evaporating the solvent at 50°C under a stream of nitrogen (N_2 gas). The films were then placed under vacuum for at least 8 h to remove residual organic solvent. To prepare the liposomes, films were hydrated with 9% sucrose (pH 5.0), heated at 45–65°C for 5–10 min, and probe sonicated for 5 min. The liposomal formulations were sterile-filtered using a 0.2- μm size membrane (Millex-GV; Millipore).

Initial screening was performed in a microtiter format (2006 Clinical and Laboratory Standards Institute Guidelines, M7-A7) with fluorescence readout using Syto 9 (LiveDead BacLight; Invitrogen), a nonfluorescent probe that diffuses freely into bacterial cells and exhibits green fluores-

cence upon binding to bacterial DNA whereby the resulting fluorescence correlates with the number of bacteria present. *P. aeruginosa* was grown for 24 h in trypticase soy broth, adjusted to McFarland 0.5 in saline, and further diluted 1/100 in 1.1-fold concentrated cation-adjusted Mueller-Hinton broth to $\sim 1 \times 10^5$ CFU/ml. Of this, 90 μl were aliquoted into wells containing 10 μl of 640 $\mu\text{g}/\text{ml}$ test liposomes or vehicle liposomes. Plates were incubated for 16 h at 37°C. Syto 9 was added (100 μl of a 1.25- μM solution in dH_2O) and fluorescence was determined after 15 min incubation at room temperature (excitation at 485 nm, emission at 530 nm). The resulting relative fluorescence units were blanked against Mueller-Hinton broth and liposomal formulations without bacteria. Liposomal formulations that demonstrated at least 80% growth inhibition at 64 $\mu\text{g}/\text{ml}$ were further diluted to determine their minimal inhibitory concentrations, subjected to CFU assay as described, and tested at 64 $\mu\text{g}/\text{ml}$ against a broader range of Gram-positive and Gram-negative bacteria using the microtiter assay. The bacterial strains were *S. aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 700802), and *Enterobacter cloacae* (ATCC 49141) cultured before testing as recommended by ATCC.

Table I. Free fatty acids, cholesterol, and cholesteryl esters are found in normal human nasal fluid in micrograms per milliliter concentrations^a

Nasal Fluid	Fatty Acids ^b						Cholesteryl Esters ^d			
	Myristic ^c (14:0)	Palmitic ^c (16:0)	Palmitoleic ^c (16:1)	Stearic (18:0)	Oleic ^c (18:1)	Linoleic ^c (18:2)	Cholesterol ^e	Palmitate	Linoleate	Arachidonate
Individual										
D1	4.8	10.1	5.8	8.0	0.3	0.2	27.8	11.4	17.9	8.6
D2	19.0	56.3	27.2	0.0	6.9	12.0	71.4	79.8	86.0	69.9
D3	1.3	3.4	1.8	0.0	0.0	0.1	6.4	4.9	4.7	4.2
D5	6.9	21.2	9.4	0.0	0.1	0.1	90.3	25.2	41.6	21.1
D6	2.7	6.0	3.5	6.6	0.1	0.1	11.2	7.2	15.6	7.1
D8	23.7	7.6	26.0	7.6	0.0	13.9	14.23	7.36	13.15	8.04
Mean	9.7	17.4	12.3	3.7	1.2	4.4	36.9	22.7	29.8	19.8
SEM	3.8	8.2	4.7	1.7	1.1	2.7	14.4	11.8	12.3	10.3
Pooled										
D1,D2,D3,D7	10.9	13.1	12.8	13.7	0.2	0.2	17.8	15.7	28.7	13.3

^a Nasal fluid collected from individual donors (D) and pooled nasal fluid were supplemented with an internal standard (heptadecanoic acid, a plant fatty acid absent in humans), and lipid extracts were subjected to rpHPLC/ELSD. Native lipids were identified and quantified according to established reference chromatograms and standard curves and recovery of the internal standard. Only lipids that have been confirmed by a second technique are included, shown as concentrations in micrograms per milliliter.

^b Confirmed by GC/MS.

^c Confirmed by enzymatic assay.

^d Confirmed by MS/MS.

^e Coelute in rpHPLC at 4.6–5 min.

^f Coelute in rpHPLC at 5.8–6.0 min. Based on GC/MS intensity distribution in nasal fluid from donors D1–D3 and D6 (see Fig. 1c) myristic acid, palmitoleic acid, and linoleic acid are assigned 10, 45, and 45% of the peak area, respectively. Palmitic acid and oleic acid are each assigned 50% of the peak area.

Data analysis

Unless stated otherwise, mean, SEM, and SD were calculated and data were graphed with SigmaPlot 9.0. Statistical significance was determined with SigmaStat 2.0.

