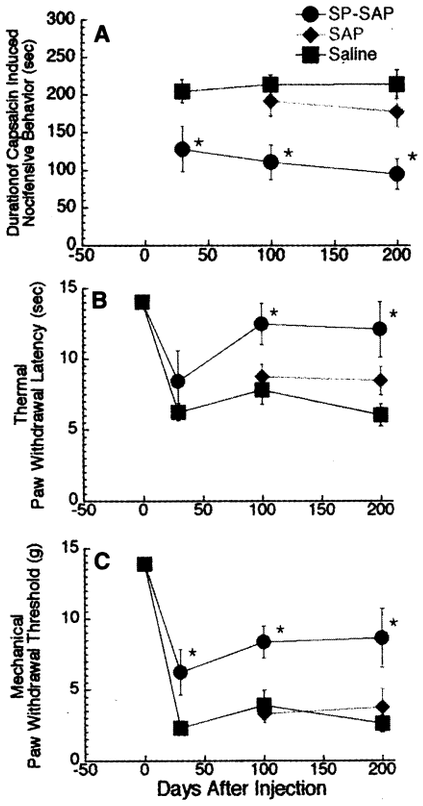

Fig. 3. Time course of antihyperalgesic effect of i.th. infusion of saline (n = 5, 10, and 6 at 30, 100, and 200 days, respectively), 10^{-6} M SAP (n = 6, 6, and 6), or 10^{-6} M SP-SAP (n = 5, 10, and 6). After SP-SAP treatment, there is a reduction in (**A**) capsaicin-induced nocifensive behavior (expressed as duration, in seconds, over 300-s observation), (**B**) thermal hyperalgesia (expressed as paw withdrawal latency, in seconds), and (**C**) mechanical allodynia (expressed as paw withdrawal threshold, in g) that does not diminish with time after injection. Infusion of saline or SAP alone did not produce any changes in these pain behaviors at any of the time points examined. Asterisks represent statistical significance from control ($P < 0.05$).

dence suggests that a uniting feature of many spinal and forebrain neurons that express the SPR is their involvement in the response to tissue injury and stress (*10*). Characterization of the gene and protein changes that SPR-expressing spinal neurons undergo during nociception should provide insight into the spinal mechanisms involved in the generation and maintenance of chronic neuropathic and inflammatory pain.

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3. Cell counts were performed with the neuronal nuclei marker NeuN and SPR antibodies [R. J. Mullen, C. R. Buck, A. M. Smith, *Development* **116**, 201 (1992); J. O. Suhonen, D. A. Peterson, J. Ray, F. H. Gage, *Nature* **383**, 624 (1996)] (i) by quantifying the total number of NeuN- and SPR-immunoreactive cells with a standard fluorescence microscope, (ii) by using the optical dissector method [R. E. Coggeshall, *Trends. Neurosci.* **15**, 9 (1992)], and (iii) by counting the total number of NeuN- and SPR-immunoreactive cells with the Bio-Rad MRC 1024 confocal microscope in a z series of 20 μm in 2-μm steps (beginning 10 μm into the tissue section). The z series was collapsed, and the number of cells was counted with Image Pro Plus (Media Cybernetics, Silver Spring, MD). With the use of these methods, the proportion of NeuN-positive cells that were also SPR-positive ranged from 2.8 to 6.3%. For similar results, see J. L. Brown *et al.* [*J. Comp. Neurol.* **356**, 327 (1995)] and N. K. Littlewood, A. J. Todd, R. C. Spike, C. Watt, and S. A. Shehab [*Neuroscience* **66**, 597 (1995)]. n sizes = 5, 6, and 10 for saline, SAP, and SP-SAP, respectively, with averages of 3 to 7 sections per animal.
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6. Rats received an s.c. injection of formalin (2.5%, 50 μl) into the dorsal surface of the hind paw or the forepaw. The number of paw flinches was quantified every 5 min for 50 min after injection [D. Dubuisson and S. G. Dennis, *Pain* **4**, 161 (1977)]. Rats receiving formalin in the forepaw were also observed for time spent licking or guarding the forepaw [B. Calvino, *Physiol. Behav.* **47**, 907 (1990); C. A. Porro, G. Tassinari, F. Facchinetti, A. E. Panerai, G. Carli, *Exp. Brain Res.* **83**, 549 (1991)].
7. Rats received λ-carrageenan (0.2 mg, 0.1 ml) or CFA (50%, 0.1 ml) s.c. into the plantar hind paw 30 days after infusion of SP-SAP (10 μl, 10^{-6} M) and were tested at 3 hours and each day for 3 days (λ-carrageenan) or 9 days (CFA) in the thermal and mechanical assays [L. Kocher, F. Anton, P. W. Reeh, H. O. Handwerker, *Pain* **29**, 363 (1987); M. J. Millan *et al.*, *Pain* **35**, 299 (1988)].
8. Rats were prepared according to the method of S. H. Kim and J. M. Chung [*Pain* **50**, 355 (1992)]. Rats were anesthetized with halothane, and the L5 and L6 spinal nerves were tightly ligated. Animals were allowed to recover for 1 week.
9. The loss of lamina III neurons at 30 days observed in the present study is a result of binning the number of SPR-positive neurons in each individual lamina. Previously, neurons from laminae III to V were analyzed as one group (*5*). If SPR-expressing neurons in laminae III to V are combined and counted as one group, there is no significant loss of laminae III to V neurons at 30 days after infusion of SP-SAP. The delayed loss of SPR-expressing neurons in laminae IV and V compared with neurons in laminae I to III may be due to the rapid diffusion and internalization of SP-SAP in laminae I and III neurons compared with the deeper laminae IV and V neurons. In contrast, the loss of SPR-expressing neurons in laminae I, III, IV, and V all appear to have reached maximal levels at 100 days.
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Specific Lipopolysaccharide Found in Cystic Fibrosis Airway *Pseudomonas aeruginosa*

