Legionella pneumophila carrying the virulence-associated lipopolysaccharide epitope possesses two functionally different LPS components

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Phase-variable expression of *Legionella pneumophila* lipopolysaccharide (LPS) has not been described in detail for strains possessing the virulence-associated epitope recognized by the monoclonal antibody (mAb) 3/1 of the Dresden Panel. About 75 % of cases of community-acquired legionellosis are caused by mAb 3/1-positive strains. In this study, the LPS architecture of the mAb 3/1-positive Corby strain was investigated during its life cycle in broth culture and inside monocytic host cells. During the exponential growth phase in broth, the highly acetylated and therefore strongly hydrophobic mAb 3/1 epitope is expressed continuously, but only 3 % of the bacteria can be detected using mAb 59/1, which recognizes a short-chain variant of the *Legionella* LPS that is less hydrophobic due to missing acetylations of the O-chain. The percentage of mAb 59/1-positive legionellae increases up to 34 % in the post-exponential growth phase. LPS shed in broth during the exponential phase is mAb 59/1-negative, and mAb 3/1-positive components do not possess short-chain molecules. The LPS pattern expressed and shed inside U937 cells and A/J mouse macrophages points to the same regulatory mechanisms. During the so-called 'pregnant pause', the period for establishment of the replicative phagosomes, the mAb 3/1-positive LPS is shed into the phagosome and seems to pass through the phagosomal membrane, while mAb 59/1-positive LPS is detectable only on the bacterial surface. After egress of the legionellae into the cytoplasm followed by host cell lysis, individual bacteria are mAb 3/1-positive and mAb 59/1-negative. Intracellularly formed *Legionella* clusters consist of surface-located mAb 3/1-positive bacteria, which are predominantly mAb 59/1-negative. They surround less hydrophobic and therefore closely packed mAb 59/1-positive bacteria. Based on the different degrees of hydrophobicity, bacteria are able to support the expression of two functionally different LPS architectures, namely more hydrophobic LPS for surviving in aerosols and more hydrophilic LPS for close-packing of legionellae inside clusters.

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INTRODUCTION

Legionella pneumophila, a bacterium ubiquitous in aquatic environments, represents the dominant *Legionella* species responsible for Legionnaires' disease. It is a facultative intracellular pathogen that can replicate in protozoa. Human infection occurs by inhalation of contaminated aerosols. Once in the lungs, *L. pneumophila* can be phagocytosed by alveolar macrophages. Virulent legionellae are able to modulate the host cells by evading fusion

with endosomes or lysosomes. The nascent phagosome becomes surrounded by mitochondria, smooth vesicles and ribosomes derived from host cell organelles (Horwitz, 1983; Abu Kwaik, 1996). After a lag phase of about 6 h, intraphagosomal bacteria have completed their differentiation from the transmissive, infectious phenotype to the replicative, non-infectious form, following the so-called 'pregnant pause' for establishing the replicative phagosome (Swanson & Fernandez-Moreira, 2002). After a few multiplication periods inside the phagosomes over a period of 14–20 h, *L. pneumophila* again enters the transmissive post-exponential (PE) growth phase, exhibiting virulence traits that promote bacterial transmission for a new round of infection. The cellular bimorphic life

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; E phase, exponential phase; MWCO, molecular weight cut-off; OMV, outer membrane vesicle; PE phase, post-exponential phase; p.i., post-infection; S phase, stationary phase.

cycle of *Legionella* can be modelled in broth. After the replicative, exponential (E) growth phase, bacteria switch to the transmissive form in the PE and stationary (S) growth phases (Byrne & Swanson, 1998).