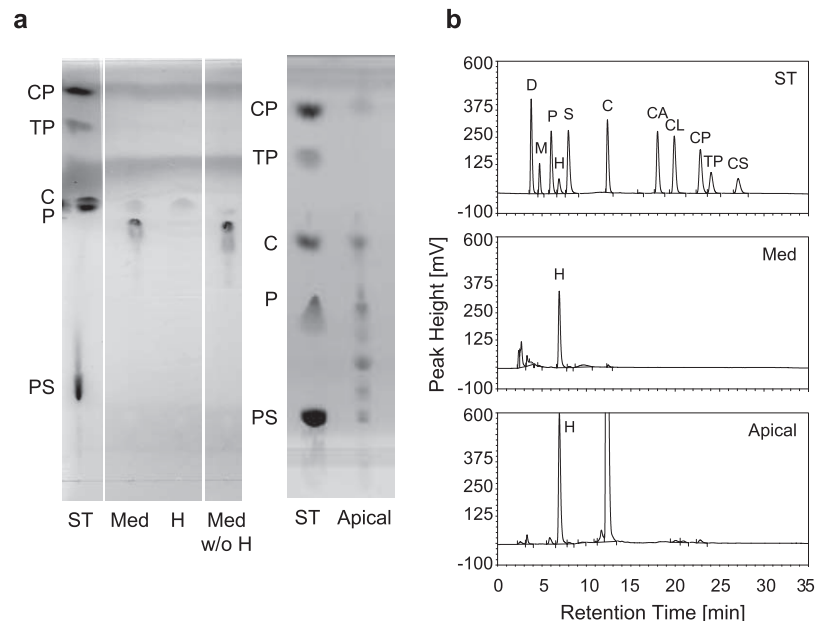
Results

Human nasal fluid is rich in lipids

By TLC (Fig. 1a) we detected all the major lipid classes in human nasal fluid: free fatty acids, glycerolipids, glycerophospholipids, sphingolipids, and sterols including cholesterol and cholesteryl esters. Retention times during rpHPLC with ELSD (rpHPLC/ELSD) were consistent with the presence of myristic or linoleic acid, palmitic or oleic acid, stearic acids, cholesterol, and a variety of cholesteryl esters and triglycerides (Fig. 1b). Additional peaks eluting shortly before cholesterol were tentatively assigned as diglycerides. Myristic (C14:0), palmitoleic (C16:1), palmitic (C16:0), linoleic (C18:2), oleic (C18:1) and stearic acid (C18:0) were identified

by GC/MS-based on retention times (Fig. 1c) and by comparing the peak mass spectra with library spectra and the spectra from authentic standards (data not shown). The presence of cholesterol was confirmed by subjecting the corresponding rpHPLC fraction to a commercial enzymatic test kit (data not shown). To identify the predominant cholesteryl esters, electrospray ionization mass spectra (Fig. 1d) were acquired from lipid extracts. The mass spectra revealed signals for the (M+ NH₄)⁺ adducts consistent with the presence of cholesteryl linoleate, cholesteryl arachidonate, and cholesteryl palmitate, and these assignments were unequivocally verified by the MS/MS spectra, which in all cases revealed a strong signal for the cholesterol fragment (m/z 369.3). This behavior was indistinguishable from that of the authentic standards. Compared with rpHPLC/ELSD peak analysis of standard lipids, the individual lipid concentrations were in the microgram per milliliter range (see Table I), free fatty acids were found at 8.1 ± 1.9 μg/ml, cholesterol at 36.9 ± 14.4 μg/ml reaching up

FIGURE 2. Apical secretions of human normal bronchial epithelial cells contain lipids similar to nasal fluid. Apical secretions of human bronchial epithelial cells (Epi-Airway kit AIR-606) accumulated over 7 days in air-liquid interface culture, and 2 ml of serum-free naive culture medium (Med), included as negative control, were mixed with the internal standard heptadecanoic acid (H, 20 μg), lipid extracted, and subjected to TLC (a) and rpHPLC/ELSD (b) analysis. a, Lipid extracts include standard lipids (ST, 25 μg each), cholesteryl palmitate (CP), tripalmitin (TP), cholesterol (C), palmitic acid (P, C16:0), phosphatidylserine (PS). Culture medium (Med) without heptadecanoic acid (H) used for comparison, and 2 ml of medium were extracted without prior addition of heptadecanoic acid. Apical lipid extracts (Apical) from 200 μl of apical secretions representing a growth area of 16.8 cm². On the left TLC plate the solvent fronts appear as homogeneously stained lines. b, rpHPLC/ELSD chromatogram with lipid standards (ST, 5 μg each), docosahexaenoic acid (D), and others shown in b. Apical lipid extract from 300 μl of apical fluid originating from a 16.8 cm² growth area.



