Chlamydial lipopolysaccharide: Chemical and antigenic structure, biosynthesis and biomedical application

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Abstract: The obligate intracellular gram-negative bacterium *Chlamydia* contains as a major surface antigen a lipopolysaccharide (LPS) which harbours in its saccharide moiety a genus-specific epitope composed of a linear trisaccharide of 3-deoxy-D-*manno*-octulopyranosonic acid (Kdo) of the sequence αKdo-(2→8)-αKdo-(2→4)-αKdo. The structure was established on LPS of recombinant *E. coli* bacteria transformed with a plasmid carrying the gene for the chlamydial Kdo transferase which is a multifunctional glycosyl transferase. The structure was determined on deacylated and dephosphorylated LPS as well as on deacylated LPS by qualitative, quantitative and methylation analyses, fast-atom-bombardment mass spectrometry, and ^{1}H-, ^{13}C-, and ^{31}P-NMR spectroscopy, and confirmed by chemcial synthesis. Artificial glycoconjugate antigens were synthesized and used for the preparation of murine monoclonal antibodies which had higher affinities than those prepared against the natural counterpart. These artificial antigens were also used as solid-phase antigens in an enzyme-immuno assay which was proven to be useful in the diagnosis of human chlamydial infections.

INTRODUCTION

To understand human infectious diseases at the molecular level, the tools of molecular microbiology, cellular biology and immunology are indispensible. However, it is less evident that, in those cases where secondary gene products are investigated, analytical, structural and organic chemistry as well as biochemistry are also necessary or desirable.

The work reviewed in the following article exemplifies how the combined tools of these different disciplines allowed us to answer questions on a surface antigen of the obligatory intracellular parasite *Chlamydia* and demonstrates the particular importance of having a profound knowledge in carbohydrate chemistry.

DESCRIPTION OF THE GENUS *CHLAMYDIA* AND CLINICAL BACKGROUND

Chlamydia psittaci, C. trachomatis, and *C. pneumoniae* are bacterial species of the monogeneric family *Chlamydiaceae*. These bacteria are pathogenic, obligatory phagosomal intracellular parasites which cause acute and chronic diseases in animals and humans (1, 2). The natural reservoir of *C. psittaci* are animals,

however, human infections are known by avian strains, particularly those from paraquets and related birds, causing severe pneumonia (ornithosis, psittacosis). *C. trachomatis* is devided into serovars of which type A through C cause trachoma, a chronic infection of the eye which, in the late stages of the disease, leads to blindness. Approximately 500 million of people suffer from this disease, of whom 6 to 7 million are blinded. Thus, chlamydial trachoma is still the World's leading cause of secondary preventable blindness (3). Serovars L1 to L3 cause lymphogranuloma venereum, a disease starting from infection of the rectal mucosa followed by infiltration and destruction of the inguinal lymph nodes. Serovars D through K cause sexually transmitted diseases in men and women and are today the most frequently diagnosed sexually transmitted infections. In man they cause urethritis, prostatitis and epidydimitis, in woman, urethritis, cervicitis, endometritis and salpingitis often resulting in infertility. The third species *C. pneumonia* is known for only a few years and our knowledge on this microorganism is still preliminary. However, it was found that *C. pneumoniae* causes diseases of respiratory tract (4).

THE MICROORGANISM

Chlamydiae undergo a unique developmental cycle inside the infected host cell. The infection is initiated by the uptake of the so-called elementary body (200 to 300 nm in diameter) by the host cell. Inside the phagosome, the elementary body differentiates into the reticulate body which measures 1 μm in diameter, is non-infectious but metabolically active, and able to multiply by binary fission making use of the host's substrate and energy pool. Late in the infectious cycle, reticulate bodies differentiate into infectious elementary bodies which are released from the lysed cell and may infect cells in the neighborhood (5). There are two major surface antigens of chlamydiae which are both located in their outer membrane namely the major outer membrane proteine OMP1 and the group-specific glycolipid antigen which is known from the early beginnings of chlamydial research of being a genus-specific antigen against which antibodies are raised by the infected host (6). In the early seventies, this antigen was reported to be a glycolipid containing as an immunodominant group a 3-deoxy-2-keto sugar acid which was similar to but not identical with Kdo (7). This observation led to the hypothesis that the chlamydial glycolipid may be related to the LPS of gram-negative bacteria. Additional evidence for the correctness of this hypothesis was obtained more than ten years later from data showing that the glycolipid was chemically and serologically related to the LPS of core-deficient enterobacterial Re-mutants (8). The definite proof that the glycolipid was an LPS was obtained from chemicial analysis of chlamydial LPS which was composed

of Kdo (we were the first showing by combined GLC/MS that the thiobarbiturate-positive material described before was indeed Kdo), glucosamine, phosphate and long chain fatty acids, the latter including ß-hydroxy fatty acids as typical LPS markers (9, 10).