A variety of virulence factors have been implicated in the pathogenesis of *L. pneumophila*. The gene products of the *dot/icm* loci are the most important ones for intracellular replication (Bitar *et al.*, 2004), but Dot-independent components have also been described (Joshi *et al.*, 2001). The involvement of LPS in *L. pneumophila* pathogenesis has been under discussion since phase-variable LPS expression in an animal model was discovered (Lüneberg *et al.*, 1998). The LPS repertoire of *L. pneumophila* strains determines their classification as serogroups and monoclonal subgroups. Epidemiological data substantiate the dominance of serogroup 1 among clinical isolates (Helbig *et al.*, 2002; Yu *et al.*, 2002), and multigenomic analysis has identified a particular LPS gene cluster that seems to be responsible for the predominance of *L. pneumophila* serogroup 1 in legionellosis (Cazalet *et al.*, 2008). The link to epidemiological data becomes still more evident because strains carrying the so-called virulence-associated LPS epitope recognized by the monoclonal antibody (mAb) 3/1 of the Dresden Panel cause 78 % of community-acquired and travel-associated but only 47 % of nosocomial legionellosis (Helbig *et al.*, 2002).

For many bacterial species the complex genotypic and phenotypic mechanisms of environmentally regulated LPS expression have been well analysed (Preston & Maskell, 2002). By contrast, only limited data are available for the most important causative agent of Legionnaires' disease, namely mAb 3/1-positive *L. pneumophila* strains. Fernandez-Moreira *et al.* (2006) have described the developmental regulation *in vitro* of mAb 3/1-positive LPS in relation to the serogroup 1-specific LPS recognized by mAb 8/5 of the Dresden Panel. The purpose of our study was to look for phase-variable expression and shedding of other LPS molecules of *L. pneumophila* Corby strain possessing the virulence-associated epitope. Helbig *et al.* (2006) found that mAb 59/1-recognized LPS is only shed during the late PE phase and not in the E phase of legionellae in broth culture. Accordingly, LPS with the mAb 59/1 epitope differs from LPS carrying the mAb 3/1 epitope and should be a candidate for developmentally regulated LPS expression. Therefore, both mAbs were first used for detailed investigations of the LPS architecture of bacteria grown in ACES-buffered yeast extract broth (AYE) to the E, PE and S phases, and of LPS components shed at the same periods. These data obtained *in vitro* were afterwards compared with the LPS repertoire expressed in two infected mammalian host cell types during the establishment of the replicative phagosome and in the replicative phase until host cell lysis.

METHODS

Legionella strain and culture. The Corby strain, which is serogroup 1, mAb 3/1-positive, was used in this study. Bacteria were grown on

charcoal yeast extract (BCYE) agar plates (Oxoid) for 2 days at 37 °C in a 5 % CO₂ atmosphere. For immunochemical analysis, bacteria were inoculated into AYE with 0.0025 % ferric pyrophosphate (Sigma) and 0.04 % L-cysteine (Oxoid) at a density of 3×10^8 bacteria ml⁻¹ and grown at 37 °C. Bacteria were incubated either for 12 h to E phase ($\sim 3 \times 10^9$ cells ml⁻¹) or for 24 h to PE phase ($\sim 1 \times 10^{10}$ cells ml⁻¹). Bacteria defined to be in the S phase were harvested 5 h after the PE phase. At this time no further multiplication steps could be measured but the browning phenomenon (Wintermeyer *et al.*, 1991) was not yet seen. Cell counts were measured by nephelometry (Turbox nephelometer, Orion Diagnostica), which agreed with the internal laboratory standard based on microscopy counting (100 nephelometric units correspond to 3×10^8 bacteria ml⁻¹). Infection assays were performed with PE phase bacteria that were highly infectious and motile (data not shown).

mAbs recognizing LPS. mAb 3/1 (Helbig *et al.*, 1995) and mAb 59/1 (Helbig *et al.*, 2006) were used for immunochemical analysis using immunoblotting and ELISA as well as for immunolabelling of legionellae in broth and host cells. mAb 3/1 (isotype IgG₃) recognizes the virulence-associated epitope which contains the 8-O-acetyl group of legionaminic acid (Helbig *et al.*, 1995), located on short and long O-specific chains of *Legionella* LPS molecules (Lück *et al.*, 2001). mAb 59/1 (isotype IgG₁) was selected for this study because previous data emphasized that the recognized LPS was not shed in broth cultures during the E phase (Helbig *et al.*, 2006). Its LPS specificity was confirmed by ELISA using purified LPS derived from agar-grown legionellae, which was prepared according to Zähringer *et al.* (1995). For more detailed analyses of the mAb 59/1 epitope, the purified LPS was deacetylated completely using sodium hydroxide (Helbig *et al.*, 1995) and also tested by ELISA. Furthermore, immunoblot analyses were performed with the non-vesicular LPS fraction (see below) from the E, PE and S phases.

