

## Molecular characterization of a virulence-associated epitope on the lipopolysaccharide of *Legionella pneumophila* serogroup 1

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### SUMMARY

For identification of lipopolysaccharide (LPS)-associated epitopes of *Legionella pneumophila* serogroup 1, LPS of strain Philadelphia 1 was investigated using monoclonal antibodies (MAbs). The O-specific chain of LPS is a homopolymer of 5-acetamidino-7-acetamido-8-O-acetyl-3,5,7,9-tetra-deoxy-D-glycero-L-galactonulosonic acid. At least four immunoaccessible epitopes were recognized by different MAbs on the intact LPS. After O-deacetylation of LPS, the reactivity of one of the MAbs (MAb 3/1) was lost, indicating thus that the corresponding epitope is associated with the 8-O-acetyl group. Since the reactivity pattern of the MAb 3/1 is identical with those of the MAb 2 which was considered as a virulence marker for serogroup 1, this epitope may be involved in mediating virulence in *L. pneumophila*. Four MAbs specific to strains of serogroup 1 other than the monoclonal subtype Philadelphia recognized epitopes on the O-deacetylated LPS of strain Philadelphia 1 and, therefore, the virulence-associated epitope blocks recognition of the immunodeterminants that are accessible on the intact LPS of the strains lacking this epitope.

### INTRODUCTION

The genus *Legionella* currently contains 39 named species [1] of which *L. pneumophila* is most important in human pathology. Up to now, strains of this species are divided into 14 serogroups and the serotype Lansing 3 [2, 3]. Strains of serogroup 1 are most frequently isolated from both environmental samples and clinical specimens [4, 5]. A substantial antigenic diversity in serogroup 1 has been discovered using MAbs [6–9], and the standard subgrouping scheme based on the reaction patterns of seven selected MAbs has been proposed by Joly and colleagues [10]. As proven statistically in representative studies, an epitope recognized by MAb 2 of this panel is more frequently expressed on clinical than on environmental isolates and is thus associated with the virulence potential of *Legionella* [11–13]. The basis of this phenomenon is unknown.

The serogroup specificity of *L. pneumophila* is defined by the structure of the outer-membrane LPS [14, 15]. Recently, the structure of the O-specific chain of LPS of *L. pneumophila* serogroup 1 strain Philadelphia 1 was elucidated [16]. In order to identify LPS-associated epitopes, a panel of 10 MAbs was employed in the present study. One of them, namely MAb 3/1, exhibited the same reaction pattern as the MAb 2 of the standard panel [17], which is considered as a virulence marker for *L. pneumophila* serogroup 1 strains. The molecular structure of the virulence-associated immunodeterminant was determined using a selected chemical modification of LPS and characterization of the epitope recognition by the MAbs before and after modification.

#### METHODS

*Strains.* The following strains of *L. pneumophila* serogroup 1 were used: Philadelphia 1 (ATCC 33152), Allentown 1 (ATCC 43016), Benidorm 030E (ATCC 43108), Knoxville 1 (ATCC 33153), France 5811 (ATCC 43112), OLDA (ATCC 43109), Oxford 4032E (ATCC 43110), Heysham 1 (ATCC 43107), Camperdown 1 (ATCC 43113), and Bellingham 1 (ATCC 43111). Strains were grown on buffered charcoal yeast extract (BCYE) agar plates for 48–72 h at 37 °C [18].

*Isolation and O-deacetylation of LPS.* LPS was isolated from dry cells of *L. pneumophila* strain Philadelphia 1 (serogroup 1) by the phenol-chloroform-petroleum ether extraction procedure [19] using a modification described previously [20]. O-deacetylated LPS was prepared from LPS (10 mg) dissolved in 1 ml 0.1 M sodium hydroxide and stirred overnight at 37 °C. The product was dialysed and the retentate was lyophilized to give 7.4 mg of the O-deacetylated LPS.

*Monoclonal antibodies (MAbs).* BALB/c mice were immunized as described by Cianfriglia and colleagues [21] with the heat-killed strains of *L. pneumophila* listed in Table 1. Cellular fusions were carried out using P3X63-Ag8/653 mouse myeloma cells as described by Peters and colleagues [22]. Hybridoma supernatants were screened for Legionella-specific antibodies by indirect ELISA. In cases of positive reactions, hybridoma cells were subcloned three times. Isotype determination was made with Sigma ImmunoType™ (Sigma, St Louis, MO).