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Cystic fibrosis (CF) patients develop chronic airway infections with *Pseudomonas aeruginosa* (PA). *Pseudomonas aeruginosa* synthesized lipopolysaccharide (LPS) with a variety of penta- and hexa-acylated lipid A structures under different environmental conditions. CF patient PA synthesized LPS with specific lipid A structures indicating unique recognition of the CF airway environment. CF-specific lipid A forms containing palmitate and aminoarabinose were associated with resistance to cationic antimicrobial peptides and increased inflammatory responses, indicating that they are likely to be involved in airway disease.

Cystic fibrosis (CF) is the most common inherited disorder of Caucasians (*1*). The respiratory tracts of most patients with CF become infected with the opportunistic gram-negative bacteria *Pseudomonas aeruginosa* (PA) shortly after birth (*2*). Chronic infection results in airwayinflammation, which is the major cause of morbidity and mortality in CF. Despite improved survival when treated with antibiotic therapy, CF patients eventually die of progressive PA pulmonary infection characterized by massive neutrophilic infiltration without bacterial destruction.

Recently, it has been demonstrated that enteric bacteria synthesize different forms of lipid A in response to environmental conditions that include magnesium-limited growth and conditions encountered during mammalian infection (*3*). Salmonellae with these modifications have increased resistance to cationic antimicrobial peptides (CAMPs) and decreased lipopolysaccharide (LPS)–mediated recognition by human cells. Because the PA-CF lung interaction is a remarkable ex-

ample of chronic bacterially induced inflammation, experiments were performed to examine whether PA could synthesize different lipid A structures within CF airways.

The dominant lipid A structure produced by the wild-type PA strains PAK and PAO-1 (*4*), which have been extensively passaged in the laboratory, was determined by gas chromatography (GC) and negative-ion mass spectrometry (MS) (*5*). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis of lipid A from culture grown in high-magnesium medium gave a dominant $[M-H]^-$ ion at a mass-to-charge ratio (*m/z*) of 1447 (Fig. 1A), which represents a penta-acylated form of lipid A. This structural interpretation is supported by the fatty acid profile obtained by capillary GC analysis and tandem MS product ion spectra from the *m/z* 1447 precursor (*6*) and is consistent with the established penta-acylated structure of PA (*7*, *8*). In contrast, lipid A from cells grown in low-magnesium medium had a substantially different structure (Fig. 1B). Collision-activated dissociation data (Fig. 1, D and E) confirmed that this environmental condition promoted lipid A modifications like those of Enterobacteriaceae, including the addition of aminoarabinose (4-amino-4- deoxy-L-arabinose) to the 1 or 4′ phosphates (or both) (*9*) and of palmitate (C16:0 fatty acid) at the 3′ 3-oxo-C10:0 (*10*). Palmitate has not been shown to be a PA lipid A constituent in previous studies.

Addition of aminoarabinose and palmitate to lipid A has been associated with bacterial resistance to CAMP. Therefore, PA grown in medium of varying magnesium concentration was compared for resistance to C18G, an alpha-helical CAMP derived from the COOH-terminus of human platelet factor IV (*11*), and to polymyxin (*12*), an acylated cyclic CAMP (*13*). As observed with *Salmonella typhimurium*, resistance to these CAMPs was promoted by growth in magnesium-limited medium (Fig. 2).

Salmonella typhimurium lipid A modifications are regulated by the *phoP/phoQ* two-component regulatory system (*3*), which is composed of the sensor kinase PhoQ and the phosphorylated transcriptional activator PhoP (*14*). PA genes similar to *phoP/phoQ* were identified by analyzing the Pseudomonas Genome Project sequence database (Pathogenesis Corporation, University of Washington). Sequence data from contig 632 (released 15 March 1998) was used to construct primers to complete the sequencing of this chromosomal region. These genes are similar to *S. typhimurium*, *Escherichia coli*, and *Yersinia pestis phoP/phoQ* sequences (>50% identity and 65% similarity) and are located 3′ to the gene encoding the PA outer membrane protein OprH1, which is expressed during magnesium-limited growth (*15*). This DNA sequence information was used to construct a PA *phoP* null mutant by insertional inactivation based on a gentamicin resistance cassette (*16*).

The phenotype of *S. typhimurium* PhoP/PhoQ mutants includes decreased resistance to CAMPs and inability to grow on magnesium-limited growth medium. In contrast to observations of the enteric bacterium *S. typhimurium* (*17*), the growth rate in magnesium-limited medium was the same for PAK and the *phoP* null mutant (*18*). PA-inducible resistance to polymyxin is mediated through PhoP/PhoQ activation, because the concentration of polymyxin at which 50% of bacteria were killed was 100 times greater for PAK (>80 μg/ml) than for the *phoP* null mutant (0.62 μg/ml) (Fig. 2C). The C18G-inducible resistance appeared to be independent of PhoP/PhoQ activation, because no difference was observed when PAK and the *phoP* null mutant were compared (*18*).