Fractionation of shed vesicular and soluble LPS. To collect supernatants, aliquots of liquid cultures containing legionellae from the E, PE and S phases were harvested and adjusted to 10^9 bacteria ml⁻¹ by addition of AYE, and bacteria were separated by centrifugation (14 000 g, 15 min). The resulting supernatants were filtered through 0.2 µm pore-size polyethersulfone syringe filters. The filtered supernatants contained LPS, which was shed as vesicular (outer membrane vesicles; OMVs) and non-vesicular components. In order to separate the non-vesicular fraction, the obtained supernatants were additionally filtered through 300 kDa molecular weight cut-off (MWCO) filters (Vivaspin, Sartorius) until the volumes were reduced to 10 % of the original. As a result, the obtained fractions of >300 kDa contained OMVs plus 10 % of the non-vesicularly shed LPS. The filtrates of <300 kDa contained non-vesicular LPS alone.

Immunoblot analysis of non-vesicularly shed LPS. The LPS fractions of <300 kDa (see above) were concentrated 50-fold by 10 kDa MWCO centrifugal concentrators (Vivaspin, Sartorius). The immunoblot analysis was performed according to a standard procedure using 10 % Bis-tris polyacrylamide gels (Invitrogen) followed by transfer onto nitrocellulose membranes (Invitrogen). Immunolabelling was performed with mAb 3/1 and mAb 59/1, diluted to 2 µg ml⁻¹, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Pierce Biotechnology). OMVs and bacterial-bound LPS were not tested.

ELISA of shed LPS components. For testing by ELISA, the shed LPS fractions (OMV and non-vesicular LPS) were heat-treated (10 min, 95 °C) and used to coat 96-well microtitre plates (Greiner). ELISA was performed as described previously (Helbig *et al.*, 1995). Bound primary antibodies mAb 3/1 or mAb 59/1 were detected by HRP-conjugated anti-mouse immunoglobulin (Pierce Biotechnology). Blank values were obtained by coating the wells with AYE broth instead of LPS fractions. Absorbance readings of blanks at 450/620 nm were

subtracted from sample absorbances. The positive cut-off value was defined as an absorbance of less than 0.2. This value was always more than three times the standard deviation of the blank.

Cell culture. Cell invasion assays were performed with (i) macrophage-like U937 cells and (ii) peritoneal macrophages from female A/J mice (Charles River Laboratories). For the infection experiments, U937 cells were diluted to 5×10^5 cells ml^{-1} and cultured in 24-well tissue culture plates (Costar) with the cell medium RPMI 1640 (PAA Laboratories) containing 10 % heat-inactivated fetal calf serum (FCS; PAA Laboratories). After addition of phorbol-12-myristate-13-acetate (Sigma) to a final concentration of 50 ng ml^{-1} and incubation for 2 days, cells had differentiated into adherent confluent cell monolayers. Peritoneal mouse macrophages were harvested by rinsing the dissected peritoneum of mice with ice-cold RPMI 1640. Cells were incubated in eight-well chamber slides (Nunc) at 37 °C in a humidified atmosphere containing 5 % CO_2 . Four hours later, the cell culture medium was changed to RPMI 1640 containing 10 % FCS. After 2 days of incubation, cells were enumerated and used for infection experiments.

Infection of host cells. Host cells were infected with *L. pneumophila* Corby strain at an m.o.i. of 10 in 200 μl of the respective host cell medium followed by initial centrifugation at 200 g for 10 min. This was defined as time zero. After 50 min incubation at 37 °C followed by centrifugation, the cells were washed three times with 1 ml of the respective medium to remove extracellular bacteria. Infected cells were further incubated at 37 °C for periods of 4 h (U937 cells alone), 6 h (macrophages alone), 15 or 22 h. After that, the A/J mouse macrophages were fixed in the chamber slides with fixative solution A (Fix & Perm, An der Grub) for 20 min. U937 cells were scraped from tissue plate wells and collected for CytoSpin slide (Shandon) preparations by centrifugation for 10 min at 75 g. The fixation procedure was the same as that described for macrophages.

