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Comparison of lipopolysaccharide structures of *Bordetella pertussis* clinical isolates from pre- and post-vaccine era

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This paper is dedicated to the memory of Malcolm B. Perry, a great scientist, mentor, and a dear friend, whose example will always inspire us

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ABSTRACT

Endotoxins are lipopolysaccharides (LPS), and major constituents of the outer membrane of Gram-negative bacteria. *Bordetella pertussis* LPS were the only major antigens, of this agent of whooping-cough, that were not yet analyzed on isolates from the pre- and post-vaccination era. We compared here the LPS structures of four clinical isolates with that of the vaccine strain BP 1414. All physico-chemical analyses, including SDS–PAGE, TLC, and different MALDI mass spectrometry approaches were convergent. They helped demonstrating that, on the contrary to some other *B. pertussis* major antigens, no modification occurred in the dodecasaccharide core structure, as well as in the whole LPS molecules. These results are rendering these major antigens good potential vaccine components. Molecular modeling of this conserved LPS structure also confirmed the conclusions of previous experiments leading to the production of anti-LPS monoclonal antibodies and defining the main epitopes of these major antigens.

1. Introduction

Bordetella pertussis, is the etiological agent of whooping-cough and a strict human pathogen.[1] In 2008, the World Health Organization estimated that around 195,000 children died from whooping cough, mostly in developing countries.[2] Whole-cell pertussis (wP) vaccine was introduced in France in 1959.[3] This efficacious vaccine was used for primary vaccination and one booster for all young French children. The change in the herd immunity of the child population led to an important decrease in the mortality and morbidity due to *B. pertussis* and to a control of the circulating clinical isolates of *B. pertussis* which were similar to the two vaccine strains.[4] However, not all isolates were controlled. The remaining isolates were close to the previous circulating isolates but exhibited changes as confirmed by using pulsed field gel electrophoresis (PFGE) in the genes encoding the S1 subunit of pertussis toxin and in the gene encoding one of the auto-transporter playing a role as an adhesion, pertactin (PRN).[4–6] No change was observed in the gene encoding the adenylate cyclase hemolysin, the other major toxin expressed by *B. pertussis*.[7] These isolates were as virulent as the vaccine strains.[8,9] These changes could not be observed in regions of low vaccine coverage like in Senegal.[10]

Acellular pertussis (aP) vaccines, containing only a few purified and detoxified bacterial proteins, such as pertussis toxin associated with filamentous hemagglutinin and with or without PRN and fimbrial proteins, were introduced in 1998 as an adolescent booster and rapidly generalized to the whole population, changing herd immunity by specifically targeting the virulence of the bacteria. We then pursued the temporal analysis of the isolates collected since 2000 under aP vaccine pressure, using PFGE, genotyping of the genes encoding the vaccine antigens and detection of expression of virulence factors. Since the introduction of the aP vaccines, there has been a steady increase in the number of *B. pertussis* isolates collected that are lacking expression of PRN but still expressing pertussis toxin and filamentous hemagglutinin. These isolates

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are as virulent as those expressing all virulence factors, according to animal and cellular models of infection,[11,12] and also according to clinicians.[13] Whereas, wP vaccine-induced immunity led to a monomorphic population of *B. pertussis*, aP vaccine-induced immunity enabled the number of circulating *B. pertussis* not expressing virulence factors to increase, thus sustaining our previous hypothesis.[1] However, other components of the bacterium, especially lipopolysaccharides,[14] were not analyzed in this perspective on different strains and clinical isolates. It was then of importance to know whether the particular structure of the *B. pertussis* LPSs temporally changed after the use of wP and aP vaccines.

We described the structure of the *B. pertussis* endotoxin (lipopolysaccharide, LPS) in detail in 2000.[14] LPS are complex glycolipids and major structural components of the Gram-negative bacterial outer membrane. The general structure of LPSs consists of three distinct domains: a hydrophobic moiety, called lipid A, a core oligosaccharide containing 3-deoxy-D-manno-2-octulosonic acid (Kdo), and a serospecific O-polysaccharide composed of repeating oligosaccharide units.[15] Lipids A anchor LPS molecules in the outer leaflet of the external asymmetric bacterial membrane. Some LPS called lipooligosaccharides (LOS), have no O-chain and this is the case for those of *B. pertussis*.[16] *B. pertussis* LPS were shown to have a dodecasaccharide core structures carrying unusual sugars in their distal part.[14] The Kdo molecules were unique in these structures, carrying PyroPhosphoryl-EthanolAmine (PPEA) molecules at C-4, in a non-stoichiometric way, approximately 50%, and there was no second Kdo molecule as observed in many other described LPS structures.[15] The *B. pertussis* LPS molecules without PPEA were called LPS-I and the others carrying PPEA at Kdo, LPS-II (Fig. 3). The lipid A moiety of these LPS is not usual, having only 5 fatty acids and with the presence of a short-chain 10:0(3-OH) ester at C-3 of the first glucosamine (Glc*p*N). These characteristics are known for leading to a low cytokine induction level and might be a good way for the bacterium to escape the host immune defense system.[17]

2. Results

The French wP vaccine strain BP 1414 is a pre-vaccine era strain producing all vaccine antigens; FR3713, FR3693, and FR3749 were collected in France and are post-vaccine era clinical isolates. FR 3713 is producing all vaccine antigens, FR3693 does not produce PRN, and FR3749 does not produce pertussis toxin. The last isolate, IS 6523, producing all vaccine antigens, was collected in Senegal and is similar to the French pre-vaccine era clinical isolates. LPS from *B. pertussis* BP1414 vaccine strain and clinical isolates were extracted by the isobutyric acid–1 M ammonium hydroxide method[18] and purified[19] as described in Section 4.

