Phase-variable Expression of Lipopolysaccharide Contributes to the Virulence of Legionella pneumophila

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Summary

With the aid of monoclonal antibody (mAb) 2625, raised against the lipopolysaccharide (LPS) of Legionella pneumophila serogroup 1, subgroup OLDA, we isolated mutant 811 from the virulent wild-type strain RC1. This mutant was not reactive with mAb 2625 and exhibited an unstable phenotype, since we observed an in vitro and in vivo switch of mutant 811 to the mAb 2625–positive phenotype, thus restoring the wild-type LPS. Bactericidal assays revealed that mutant 811 was lysed by serum complement components, whereas the parental strain RC1 was almost serum resistant. Moreover, mutant 811 was not able to replicate intracellularly in macrophage-like cell line HL-60. In the guinea pig animal model, mutant 811 exhibited significantly reduced ability to replicate. Among recovered bacteria, mAb 2625–positive revertants were increased by fourfold. The relevance of LPS phase switch for pathogenesis of Legionella infection was further corroborated by the observation that 5% of the bacteria recovered from the lungs of guinea pigs infected with the wild-type strain RC1 were negative for mAb 2625 binding. These findings strongly indicate that under in vivo conditions switching between two LPS phenotypes occurs and may promote adaptation and replication of L. pneumophila. This is the first description of phase-variable expression of Legionella LPS.

Key words: Legionella pneumophila • LPS • phase-variation • serum resistance • virulence

Legionella pneumophila is the causative agent of Legionnaires’ disease, a severe pneumonia with frequently fatal progression (1). The habitat of Legionella species are natural or man-made water reservoirs where the bacteria survive and multiply intracellularly in amebae (2–4) in tight association with biofilms (5–7). Infection of man occurs by inhalation of Legionella-containing aerosols, but person to person transmission has never been observed (1, 8). In the human lung attachment of L. pneumophila and internalization into alveolar-macrophages is mediated by the major outer membrane protein, MOMP,1 the complement factors C3b and iC3b and the corresponding receptors (9, 10). In phagocytes fusion of Legionella-containing phagosomes with lysosomes is prevented and L. pneumophila survives and multiplies within macrophages (11, 12).

Several virulence factors of L. pneumophila have been identified and characterized. The macrophage infectivity potentiator protein (Mip) plays an important role in infection of macrophages, although its precise function is unclear (13–20). The products of the icm and dot loci are required for intracellular multiplication. Again, their role in the pathogenesis of disease is unresolved (21–25). Likewise, LPS of L. pneumophila is considered a factor mediating pathogenicity (8). It is the major immunodominant antigen and represents the basis for the classification of serogroups (26–29). In contrast to enterobacterial LPS activation it has been shown that Legionella LPS is able to activate both the classical and the alternative complement pathway (30). Due to the exceptional chemical structure of the L. pneumophila LPS, it is likely that this molecule participates in a number of essential legionellae capabilities, such as adaptation to

1Abbreviations used in this paper: BYCE, buffered CYE; Cl, chemical ionization; CYE, charcoal yeast extract; EI, electron impact; GLC, gas–liquid chromatography; MAC, membrane attack complex; MOMP, major outer membrane protein; NHS, normal human serum; NMR, nuclear magnetic resonance; SG, serogroup.

This work is dedicated to Dieter Bitter-Suermann on the occasion of his 60th birthday.
various environmental challenges (31). The L. pneumophila serogroup (SG) 1 (strain Philadelphia) LPS differs from that of other Gram-negative bacteria in that its lipid A section consists of long chain fatty acids which may account for the weak endotoxicity of the molecule (31). The O-specific chain is composed of an \( \alpha \)-(2→4) linked 5-acetamido-7-acetamido-8-O-acetyl-3,5,7,9-tetraideoxy-L-glyero-D-galacto-nonulosonic acid (legionaminic acid) homopolymer. This unusual sugar molecule completely lacks free hydroxyl groups and is thus very hydrophobic (31, 32). In addition, an iso-mer of legionaminic acid hypothesized to be a terminal sugar of the LPS O-chain has been recently detected (33). The outer core oligosaccharide also exhibits hydrophobic properties (34). Based on these findings, it can be assumed that L. pneumophila possesses a hydrophobic cell surface that may support concentration of the bacterium in aerosols as well as adherence to host cells (31, 35).

To further elucidate the role of the LPS molecule and the surface properties of L. pneumophila in adaptation to various exogenous conditions, we raised mAb against the LPS of L. pneumophila SG 1 (subgroup OLDA). In this study, we describe mAb 2625 which binds to this LPS. Moreover, we show that the O-chain as well as the core are required for binding of mAb 2625. With the aid of mAb 2625, we isolated an LPS mutant from the virulent patient isolate R C1 (subgroup OLDA). Here we report for the first time that the LPS structure appears to be a virulence determinant of L. pneumophila and that expression of L. pneumophila LPS occurs in a phase-variable manner.

Materials and Methods

Bacterial Strains and Cultivation. L. pneumophila SG 1 strain R C1 (OLDA), a clinical isolate, was a generous gift from B. Wight (Righospitalet, Copenhagen, Denmark). All other Legionella strains were obtained from the American Type Culture Collection (Rockville, MD) and the National Collection of Type Cultures (London, UK), respectively. Strains and sources are listed in Table 1. Legionella strains were cultivated on charcoal yeast extract (CYE) agar supplemented with buffered charcoal yeast extract (BCYE) growth supplement and MWY selective supplement (Unipath-Oxoid, Wesel, Germany). Plates were incubated at 37°C for 48–72 h unless otherwise stated. Propagation in liquid media (1% wt/vol yeast extract supplement (Unipath-Oxoid, Wesel, Germany). Plates were incubated at 37°C under 5% CO\(_2\) for 48–72 h unless otherwise stated. Propagation in liquid media (1% wt/vol yeast extract supplemented with BCYE growth supplement) was carried out at 37°C under constant agitation.