Immunofluorescence staining and microscopy. To identify LPS on bacteria grown in broth or in host cells, or LPS shed inside the host cells, samples were triple-stained. Aliquots of bacteria multiplied in broth culture to the E and PE phases were collected by the CytoSpin technique (Shandon) using centrifugation at 250 g for 10 min and fixed as described for infective host cells (see above). For blocking and subsequent immunolabelling of host cell preparations, all reagents additionally contained 50 % (v/v) permeabilization medium (Fix & Perm, An der Grub). RPMI 1640 containing 10 % (v/v) FCS was used as blocking solution. The slides were rinsed three times with PBS and then incubated for 45 min at 37 °C with mAb 59/1. Bound mAb 59/1 was detected after incubation of washed slides with goat anti-mouse Cy3.5 (red fluorescence)-labelled IgG (Sigma) for another 45 min at 37 °C. Following a thorough washing step, mAb 3/1 was added. Bound mAb 3/1 was detected by FITC-labelled (green fluorescence) goat anti-mouse IgG₃ (Sigma). Bacterial DNA and host cell nuclei were additionally stained with 4',6-diamidino-2-phenylindole (DAPI). The slides were embedded in SlowFade Gold reagent (Invitrogen). A Zeiss LSM 510 laser-scanning confocal microscope with a Plan-Apochromat $\times 63/1.40$ DIC oil objective (Zeiss) was used for fluorescence microscopy. For 3D reconstruction, the image data were processed with Velocity 4 software (Improvision).

RESULTS AND DISCUSSION

LPS is the major surface component of bacteria and acts as mediator between pathogens and potential host cells. The LPS structure undergoes modifications in response to environmental changes, as described for many genera (Preston & Maskell, 2002). For *L. pneumophila*, Lüneberg

et al. (1998, 2001) have described a mAb 2625-negative mutant that exhibits an unstable LPS phenotype in a mAb 3/1-negative strain *in vivo*. LPS switching of other *L. pneumophila* strains during their intracellular life cycle has not been investigated so far. Therefore, the aim of our study was to examine whether there are bacterial or host cell-regulated mechanisms that cause LPS switching and shedding. For this, we chose a mAb 3/1-positive *L. pneumophila* strain representative of the *L. pneumophila* strains mainly isolated from cases of legionellosis.

Liquid cultures of legionellae are accepted as a representative model of the intracellular life cycle with respect to the growth phases and infectivity of the bacteria; this has been described, for example, for investigations of the Dot/Icm apparatus and the LetA/LetS regulatory system (Hammer *et al.*, 2002; Molofsky & Swanson, 2004). The question as to whether the broth model is also applicable to intracellular LPS biogenesis was answered by our LPS staining inside host cells.

Shed and cell wall-bound LPS recognized by mAb 59/1 is expressed in a phase-variable manner in broth cultures

Using ELISA, mAb 59/1-positive LPS is not detectable as a shed component in broth cultures (Helbig *et al.*, 2006). In the present study we investigated by immunoblot analysis the shed LPS fractions of <300 kDa after 50-fold concentration. The mAb 59/1 epitope was not significantly detectable in the E phase but was detectable in the PE and S phases. Interestingly, only short-chain LPS corresponding to 11–17 kDa protein molecular mass markers carried this epitope (Fig. 1a). Bacteria-bound mAb 59/1-positive LPS of the Corby strain had the same chain length, whereas shed and bound fractions with this LPS specificity of mAb 3/1-negative serogroup 1 strains had long-chain molecules also (data not shown). In order to characterize this epitope in more detail, LPS was purified according to Zähringer *et al.* (1995) and completely deacetylated using sodium hydroxide. Deacetylated LPS does not react with mAb 3/1 (Helbig *et al.*, 1995), whereas the reaction with mAb 59/1 remains positive (data not shown).