With these results (Table 1) it became generally accepted that the chlamydial genus-specific antigen reported so far as the "group-specific glycolipid antigen" was an LPS with a chemical composition similar to that of well characterized enterobacterial core-deficient rough mutants.

We then started to determine the immunogenic and antigenic properties of chlamydial LPS in comparison to Re-type LPS (9, 11). Thereby the following results were obtained (Table 2). Rabbit antisera prepared against Re-type bacteria cross-reacted with Re and chlamydial LPS and absorption with either antigen abolished the reactivity towards both of them. Antisera against chlamydiae also reacted with both LPS, however, absorption with Re LPS destroyed only the homologous reaction whereas absorption with chlamydial LPS removed both reactivities from the serum. These data indicated that there existed structural elements shared between chlamydiae and Re-type bacteria on the one hand and those which were specific for chlamydial LPS.

TABLE 1. Chemical composition of *Chlamydia-* and Re-LPS

Constituent	Amount (nmol/mg) present in LPS of:		
	C. trachomatis L2	*C. psittaci*	*E. coli* Re
Glucosamine	480 (2.0)*	513 (2.0)	720 (2.0)
Galactosamine		257 (1.0)	
Kdo	715 (3.0)	1190 (4.6)	732 (2.0)
Phosphate	625 (2.6)	578 (2.3)	748 (2.1)
Fatty acids	1185 (4.9)	1190 (4.6)	1955 (5.4)

* Molar ratios in parenthesis.

TABLE 2. Serological cross-reaction between *Chlamydia-* and Re-LPS in the passive hemolysis assay.

Antiserum	Reactivity with LPS of:	
	Re-type	*Chlamydia*
Anti-Re	+	+
abs. Re-LPS	—	—
abs. *Chlamydia*-LPS	—	—
Anti-*Chlamydia*	+	+
abs. Re-LPS	—	+
abs. *Chlamydia*-LPS	—	—

CHEMICAL STRUCTURE OF CHLAMYDIAL LPS (A DEVIATED APPROACH)

Since the structure of chlamydial LPS could not be determined directly (the preparation of adequate amounts allowing structural analysis is not feasable) we asked the question whether the common elements in these two LPS could be defined serologically. Due to our extensive work on the specific chemistry of Kdo and the acquired experience (12-32), we were in the position to determine all structural details of the Re LPS (33-36) and then prepared monoclonal antibodies against it (32, 33, 39, 40) which cross-reacted with Re- and chlamydial LPS. Using chemically synthesized Kdo oligosaccharides and analogues
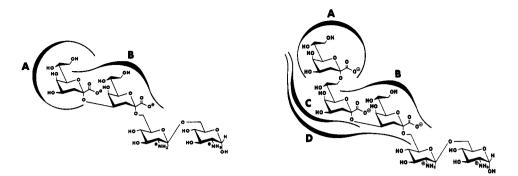
Fig. 1 Schematic representation of antibody specificities binding to Re-type (left) or chlamydial LPS (right). Antibodies of type A and B recognize an α-pyranosidically linked Kdo residue or the α2→4-linked Kdo disaccharide, present in both LPS, those of type C and D recognize the disaccharide αKdo-(2→8)-αKdo and the trisaccharide αKdo-(2→8)-αKdo-(2→4)-αKdo, respectively. The 2→8-linkage has not been detected in other bacteria or natural products, and thus, is a chemotaxonomical marker for *Chlamydia.*

(41-47), we defined one antibody which recognized a terminal α-pyranosidically linked Kdo residue whereas another required an α2→4-linked Kdo disaccharide for binding [Fig. 1, antibody type A and B; (38)].