Pseudomonas aeroginosa (ATCC 49266) was obtained from the American Type Culture Collection. The following strains were isolates from the Institut für Medizinische Mikrobiologie (Medizinische Hochschule Hannover, Germany): P. fluorescens, Bordetella pertussis, Aeromonas iwoffii, and Esherichia coli.

Production of Mononodal Antibodies. 6-wk-old female BALB/c mice (Zentralinstitut für Versuchstierkunde, Hannover, Germany) were immunized intraperitoneally for four times once a week with \( 2 \times 10^8 \) L. pneumophila SG 1 strain R C1 viable cells as previously described (36). Before injection, bacteria were passaged once in a guinea pig as described below. At the end of the immunization regimen, mice were splenectomized and the spleen cells were fused with X63-Ag8.653 myeloma cells as described elsewhere (37). The culture supernatant fluids of growing clones were screened by ELISA with whole L. pneumophila SG 1 (strain R C1) cells as antigens. The resulting hybridomas were cloned by limiting dilution.

Immunoelectron Microscopy. Bacteria were fixed with 0.5% formaldehyde and 0.2% glutaraldehyde (final concentrations) in 0.1 M PBS for 1 h on ice. After three washes with 0.1 M PBS containing 10 mM glycine to block free aldehyde groups, the cells were embedded by progressively lowering the temperature with Lowicryl K4M resin (38). The following modifications of the method were made: (a) after dehydration in 10% ethanol, the samples were treated with 0.5% uranyl acetate in 10% ethanol for 1 h on ice; (b) the infiltration step with 1 part ethanol and 1 part K4M resin was performed overnight; (c) the infiltration step with one part ethanol and two parts K4M resin lasted for 12 h; and (d) infiltration with pure K4M resin lasted for 2 d. After polymerization of the samples for 2 d at −35°C, samples were trimmed and polymerized for another day at room temperature. Ultrathin sections were incubated overnight with 200 μg of IgG per ml of mAb 2625 or mAb LPS-1 (39) at 4°C. mAb LPS-1 was purchased from Progen (Heidelberg, Germany). After washing with 0.1 M PBS, sections were incubated with protein A-gold complexes (10-nm diam; concentration giving an A\(_{280}\) of 0.02). The sections were subsequently rinsed with 0.1 M PBS containing 0.01% Twen 20 and then with distilled water. After air drying, the sections were counterstained with 4% aqueous uranyl acetate (pH 4.5) for 5 min. Samples were examined with a Zeiss EM 910 electron microscope at an acceleration voltage of 80 kV at calibrated magnifications.

Western Blot. 1 ml of bacterial cell suspensions (OD\(_{550nm}\) 1.2) were centrifuged and the resulting pellet was resuspended in 100 μl sample solution (20% glycerol, 3% sodium dodecyl sulfate, 3% mercaptoethanol, 1% bromphenol blue). The suspensions were heated to 100°C for 5 min before 5-μl aliquots were applied to 12.5% polyacrylamide gels. To ensure equal protein concentrations in each lane, control gels were stained with Coomassie Blue dye (Sigma Chemical Co., Deisenhofen, Germany). For analysis of LPS samples, 2 μg purified LPS was applied to gels after boiling in sample solution. Western blotting onto nitrocellulose filters was carried out as described by Towbin et al. (40). Filter membranes were blocked with 3% dried milk powder suspended in PBS. Immunostaining was performed with mAb 2625 and mAb LPS-1 (39), respectively, and subsequent incubation with alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Dianova, Hamburg, Germany).

Colony Blot. Nitrocellulose filters were soaked in sterile PBS before colonies grown on BCYE agar were blotted. Filter membranes were then placed for 5 minutes on paper filter sheets (Schleicher and Schuell, Dassel, Germany) soaked with 70% ethanol, 1% bromphenol blue. The suspensions were heated to 100°C for 5 min before 5-μl aliquots were applied to 12.5% polyacrylamide gels. To ensure equal protein concentrations in each lane, control gels were stained with Coomassie Blue dye (Sigma Chemical Co., Deisenhofen, Germany). For analysis of LPS samples, 2 μg purified LPS was applied to gels after boiling in sample solution. Western blotting onto nitrocellulose filters was carried out as described by Towbin et al. (40). Filter membranes were blocked with 3% dried milk powder suspended in PBS. Immunostaining was performed with mAb 2625 and mAb LPS-1 (39), respectively, and subsequent incubation with alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Dianova, Hamburg, Germany).