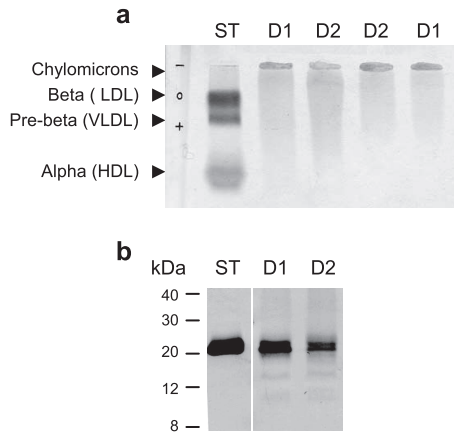


FIGURE 3. Nasal fluid contains apolipoproteins including ApoA-I. Individual nasal fluid samples were subjected to agarose gel electrophoresis with Fat Red 7B staining (*a*), and 12% tricine SDS-PAGE followed by Western immunoblotting (*b*) using mouse mAbs against apoA-I and an alkaline phosphatase and BCIP/NBT detection system. Serum standard (ST, 2 μ l) and donors D1 and D2 for nasal fluid (20 μ l each) collected from donors 1 and 2.

to 90 μ g/ml, and individual cholesteryl esters at 24.1 ± 6.3 μ g/ml (mean \pm SEM, $n = 36, 6,$ and 18 samples, respectively).

Lipid compositions of human respiratory epithelial cell secretions and nasal fluid are similar

To further substantiate that lipids detected in nasal fluid represent respiratory epithelial cell secretions, we harvested apical secretions from commercial normal human bronchioepithelial cells grown at air-liquid interface and determined their lipid profile using TLC (Fig. 2*a*) and rpHPLC/ELSD (Fig. 2*b*). As in nasal fluid, cholesterol was the predominant nonpolar lipid in the apical secretions, reaching concentrations of ~ 250 – 300 μ g/ml in both batches tested. A precholesterol peak in rpHPLC/ELSD (see Fig. 1*b*) as well as lipids with chromatographic behavior characteristic of cholesteryl esters was also detected at the low microgram per milliliter range. In contrast, naive cell culture medium contained fatty acids and lipids that behaved like phospholipids in TLC (Fig. 2*a*) at much reduced concentrations and, importantly, cholesterol and cholesteryl esters were not detectable (Fig. 2). This was despite

analysis of a 10-fold larger volume. Hence, it appears that lipids in nasal fluid originate at least in part from local production by epithelia in the upper airways.

Lipoproteins are also found in nasal fluid

In bodily fluids, an aqueous medium, the vast majority of lipids is bound to lipoproteins. Therefore, we surmised that lipoproteins should also be present in nasal fluid, and we subjected nasal fluid to Fat Red 7B-stained gel electrophoretic lipoprotein analysis (Fig. 3*a*). All samples tested contained one stronger band, which was located where chylomicrons in serum typically appear, according to the manufacturer, and also showed diffuse staining across the gel toward the anode. Subsequent immunoblotting using mAbs against the apolipoproteins apoA-I, apoB100, and apoE demonstrated the presence of apoA-I (Fig. 3*b*), but not apoB100 and apoE (data not shown). In addition to two major bands appearing between 20 and 30 kDa (nominal molecular mass of apoA-I is 28 kDa) minor bands of smaller mass were detected possibly reflecting fragmentation of apoA-I.

Partial removal of lipids from nasal fluid results in decreased antimicrobial activity, which can be restored by lipid resupplementation

To ascertain whether lipids contribute to the inherent antimicrobial activity of nasal fluid, we selectively removed lipids from nasal fluid (Fig. 4) and compared the antimicrobial activity of complete nasal fluid and lipid-depleted nasal fluid (Fig. 5). We focused on the removal of less polar lipids (fatty acids, cholesterol, diglycerides and triglycerides, and cholesteryl esters) because pilot studies suggested an association between higher concentrations of cholesteryl esters in nasal fluid and stronger inherent antibacterial activity (data not shown). For this experiment, we developed a SPE protocol using a C18 matrix that achieved a reduction of fatty acids, cholesterol/diglycerides, and cholesteryl esters/triglycerides by $54.6 \pm 11.1\%$, $59.6 \pm 19.2\%$, and $71.9 \pm 15.3\%$, respectively (mean \pm SEM, $n = 4$ samples). When comparing the rpHPLC/ELSD chromatograms of complete nasal fluid before and lipid-depleted nasal fluid after SPE, $61.57\% \pm 11.24\%$ of the less polar lipids (mean \pm SEM, $n = 4$ samples) were removed with this method and shown as representative chromatograms reflecting an