To analyse the expression of bacteria-bound LPS components, legionellae fixed on microscope slides were fluorescently labelled with mAb 59/1 and subsequently with mAb 3/1, and then enumerated. The LPS pattern of bacteria sampled in the E phase was characterized by the expression of mAb 3/1 epitopes uniformly on the surface of all organisms. Only 3 % (SD ± 2.8 %) of the enumerated bacteria were also coevally positive for mAb 59/1 in this growth phase. The LPS pattern of the bacteria changed in the PE phase when legionellae switched to the infectious transmissible form. Whereas long- and short-chain LPS molecules recognized by mAb 3/1 were expressed consistently and continuously until the PE phase was reached, it became apparent that expression of short-chain non-acetylated mAb 59/1-positive LPS components increased over time, reaching a plateau in

Fig. 1. Immunoblot analysis of *L. pneumophila* non-vesicular LPS fractions from broth supernatants. Samples were collected at different growth phases and concentrated by 10 000 Da MWCO Vivaspins centrifugal concentrators. The LPS carrying the epitope recognized by mAb 59/1 is only present on the short-chain LPS of the PE and S phases (a). The E phase fraction is lacking in short-chain mAb 3/1-positive LPS (b). The PE and S phases do not differ in LPS composition (a, b).

the PE phase with a significantly higher ($P < 0.001$) percentage of mAb 59/1-positive bacteria ($34 \pm 10.1\%$). The distribution of mAb 59/1-positive LPS epitopes appeared irregular and non-homogeneous at the surface of the legionellae. Bacteria expressing mAb 59/1 epitopes seemed to deliver mAb 3/1-positive LPS components into broth (see below) and were characterized by attenuated mAb 3/1 fluorescence, whereas for bacteria not expressing mAb 59/1-positive epitopes, a strong mAb 3/1 signal was obtained (Fig. 2). Therefore, it can be assumed that, due to phase regulation, the density of mAb 3/1-positive LPS on the bacterial surface is decreased by shedding into liquid media (see below, Fig. 3) and insufficient re-expression, whereas mAb 59/1-positive LPS is upregulated.

LPS carrying the mAb 3/1 epitope is shed in broth cultures during all growth phases

The epitope repertoire of LPS shed in non-vesicular form during the bacterial life cycle in broth was analysed by immunoblotting. To achieve this, the 300 kDa fractions of

Fig. 2. Distribution of mAb 3/1 and mAb 59/1 epitopes on the surface of broth-cultured bacteria in the PE phase detected by merged images. Green fluorescence (mAb 3/1) shows a homogeneous distribution, while LPS recognized by mAb 59/1 (Cy3.5, red) is dispersed irregularly on the bacterial surface. DNA was stained by DAPI (blue fluorescence). Bar, 5 µm.

the broth supernatants were collected, concentrated 50-fold, blotted and analysed using mAb 3/1 and mAb 59/1. The results confirmed that mAb 3/1-positive LPS is shed during all growth phases. In the PE and S phases, LPS with high and low molecular weight protein marker components ranging from 98 to 15 kDa were detected, but in the E phase, only LPS components between 70 and 30 kDa were seen (Fig. 1b). These data differ from results obtained for the mAb 59/1 epitope (see above, Fig. 1a).

In addition to immunoblot analysis, shed LPS components were analysed by ELISA. Fig. 3 shows ELISA data representing the distribution of shed LPS components recognized by mAb 3/1 and mAb 59/1. The accumulation of shed LPS in the later growth phases was always greater in the 0.2 µm fraction compared with non-vesicular LPS, which shows that most of the shed LPS in liquid culture is incorporated into vesicles.

mAb 59/1-positive LPS was found in the <0.2 µm fractions of supernatants from the PE and S phases but not in E phase bacteria. Non-vesicular mAb 59/1-positive LPS was only shed in the S phase. This finding confirms that mAb 59/1-positive LPS components remain mainly on the bacterial surface. Furthermore, the examined supernatant samples additionally showed a clear increase of the absorbance values in the PE and S growth phases, indicating that the delivery of mAb 3/1-positive LPS components affects the increased expression of cellularly bound LPS epitopes recognized by mAb 59/1 during these growth phases. While mAb 3/1-positive LPS components

Fig. 3. ELISA for detection of shed LPS carrying the epitopes for mAb 3/1 and 59/1. The Corby strain was grown to the E, PE and S phases. The supernatant was fractionated into $\leq 0.2 \mu\text{m}$ and $\leq 300 \text{ kDa}$ fractions. Samples from the different growth phases were adjusted to $10^9 \text{ bacteria ml}^{-1}$ by addition of AYE medium before separation by centrifugation.

were released accretively by the bacteria, the expression of LPS harbouring mAb 59/1 epitopes increased from 3 % up to 34 % of the bacteria (see above).