Extraction of LPS and Isolation of O-Chain Polymers. LPS of L. pneumophila SG 1 strain R C1 (subtype OLDA) and mutant 811 was isolated from dry cells by a modified phenol-chloroform-petroleum ether procedure as described (32, 41). Starting from washed and enzymatically degraded dried cells excellent yields were obtained ranging between 8.1% (wt/wt) for the wild-type and 9.9% (wt/wt) for the mutant, respectively. LPS (155 mg of the wild-type, 144 mg of the mutant) was degraded with 0.1 M NaOAc-HOAc buffer (pH 4.4, 30 ml) at 100°C for 4 h and the resulting precipitate was removed by centrifugation. The supernatant was freeze-dried and fractionated by gel permeation chro-
Table 1. Legionella Strains Used in this Study and Indication of the Source

| Strain Source | Strain Source |
|---------------|---------------|
| L. pneumophila SG 1 (Oxford) | ATCC 43109 |
| L. pneumophila SG 1 | ATCC 33152 |
| L. pneumophila SG 1 | ATCC 43108 |
| L. pneumophila SG 1 | ATCC 43106 |
| L. pneumophila SG 1 | NCTC 11191 |
| L. pneumophila SG 1 | NCTC 11201 |
| L. pneumophila SG 1 | NCTC 11378 |
| L. pneumophila SG 1 | ATCC 33154 |
| L. pneumophila SG 1 | ATCC 33156 |
| L. pneumophila SG 1 | ATCC 33215 |
| L. pneumophila SG 1 | ATCC 335096 |
| L. pneumophila SG 1 | ATCC 43283 |
| L. pneumophila SG 1 | ATCC 43290 |
| L. pneumophila SG 14 | ATCC 43703 |
| L. cherrii | ATCC 35252 |
| L. birminghamensis | ATCC 43702 |
| L. dumoffii | ATCC 33279 |
| L. miidadei | ATCC 33204 |

Chemical LPS Analysis by Gas–Liquid Chromatography and Nuclear Magnetic Resonance. Gas–liquid chromatography (GLC) was performed with a Varian Model 3700 chromatograph equipped with a capillary column of SPB-5 using a temperature gradient 150–320°C at 5°C/min. GLC-mass spectrometry was carried out with a Hewlett-Packard Model 5989 instrument equipped with a capillary column of HP-1 under the same chromatographic conditions as in GLC. Monosaccharides were analyzed by GLC after methanolysis with 2 M HCl/MeOH at 60°C for 40 h and acetylation with Ac₂O in pyridine (70°C, 0.5 h). 1H- and 13C-nuclear magnetic resonance (NMR) spectra were obtained with a Bruker AM-360 spectrometer for solutions in D₂O at 30°C with acetone (δH 2.225, δC 31.45) as internal standard. Standard Bruker software was used in all 1H- and 13C-NMR experiments.

Competitive ELISA. 1 µg purified LPS from L. pneumophila strain RC1 in 20 µl 0.2 M sodium carbonate buffer, pH 9.6, per well was adsorbed to microtiter plates (Microlon, Greiner, Nürtingen, Germany) overnight at 4°C. Plates were subsequently blocked with 2% (wt/vol) dried milk powder in PBS for 1 h at room temperature. mAbs 2625 and LPS-1, respectively, were preincubated with carbohydrate fractions from wild-type strain RC1 and mutant strain 811, respectively. These sugar fractions containing O-chain polysaccharide with attached core oligosaccharide were obtained from gel permeation chromatography as described above. The polysaccharides were redissolved in distilled water to a concentration of 50 µg/µl. 1 µl of serial dilutions of carbohydrate fractions was added to 19 µl dilutions of mAb 2625 and mAb LPS-1, respectively. Before addition of the sugars the antibodies were diluted to a suitable concentration in PBS. In control reactions 1 µl distilled water was added to the antibody dilutions instead of carbohydrate solutions. Coincubation of mAbs and carbohydrate fractions was performed at 4°C overnight. After blocking and three washing steps with PBS, microtiter plates were incubated with the antibody–carbohydrate mixture for 2 h at room temperature and subsequently washed with PBS for three times. Detection was carried out by incubation of the plates with peroxidase-labeled goat anti-mouse antibody (Dia-anoa) for 1 h at room temperature. After three washing steps as before the substrate H₂O₂ and azino-di-ethylbenzthiazolinsulfonate (ABTS) was added and after a 30-min incubation at room temperature absorbance was determined in a microplate reader at 405 nm. Inhibition of antibody binding was calculated as percentage of the control reactions (no carbohydrates added). All reactions were carried out in duplicates.