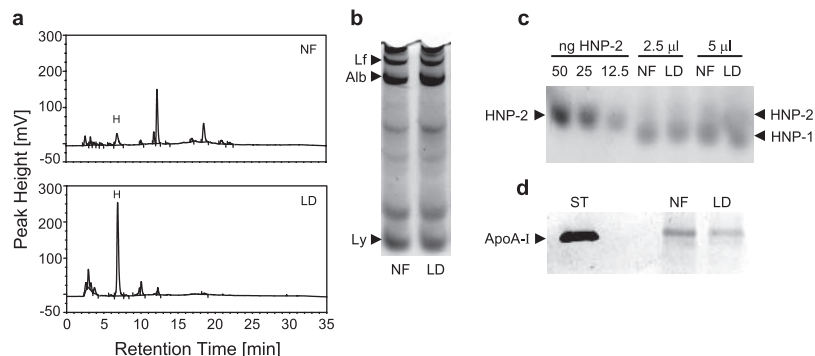


FIGURE 4. Nonpolar lipids can be selectively removed from nasal fluid by SPE. Nasal fluid was pooled from three donors (D1–D3) and subjected to SPE using a tC18 Sep-Pak cartridge to selectively remove less polar lipids. Aliquots from nasal fluid (NF) and lipid-depleted nasal fluid (LD) were adjusted to the same protein concentration and analyzed by rpHPLC/ELSD (*a*), silver-stained AU-PAGE (*b*), Western immunoblotting probing for human neutrophil peptides HNP-1–3 (*c*) and apoA-I (*d*). *a*, rpHPLC/ELSD chromatograms of lipid extracts from 200 μ l of nasal fluid and lipid-depleted nasal fluid with heptadecanoic acid (H, C17:0). In this experiment, an overall lipid reduction of 89.5% was achieved. *b*, A total of 2 μ l of nasal fluid and lipid-depleted nasal fluid were loaded, with lactoferrin (Lf), albumin (Alb), and lysozyme (Ly). *c*, Samples were probed with a polyclonal rabbit antiserum against HNP-1–3 and HNP-specific bands were visualized with an alkaline phosphatase and BCIP/NBT detection system. *d*, A total of 2 μ l of serum standard (ST) and 25 μ l of nasal fluid and lipid-depleted nasal fluid each were probed for apoA-I.

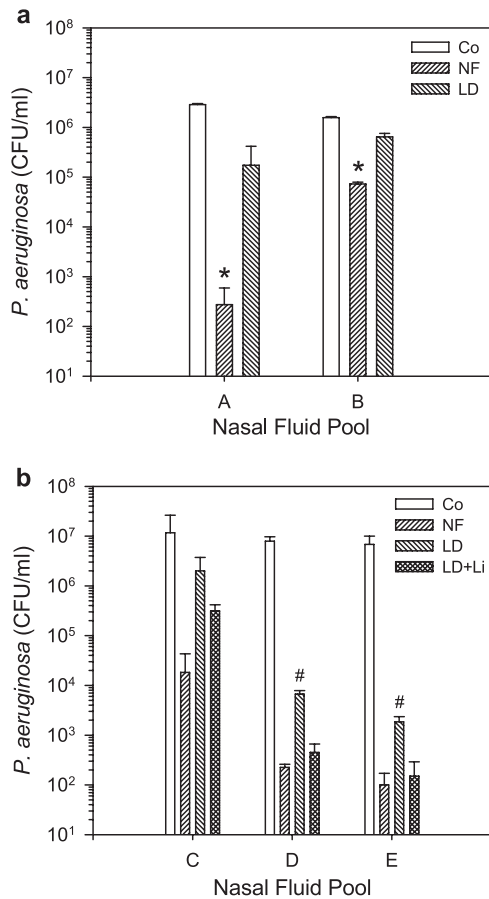


FIGURE 5. Lipids contribute to the inherent antimicrobial activity of nasal fluid. Nasal fluid (NF), lipid-depleted nasal fluid (LD), and lipid-depleted nasal fluid supplemented with lipids extracted from the corresponding nasal fluid (LD + Li) were mixed with *P. aeruginosa* and CFU per milliliter was determined after 30 min of incubation at 37°C. Bacteria were incubated in buffer control (Co). *a*, Nasal fluid pooled from donors D1–D3 (A), and nasal fluid pooled from donors D1, D2, and D5 (B) are shown. *, $p = 0.022$ for nasal fluid significantly reduced CFU per milliliter compared with the control by paired *t* test (for A) and *, $p = 0.023$ (for B), in contrast to lipid-depleted nasal fluid. *b*, Nasal fluid pooled from donors D1 and D3 (C), donors D1, D6, D9, and D10 (D), and from donors D1, D5, D6, D9, and D11 (E). Data are mean + SD of two independent experiments conducted in duplicates for each nasal fluid pool. #, $p = 0.016$ and $p = 0.018$, CFU per milliliter in lipid-depleted nasal fluid are significantly increased compared with nasal fluid and lipid-depleted nasal fluid plus lipids extracted by *t* test, respectively (for C); #, $p = 0.038$ and $p = 0.043$, respectively (for D); while nasal fluid and lipid-depleted nasal fluid supplemented with lipids extracted from the corresponding nasal fluid are statistically not different.

89% reduction (Fig. 4a), whereas the overall protein profile appeared unaltered (Fig. 4b). Importantly, this procedure did not reduce the contents of antimicrobial polypeptides as indicated by silver-stained AU-PAGE and by immunodetection of HNP-1–3 (nasal fluid: $6.4 \pm 1.1 \mu\text{g/ml}$; lipid-depleted nasal fluid: $6.2 \pm 1.3 \mu\text{g/ml}$, mean \pm SEM, $n = 4$ samples) (Fig. 4c). However, apoA-I appeared to be somewhat reduced in lipid-depleted nasal fluid at a 32% reduction of apoA-I in lipid-depleted nasal fluid (Fig. 4d).