Based on these data we hypothesized that the chlamydial LPS must contain an α-pyranosidically linked terminal Kdo residue and an α2→4-linked Kdo disaccharide. In the meantime, other groups had reported on the preparation of monoclonal antibodies which reacted in a chlamydia-specific way, i.e. they did not bind to Re-type LPS (48). Finally, making use of such antibodies, the molecular cloning of a chlamydial DNA fragment (plasmid pFEN207) was reported (49) which led to the expression of the chlamydial genus-specific epitope in *E. coli* K-12. These transformants synthesized two LPS populations one of which corresponded to the parent LPS and the second migrated faster in SDS-PAGE and was thus smaller in size. Western blots using monoclonal antibodies revealed that the modified LPS population contained the genus-specific epitope. Since LPS is a secondary gene product these experiments did not provide any information on the molecular structure of the modified LPS. Although Caldwell and Nano (49) had speculated that the cloned gene encoded for a glycosyl transferase there was no experimental evidence for this suggestion. We used a set of core-defective mutants of *Salmonella minnesota* and *S. typhimurium* for transformation with pFEN207 and found that the Re mutants could still be modified into chlamydia-reactive LPS (50). Comparative chemical analysis on LPS of the parent and the recombinant strain indicated that they contained 2 and 3 moles of Kdo per mole, respectively. We had reported earlier on the release of Kdo oligosaccharides from LPS by very mild acid hydrolysis and on their analysis by GLC/MS. This method, when applied to Re LPS, yielded spectra for Kdo monosaccharide and the

α2.4-linked Kdo disaccharide. The recombinant LPS yielded two additional signals which corresponded to a 2.8-linked Kdo disaccharide and a Kdo trisaccharide of the sequence Kdo2$\rightarrow$8Kdo2$\rightarrow$4Kdo of unknown anomeric configurations (51). These data suggested that the genus-specific epitope was related to a distinct Kdo oligosaccharide and that the cloned chlamydial gene encoded most likely for a Kdo transferase. Interestingly, the 2.8-linkage between two Kdo residues has not been found in other bacterial LPS or any other natural compound. **Therefore, it seemed likely that the unique chemical structure was the molecular basis for its unique antigenic properties.**

To determine the anomeric configuration of the terminal Kdo residue the two possible isomers Kdoα2$\rightarrow$8Kdo and Kdoβ2$\rightarrow$8Kdo were synthesized and compared by GLC/MS as acetylated or methylated derivatives. The data indicated that the terminal Kdo was α-linked in the natural compound (43, 52). The determination of the anomeric configurations of the reducing Kdo residue and of those in the trisaccharide required a different analytical approach in which the glycosidic linkage of the first Kdo residue was left intact. At the same time this approach should show whether the Kdo trisaccharide was directly linked to lipid A (as expected) or whether a so far not detected component was present between them. The LPS was de-O-acylated with hydrazine, dephosphorylated with hydrogen fluoride, reduced, and finally de-N-acylated with hydrazine at elevated temperature. Among others, one major product was composed of Kdo, GlcN, and glucosaminitol in the molar ratio of 3:1:1. This compound could be separated and purified to homogeneity by high-voltage paper electrophoresis, ion exchange and gel-permeation chromatography. The final product was analyzed by ^{1}H- and ^{13}C-NMR spectroscopy whereby it was identified as αKdo-(2$\rightarrow$8)-αKdo-(2$\rightarrow$4)-αKdo-(2$\rightarrow$6)-GlcN-(1$\rightarrow$6)-GlcNol (53). Additional NMR experiments, synthetic standards, and computer modelling allowed a preliminary determination of the three-dimensional conformation of the molecule (54). Only recently, we succeeded to get the partial structure αKdo-(2$\rightarrow$8)-αKdo-(2$\rightarrow$allyl crystallized and resolved its structure by X-ray crystallography (55). Since we knew, from other serological investigations on deep rough LPS (39, 56, 57) and lipid A (58-62) and the corresponding antisera against them, that there exist also epitopes comprising phosphorylated sugars, we wanted to isolate the carbohydrate backbone with the phosphate groups attached to it. This goal could so far not be reached since there was no method to deacylate LPS without removing the phosphate groups at the same time, however, an appropriate method was developed in our laboratory. LPS was de-O-acylated with hydrazine at low temperature followed by de-N-acylation with strong alkali