Bactericidal Assay. For investigation of serum resistance of L. pneumophila wild-type strain RC1 and mutant strain 811 normal human serum (NHS) was obtained from 10 healthy volunteers. Blood was allowed to clot for 30 min at room temperature. After centrifugation for 5 min at 2,000 g, the sera were pooled, quick-frozen in liquid nitrogen and stored at −80°C. Pool serum was negative for anti-L. pneumophila antibodies as determined by standard diagnostic serology methods (immune fluorescence test). Bacteria were plated on BCYE agar from frozen stocks and suspended in 0.9% saline after 40 h of incubation. Spectral density was determined at 600 nm and bacterial suspensions were appropriately diluted in 0.9% saline. 40% NHS was incubated with 10⁶ bacteria in a final reaction volume of 1 ml. The reaction mixture was incubated at 37°C in a water bath and stopped on ice at 0, 15,
ditions, HL-60 were infected with 10⁶ bacteria per well. At time
again and incubated in antibiotic-free medium. Under these con-
After a 2-h incubation time with gentamicin, cells were washed
strains were plated on BCYE agar from frozen stocks and were
tral neck and the bacterial suspension was injected into the ex-
were pooled and aliquots of serial dilutions were plated on BCYE
In a second assay, extracellular remaining bacteria were killed
by adding gentamicin (GIBCO BR L) to a final concentration of
40 μg/ml after 2 h of incubation of H L-60 cells and bacteria. After
incubation time with gentamicin, cells were washed again and incubated in antibiotic-free medium. Under these con-
L. pneumophila were infected with 10⁶ bacteria per well. At the
segments in 0.9% saline was determined at 600 nm and appropri-
dilutions were prepared in RPMI medium. For determination
results were also plated on blood agar to control for contaminating
mL xylazinhydrochloride (2% solution) per 100 g body
in the range of 30–65 kD. This molecular mass range does not
non-L pneumophila strains as well as several bacteria from other genera were tested and
did not bind to mAb 2625. All of the investigated strains are listed in Materials and Methods.
In electron microscopy experiments, the epitope on the antigen bound by mAb 2625 could be localized to the cell
surface of L. pneumophila (Fig. 1). In Western blot analysis of whole cell lysates and purified LPS, mAb 2625 exhibited a ladder-like binding pattern characteristic of LPS (Fig. 2A, lanes 1 and 3). With the aid of molecular mass standards for SDS-PAGE, the ladder-like bands were estimated to be in the range of 30–65 kD. This molecular mass range does not correspond to the molecular mass of L. pneumophila
LPS, but permitted a relative comparison of LPS banding patterns in Western blots. In control experiments, we incubated Western blots with mAb LPS-1 (39), an mAb specific to serogroup 1 of L. pneumophila. As shown in Fig. 2B (lanes 1 and 3), LPS-1 exhibited a ladder-like banding pattern in the range of 20–30 kD and in addition, faint bands in the range of 35–45 kD. This binding pattern corresponds well to the image of L. pneumophila LPS bands which become visible after silver staining (Fig. 3). From the binding characteristics of mAb 2625, that is the banding pattern in a higher molecular mass range than observed with mAb LPS-1, we conclude that mAb 2625 binds a conformational epitope, which is not generated before a distinct O-chain length is achieved.
Isolation of LPS-M mutant Strain 811. Next, we wondered whether mAb 2625-reactive epitope is present on all bacteria within a population. Therefore, we performed colony blots on the virulent L. pneumophila strain R C1, a clinical isolate of the OLDA subtype of serogroup 1, with mAb 2625. Using this strategy, we could show that mAb 2625-negative colonies could be detected in a frequency of 10⁻⁴. We isolated one of the mAb 2625-negative colonies for further analysis. Interestingly, the phenotype of this LPS variant, termed 811, was unstable and exhibited a remarkable switching back to the LPS phenotype of the parent strain. This could be shown using mAb 2625. When a single colony of the mutant 811 with mAb 2625 nonreactivity confirmed by colony blot analysis was restreaked on BCYE agar, 80–100% of the grown colonies were again positive for binding of mAb 2625. Colony morphology of mutant strain 811 did not exhibit any differences compared with that of the wild-type strain, but the colony material appeared extremely viscous and sticky. By repeating the pro-

Results

Characterization of mAb 2625. To generate mAbs, mice were immunized with whole cells of L. pneumophila SG 1
strain R C1 (OLDA). Before the immunization, strain R C1 was subjected to a single guinea pig passage. Screening of hybridoma supernatants in an ELISA with the same strain used for immunization led to the isolation of IgG3 mAb 2625. The reactivity of mAb 2625 was not eliminated after treatment with proteinase K, suggesting that a carbohydrate epitope was recognized. Binding of mAb 2625 occurred exclusively in strains belonging to the OLDA and Oxford subgroups of L. pneumophila SG 1, respectively, as determined by ELISA and Western blot analysis. There was no binding reaction observed in any other of the 14 tested SG 1 strains. This was also the case for type strains from serogroup 2 to 14. Likewise, eight non-L pneumophila strains as well as several bacteria from other genera were tested and did not bind to mAb 2625. All of the investigated strains are listed in Materials and Methods.

Infection of HL-60 Cells with L. pneumophila. Infection of HL-60 cells was performed essentially as described (25, 43). Human macrophage-like cell line HL-60 was propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO BRL, Eggenstein, Germany). Differentiation of the cells was induced with PMA (Sigma Chemical Co.) at a final concentration of 10⁻⁸ M. After 48 h of incubation with PMA cells were washed and 0.5 × 10⁶ adherent HL-60 cells per well were infected with 10⁶ bacteria of the appropriate L. pneumophila strain. Bacteria were plated on BCYE agar from frozen stocks and harvested after 40 h incubation. The optical density of bacterial sus-

Infection of Guinea Pigs. Male and female guinea pigs strain 2BS (400–600 g) obtained from the Zentrales Tierlaboratorium (Medizinische Hochschule Hannover, Germany) were infected with L. pneumophila by intratracheal application of the bacteria as described (15, 44). Animals were anesthetized by intra-
muscular injection of a mixture of 10 μl ketamine (10% solution) and 10 μl xylazinhydrochloride (2% solution) per 100 g body weight. Subsequently, a small skin incision was made in the ventral neck and the bacterial suspension was injected into the ex-
posed trachea. Before inoculation, the appropriate L. pneumophila strains were plated on BCYE agar from frozen stocks and were incubated for 40 h. Bacteria were harvested and suspended in 0.9% saline to a concentration of 10⁸/ml. 0.3 ml (corresponding to 3 × 10⁶ CFU) of this suspension was injected into the tracheal lumen with a 24-gauge needle and the incision was closed with sutures. In previous experiments, 3 × 10⁶ CFU had been deter-
mained as the 50% lethal dose (LD₅₀) of strain R C1 to guinea pig strain 2BS within 3–5 d after infection. After the infection, ani-
mals were observed several times daily for signs of illness and re-
spiratory disease. 48 h after infection, animals were killed and the lungs were removed. Lung tissue was homogenized in 0.9% saline and aliquots of serial dilutions of the lung suspensions were plated on BCYE agar. For determination of recovered Legionella, CFU were counted after 72 h incubation. Aliquots of the lung suspensions were also plated on blood agar to control for contaminating bacteria. Binding of injected and recovered bacteria to mAb 2625 was in all experiments monitored by colony blot assay. Animal experiments were carried out with the permission and according to the guidelines of local authorities.