Negative-ion MALDI-TOF MS analysis of lipid A from *phoP* null PA did not show the addition of aminoarabinose and palmitate

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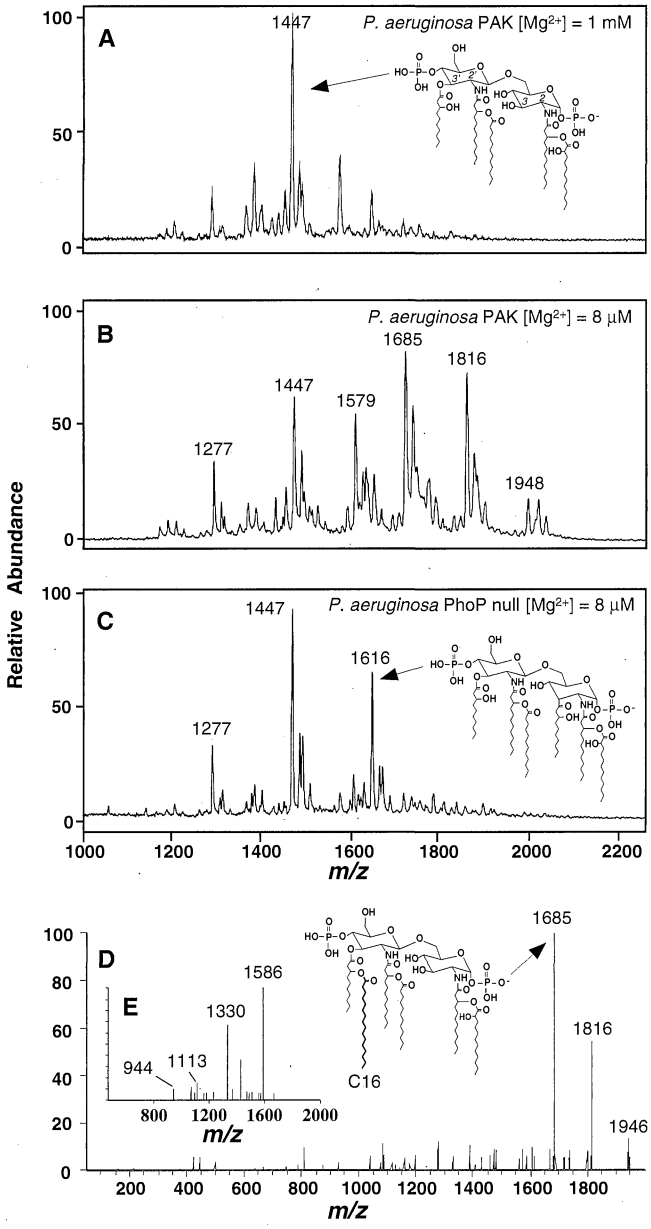

Fig. 1. Characterization of structural modifications of PA lipid A by negative-ion MS. All values given are the average mass rounded to the nearest whole number for singly charged deprotonated molecules. (**A**) MALDI-TOF mass spectrum of lipid A from strain PAK grown under high-magnesium conditions, with the dominant penta-acylated form yielding the $[M-H]^-$ at *m/z* 1447 (note also the ring carbon numbering scheme). (**B**) MALDI-TOF mass spectrum of strain PAK grown under low-magnesium conditions, showing additions of C16:0 (*m/z* 1685), C16:0 with one aminoarabinose group (*m/z* 1816), and C16:0 with two aminoarabinose groups (*m/z* 1948). (**C**) MALDI-TOF mass spectrum from the *PhoP* null mutant grown under low-magnesium conditions, showing the hexa-acylated form containing the 3-OH-C10:0 at the 3 position. The location was assigned on the basis of prior work (*8*) and of MS^2 or MS^3 mass spectral fragmentation patterns (*25*). (**D**) Electrospray triple quadrupole MS^2 mass spectrum of fragments from the precursor ion at *m/z* 1948 [see (B)], the $[M-H]^-$ for the hexa-acylated form containing two aminoarabinose groups and C16:0 (*10*). (**E**) Fragments of the C16:0-modified hexa-acylated precursor ion at *m/z* 1685; for details with respect to the location of the C16:0 acyl group, see (*10*).

(Fig. 1C), which is consistent with a role for PA PhoP/PhoQ in magnesium-regulated lipid A modifications. The absence of aminoarabinose and the susceptibility of the PA *phoP* null mutant to polymyxin were consistent with previous results from *S. typhimurium* (*19*). For enteric bacteria, increased acylation of lipid A by palmitoylation promotes resistance to C18G. However, palmitoylation does not appear to be required for PA resistance to C18G, because the *phoP* null mutant and the wild type demonstrated similar resistance. The formation of a PhoP-independent hexa-acylated lipid A structure could promote resistance to C18G in a fashion similar to PhoP-dependent palmitoylation. The presence of the ion at *m/z* 1616 was consistent with the existence of such a hexa-acylated structure substituted with a 3-OH-C10:0 group at the 3 position (*8*) of the diglucosamine backbone (Fig. 1C). Fatty acid profiles and tandem MS analyses of *m/z* 1616 (*6*) were consistent with this hypothesis. Because a specific 3-deacylase activity has recently been demonstrated in PA (*20*), it is possible that the hexa-acylated lipid A (*m/z* 1616) is a precursor of the penta-acylated lipid A (*m/z* 1447). These results indicated that a laboratory PA strain, originally isolated from a CF patient, could synthesize a variety of lipid A structures in response to different environmental conditions.