Fernandez-Moreira *et al.* (2006) have quantified the mAb 3/1 LPS component in relation to a common *L. pneumophila* serogroup 1 LPS component recognized by a mAb that is not included in the present study. Their values for cell wall-bound LPS do not differ between the early E phase and the PE phase. This result does not seem to be in accord with the increase in mAb 59/1-positive bacteria in our study, although this was caused by the non-quantitatively obtained fluorescence signals. Furthermore, Fernandez-Moreira *et al.* (2006) have shown that the relative amount of mAb 3/1-positive LPS shed into liquid media is upregulated in PE phase. This also was not reflected in our study. To explain this, firstly, it may be pointed out that shed mAb 59/1-positive LPS also contains the common LPS component. Moreover, it may be that LPS components were shed in this growth phase as well that did not contain mAb 3/1 and 59/1 epitopes but did carry the common serogroup 1 LPS epitope, which is located in the core region (Kooistra *et al.*, 2002).

LPS carrying the mAb 3/1 epitope is shed during the 'pregnant pause' and replication in host cells

Shedding of LPS and LPS-enriched OMVs by Gram-negative bacteria has been demonstrated elsewhere, e.g. for *Salmonella typhimurium* (Garcia-del Portillo *et al.*, 1997) and *Escherichia coli* (Horstman & Kuehn, 2000; Balsalobre *et al.*, 2006). For *L. pneumophila* it has been shown that shed OMVs inhibit phagosome-lysosome

fusion in mouse macrophages (Fernandez-Moreira *et al.*, 2006). Using infection assays we examined the growth-regulated expression of mAb 3/1- and mAb 59/1-positive LPS epitopes by immunofluorescence of infected U937 cells and A/J mouse macrophages using confocal laser-scanning microscopy. Microscopy samples were taken at different stages of the *Legionella* infective cycle, that is (i) the lag phase [4–6 h post-infection (p.i.)], (ii) the replication period (15 h p.i.) and (iii) the egress phase followed by host cell lysis (22 h p.i.).

Altogether, in both host cell types examined, legionellae displayed an almost identical LPS pattern during the course of infection. In the early stage up to 1 h p.i., infectious bacteria were only detectable by immunofluorescence when stained with mAb 3/1 (data not shown). The fluorescence signal of this mAb was strongly visible on the surface of bacteria inside host cells until 4–6 h p.i. (Figs 4a and 5a), and the shedding of components containing mAb 3/1-positive LPS was initiated at this stage. The delivery of mAb 3/1-positive LPS components into the *Legionella*-containing phagosome and the expression of mAb 59/1-positive LPS seem to be connected in a time-dependent manner (Fig. 5a). The expression of LPS recognized by mAb 59/1 is apparently triggered by the intracellular shedding of mAb 3/1-positive LPS molecules observed at the early stages of the infective cycle.

During the formation of the replicative phagosome and the replication period of the intracellular bacteria the amount of delivered mAb 3/1-positive LPS increased around the replication vacuole, while the expression of LPS epitopes recognized by mAb 59/1 was visible only at the surface of intracellular bacterial clusters (Fig. 4b).

Fig. 4. Infection of A/J mouse macrophages with *L. pneumophila* Corby strain. (a) Single bacterium inside the host cell 6 h p.i. Epitopes for mAb 3/1 (green) and mAb 59/1 (red) are expressed on the bacterial surface; mAb 3/1-positive LPS has already been shed. (b) Replication of intracellular legionellae 15 h p.i. mAb 3/1-positive LPS is shed into the cytoplasm, while mAb 59/1 LPS epitopes are expressed only inside the bacterial cluster. (c) Host cell lysis is initiated 22 h p.i. Transmissible bacteria are widespread in the cytoplasm. mAb 59/1-positive LPS epitopes are distributed irregularly on the bacterial surface and are only slightly visible on the bacteria. Nuclei are stained with DAPI. Bars, 10 μ m.