(4 M potassium hydroxide) at 120°C over night. After neutralization and desalting, the phosphorylated

oligosaccharides were separated by high-performance anion exchange chromatography yielding

homogenous preparations suitable to be analyzed by NMR-spectroscopy. Thus, the two oligosaccharides

αKdo-(2→8)-αKdo-(2→4)-αKdo-(2→6)-βGlcN4P-(1→6)-αGlcN1P and αKdo-(2→4)-αKdo-(2→6)-

-βGlcN4P-(1→6)-αGlcN1P were obtained and analyzed by ^{1}H-, ^{31}P-, and ^{13}C-NMR spectroscopy and by

fast-atom-bombardment mass spectroscopy. Fig. 2 shows the ^{13}C-NMR spectrum of the pentasaccharide

(63). Most structures described so far as well as partial structures thereof have been chemically

synthesized ascertaining the analytical data obtained (42-45). It is noted that these natural compounds

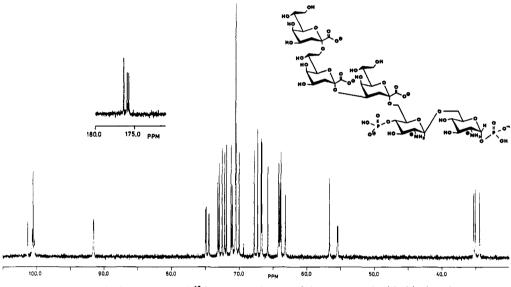have been prepared in quantities up to 250 mg.

Fig. 2 Chemical structure and ^{13}C-NMR spectrum of the pentasaccharide bisphosphate

αKdo-(2→8)-αKdo-(2→4)-αKdo-(2→6)-βGlcN-4P-(1→6)-αGlcN-1P.

ANTIGENIC STRUCTURE OF CHLAMYDIAL LPS

Now, with the profound knowledge of the chemical structure on the molecule, we investigated its

antigenic and immunogenic properties. First, the synthetic oligosaccharide monomeric haptens

(allylglycosides) were converted into polymeric antigens by copolymerization with acrylamide or by

conjugation to proteins after derivatization into cysteamine-spacered ligands. These artificial

glycoconjugate antigens were used in inhibition experiments with *Chlamydia*-specific monoclonal

antibodies. Whereas Kdo monosaccharide and the 2→4- and 2→8-linked disaccharides were inactive, the

trisaccharide was an efficient inhibitor. The addition of one or two glucosamine residues (as present in

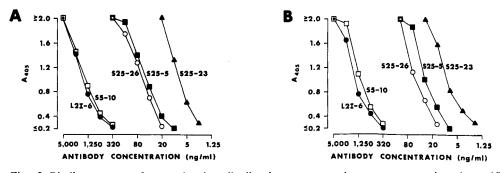the natural LPS) did not improve inhibition (53). These data clearly allowed the conclusion that the Kdo

Fig. 3 Binding curves of monoclonal antibodies in an enzyme-immuno assay using the artificial glycoprotein antigens αKdo-(2→8)-αKdo-(2→4)-αKdo-BSA (A) or αKdo-(2→8)-αKdo-(2→4)-αKdo-ßGlcNAc-BSA (B) as a solid phase antigen. Monoclonal antibodies L2I-6 and S5-10 were obtained after immunization with elementary bodies of *C. trachomatis* and *C. psittaci*, respectively; the others were obtained after immunization with the glycoconjugate αKdo-(2→8)-αKdo-(2→4)-αKdo-ßGlcNAc-BSA.

trisaccharide was the minimal structure to which the antibody bound and showed that the antigenic properties of the synthetic antigen were similar to those of its natural counterpart. To investigate the immunogenic properties, the glycoconjugate αKdo-(2→8)-αKdo-(2→4)-αKdo(2→6)-ßGlcNAc-BSA was used to immunize mice and to prepare monoclonal antibodies (64). The results indicated that antibodies had been generated which cross-reacted between Re-type and chlamydial LPS and those which were *Chlamydia*-specific. Among the latter were those which did not require the complete Kdo trisaccharide but also reacted with the α2→8-linked Kdo disaccharide. This shows that our hypothesis was correct that the unique chemical structure also represents a unique epitope.