PA strains isolated from CF patients have been shown to have a variety of virulence-associated properties when compared to laboratory-passaged strains; properties that are often lost after growth in vitro. The expression of virulence properties immediately after a strain's isolation from CF patients probably reflects the properties' selection in vivo. Therefore, minimally passaged PA isolates from bronchoalveolar lavage and oropharyngeal swabs from clinically stable CF infants were tested to determine their lipid A structure. Lipid A was isolated and analyzed by ion-trap MS, after growth under conditions in which it is not modified by laboratory strains (*4*). As described earlier, the well-studied laboratory strains PAO-1 and PAK had *m/z* 1447 as the dominant ion (Fig. 3A). Lipid A from seven minimally passaged clinical PA isolates was analyzed and gave mass peaks at *m/z* 1447, 1685, 1816, and 1948. These ions were consistent with the synthesis of lipid A containing palmitate (*m/z* 1685) and aminoarabinose (*m/z* 1816 and 1948); tandem MS studies (*6*), amino sugar analysis, and fatty acid profiles supported these assignments. All seven of the CF clinical isolates had penta-acylated lipid A, *m/z* 1447, and more than 50% of the CF clinical isolates showed increased palmitoylation when compared to PAK, as measured with electrospray MS (*21*). One CF isolate, CF1188, an oropharyngeal isolate from a CF patient who later developed chronic sinusitis, demonstrated the highest level of modified lipid A, with an estimate that more than 33% of the lipid A molecules contained palmitate (Fig. 3B). When CF1188 was continuously passaged in Luria broth (LB) medium and analyzed by MS, these modifications were lost (*6*).

Lipid A was analyzed from minimally passaged PA isolated from two patients with sepsis and three patients with bronchiectasis, a chronic non-CF lung infection, to test the specificity of our observations for CF. Nei-

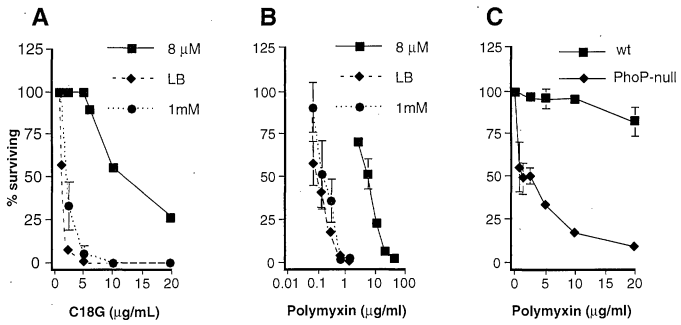

Fig. 2. Bacterial growth in different magnesium conditions and resistance to CAMPs. (**A** and **B**) Increased resistance of PAK bacteria to killing by C18G (0 to 20 μg/ml) and polymyxin (0.1 to 80 μg/ml), respectively, when grown under magnesium-limited conditions. Each assay was performed in triplicate, and the mean ± SD is presented. (**C**) PAK PhoP-null strain has increased susceptibility to polymyxin (0.1 to 20 μg/ml; wild-type survival did not diminish further at 80 μg of polymyxin per milliliter). Both PAK and PhoP-null strains were grown in limited magnesium conditions for survival assays. Each assay was performed in triplicate, and the mean ± SD is presented.