2.1. Comparison of the four *B. pertussis* clinical isolates' LPS structures with that of the BP 1414 vaccine strain

2.1.1. LPS thin layer chromatography (TLC) analyses

As previously shown by Caroff and Karibian,[20] it was possible to obtain the profile of LPS molecular species by migration on TLC after deposition of lyophilized bacterial cells suspended in the solvent and lined down on the TLC plate baseline. A dark deposit corresponding to remaining bacterial cells was revealed on the baseline by charring, then two main spots were separated with respective 0.28 and 0.32 R_f and a minor species developed with a 0.34 R_f (Fig. 1A). These spots did not correspond to the previously so-called SDS–PAGE A- and B-bands,[20] so in order to characterize them, the corresponding products were eluted from a preparative scrapped TLC plate after non-destructive revelation by spray with a mixture of MeOH–water (1:1). They were further analyzed by MALDI-MS. They corresponded to the dodecasaccharide LPS with PPEA for the faster major migrating spot, and for the non-substituted Kdo LPS form for the first one, respectively. The minor species corresponded to a LPS-I with penta-acylated lipid A structure carrying a core missing a terminal heptose (Hep). This new way to separate LPS molecules present in the whole endotoxin extract is interesting, as the only described method for such separation was time consuming.[21]

2.1.2. LPS SDS–PAGE analyses

As shown in Figure 1B, all LPS of clinical isolates and the reference vaccine strain migrated alike, they showed a major molecular species corresponding to the A-band LPS. Some fast migrating minor molecular species were observed below. However they did not migrate like the B-band corresponding to the described nonasaccharide LPS missing the three distal sugars.[16] The molecular heterogeneity of the minor species will be described in more details in the corresponding LPS, its derived lipid A and core MALDI spectra.

The two isolated TLC spots migrated alike and gave a similar SDS–PAGE migration to that of the A-band LPS (data not shown). This result was consistent to the MALDI-MS analyses (Fig. 1C).

2.1.3. Lipid A structures

Structures of lipid A moieties obtained for the four clinical isolates and the reference strain mentioned above were established by MALDI-MS analysis of the isolated lipid A. This was followed by sequential fatty-acid liberation using alkaline treatments[22] and by MALDI-MS analysis (negative ion-mode) supplemented by fragmentation analysis of starting samples in MALDI-MS[23] (positive ion-mode).

The negative-ion MALDI mass spectra shown in Figure 2B for the five lipid A samples displayed major peaks at *m*/*z* 1559 and 1333; they were doubled by small peaks at *m*/*z* 1614 and 1388 respectively. Mass differences between most of the adjacent molecular ion peaks gave a first idea of the fatty acid components like hydroxytetradecanoic acid [14:0(3-OH); 226 units]. Some heterogeneity was observed with minor peaks surrounding *m*/*z* 1559 at plus $-CH_2$ (*m*/*z* 1587), and $2\times -CH_2$ (*m*/*z* 1614) and minus $-CH_2$ (*m*/*z* 1531). They corresponded to molecular species in which the 14:0(3-OH) ester was replaced by 10:0(3-OH) or 12:0(3-OH) due to relaxed fatty acid specificity as already described.[24,25] Molecular species containing additional free Glc*p*N substituting the phosphate groups were observed giving minor peaks at *m*/*z* 1720. The latter were recently described in the Bordetella genus and never observed in other genera.[22]

The exact position of each fatty acid was determined by the lipid A liberation patterns obtained after alkaline treatments. Under the conditions of this method,[22] all substituents at the C-3 and C-3′ positions are liberated by ammonium hydroxide treatment (not shown). In all the examined *B. pertussis* lipid A strains and isolates, a total release of the 10:0(3-OH) fatty acid at position C-3 and 14:0(3-OH) at C-3′ was observed.

The positive-ion MALDI mass spectra (not shown) of all samples had a prominent fragment peak at *m*/*z* 904 and a minor one at *m*/*z* 678. In accordance with a previously published fragmentation pattern,[23] these peaks were attributed to lipid A fragments composed by the distal glucosamine (Glc*p*N II) substituted with one phosphate group and different numbers of fatty acids: one 14:0(3-OH) with a 14:0 for the *m*/*z* 678 fragment peak, and an additional 14:0(3-OH) for *m*/*z* at 904. In the higher masses part of the spectra, Glc*p*N disaccharide fragment peaks appeared, they corresponded to: three 14:0(3-OH), one 10:0(3-OH), and one 14:0 for pentaacyl molecular species at *m*/*z* 1478.

Taken together, these data established the structures presented in Figure 2A for the lipid A moieties of the vaccine strain BP1414 and all clinical isolates, FR3693, FR3713, FR3749, and IS 6523. No