*ELISA.* For indirect ELISA, microtitre plates (Polysorb, Nunc GmbH, Wiesbaden, Germany) were coated with heat-killed sonicated Legionella cells ( $10^8$  cells/ml) or purified LPS (0.5 µg/ml) in PBS (pH 7.2) overnight at 4 °C. In order to prove the LPS nature of the epitopes recognized, the sonicated antigens were treated with Proteinase K (2 mg/ml, 60 min, 60 °C, followed by 10 min at 100 °C) and used for coating as well. After washing, wells were saturated with 10% foetal calf serum in PBS containing 0.05% Tween 20 for 60 min. After incubation with the MAbs applied as native culture supernatant, bound antibodies were detected by anti-mouse (polyvalent)-HRPO (Sigma, Munich). O-Phenylenediamine was used as chromogen. Measured optical density (o.d.<sub>492 nm</sub>) was evaluated semiquantitatively according to the following values: positive o.d. > 0.6, weak positive 0.15 < o.d. < 0.6, negative o.d. < 0.15. O.d. of blanks (without antigen for coating and without MAb, respectively) did not exceed 0.075.

Capture ELISA was used as described previously [23]. Briefly, wells were coated with rabbit polyclonal anti-*L. pneumophila* (serogroup 1) IgG. After saturation of

Table 1. Monoclonal antibodies against LPS epitopes of *L. pneumophila* serogroup 1 strains: immunogen, isotype, and reaction pattern

MAb	Immunogen (ATCC strain*)	Isotype	Reactivity† with ATCC type strains*									
			Ph	Al	Be	Kn	Fr	OL	Ox	He	Ca	Bh
3/1‡	Ph	IgG <sub>3</sub>	++	++	++	++	++	-	-	-	-	-
8/5‡	Ph	IgM	++	++	++	++	++	++	++	++	++	++
8/4	Ph	IgG <sub>2b</sub>	++	+	-	++	-	++	++	+	+	-
12/2	Ca	IgM	++	+	-	++	-	++	++	++	++	++
26/1	Ca, Bh, Fr	IgG <sub>3</sub>	-	-	-	+	-	++	++	+	++	++
26/2	Ca, Bh, Fr	IgG <sub>3</sub>	-	+	-	-	++	++	-	-	-	-
26/3	Ca, Bh, Fr	IgG <sub>2b</sub>	-	-	-	+	-	-	++	++	++	+
30/1	Ca, Al, OL	IgG <sub>2b</sub>	-	-	-	-	-	++	++	+	+	-
10/1	Bh	IgM	-	-	-	-	-	-	-	-	-	++
20/1‡	Bh	IgG <sub>3</sub>	-	-	++	-	-	+	-	-	-	++

\* Ph, Philadelphia 1; Al, Allentown 1; Be, Benidorm 030E; Kn, Knoxville 1; Fr, France 5822; OL, OLDA; Ox, Oxford 4032E; He, Heysham 1; Ca, Camperdown 1; Bh, Bellingham 1.

† Tested by indirect ELISA: ++, positive; +, weak positive; -, negative (for explanation see Material and Methods).

‡ At least one other MAb exhibiting the same reaction pattern was found.

the wells with 10% foetal calf serum in PBS and incubation with LPS or the *O*-deacetylated LPS, bound antigens were detected by MAbs followed by anti-mouse(polyvalent)-HRPO (Sigma, Munich, Germany). The lowest antigen concentration producing an o.d.<sub>492 nm</sub> higher than the mean value plus threefold standard deviation of the blank was taken as value for sensitivity of the assay.

## RESULTS

In order to obtain MAbs recognizing diverse antigens of *L. pneumophila* serogroup 1 strains, seven fusion experiments using spleen cells of mice, immunized with either individual *Legionella* strains or two or three strains in combination, were carried out. A total of 18 MAbs reacting with *L. pneumophila* serogroup 1 strains was selected. No reactivity of these MAbs with strains of serogroups 2-14 and serotype Lansing 3 was observed (data not shown). The MAbs were tested by indirect ELISA with the ten *L. pneumophila* serogroup 1 reference strains as described by Joly and colleagues [10]. The observed reactivity pattern allowed discrimination of at least ten different epitopes on the LPS of the various strains (Table 1). Of the corresponding ten MAbs, four (3/1, 8/5, 8/4, and 12/2) recognized epitopes located on the sonicated cells of strain Philadelphia 1 as well as on its purified LPS. As shown in Table 1 as well as demonstrated previously (17), one of them (MAb 3/1) exhibited the same reaction pattern as MAb 2 of the standard subgrouping scheme [10], which recognizes an epitope known as a virulence marker for *L. pneumophila* serogroup 1 strains [11, 13, 17].