When we compared the monoclonal antibodies obtained after immunization with the synthetic glycoconjugate to those obtained after imunization with chlamydial elementary bodies from either *C. psittaci* or *C. trachomatis*, we found that the latter were 100-fold less affine than the former. Thus, the immunogenic and antigenic properties of the synthetic compounds are not only similar but superior to their natural counterparts (64).

BIOSYNTHESIS OF CHLAMYDIAL LPS

For the following section, an introduction into the biosynthesis of LPS in general and of the Kdo region in particular is required. The three regions of LPS, i.e. O-side chain, core region, and lipid A, are generated through the action of enzymes the encoding genes of which are clustered on the chromosome (65, 66). The enzymes for the core biosynthesis are located in the so-called *rfa* locus which has been cloned and sequenced in *E. coli* K-12 and *S. typhimurium*. Core biosynthesis starts with the transfer of Kdo to a precursor of lipid A, called precursor Ia (composed of the 1,4'-bisphosphorylated ß1→6-linked glucosamine disaccharide and four mole equivalents of 3-hydroxytetradecanoic acid linked to positions

2, 3, 2', and 3'). Kdo is activated into Kdo-CMP by Kdo-CMP synthase encoded by the gene *kdsB*. The activated sugar is transferred by a Kdo transferase encoded by the gene *kdtA*. This enzyme has the unusual property to catalyze two different glycosylation steps namely the transfer of Kdo to position 6' of the lipid precursor and the transfer of a second Kdo residue to position 4 of the former. Thus, the enzyme is bifunctional, a behavior which has not been described so far for any of the known glycosyl transferases. Even more surprising to us was the report that the Kdo transferase in *C. trachomatis* is able to catalyze the transfer of three Kdo residues (67). By these unexpected data we became interested in this enzyme and started with the molecular cloning of this gene from *C. psittaci* strain 6BC (68). Sequence analysis of the cloned gene revealed a homology of only 67% when aligned to the sequence of the corresponding gene in *C. trachomatis* L2. Considering that these genes are from two species of the same genus and that e.g. the Kdo transferases of *E. coli* and *S. typhimurium* (two different genera) exhibited a homology of more than 95%, this result was unexpected. We are currently investigating the enzymatic activity of these enzymes and their products in vitro. Preliminary data indicate that there exist molecular species with more than three Kdo residues, the structures of which are presently determined.

TECHNOLOGY TRANSFER AND BIOMEDICAL APPLICATION

Finally, we succeeded to transfer our knowledge to biomedical application. The diagnostic principles for chlamydial infections are based on the detection of the microorganism or its "traces" in the host. The former is achieved by culture and identification with specific antibodies. Here, our LPS antibodies are useful reagents to identify chlamydiae at the genus level and many laboratories are using our antibodies presently in routine diagnosis. Other possibilities are i) the vizualization of chlamydiae in swabs from the infected site by immunofluorescence, ii) the detection of chlamydial antigens by ELISA or iii) the detection of specific chlamydial DNA or RNA. Recording the immune response against chlamydiae includes the measurement of antibodies in serum or secretions and the detection of specifically primed immune cells. With the availability of a defined LPS antigen, we investigated different groups of patients and healthy blood donors for the presence of antibodies against the chlamydial LPS (69). The data shown in Table 3 demonstrate that LPS antibodies of the IgG, IgA, and IgM type occur more frequently in the serum of patients than in blood donors. Most significant is the occurrence of IgA and IgM serum antibodies. In cooperation with a pharmaceutical company we have developed an enzyme-immuno assay which has been clinically evaluated and, at the end of last year, got approval by the Paul-Ehrlich-Institut,

TABLE 2. LPS antibodies in patients with chlamydial infections and blood
donors using artificial glycoconjugates in ELISA

		Percent positive for		
Serum source	n =	IgG	IgA	IgM
Genital infections[a]	111	86	65	31
Respiratory infections[b]	99	89	93	76
Blood donors	200	53	28	7

[a] Positive for chlamydiae by culture

[b] Positive for *C. pneumoniae*-specific antibodies in the micro-immunofluorescence assay

the german controlling authority for reagents used in the diagnosis of sexually transmitted diseases. Now

with the commercialization of this test, the clinical experience is rapidly increasing and we expect a better

diagnosis and record of chlamydial and related diseases and a more precise epidemiology of these

infections.

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