After several cycles of replication before host cell lysis, mAb 3/1-positive LPS was detectable only at the surface of marginally located bacteria and as delivered LPS components around the phagosome (Fig. 5b). The observation that bacteria in the centre of the phagosome exclusively express LPS recognized by mAb 59/1 (Figs 4b and 5b) suggests on the one hand that bacteria within the phagosome turn off the expression of mAb 3/1 epitopes and on the other hand that these replicative bacteria had released all of the cell wall-bound mAb 3/1-positive LPS components into the phagosome and the host cell cytoplasm. Some of the phagosomal bacteria showed an absence of both examined LPS epitopes and these bacteria were localizable by DAPI staining alone (Figs 5c and 6).

To conclude, the switching and shedding of the described *Legionella* LPS components take place during the exponential and transmissive growth phases *in vivo* and *in vitro*. The investigated LPS pattern *in vitro* is similar to that seen in monocytic host cells. Moreover, the phagosomal environment stimulates the bacteria to suppress the expression of acetylated and therefore hydrophobic LPS, which is obstructive to bacterial attachment in replicative phagosomes. For this, the expression of the mAb 59/1-positive short-chain non-acetylated LPS is upregulated.

The target of shed *Legionella* LPS components has remained speculative up to now; however, the cytoskeleton is the most likely target, as has been substantiated for many Gram-negative bacteria (Belyi, 2002). Interestingly, mAb 3/1-positive LPS shed in the non-vesicular fraction from the E

phase does not contain very long O-specific chains (Fig. 1b) and is visible outside the phagosomes just 4–6 h after phagocytosis (Figs 4a and 5a). During the pause for phagosome maturation it can be assumed that OMVs with a diameter of about 200 nm (Fernandez-Moreira *et al.*, 2006) shed from the phagocytosed transmissible bacteria cannot be transported through the intact phagosomal membrane. Therefore, in a manner similar to the gene products of the *dot/icm* loci, the soluble LPS may also be involved in host cell modulation.

Phase-variable expression of LPS architecture modulates the best conditions for survival and infection of new host cells

The shedding of LPS into the cytoplasm before egress of the bacteria into this cell compartment is closely connected with the bacteria switching to the transmissive part of their life cycle. The pattern of widespread shedding into infected host cells is visible from the 3D reconstruction of stacked images from 0.3 μ m confocal z-sections (Fig. 6). These data from closely packed legionellae again convincingly demonstrate that only bacteria from outer phagosomal regions express mAb 3/1-positive LPS on their surface, while mAb 59/1-positive non-acetylated LPS remains on the bacterial surface inside the phagosome and facilitates close contact. In contrast to bacteria in closely packed phagosomes, single bacteria predominantly express mAb 3/1 epitopes before host cell lysis, and the fluorescence intensity of mAb 59/1 is very low (Figs 4c and 5d). During this phase, the shedding

Fig. 5. Infection of U937 cells with *L. pneumophila* Corby strain. During infection of host cells and the beginning of bacterial replication (a) (4 h p.i.), bacteria are positive for mAb 3/1 that is shed into the phagosome, while mAb 59/1 is expressed only slightly and at discrete points on the bacterial surface. (b) Bacteria of late replicative phagosomes (15 h p.i.) express and shed mAb 3/1-positive LPS, while mAb 59/1-positive epitopes remain at the bacterial surface. (c, d) Cytoplasmic bacteria (22 h p.i.) are localizable predominantly as clusters of the former intact phagosomes (c) or are already packed throughout the cytoplasm (d). LPS is detectable by mAb 3/1 on nearly all bacteria, while the epitope recognized by mAb 59/1 is only localizable on a few bacteria. Nuclei were stained with DAPI. Bars, 10 µm.

of mAb 3/1-positive LPS components seems to be suppressed. The highly acetylated LPS molecules remain on the bacterial surface and account for the increased bacterial hydrophobicity before the lysis of infected host cells is initiated after 20 h p.i.