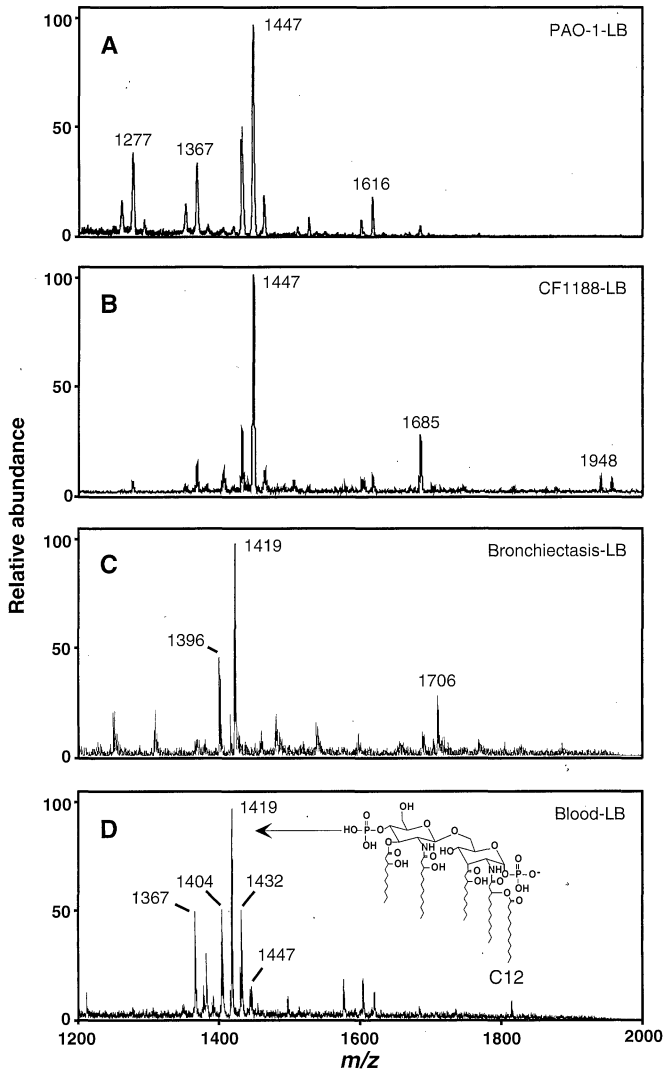

Fig. 3. Characterization of structural modifications of CF and non-CF PA lipid A by negative-ion quadrupole ion trap MS. (**A**) Mass spectrum (MS^1) of lipid A from strain PAO-1 grown in LB medium (a growth condition under which laboratory strains do not modify lipid A), showing the dominant penta-acylated form yielding the $[M-H]^-$ at *m/z* 1447. (**B**) CF clinical isolate CF1188 grown in LB medium, showing additions of C16:0 (*m/z* 1685) and C16:0 with two aminoarabinose groups (*m/z* 1948). (**C**) Representative bronchiectasis isolate grown in LB medium, showing the dominant penta-acylated form at *m/z* 1419, containing 3-OH-C10:0 and lacking 2-OH C12:0 (*22*). (**D**) Representative non-CF blood isolate grown in LB medium, showing the dominant penta-acylated form at *m/z* 1419.

ther mucoid PA from bronchiectasis (Fig. 3C) nor nonmucoid PA from blood (Fig. 3D) showed the dominant penta-acylated form, *m/z* 1447, that was observed for PAK (Fig. 1A), PAO-1 (Fig. 3A), or the CF clinical isolate CF1188 (Fig. 3B). The dominant ion observed for these clinical isolates was *m/z* 1419, which represents a penta-acylated form of lipid A containing 3-OH-C10:0 and lacking 2-OH C12:0 (*22*). Addition of palmitate or aminoarabinose to this penta-acylated lipid A (*m/z* 1419) was not observed for any of these non-CF clinical isolates, even when grown in magnesium-limited medium. However, lipid A prepared from both the bronchiectasis and blood isolates after growth in magnesium-limited medium contained *m/z* 1447, 1685, and 1816 ions (*6*), indicating that the pathways necessary for the synthesis of CF-specific lipid A were intact and inducible.

Because the lipid A acylation state can affect biological activity, LPS from different PA clinical isolates was tested for the ability to stimulate interleukin-8 (IL-8) production by human umbilical cord endothelial cells (HUVECs) (*23*). LPS from five of seven CF strains showed significantly increased stimulation of IL-8 expression when compared with the clinical bronchiectasis isolate (Fig. 4D) (*24*). Again, the most dramatic results were obtained with CF1188, the strain with the highest level of palmitate, which suggests that structures with this fatty acid may stimulate increased responses.