Characterization of mAb 2625. To generate mAbs, mice were immunized with whole cells of L. pneumophila SG 1
puriﬁed LPS; lane m wild-type RC1 2

Figure 2. Western blot analysis of wild-type strain RC1 and mutant 811 with mAb 2625 (A) and mAb LPS-1 (B), respectively. Lane 1, wild-type RC1 whole cell lysate; lane 2, mutant 811 whole cell lysate; lane 3, wild-type RC1 2 μg puriﬁed LPS; lane 4, mutant 811 2 μg puriﬁed LPS. Numbers on the left side indicate molecular masses of a standard protein marker. The molecular mass of L. pneumophila LPS does not correspond to that of the marker proteins, but determination of a relative range of LPS bands was achieved by this method.

procedure of colony blots and restreaking single mAb 2625-negative colonies (three passages), we could reduce the proportion of mAb 2625-positive (wild-type) colonies of the mutant 811 to ~10%. For all experiments described in the following paragraphs, the mutant 811 was subjected to this treatment. The ~10% portion of wild-type cells in the mutant 811 population was controlled in all experiments and conﬁrmed by colony blot analysis with mAb 2625. Mutant 811 replicated with the same growth rate in liquid media as the wild-type RC1. As could be expected from this treatment. The ~10% wild-type cells present in the bacterial suspension. In contrast, mAb LPS-1 revealed enhanced binding to mutant 811 in comparison to the parental wild-type strain RC1, resulting in a more sensitive staining of high molecular mass LPS populations. It is conceivable that LPS of mutant 811 is altered in a way that the epitope bound by mAb LPS-1 becomes more accessible and antibody binding is thereby promoted.

Chemical analysis of the LPS O-chain of OLDA Wild-type RC1 and Mutant 811. To determine the chemical alteration in LPS composition of mutant 811, which was indicated by the antibody binding characteristics, we analyzed the LPS O-chain as well as the core oligosaccharide of both strains. For this purpose, the LPS from strains RC1 and mutant 811 was isolated. In Western blot analysis, puriﬁed LPS from both strains exhibited the same binding characteristics with mAb 2625 and mAb LPS-1 as has been determined for cell lysates of the strains (Fig. 2, A and B, lanes 3 and 4). As recently published, the core oligosaccharide of L. pneumophila SG 1 strain Philadelphia is composed of Rha/QuINAc/GlcNAC/M an/Kdo in a relative ratio of ~2:1:2:2 (34, 45). Analysis of the core sugar components of strains RC1 and mutant 811 by GLC-MS revealed that the same sugars were present in both strains. However, complete analysis of the core structure has not yet been accomplished. In particular, modiﬁcation of sugars, conformational structures, and ketosidic linkages within the core remain to be established. Chemical analysis of the carbohydrate composition of the O-chain was performed by 1H- and 13C-NMR spectra analysis. It was found that the O-chain was composed of a homopolymer of α-(2→4) interlinked 5-acetamidino-7-acetamido-3,5,7,9-tetraoxyl-D-glycero-D-manno-nonulosonic acid (8-deoxyacyl derivative of legionaminic acid). No structural difference was observed between the O-deacylated legionaminic acid in the two strains investigated. In addition, length distribution of the LPS O-chain was identical in wild-type RC1 and mutant 811, as was determined by SDS-PAGE and gel permeation chromatography. SDS-PAGE of puriﬁed LPS from RC1 and 811 revealed a ladder-like banding pattern with a bimodular distribution of O-chain length which is typical for Legionella LPS (Fig. 3). No difference in banding pattern was observed between wild-type and mutant. Moreover, both strains exhibited a banding pattern very similar to that of L. pneumophila SG 1 strain Philadelphia. In conclusion, no difference in O-chain structure and length was observed between wild-type RC1 and mutant 811, further conﬁrming chemical data indicating the O-chain not to be changed in the mutant.

Epitope Mapping of mAb 2625 and mAb LPS-1. To determine the region of the LPS molecule where a structural difference between wild-type RC1 and mutant 811 could be located, we attempted to map the epitopes bound by mAb 2625 and mAb LPS-1, respectively. For this purpose, we investigated the binding capacity of sugar fractions obtained from the gel ﬁltration assay. Before separation on Sephadex columns, the lipid A moiety had been removed from LPS by mild acid hydrolysis. After gel ﬁltration three
Figure 3. SDS-PAGE and silver staining of LPS from L. pneumophila SG 1, 2 μg purified LPS were applied to each lane. Lane 1, mutant 811; lane 2, wild-type R C1; lane 3, strain Philadelphia. All strains exhibit a characteristic bimodal distribution of LPS O-chain representing 10-35 and 45-100 carbohydrate units, respectively. On the left side, LPS bands corresponding to fractions I and II of wild-type RC1 are indicated. These fractions were employed for epitope mapping by competition ELISA. As a control, 2 μg purified LPS from P. aeruginosa subgroup Fisher 2 was applied to lane 4.