The phenotypic modifications of the LPS pattern shown in the present study benefit the bacteria in two ways. Increased expression of non-acetylated mAb 59/1-positive short-chain LPS molecules in conjunction with the suppression of highly hydrophobic mAb 3/1-positive LPS enables the replicative bacteria to have close contact with each other inside phagosomes (Fig. 5b). The attachment theory is supported by the observation that single released bacteria do not express the mAb 59/1-positive epitopes on the cell wall and that surface-exposed bacteria of *Legionella*-containing vesicles are only mAb 3/1-positive (Fig. 6).

The LPS architecture of clusters formed within phagosomes is particularly interesting with respect to the release of host cell vesicles containing large numbers of legionellae as potentially highly infectious particles. Rowbotham

(1986) coined the term 'large and small particles' for particles that can be released from natural host cells. Berk *et al.* (1998) described the production of respirable vesicles containing hundreds of live *L. pneumophila* cells by two species of *Acanthamoeba*. Our investigations have shown that *Legionella* clusters produced in natural host cells are characterized by the same architecture with respect to mAb 3/1- and mAb 59/1-positive LPS (data not shown). The strongly hydrophobic mAb 3/1-positive LPS of surface-located legionellae allows them to survive very well in aerosols, and therefore infective bacteria can be transported over long distances from the source of contaminated aerosols to susceptible humans. Interestingly, large outbreaks of legionellosis have been caused by mAb 3/1-positive legionellae that belong to five monoclonal subgroups of serogroup 1. The inhalation of so-called 'large particles' implies a high infective dose, so that not only humans with an impaired host defence are infected.

In our study using monocytic host cells only about 5 % of the infected cells released *Legionella*-containing vesicles, and these were characterized by the LPS architecture seen

Fig. 6. Infection of U937 cells with *L. pneumophila* Corby strain 22 h p.i. and distribution of shed mAb 3/1-positive (green) LPS components into the cytoplasm of the host cell. While marginally located bacteria in the phagosome express and shed LPS epitopes recognized by mAb 3/1, bacteria in the inner regions are positive for mAb 59/1 (red). Extracellularly located legionellae are only recognized by mAb 3/1. The 3D reconstruction of the image data was performed with Volocity 4 software (Improvision). Unit cell, 5 μ m. N, nucleus of U937 cell; Ph, phagosome containing legionellae; eL, extracellular legionellae.

in Fig. 6. Nevertheless, it cannot be ruled out that these structures are typical for the lysis of infected human alveolar macrophages. The legionellae within this structure are much better protected against environmental conditions than individual bacteria, and so they might be able to persist in the lung and display increased resistance to antibacterial treatment. It is conceivable that the increased persistence followed by repeated disintegration of such *Legionella*-containing structures is one reason for the often prolonged excretion of *Legionella* urinary antigen (our unpublished data). It is so far not known how, and in particular how long, such structures are able to survive under conditions of host resistance in the lung.

Taken together, for the intracellular life cycle, infection assays of A/J mouse macrophages and U937 cells only partially confirm the findings of liquid cultures. It was shown that the expression and shedding of the investigated LPS components are phase-regulated. Host modulation, however, is already evident during the E phase and lasts until the legionellae are released. In liquid cultures, the PE phase is characterized by one-third of bacteria having mAb 59/1-positive LPS. Whether this is effective in increasing infectivity remains an open question, but it is certain that the decreased hydrophobicity does not aid survival and transmission in aerosols. The expression of virulence-associated mAb 3/1-positive LPS seems to have two

advantages for the bacteria. First, LPS recognized by mAb 3/1 shed into host cells enables the bacteria to modulate their environment via their own LPS pattern. The finding that mAb 59/1-positive LPS epitopes are at first detectable predominantly in the replicative growth phase of the bacteria, with positive effects on bacterial attachment, correlates temporally with the initiation of intracellular shedding of mAb 3/1-positive LPS molecules. Second, the constitutive expression of the virulence-associated epitope on single infectious bacteria and marginally located bacteria in clusters (Fig. 6) increases the hydrophobicity due to a lack of free hydroxyl groups in the long and short O-specific chains of the mAb 3/1-positive LPS molecules.

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