We tested then the antibacterial activity of nasal fluid and lipid-depleted nasal fluid and found that the antibacterial activity of lipid-depleted nasal fluid against *P. aeruginosa* was diminished (Fig. 5a), but could be partially restored after replenishing lipid-depleted nasal fluid with lipid extracts prepared from homologous

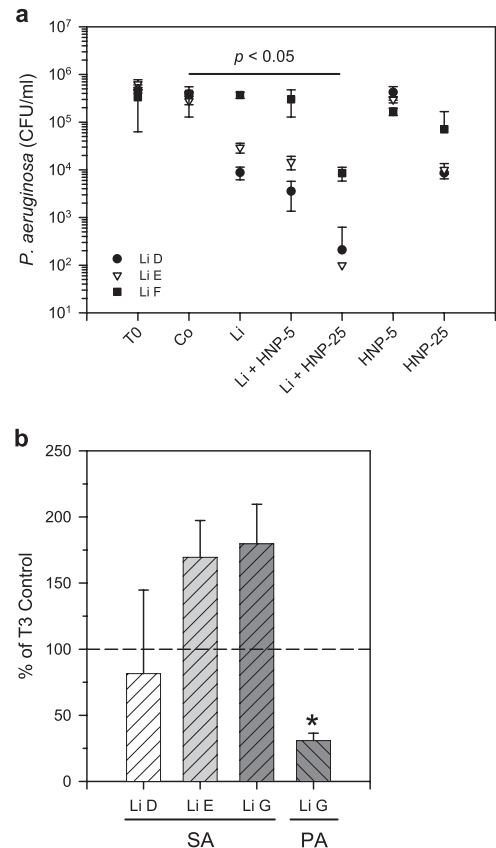


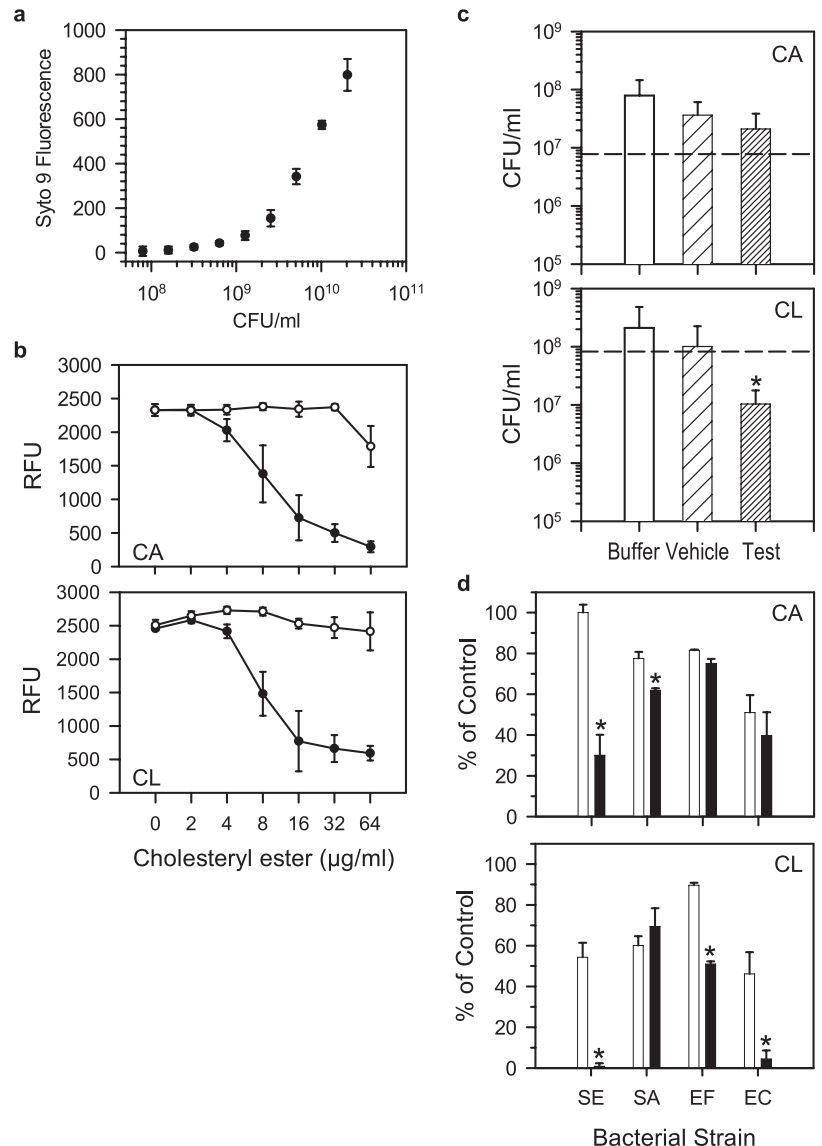
FIGURE 6. Lipids extracted from nasal fluid exert antibacterial activity against *P. aeruginosa* but not *S. aureus*. Lipids (Li) were extracted from nasal fluid pooled from donors D1, D6, D9, and D10 (Li D), from donors D1, D5, D6, D9, and D11 (Li E), from donors D1 and D6 (Li F), and from donors D1, D5, and D6 (Li G). Li C and Li D originate from nasal fluid pool from donors D3 (C) and donors D1, D6, D9, and D10 (D), respectively, as described in Fig. 5. Extracted lipids or solvent control (Co) were heated and sonicated in assay buffer to yield liposomal preparations. Extracts were mixed with bacteria, and CFU per milliliter was determined immediately (T0) or after 3 h (T3) incubation at 37°C. *a*, Activity of lipids against *P. aeruginosa* in the presence or absence of 5 and 25 $\mu\text{g/ml}$ HNP-2 (HNP-5 and HNP-25, respectively). *, $p < 0.05$ in Kruskal-Wallis one-way ANOVA on Ranks for control vs treatments. *b*, Activity of lipids against *S. aureus* and, for comparison, against *P. aeruginosa*. Mean + SD of two independent experiments conducted in duplicates is shown. *, $p = 0.040$ in *t* test for donors D1, D5, and D6 vs solvent control when tested against *P. aeruginosa*.