fractions of O-chain with attached core oligosaccharide were eluted from the column: fraction I represented the long O-chain (45-100-mer); fraction II the short O-chain (10-35-mer); and fraction III the isolated core oligosaccharide. The LPS bands separated by SDS-PAGE, which correspond to fractions I and II, are indicated in Fig. 3. All fractions eluted from the column contained the carbohydrate moiety of LPS (O-specific chain attached to the core) alone and therefore could not be analyzed by Western blot or conventional ELISA techniques. For this reason we performed a competition ELISA which is based on the principle of inhibition of antibody binding to LPS by LPS carbohydrate fractions. Purified LPS from L. pneumophila SG 1 wild-type RC1 was adsorbed to microtiter plates. mAb 2625 and LPS-1, respectively, were preincubated with serial dilutions of sugar fractions obtained from Sephadex gel filtration. In this way we investigated fractions I and II from wild-type RC1 and mutant 811 as well as the core portion of both strains. The results of the competition ELISA are illustrated in Fig. 4. Binding of mAb 2625 is inhibited in a concentration dependent manner by carbohydrates from fractions I and II of wild-type RC1, but not by the core oligosaccharide, indicating that the epitope bound by mAb 2625 involves the O-specific chain. It is noteworthy, that carbohydrates from fraction II exhibited a decreased binding to mAb 2625 in comparison to those from fraction I. O-chain molecules of the required length are presumably only present as a minor part of fraction I. For mutant 811, weak binding of carbohydrates from fraction I to mAb 2625 is due to wild-type bacteria (~10%, see above) that are inherent to the unstable character of 811. Inhibition of binding of mAb LPS-1 occurred in a likewise dose-dependent way. Carbohydrates from fractions I and II, as well as the core fractions from wild-type RC1 and mutant 811, were bound by mAb LPS-1. As already shown in Western blot analysis (see Fig. 2), binding of mAb LPS-1 to 811 is increased in comparison to wild-type RC1 (Fig. 4, C and D). In conclusion, mAb 2625 binds the LPS O-chain, but not the isolated core oligosaccharide. However, we cannot exclude that the core moiety is also required for mAb 2625 binding, since carbohydrates from fractions I and II contain the core portion as well as the O-chain. Since no differences in the O-antigen composition by chemical analysis could be observed, we conclude that the epitope bound by mAb 2625 is a conformational epitope which involves both the O-chain and the core. In contrast, the epitope bound by mAb LPS-1 is clearly located in the core oligosaccharide, and the O-chain is not required for binding. Together with the results obtained from chemical analysis, these data strongly support the idea, that alterations in the core oligosaccharide of mutant 811 are responsible for loss of mAb 2625 binding and increased binding to mAb LPS-1.

Intracellular Replication of RC1 and 811 in HL-60 Cells. Virulence of mutant 811 in comparison to the parent strain RC1 was determined by infection of human macrophage-like cell line HL-60 and determination of CFU on days 1 to 3 after infection. 2 × 10^6 HL-60 cells were infected with 10^4 bacteria from frozen stocks. Wild-type strain RC1 proved to be virulent and replicated in HL-60 cells by 2 orders of magnitude within 72 h (Fig. 5). A mAb 2625-positive revertant of mutant 811 (811-rev.) served as a control in these experiments. It is well known that virulence of pathogenic bacteria is attenuated or even abolished after laboratory passage on artificial media, strain 811-rev. was therefore employed to identify those effects due to agar passage. In comparison with the animal passage wild-type strain RC1, 811-rev. exhibited a slightly reduced growth rate, but proved to be virulent (Fig. 5). In contrast, mutant 811 failed to replicate intracellularly in HL-60 cells. The number of recovered bacteria at all time intervals remained in the range of the inoculum (Fig. 5). In conclusion, wild-type RC1 proved to be able to replicate in HL-60 cells, whereas mutant 811 showed no replication and consequently proved to be avirulent. Moreover, by investigating 811-rev., it became evident that switching of 811 to the wild-type LPS-phenotype (mAb 2625 binding) also restored virulence. These findings indicate that alteration of LPS carbohydrate moiety was the only mutation that had occurred in 811. When gentamicin was added to kill extracellular bacteria after 2 h of coincubation of host cells and bacteria, mutant 811 was found intracellularly to approximately the same extent as wild-type RC1 and 811-rev. (Fig. 6). These data show that mutant 811 entered HL-60 cells as efficiently as the wild-type strain, but was unable to replicate in the host cell. Colony blot analysis of mutant 811 revealed that the percentage of mAb 2625-positive revertants among bacteria recovered from the HL-60 infection assay was identical to that of the inoculum.

Investigation of Serum Resistance. To determine the bactericidal activity of complement present in NHS on wild-type RC1, mutant 811 and 811-rev., we performed bactericidal assays as described in Materials and Methods. After a 1-h incubation in 40% serum, number of bacteria from wild-type strain RC1 declined by >1 log. Thus, wild-type RC1 was not completely, but almost serum resistant. In contrast, the number of viable bacteria from mutant 811 declined by 3 logs (from 6.1 log to 3.2 log) within 15 min incubation in 40% NHS (Fig. 7). After 30 min no viable bacteria were recovered (detection limit 10^2 CFU/ml). Therefore, mutant 811 was serum-sensitive and the sensi-
Activity is related to an altered LPS conformation. Incubation of 811-rev. in 40% NHS revealed that the number of viable bacteria was reduced by 2 logs within 1 h (Fig. 7). These findings indicate that agar passage does indeed have an influence on serum resistance, even though its effect is minor. From the results of the bactericidal assays, we conclude that the alteration in LPS carbohydrate moiety of mutant 811, which is presumably located in the core oligosaccharide, results in deprivation of resistance against serum complement activity. In addition, our data show that serum resistance of *L. pneumophila* is mediated by the LPS carbohydrate moiety.