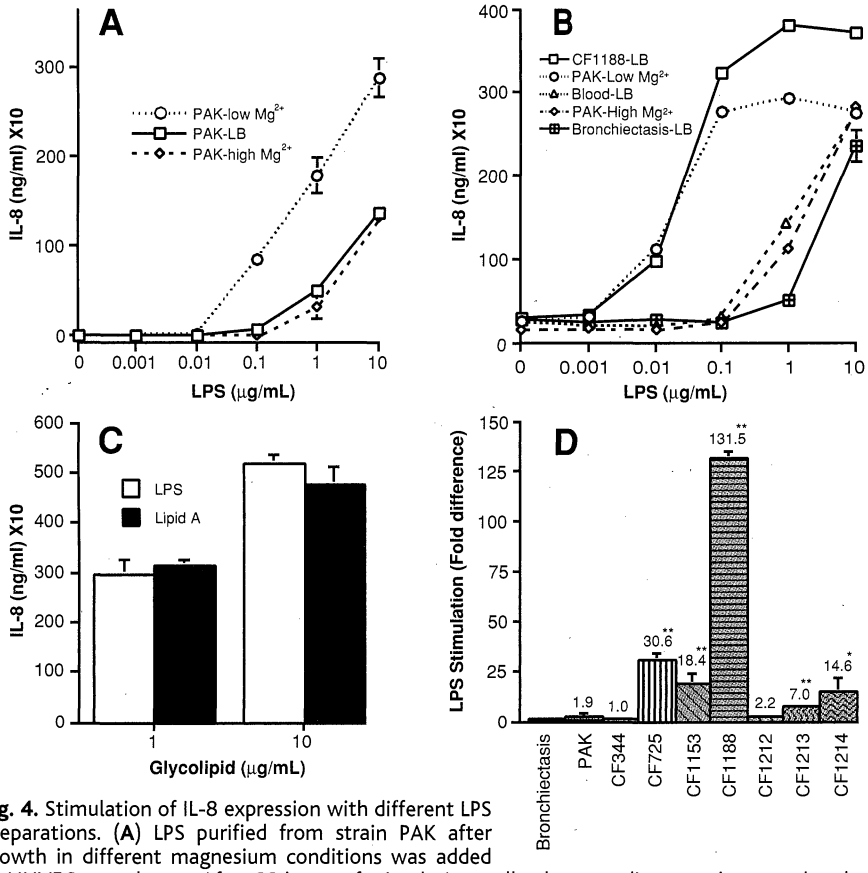

Fig. 4. Stimulation of IL-8 expression with different LPS preparations. (**A**) LPS purified from strain PAK after growth in different magnesium conditions was added to HUVEC monolayers. After 22 hours of stimulation, cell culture medium was harvested and was assayed for the presence of IL-8 by ELISA. Each stimulation assay was performed in triplicate and the mean ± SD is presented. GC analysis of the various LPS fatty acid derivatives indicated that the quantity of 3-OH-C12:0 (the amide-linked and least variable fatty acid in lipid A per milligram of LPS dry weight was 92.6, 71.5, and 88.7 nM for PA LPS from growth in LB, in 8 μM Mg^{2+}, and in 1 mM Mg^{2+}, respectively. (**B**) LPS purified from PAK after growth in different magnesium conditions and from PA clinical isolates (CF1188, bronchiectasis, and blood) after growth in LB medium. Stimulation assays were performed as above. GC analysis of the various LPS fatty acid derivatives indicated that the quantity of 3-OH-C12:0 was 44.4 and 37.7 nM for PAK after growth in 8 μM Mg^{2+} and in 1 mM Mg^{2+}, respectively; and was 40.6, 27.0, and 27.3 nM for clinical isolates CF1188, bronchiectasis, and blood, respectively, after growth in LB medium. (**C**) LPS and lipid A purified from CF1188 after growth in LB medium. Stimulation assays were performed as above. GC analysis of LPS fatty acid derivatives indicated that the quantity of 3-OH-C12:0 was 13.8 nM for LPS and 17.4 nM for lipid A. (**D**) LPS purified from all clinical isolates after growth in LB medium. Stimulation assays were performed as above. LPS stimulation relative to bronchiectasis LPS (100 ng/ml corrected for background) is presented. Statistical analysis (*t* test, $*P > 0.05$, $**P > 0.005$) is also shown.

The results from the clinical isolates indicated that LPS stimulatory activity could be increased as a result of synthesis of different lipid A structures. Because laboratory strains grown in low-magnesium medium show LPS modifications similar to those observed in the clinical isolates (Fig. 1A), LPS from PAK was tested for the ability to stimulate IL-8 production by HUVECs. LPS from cells grown in low-magnesium medium also stimulated an increase in IL-8 (with a range of 10- to 100-fold from different LPS preparations) when compared with LPS derived from cells grown in magnesium-replete conditions (Fig. 4A). Similar differences were observed for LPS-induced E-selectin expression, an outer membrane adhesion molecule (*18*). HUVEC IL-8 and E-selectin–induced expression by LPS was CD14-mediated, because HUVEC preincubation with a monoclonal antibody to CD14 abolished the LPS effect (*18*). Additionally, the stimulatory activity of LPS prepared from bronchiectasis and blood isolates after growth in LB medium was similar to that seen for PAK LPS derived from cells grown in magnesium-replete conditions (Fig. 4B). These results provide further evidence to indicate that CF-specific lipid A structures induce increased inflammatory responses.

Finally, to confirm that the increased stimulation observed with LPS from clinical isolates and laboratory strains grown in low-magnesium medium was caused by the lipid A component, HUVECs were stimulated with either LPS or lipid A isolated from the PA clinical isolate CF1188 (Fig. 4C). Similar levels of IL-8 expression were observed for both LPS and lipid A, indicating that only the lipid A portion of LPS was required for the IL-8 expression observed. Furthermore, the higher stimulatory activity of CF1188 LPS was lost after serial passage concurrent with the loss of modified lipid A (*6*, *18*). Taken together, these data indicate that the mixture of CF-specific lipid A structures promoted increased CD14-dependent LPS recognition by HUVECs when compared to LPS from PA isolated from humans with bronchiectasis.

These data suggest that PA lipid A structure can influence two aspects of the pathogenesis of CF chronic lung disease. First, the unique PA lipid A structures may promote bacterial survival or colonization. Because certain lipid A structures promote resistance to CAMPs and other membrane active components of the innate immune system, strains with increased ability to synthesize modified lipid A are selected within the CF lung. Second, CF-specific PA lipid A structures may then generate increased or unique inflammatory responses. Therefore, compounds that block the synthesis of the modified PA lipid A forms may have utility in the treatment of

CF lung disease by increasing susceptibility to innate immune killing and by decreasing the inflammatory response.