nasal fluid (Fig. 5b). These data suggest that less polar lipids contribute to the inherent antibacterial activity of nasal fluid.

Lipids extracted from nasal fluid exert direct antibacterial activity against *P. aeruginosa* in synergism with HNP-2

To further substantiate the antibacterial role of lipids in nasal fluid we subjected native liposomes prepared from lipid extracts from three different nasal fluid pools to CFU assays with *P. aeruginosa*. Considering the presence of HNP-1–3 in nasal fluid (see Fig. 4c) and previous reports on synergism between fatty acids and antimicrobial peptides (25), we also included testing the effect of HNP-2 on the antibacterial activity of lipids. We found that native liposome preparations from two nasal fluid pools reduced the number of CFU per milliliter by over 1 log and that all three native liposome preparations acted in synergism with HNP-2 leading to a significant reduction of colonies of up to 4 log in the presence of 25 $\mu\text{g/ml}$ HNP-2 (Fig. 6a).

FIGURE 7. Cholesteryl arachidonate and cholesteryl linoleate exhibit antibacterial activity against Gram-negative and Gram-positive bacteria in vitro. *a*, Staining with the DNA probe Syto 9 as measurement for bacterial growth. *P. aeruginosa* were grown in trypticase soy broth and serially diluted. Syto 9 relative fluorescence units (RFU) and the number of CFU per milliliter were determined in parallel. Shown is mean \pm SD for $n = 4$ samples. In a minimal inhibitory concentration assay, uninhibited bacteria reach concentrations of 10^{11} CFU/ml. A reduction of relative fluorescence units of 50% and 80% relates to a decrease in the number of CFUs by 1 and 1.5 log, respectively. *b*, Dose-dependent inhibitory activity of cholesteryl arachidonate (CA) and cholesteryl linoleate (CL) against *P. aeruginosa*. Bacteria were incubated in cation-adjusted Mueller-Hinton broth for 16 h with and without liposomes containing cholesteryl esters (●) and vehicle liposomes (○) and relative fluorescence units were measured after addition of the DNA probe Syto 9. Shown is mean \pm SD for $n = 3$ samples. *c*, CFU assay with *P. aeruginosa* and the cholesteryl esters cholesteryl arachidonate and cholesteryl linoleate. Bacteria were incubated for 3 h in saline-supplemented, low-nutrient phosphate buffer in the presence or absence of vehicle, and test liposomes and the number of CFU per milliliter were determined. Shown is mean \pm SD for $n = 4$ samples with cholesteryl arachidonate and $n = 3$ samples with cholesteryl linoleate. *, $p = 0.002$, concentration in CFU per milliliter were significantly reduced to $20 \pm 16.99\%$ of the inoculum, determined by one-way ANOVA for buffer vs cholesteryl linoleate. *d*, Extended antibacterial activity testing of cholesteryl arachidonate and cholesteryl linoleate in microtiter assay with Syto 9 readout. Cholesteryl esters were tested at $64 \mu\text{g/ml}$ against *Staphylococcus epidermidis* (SE), *Staphylococcus aureus* (SA), *Enterococcus faecalis* (EF), and *Enterobacter cloacae* (EC). Data are expressed as a percentage of relative fluorescence unit values for untreated bacteria. Mean \pm SD is shown for $n = 3$ samples. *, $p < 0.001$ by *t* test for test liposomes compared with vehicle liposomes.



We also tested the activity of initially two native liposome preparations from nasal fluid against *S. aureus* (Fig. 6b), a microorganism that is often found in the nostrils unlike *P. aeruginosa*. No significant activity against *S. aureus* was observed. To rule out a loss of lipid activity due to prolonged storage of lipid extracts before liposome preparation, we made native liposomes from lipids extracted from an additional nasal fluid pool (Fig. 6, Li G) and tested the antibacterial activity against *S. aureus* and *P. aeruginosa* in parallel. As before, the number of *S. aureus* CFU was not significantly reduced in contrast to the number of *P. aeruginosa* CFU, thus suggesting a differential antibacterial activity of host-derived lipids. The reduction of antibacterial activity of lipid-depleted nasal fluid appeared to be mainly linked to the removal of cholesterol and cholesteryl esters. Therefore, to begin characterizing the lipids that carry the antibacterial function in nasal fluid, we investigated the antibacterial activity of cholesterol and cholesteryl esters packaged in liposomes in vitro.