**In Vivo Virulence of Wild-type RC1 and Mutant 811.**

Next, we were interested to study virulence of mutant 811 under in vivo conditions in the guinea pig animal model. Animals (n = 4 for each bacterial strain) were infected by intratracheal injection of bacteria. On day 2 after infection, animals infected with the wild-type strain RC1 exhibited signs of severe illness such as fever, ruffled fur, almost no motion and reaction and respiratory distress. Animals were killed on day 2 after infection and lungs were removed. The lungs appeared greatly enlarged and were completely hemorrhagic. After homogenization of the lungs, aliquots of the homogenates were plated for determination of the number of bacteria. $6.5 \times 10^9$ (mean value) bacteria were recovered from the animals (Fig. 8). The control blood agar plates were found to be sterile. In contrast, the animals infected with the mutant strain 811 showed moderate signs of illness, such as elevated body temperature and limited motion, but did not show signs of respiratory distress. These animals were also killed on day 2 after infection. Lungs were only slightly enlarged and hemorrhagic patches were visible, but were not distributed over the entire lung tissue. $1.8 \times 10^8$ viable bacteria (mean value) were recovered from the animals, a significantly lower number than was determined for the wild-type strain (Fig. 8). Interestingly, colony blot analysis of strain 811 isolated from animal lungs revealed that 35% of the recovered bacteria were mAb 2625-positive, whereas only 8% of the inoculated bacteria were mAb 2625-positive. These results indicate that under in vivo selective pressure the 2625-positive phenotype (wild-type phenotype) is evidently advantageous over the
mAb 2625-negative phenotype of mutant 811. It remains unclear if a preferential replication of the mAb 2625-positive portion of mutant 811 occurred or if switching back to the wild-type phenotype is promoted under in vivo conditions.

On the other hand, for the wild-type strain RC1 we observed a significantly increased switching frequency under in vivo conditions to the mAb 2625-negative phenotype. A frequency of $5 \times 10^{-2}$ among recovered bacteria from guinea pig lungs was determined by colony blot assay, whereas mAb 2625-negative clones could be detected among agar plated bacteria in a frequency of only $10^{-4}$ (see above).

In conclusion, mutant 811 was not able to cause severe pneumonia as the wild-type RC1 did in the animal host and showed a significantly reduced replication in comparison to the wild-type strain. Even though mutant 811 was completely serum sensitive, it was not cleared from the animal lung. Moreover, our data strongly indicate that two LPS phases of L. pneumophila are expressed in vivo.

**Discussion**

We here describe the isolation and investigation of an LPS-mutant of Legionella pneumophila SG 1, subgroup OLDA. In comparison to other Gram-negative bacteria, L. pneumophila exhibits an unusual LPS structure (31, 32, 34). The chemical structure has recently been analyzed for L. pneumophila SG 1, subgroup Philadelphia. It was found that the lipid A moiety consists of long-chain fatty acids which may account for its low endotoxic activity (31). The core oligosaccharide lacks heptose and phosphate groups and exhibits hydrophobic properties due to the presence of four O-acetyl groups and three deoxy-sugars (Rha 2, QuiNAc) (31, 34), which has so far not been found in any other bac-
The O-chain is composed of an unbranched homopolymer with α-(2→4) interlinked 5-acetamido-7-acetamido-8-0-acetyl-3,5,7,9-tetrahydro-L-glycero-D-galacto-nonulosonic acid, termed legionaminic acid. Due to the lack of free hydroxyl groups and characteristic substituents, the O-chain is highly hydrophobic (31, 32). In this study we determined the chemical structure of the LPS O-antigen of L. pneumophila SG 1, subgroup O LDA. The O-chain was found to be of the same structure as the one from Philadelphia (31, 32), except that it lacks the 8-0-acetyl group and therefore is termed 8-0-deacetyl-legionaminic acid. The finding that O LDA strains all lack the 8-0-acetyl group was expected after the serological data gained by investigating antibody reactivities: the 8-0-acetyl group is known to be involved in binding of mAb 2 (27) and mAb 3/1 (46). In contrast, both antibodies do not bind to strains of the subgroup O LDA. The length of the O-chain of subgroup O LDA shows a bimodal distribution with maxima at 10-35 and 45-100 carbohydrate units, respectively. This banding pattern is very similar to that obtained for subgroup Philadelphia (32). LPS-mutant 811 did not show any differences in O-chain structure and length when compared with its parent wild-type strain R C1. Therefore, we conclude that LPS of mutant 811 is altered in the core oligosaccharide.

Mild acid hydrolysis in acetate buffer was used to cleave the lipid A moiety from the carbohydrate moiety of the LPS molecule. Under these conditions the ketodic linkages of Kdo and iso-legionaminic acid are as well cleaved (32-34, 45), whereas O-acetyl groups remain intact (34, 45). It can be excluded that artifacts created by the acid hydrolysis treatment prevented the identification of modifications of the mutant 811 LPS compared with the wild-type LPS. Epitope mapping experiments showed that carbohydrate fractions isolated after mild acid hydrolysis and gel permeation chromatography still were able to compete for epitope binding by mAbs 2625 and LPS-1. If artifacts were generated by the degradation procedure, they did not interfere with those substituents important for formation of the epitopes bound by mAbs 2625 and LPS-1.

Our idea that the core sugar composition of mutant 811 differs from that of the parent wild-type R C1 is supported by the results of epitope mapping of mAb 2625 and mAb LPS-1. With the aid of a competition ELISA method we could show that mAb LPS-1 binds to the core oligosaccharide of L. pneumophila SG 1. mAb LPS-1 shows a stronger binding to mutant 811 than to wild-type R C1, indicating that alterations in the core structure of 811 enhance the accessibility of the LPS-1 epitope. In contrast, mAb 2625 binds to the LPS O-chain and the core oligosaccharide is presumably also involved in formation of the epitope. Moreover, short-chain polysaccharides showed a reduced binding to mAb 2625 in comparison to long-chain fractions. This finding supports our hypothesis that a distinct O-chain length is required for mAb 2625 reactivity.