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4. The laboratory CF strains used were PAK and PAO-1. Clinical isolates CF344, CF725, CF1153, CF1188, CF1212, CF1213, and CF1214 were collected as part of a multicenter study on infection and inflammation in young infants with CF. Non-CF clinical isolates (from patients with bronchiectasis, RKE024-026; from blood, RKE020) were from J. Burns and PathoGenesis Corporation. Cultures were grown at 37°C with aeration in either LB or N-minimal medium supplemented with 38 mM glycerol, 0.1% casamino acids, and either 8 μM or 1 mM $MgCl_2$ [E. Garcia-Vescovi, F. C. Soncini, A. A. Groisman, *Cell* **84**, 165 (1996)]. LPS was isolated by means of Mg^{2+} ethanol precipitation as described by Darveau and Hancock [R. P. Darveau and R. E. Hancock, *J. Bacteriol.* **155**, 831 (1983)]; lipid A was isolated after hydrolysis in 1% SDS at pH 4.5 [M. Caroff, A. Tacken, L. Szabo, *Carbohydr. Res.* **175**, 273 (1988)]. LPS fatty acids were derivatized to fatty acid methyl esters and analyzed by GC [J. E. Somerville, L. Cassiano, B. Bainbridge, M. D. Cunningham, R. P. Darveau, *J. Clin. Invest.* **97**, 359 (1996); R. P. Darveau *et al.*, *Infect. Immun.* **63**, 1311 (1995)].
5. Negative-ion MALDI-TOF and electrospray experiments were performed as described, with the following modifications [*Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics*, American Society for Mass Spectrometry (ASMS), Orlando, FL, 31 May to 4 June 1998 (ASMS, Santa Fe, NM, 1998)]. Lyophilized lipid A was dissolved with 5 μl of 5-chloro-2-mercaptobenzothiazole (CMBT) MALDI matrix in chloroform/methanol, 1:1 (v/v) and then applied (1 μl) onto the sample plate [N. Xu, Z.-H. Huang, *et al.*, *J. Am. Soc. Mass Spectrom.* **8**, 116 (1997)]. All MALDI-TOF experiments were performed with a Voyager DE mass spectrometer (PerSeptive Biosystems, Framingham, MA). The electrospray work was performed with either a TSQ 7000 triple quadrupole mass spectrometer or an LCQ ion trap (Finnigan/Thermoquest, San Jose, CA). Both electrospray instruments were equipped with an experimental low flow (nanoliters per minute) capacitive ion source [H. Wang and M. Hackett, *Anal. Chem.* **70**, 205 (1998)].
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10. Based on the published lipid A structure for the penta-acylated form (7, 8), there are only two possible locations where the C16:0 can be attached to yield hexa-acylated lipid A, *m/z* 1685: (i) directly onto the 3-hydroxy position of the diglucosamine backbone or (ii) piggyback on the 3′ 3-oxo-C10:0 (see Fig. 1A for the numbering of the carbons on the sugar backbone). Fragmentation of the *m/z* 1685 precursor ion showed a peak at *m/z* 944 as a result of the loss of a phosphate, C10:0, 2-OH-C12:0, and C16:0 groups to yield triacyl monophosphate lipid A (Fig. 1, D and E). From the two possibilities discussed above, only the assignment of the C16:0 group on 3′ 3-oxo-C10:0 would yield the observed *m/z* 944 fragment. When similar logic was applied, with the C16:0 group arbitrarily assigned to the 3-hydroxy position of the diglucosamine backbone, mass peaks related to the triacyl and tetra-acyl monophosphate lipid A fragments were predicted to occur at *m/z* 926 and 1183 respectively, but were not observed in the product ion (MS^2) spectra. Furthermore, the MS^2 spectra from the *m/z* 1447 precursor ion (with C16:0 absent) also yielded the *m/z* 944 fragment, which is the expected triacyl monophosphate product ion, and further confirmed that the location of C16:0 was not at the 3-hydroxy position. Finally, the presence of an ion at *m/z* 1948 (Fig. 1B) indicated that two aminoarabinose moieties could be added to the hexa-acylated form of lipid A. Because of the facile sequential loss of aminoarabinose residues during fragmentation in the mass spectrometer, our studies using electrospray tandem MS were unable to confirm the precise location on the molecule of the two amino sugar groups. We sometimes observe low–signal-to-noise ratio (S/N) ions in our MS work (*m/z* 1685), which are attributable to a C16:0-containing hexa-acylated lipid A in the PAK and PAO-1 reference strains. Such signals for *m/z* 1948 have not been detected in the reference strains.
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12. Polymyxin B sulfate was purchased from USB (Cleveland, OH). Assays were performed as described [D. A. Steinberg *et al.*, *Antimicrob. Agents Chemother.* **41**, 1738 (1997)].
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16. The plasmid vector pBSphoP was constructed by cloning a 2.7-kb Bam HI–Mse I fragment [nucleotide 8054 through 10784 (nt 8054–10784)] containing the complete *oprH1* (nt 8755–9354) and *phoP* (nt 9437–10110) genes and a partial *phoQ* (nt 10138–11454) gene into the vector pBluescript KS (+) digested with Eco RV. Nucleotide numbers are derived from contig 632 from the Pseudomonas Genome Project sequence database, release 3/15/98 (Pathogenesis Corporation, Univ. of Washington; available at www.pseudomonas.com). A 1.7-kb gentamicin resistance cassette with Eco RI ends was blunt-end cloned into a unique Sfi I (nt 9716) site located in the *phoP* gene. This plasmid, called pBSphoP-Gm, was used for the insertional inactivation of the *phoP* gene in PA strain PAK (RKE004).