Cholesteryl arachidonate and cholesteryl linoleate have antibacterial activity

Initial screening of numerous liposomal formulations of cholesterol, and various cholesteryl esters using a minimal inhibitory concentration-based assay with *P. aeruginosa* and a fluorescence

readout (Fig. 7a) yielded two lipids, cholesteryl arachidonate and cholesteryl linoleate that effected fluorescence inhibition of at least 80%, which translates to a 1.5 log difference in colony count (CFU per milliliter) between treated and untreated bacteria. This activity was dose-dependent, and growth inhibition of *P. aeruginosa* was demonstrable at concentrations as low as $4 \mu\text{g/ml}$ (Fig. 7b), which is within the lower range of what we have measured in nasal fluid ex vivo. When cholesteryl arachidonate and cholesteryl linoleate were subjected to a 3-h CFU assay in a low nutrient phosphate buffer with a higher bacterial inoculum (Fig. 7c), cholesteryl arachidonate appeared to slow bacterial growth, whereas cholesteryl linoleate was bactericidal, significantly reducing the number of CFU per milliliter to $20 \pm 16.99\%$ of the inoculum ($p = 0.002$ in one-way ANOVA for buffer vs cholesteryl linoleate liposome). When testing cholesteryl arachidonate and cholesteryl linoleate against a broader range of bacterial strains, both cholesteryl esters inhibited growth of *S. epidermidis* and, furthermore, cholesteryl linoleate inhibited also *E. faecalis* and *E. cloacae* (Fig. 7d). The vehicle liposomes, which are composed mainly of phospholipids, demonstrated some inhibitory activity as well.

Taken together, we have demonstrated that cholesteryl arachidonate and cholesteryl linoleate exhibit direct antibacterial activity in low and high nutrient medium and that cholesteryl linoleate

appears to be the more potent antibacterial lipid with a broader spectrum.

Discussion

This study provides evidence that lipids are secreted to mucosal surfaces and contribute to the inherent antimicrobial activity of mucosal secretions. To examine the potential role of lipids in innate mucosal host defense, we used nasal mucosal secretions. Nasal mucosa is a primary microbial exposure site; nasal mucosa is not exposed to alimentary lipids, its secretions are easily accessible and its antibacterial activity has been previously established in respect to antimicrobial polypeptides (27).

We found all major lipid classes in nasal fluid collected from healthy adults and, to our knowledge, this study is the first quantification of lipids in human nasal fluid. Glycerophospholipids and cholesterol, as well as to a lesser extent triglycerides and free fatty acids, have already been described in bronchioalveolar and nasal lavages, whereby the lipids were mainly thought to originate from lung surfactant reaching the upper airways through mucociliary propulsion (35–37). However, the presence of cholesteryl esters in native nasal fluid and their identification in the apical secretions of human bronchial epithelial cells suggest that the epithelial cells of the upper airways contribute to the lipids found in nasal fluid. This is also supported by the recent discovery of surfactant lamellar bodies in normal sinus mucosa (38). Nonetheless, we cannot exclude transudation of plasma lipids through endothelial cells of the upper respiratory tract (39, 40).

In aqueous environments, at submicrocellular concentrations, lipids require carrier molecules such as albumin or lipoproteins. Our results demonstrate the presence of lipoproteins in nasal fluid. Specifically, by using immunoblotting, apoA-I was identified, which has also been detected by Ghafouri et al. (41) in nasal fluid lavage. We found apoA-I in individual samples in multiple forms possibly reflecting proteolysis of apoA-I by enzymes in nasal fluid (42). ApoA-I is mainly found in HDL and one of the major functions of apoA-I is cholesterol binding and reverse cholesterol transport from tissue to bile (43). This finding is consistent with our showing the presence of both cholesterol and apoA-I. However, we were not able to detect distinct lipoprotein bands comigrating with HDL in the lipoprotein gels, but in contrast, observed diffuse staining. Such diffuse mobility in agarose gels has been also shown for protease-modified HDL particles (42). Furthermore, our results could also reflect the presence of other lipid binding proteins. For example, lipocalin, which is produced by nasal mucosa (44, 45), has been previously detected in nasal fluid (27, 46). Alternatively, nasal fluid may contain unique lipoprotein particles synthesized by epithelial cells in the upper airways. The specific lipoproteins in nasal fluid and their origins remain to be defined.

To achieve reasonably selective lipid depletion from nasal fluid while minimally altering its natural, highly complex composition, we developed an SPE procedure. Though there are several widely accepted SPE protocols for the purification of lipids from biological fluids (reviewed in Ref. 47), these do not aim to preserve the depleted fractions for further testing. Initial pilot studies suggested an association between inherent killing capacity of nasal fluid with cholesteryl ester contents and we focused on removing primarily nonpolar lipids while allowing the presence of phospholipids. We found that the inherent antibacterial activity of nasal fluid was significantly diminished when nonpolar lipid concentrations were reduced. Even though the SPE procedure may have altered other constituents of the nasal fluid, such as electrolytes and mucins, the observation that the re-addition of lipids restored in part the antibacterial activity, and the direct antibacterial activity of lipid extracts and commercial cholesteryl esters strongly sug-

gest that host-derived antimicrobial lipids contribute to the inherent antimicrobial activity of nasal fluid, which was previously speculated by Widdicombe (48). Antimicrobial functions of cholesteryl esters are supported by a study by Georgel et al. (49), in which mice with a mutation in the stearoyl coenzyme A desaturase 1 gene develop spontaneous chronic dermatitis and also exhibit a decrease in cholesteryl ester content in the affected skin.