For molecular identification of the LPS-associated epitopes, the LPS of strain Philadelphia 1 was chemically modified by treatment with 0.1 M sodium hydroxide (37 °C, 14 h). As judged by the <sup>1</sup>H-NMR spectroscopy data, this treatment resulted in the complete removal of the 8-*O*-acetyl group from the *O*-specific chain of LPS, which has been previously identified as an a-(2 → 4)-interlinked homo-

Table 2. Detection of LPS of *L. pneumophila* serogroup 1, strain Philadelphia 1, using MAbs. Comparison of intact LPS with LPS after *O*-de-acetylation (LPS-OH)

Mab	Indirect ELISA reactivity* after coating on polystyrene plates		Capture ELISA sensitivity (ng/ml)	
	LPS	LPS-OH	LPS	LPS-OH
3/1	++	—	< 0.2	> 1000
8/5	++	++	< 0.2	20
8/4	++	++	20	2
12/2	++	++	200	2
26/1	—	++	> 1000	20
26/2	—	++	> 1000	20
26/3	—	++	> 1000	20
30/1	—	++	> 1000	20
10/1	—	—	> 1000	> 1000
20/1	—	—	> 1000	> 1000

\* For explanation see Table 1.

polymer of 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetra-deoxy-D-glycero-L-galacto-nonulosonic acid (di-*N*-acyl-8-*O*-acetyllegionaminic acid) [16]. When the intact LPS and the *O*-deacetylated LPS were used in both indirect and capture ELISA, differences in the MAb reactivity were noticed (Table 2). In particular, *O*-deacetylation resulted in the loss of the reactivity of MAb 3/1, thus indicating that the corresponding epitope involves an *O*-acetyl group, most likely the 8-*O*-acetyl group of the di-*N*-acyl-8-*O*-acetyllegionaminic acid.

It has, however, been found that not only the *O*-specific chain but also the core oligosaccharide of the strain Philadelphia 1 LPS is highly *O*-acetylated [24]. To exclude a possibility of recognition by MAb 3/1 of an *O*-acetyl-containing epitope on the core, we analysed the structure of LPS of *Pseudomonas fluorescens* ATCC 49271, which reacts with MAb 3/1 as strongly as the strain Philadelphia 1 LPS (authors' unpublished data). It was found that the *P. fluorescens* LPS has the same homopolymer *O*-specific chain as *L. pneumophila* with the only difference in the degree of *O*-acetylation of the di-*N*-acyl-8-*O*-acetyllegionaminic acid which is quantitative in the latter but reaches only *c.* 70% in the former [24, 25]. In contrast, although also *O*-acetylated, the core of the *P. fluorescens* LPS has a quite different structure [25]. Therefore, the cross-reactivity between these two microorganisms is based on the similarity of the *O*-specific polysaccharides, and MAb 3/1 recognizes an epitope on the *O*-specific chain of LPS that is associated namely with the 8-*O*-acetyl group of the di-*N*-acyl-8-*O*-acetyllegionaminic acid.

Unlike MAb 3/1, four MAbs (26/1, 26/2, 26/3, and 30/1) which reacted with *L. pneumophila* serogroup 1 strains other than the monoclonal subtype Philadelphia recognized epitopes on the *O*-deacetylated LPS of strain Philadelphia 1. This finding suggested that complete 8-*O*-acetylation of legionaminic acid blocks recognition of the immunodeterminants that are accessible on the intact LPS of the other *Legionella* strains lacking the MAb 3/1 epitope.

In the case of MAb 8/5, the capture ELISA turned out to be more sensitive for the intact LPS than for the *O*-deacetylated LPS. This relation was reversed when MAb 8/4 or 12/2 were used. Such influence of *O*-deacetylation on the reactivity of

the MAbs showed that the corresponding epitopes are located on the environment of the 8-*O*-acetyl group of the di-*N*-acyl-8-*O*-acetyllegionaminic acid or the *O*-acetyl groups of the core oligosaccharide.

#### DISCUSSION

It is known that MAb 3/1 studied in this work has the same reactivity with the reference strains [17] as MAb 2 of the standard subgrouping scheme [10]. The epitope on the outer-membrane LPS recognized by these MAbs is considered to be associated with the virulence of *L. pneumophila* serogroup 1 strains [11, 13]. Furthermore, of 18 selected MAbs, 3 other MAbs exhibited the same reaction pattern emphasizing a high immunogenic potential and/or the predominance of the MAb 2/MAb 3/1 epitope.