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20. S. S. Basu, K. A. White, N. L. Que, C. R. Raetz, *J. Biol. Chem.* **274**, 11150 (1999). PA $lipid_{IVA}$, a tetra-cylated lipid A precusor [C. R. H. Raetz, in Escherichia coli and Salmonella, F. C. Neidhardt, Ed. (ASM Press, Washington, DC, 1996), pp. 1035–1063] can be generated by the addition of C12 and 2-OH-C12 to generate the hexa-acylated species of lipid A, *m/z* 1616. The CF-specific dominant penta-acylated species *m/z* 1447 can be generated by the deacylation of the hexa-acylated species *m/z* 1616; then the species *m/z* 1447 can be palmitoylated to generate the hexa-acylated species at *m/z* 1685.
21. Because of the qualitative nature of our of main beam (MS^1) MALDI-TOF mass spectra, quantitative analyses of the lipid A signals at *m/z* 1447 (penta-acyl), 1685 (penta-acyl plus C16) 1816 (hexa-acyl plus one aminoarabinose residue), and 1948 (hexa-acyl plus two aminoarabinose residues) for each minimally passaged clinical isolate were performed on a Finnigan LCQ ion trap using isopentenyl pyrophosphate (IPP, Sigma) (5 pmol/μl) as an internal standard (*m/z* 245). Approximately 10 pmol of each sample per microliter was prepared in 1:1 chloroform/methanol, which also contained IPP. Samples were infused (at a rate of 0.7 μl/min) into the capacitive electrospray source. The spray voltage and heated capillary temperature were set at 2.4 kV and 250°C; 20 scans were acquired per analysis; and 10 replicate analyses were performed and averaged for each sample. The relative standard deviation values for replicate analyses were 5% or better. Signals from the ions given above were normalized to the signal for IPP and summed, and the percentage of the total signal due to C16:0-containing structures was calculated as follows: PAK, 8.8; CF1188, 33.7; CF344, 21.1; CF725, 11.2; CF1213, 6.6; CF1153, 13.1; CF1214, 18.9; and CF1212, 5.4.
22. Tandem MS data (6) from the *m/z* 1419 precursor were consistent with prior work [R. C. Goldman, C. C. Doran, S. K. Kadam, J. O. Capobianco, *J. Biol. Chem.* **263**, 5217 (1988)].
23. Growth media for the HUVECs consisted of Medium 199 (Gibco-BRL Gaithersburg, MD) supplemented with 4 mM L-glutamine, heparin (90 μg/ml), 1 mM Na pyruvate, endothelial cell growth stimulant (30 μg/ml) (Biomedical Products, Bedford, MA), and 20% fetal bovine serum (Summit Biotechnology, Fort Collins, CO). Stimulation medium consisted of Medium 199 plus 4 mM L-glutamine, heparin (90 μg/ml), 1 mM Na pyruvate, human serum albumin (1 mg/ml), and 5% pooled human serum (Gemini Bioproducts, Calabasas, CA).
24. HUVEC stimulation assays were performed as described [R. P. Darveau *et al.*, *Infect. Immun.* **63**, 1311 (1995)], with the following modifications. IL-8 samples (75 μl) were removed after the stimulation interval (22 hours) and stored at −20°C until assayed. IL-8 immunodetection was performed as follows: Microtiter plates (Immulon II, Dynex Technologies, Chantilly, VA) were coated using 50 μl of IL-8 capture monoclonal antibody (2.0 μg/ml) (M-801, Endogen, Woburn, MA) in phosphate-buffered saline (PBS) overnight at 4°C. Free binding sites were blocked with 200 μl of 2% bovine serum albumin in PBS at room temperature for 1 hour. Washes were performed with PBS containing 0.2% Tween-20 between incubations. Fifty microliters of sample and 50 μl of IL-8 biotinylated monoclonal antibody (0.2 μg/ml) (M-802-B, Endogen, Woburn, MA) were incubated at room temperature. After being shaken for 2 hours, wells were washed and were incubated with 50 μl Vectastain (PK6100, Burlingame, CA) at 37°C for 1 hour. Plates were developed according to manufacturers' recommendations using EIA chromogen reagent (R6, Redmond, WA) and were read with a Molecular Devices Thermomax microplate reader (Sunnyvale, CA) at an optical density of 450 nm. MY4, the monoclonal antibody to human CD14, was obtained from Coulter Immunology (Hialeah, FL).
25. MS^2 refers to a single stage of the sequential product ion experiment in which a precursor ion (for example, *m/z* 1616) is isolated from others present in the main beam spectrum (MS^1) and fragmented by colliding the precursor ion with a neutral target gas, such as argon (~3 mTorr) in the triple quad collision cell [R. A. Yost and R. K. Boyd, *Methods Enzymol.* **193**, 154 (1990)] or the helium bath gas (~1 mTorr) in the ion trap [K. R. Jonscher and J. R. Yates 3rd, *Anal. Biochem.* **244**, 1 (1997); P. S. H. Wong and R. G. Cooks, *Curr. Sep.* **16**, 85 (1997); M. E. Bier and J. C. Schwartz, in *Electrospray Ionization Mass Spectrometry*, R. B. Cole, Ed. (Wiley, New York, 1997), pp. 235–289]. MS^3 refers to an additional stage of this same process, typically performed in an ion trap, in which a structurally informative fragment found in the MS^2 spectrum is in turn isolated and subjected to fragmentation.

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LINKED CITATIONS

References and Notes

[3] **Regulation of Lipid A Modifications by Salmonella typhimurium Virulence Genes phoP-phoQ**

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[14] **A Two-Component Regulatory System (phoP phoQ) Controls Salmonella typhimurium Virulence**

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