The contributions of apoA-I and possibly other lipoproteins to the observed antimicrobial activity of lipids is not yet clear. ApoA-I was partially codepleted during SPE, in contrast to other proteins, and lipid supplement alone did not fully restore the antibacterial activity of lipid-depleted nasal fluid. Hence, apoA-I may exert antimicrobial activity alone (50, 51) or by enabling host-derived lipids to function similarly to the reported antibacterial activity of lipoproteins in conjunction with antimicrobial peptides (52).

Host-derived antimicrobial lipids may exert their activity in conjunction with antimicrobial polypeptides, which have a well documented membrane-perturbing activity (53). It is conceivable that antimicrobial lipids disrupt microbial membranes by embedding their hydrophobic acyl chains or side chains, and this activity could be facilitated by lesions initially created by antimicrobial polypeptides. In this study, we have shown that lipids extracted from nasal fluid act synergistically with the antimicrobial peptide HNP-2. Tollin et al. (25) reported *in vitro* synergistic activity between selected fatty acids and the antimicrobial peptide LL37, and we are currently addressing in our laboratory the mode of synergistic action between fatty acids and lysozyme (J. Martinez, M. Waldon, S. Alvarez, A. Oren, M. Du, F. Zhou, A. Zenz, K. Lohner, R. Desharnais, and E. Porter, manuscript submitted). Similarly, lipopeptabols, naturally occurring lipopeptides with antimicrobial activity (54), and *in vitro* modified antimicrobial peptides that contain acyl chains and exhibit enhanced antimicrobial activity have been described (55). The antimicrobial properties of host-derived lipids may also extend to intracellular targets (56) and direct or indirect toxicity due to oxidation of lipids. Polyunsaturated fatty acids, such as linoleic acid and arachidonic acid, which have been found in our study either as free fatty acid or esterified in cholesteryl linoleate and cholesteryl arachidonate, are prone to peroxidation in the presence of oxygen and can form cytotoxic by-products (57–59).

Cole et al. (27) reported previously that the cationic protein extract of nasal fluid retained most of the bactericidal activity of nasal fluid. However, the lipid contents have not been analyzed in this study and it cannot be ruled out that some antibacterial lipids associated with highly hydrophobic antimicrobial proteins such as lysozyme may have been coextracted. Even though lysozyme exerted rapid bactericidal activity and was able to fully restore the bactericidal activity of cationic protein-depleted nasal fluid when tested at high concentrations *in vitro* suggesting a predominant role in mucosal defense, its relative contribution to the inherent antibacterial activity of nasal fluid (and other bodily fluids) may be more complex *in vivo*. Cholesteryl esters exhibited prolonged activity in a cation adjusted, nutrient rich medium, unlike most antimicrobial peptides (60), and it is conceivable that antimicrobial peptides and antimicrobial lipids cover different aspects of mucosal defense, rapid initial mass destruction and retardation of the proliferation of survivors, respectively. Similarly, considering the varying spectrum of activity among both, antimicrobial polypeptides and cholesteryl esters, mucosal defense may relay on both components to cover the entire spectrum of potential invaders. A careful analysis of antimicrobial polypeptides and antimicrobial lipids in mucosal secretions and their *in vitro* activities will reveal their respective relative contributions to mucosal defense.

Lipid secretion may contribute to shaping the resident microbiota on mucosal surfaces like antimicrobial peptides (61). We did

not observe a significant activity of nasal fluid lipids against *S. aureus*, which often colonizes the nostrils (62), but a pronounced activity against *P. aeruginosa*, which is not routinely present in normal subjects. Furthermore, secretion of antimicrobial lipids may be also important for keeping the total number of the resident microbiota low. Mucosal surfaces populated by squamous epithelial cells with less secretory functions such as in the oral and vaginal cavity are typically heavily colonized. Hence, antimicrobial lipid secretion may be a feature of columnar epithelial cells in the airways and possibly other body sites with little colonization.

In conclusion, we have shown that lipids are present in mucosal secretions of the upper airways in significant quantities, and have provided evidence that lipids contribute to the inherent antibacterial activity of nasal fluid alone and in synergism with antimicrobial peptides. This suggests a role of host-derived lipids as direct antimicrobial effector molecules in innate mucosal immunity. The concept of antimicrobial lipids may unveil new mechanisms of host resistance to infections and microbial pathogenesis, as well as new avenues for prophylactic and therapeutic strategies in infectious diseases.

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Disclosures

The authors have no financial conflict of interest.

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