Molecular identification of this epitope required the knowledge of the exact structure of the LPS, in particular, of the *O*-specific polysaccharide chain. Despite a number of attempts to determine the chemical composition and structure of the LPS of *L. pneumophila* [26, 27, 28], the detailed structure of the *O*-specific chain of LPS of *L. pneumophila* serogroup 1 strain Philadelphia 1 has been established only recently [16]. It was found to be an  $\alpha$ -(2  $\rightarrow$  4)-interlinked homopolymer of 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetra-deoxy-D-glycero-L-galactonulosonic acid (di-*N*-acyl-8-*O*-acetyllegionaminic acid) [16]. Selective removal of the 8-*O*-acetyl group from legionaminic acid resulted in the loss of the reactivity with the MAb 3/1 recognizing the virulence-associated epitope. Therefore, the 8-*O*-acetyl group is the immunodominant part of the virulence-associated epitope and may be considered as a marker for virulence.

On the other hand, *O*-deacetylation of the LPS uncovered some additional immunodeterminants which are present in the immunoinaccessible form on LPS of *L. pneumophila* strains lacking the virulence-associated epitope. In addition, although not lost after *O*-deacetylation, the reactivity of three MAbs other than MAb 3/1 is influenced by the presence of the 8-*O*-acetyl residue. It can be, thus, suggested that the antigenic diversity of *L. pneumophila* serogroup 1 strains is attributed to a great extent to the *O*-acetyl substituent of the *O*-specific chain of LPS (or, less probable, of the core oligosaccharide) rather than to the *N*-acyl groups and legionaminic acid itself.

Up to now, the molecular basis for the higher virulence potential of strains carrying the epitope recognized by MAb 2/MAb 3/1 remains obscure. Possible explanations are: (i) legionellae bearing this epitope are preferentially taken up by phagocytic cells in some way and multiplied intracellularly; (ii) factor helping combating host defence mechanism; and (iii) increased survival of legionellae in aerosols as a result of a protective effect of the possession of MAb 2 epitope thus increasing the dose inhaled by exposed individuals. While the first point is based on laboratory and animal studies of limited numbers of strains [29–31], Edelstein and Edelstein [32] tested the intracellular growth of ten randomly selected strains of *L. pneumophila* serogroup 1 lacking the epitope recognized by MAb 2. All of the ten MAb 2-negative isolates grew in guinea-pigs' alveolar macrophages as well as the virulent strain. However, the ability to infect guinea-pigs' macrophages cannot be taken to imply virulence for humans. The second point deals with a

property depending on the *in vivo* behaviour of the strains. It has been shown that isogenic mutants of a MAb 2-positive strain which no longer expressed the MAb 2 epitope became serum sensitive but their multiplication in human macrophage-like cells U937 was not affected [33]. On the other hand, as shown in amoebae, U937 cells, and guinea-pigs, virulent and avirulent variants of Philadelphia 1 strain exhibit the same reactivity with MAb 2 [34].

With reference to the third point mentioned, the survival of *Legionella* following aerolization is very important in causing illness. Dennis and colleagues [35] demonstrated that strains of *L. pneumophila* serogroup 1 which are epidemiologically associated with Legionnaires' disease had better survival in small particle aerosols than strains not associated with the disease. Concurrently, the presence or absence of the epitope described as a virulence marker is connected with the ability to survive [35]. Epidemiological data support this observation since MAb 2-positive strains were isolated from all kinds of patients, both sporadic cases and in outbreaks. MAb 2-negative strains and strains of other *Legionella* serogroups and species often cause infections in immunosuppressed patients, who may acquire the disease by both ingestion and inhalation [30, authors' unpublished data].

At present, the basis for the association of strains bearing the MAb 2/MAb 3/1 epitope with survival in aerosols cannot be accounted for with certainty. Chemical characterization confirmed the hydrophobicity of *L. pneumophila* LPS which is caused by a high lipid A content and its unusual fatty acid composition (the predominance of long-chain and branched-chain fatty acids [26, 26, 28]), but also by the presence of hydrophobic components (*N*- and *O*-acyl groups, deoxy units) in the repeating unit of the O-specific chain devoid of free hydroxy groups and in the outer core [16, 24]. In the absence of the 8-*O*-acetyl group of legionaminic acid, free hydroxy groups appear in the O-specific chain and the hydrophobicity of LPS decreases. However, it remains undetermined as to whether strains lacking the virulence-associated epitope have non-*O*-acetylated O-specific chain with the free hydroxy group or with another acyl substituent at position 8 of the di-*N*-acyl-8-*O*-acetyllegionaminic acid.

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