In vitro and in vivo experiments revealed that virulence of mutant 811 was significantly reduced in comparison to the wild-type strain R C1. In in vitro assays, LPS-mutant 811 was unable to replicate intracellularly in macrophage-like cells even though the mutant bacteria were able to enter the host cells. In addition, mutant 811 was rapidly killed by serum complement factors, whereas the corresponding wild-type strain was almost resistant to complement lysis. By comparative analysis of LPS-mutant 811 and the parent wild-type R C1, these data show for the first time that resistance to serum complement of L. pneumophila is mediated by LPS carbohydrate moiety. However, we cannot exclude that the LPS variation of mutant 811 leads to additional modifications in the formation of the outer membrane. Such alterations could for example affect surface molecules which might as well be required for serum resistance and virulence of L. pneumophila. For many bacterial pathogens, surface polysaccharide structures such as capsules and LPS were found to be involved in complement inhibition (reviewed in references 47, 48). Currently we do not know whether killing of mutant 811 by serum complement factors is due to an increase in insertion of membrane attack complex (MAC) into the membrane of mutant 811 or if insertion of MAC occurs to the same extent in both, wild-type and mutant, but does not lyse wild-type cells. Therefore, future experiments should address the ability of wild-type R C1 and mutant 811 to prevent or promote insertion of MAC. In particular, activation of complement and deposition of complement factors C3b and C3bi on the cell surface is of special interest, since these molecules are known to mediate uptake into the host cells via complement receptors CR 1 and CR 3 (9, 10).

In the guinea pig animal model, mutant 811 was not able to cause severe pneumonia in guinea pigs infected with a dose that corresponds to the LD50 of wild-type strain R C1. Moreover, number of wild-type bacteria recovered from the lungs of infected animals exceeded the number of mutant bacteria recovered from infected animals by 1 to 2 orders of magnitude. Most interestingly, LPS-mutant 811 exhibited an unstable phenotype. The majority of cells from the originally isolated clone 811, which was negative for mAb 2625 binding, switched back to the wild-type phenotype. This phase variation between two LPS phenotypes was found to be immensely promoted in vivo in the animal host. Inoculated bacteria of mutant 811 included 8% cells positive for mAb 2625 binding. This phase variation of mutant 811 was increased to 35%, indicating a selective advantage of the wild-type phenotype over the mutant phenotype. Bacteria recovered from animals infected with the wild-type R C1 contained 5% cells negative for mAb 2625 binding and therefore exhibiting the phenotype of mutant 811. We observed the same phase variation of wild-type R C1 and mutant 811 when the bacteria were incubated with heat-inactivated human serum for 1 h (data not shown). Our results strongly indicate that phase variation of L. pneumophila LPS is induced and promoted by the animal host and by human serum. This is the first description of a phase-variable expression of surface polysaccharides of L. pneumophila. Diversity of surface carbohydrates...
achieved by means of high-frequency, reversible switching of sugar epitopes has been described and intensively studied in H. aemophilus influenzae (49–58), N. gonorrhoeae (59, 60) and N. meningitidis (61–64). In H. influenzae, expression of enzymes involved in LPS biosynthesis is controlled by multiple repeats of tetrameric nucleotides within the lic, lex2, and IgtC loci (50, 56, 58). A change in the number of tetrameric repeats, arising through slipped-strand mispairing, results in a frame shift mutation, thus preventing expression of the encoded enzyme. Generation of phenotypic LPS variation by these intragenic alterations is considered as a virulence mechanism, enabling the bacteria to adapt to different environmental conditions (55). Essentially the same slipped-strand mispairing mechanism is found in N. meningitidis. Genes of the lgt locus, encoding glycosyl-transferases responsible for LPS biosynthesis, are expressed or not depending on the number of guanosine residues within a poly-G stretch in the coding sequence (59–61). In N. meningitidis, expression of the terminal lacto-N-neotetraose on the LPS, which requires the glycosyl-transferase encoded by lgtA, is correlated with serum resistance, non-invasiveness and predominance in the blood of infected mice. In contrast, strains with the lacto-N-neotetraose negative LPS phenotype are serum-sensitive, invasive and predominantly found in the nasopharynx (61, 62, 65, 66). For L. pneumophila, it remains to be established by which molecular mechanism LPS phase variation is determined. A prerequisite to further investigate this question is the characterization of genes involved in LPS biosynthesis. Except for an O-acetyl-transferase gene (Mintz, C.S., unpublished data, accession number U32118) such genes have not yet been identified in Legionella.

Species of the genus Legionella do not express a capsule or an exopolysaccharide. Therefore the LPS carbohydrate moiety is the predominant molecule on the cell surface of these bacteria which contributes to the cell surface properties in an exceptionally important way. Nothing is known about adherence of legionellae to the lung epithelium. The ability of L. pneumophila to replicate in alveolar epithelial cells has been reported (67, 68). It is conceivable that adhesion and tight attachment to epithelial cells is a crucial step in infection before the target host cell can be invaded. Attachment and adhesion could be mediated by surface carbohydrates such as LPS, which has been suggested to act as an adhesin of numerous pathogenic bacteria (reviewed in reference 69). Moreover, LPS of L. pneumophila may also be involved in attachment to its host cell. In a very recent study, a 170-kD lectin of Hartmanella vermiformis has been identified as a potential receptor used by L. pneumophila to invade the protozoan cell (70). However, the ligand on the bacterial surface remains to be identified. Future studies should therefore focus on the role of LPS in attachment and adhesion to different host cells and environments that are exploited by L. pneumophila.

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