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Bactericidal Monoclonal Antibody Against *Moraxella catarrhalis* Lipooligosaccharide Cross-Reacts with *Haemophilus Spp.*

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Abstract. Monoclonal antibodies (MAbs) against lipooligosaccharide (LOS) determinants after immunization of BALB/c mice with heat inactivated *Moraxella catarrhalis* serotype A were generated. MAb 219A9 was specific for a common epitope of A, B, and C *M. catarrhalis* serotypes in ELISA and immunofluorescent test (IFT). In both tests it also cross-reacted with whole bacteria and LPS antigens isolated from non-typeable *H. influenzae* and *H. parainfluenzae* strains. IgM antibody clone 219A9 possessed a strong bactericidal effect against the three serotypes in the presence of complement. Our results demonstrate that antibodies directed to a single LOS epitope common for A, B, and C serotype could be highly protective. This suggests that the common determinants are very promising in the development of LOS-based vaccine against *M. catarrhalis*. The cross-reactions of MAb 219A9 with *Haemophilus spp.* also show that immunization could result in immune response to epitopes conserved in other important respiratory pathogens.

Moraxella catarrhalis is considered to be an important human pathogen [1, 2]. It is associated with lower respiratory tract infection in adults, especially in patients with chronic obstructive pulmonary disease [3–5]. In children it is a common causative agent of sinusitis, otitis media, bronchitis, and pneumonia [6, 7]. Outer membrane proteins, pili, and lipopolysaccharide (LPS) have been proven as essential virulence factors of *M. catarrhalis* strains [8, 9]. Novel virulence determinants associated with a phase variable expression of the LPS genes were identified in *Haemophilus spp.* and *M. catarrhalis* [10, 28]. LPS of *M. catarrhalis* is composed of a lipid A and oligosaccharide part without repeated O-side chain units [11, 12]. In fact it is lipooligosaccharide (LOS) with a structure typical for other non-enteric Gram-negative pathogens such as *Haemophilus spp.* and *Neisseria spp*. [10, 14]. *Moraxella* LOS exhibits toxicity and can induce pathological reactions similar to those of the *Enterobacteriaceae* endotoxins [14]. Serological typing of *M. catarrhalis* is based on LOS antigens [15]. More than 95% of all isolates belong to the three major serotypes: A, B, and C. Serotype-specific and cross-reactive epitopes have been demonstrated by monoclonal antibodies (MAbs) and polyclonal rabbit sera [10, 16–18]. Chemical structure analysis of LOS molecules shows the relationship and the antigen specificity of the three serotypes [12, 15]. It has been established that the humoral immune response to *M. catarrhalis* infections is directed mainly against the LOS [9, 19]. Immunization of rabbits results in a serotype-specific response, while in patients with respiratory infections the antibodies are cross-reactive. Thus, detoxified *M. catarrhalis* LOS and LOS-protein conjugates are considered as prospective vaccine candidates [11, 20, 21]. Strains expressing complete smooth LPS forms with long O-side specific polysaccharide chains are usually resistant to the complement mediated killing. Although *M. catarrhalis* produce rough type LPS, most of the isolates are complement resistant [22]. The mechanism of complement resistance in moraxellae is very complex and mediated by different components in the outer membrane. Several surface factors are identified as membrane-attaching complex inhibitors. It has been established that the LOS molecule also participates in *M. catarrhalis* complement resistance [23]. However, the mechanism of LOS-mediated resistance is

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still not well understood [2]. Elucidation of the antigenic properties of key surface molecules and identification of their protective epitopes is essential for successful vaccine development.

In this study, we characterized a MAb specific for a common LOS epitope of *M. catarrhalis*, which induced complement-mediated killing of bacteria from the three major serotypes, A, B, and C, and cross-reacted with *Haemophilus* strains. The results confirm the important role of LOS as a protective antigen and support the concept for the development of LOS-based vaccines against *M. catarrhalis* infections in humans.

Materials and Methods

Bacterial Strains and Antigens. *M. catarrhalis* serotype A (strain 353CCUG), B (strain 26400CCUG), and C (strain 26391CCUG) were purchased from the Belgium Microbial Collection. *H. influenzae* ATCC49766 and *H. influenzae* nontypable strain 5156 (isolated from blood culture) and *H. parainfluenzae* strain 788 (isolated from sputum) were from our laboratory collection. Moraxellae were grown in tryptic broth and tryptic-soy agar (BBL Becton Dickinson) and LOS from the three serotypes were purified by the hot phenol-water extraction as described previously [24]. The same method was applied for LPS extraction from both *Haemophilus* strains. Alkaline-treated LPS antigens was prepared by boiling 100×10^9 bacteria ml^{-1} suspension in 0.1N NaOH, followed by pH neutralization and dialysis of the supernatant obtained. Purified LPS from R-mutants of *S. minnesota*, *A. calcoaceticus*, *P. gingivalis*, and lipid A from Re mutant of *E. coli* were a kind gift of C. Galanos, Max-Planck-Institute of Immunobiology, Freiburg, Germany. Whole bacteria from a large number of *M. catarrhalis*, *H. influenzae*, *H. parainfluenzae*, *K. pneumoniae*, *S. marcescens*, *P. aeruginosa*, *A. baumannii*, *B. cepacia*, and *S. maltophilia* clinical isolates were used in the immunological tests.

Production of MAbs. BALB/c mice were immunized at days 0, 14, and 28 intraperitoneally (i.p.) with 10^9 heat-killed bacteria in 0.5 ml phosphate-buffered saline (PBS), pH 7.4. A week after the last application, the animals were bled from the retroorbital venous plexus and sera were tested in ELISA and passive hemagglutination assay (PHA). The mouse with the highest antibody response was boosted intravenously (i.v.) with 10^9 bacteria. Three days later, spleen cells were harvested and fused with P3X63-Ag8.653 myeloma cells, according to the method of Köhler and Milstein [24]. Supernatants of the cultured hybridomas were tested in ELISA, PHA, passive hemolysis (PH), and immunofluorescent test (IFT). MAbs were isotyped by antigen-mediated ELISA using ISO-2 kit (Sigma). Specific hybridomas producing slide agglutinating MAbs were expanded and frozen. Ascitic fluid from hybridoma clone 219A9 was obtained after i.p. injection of 10×10^6 hybridoma cells in pristane-primed BALB/c mice [25].

Enzyme-Linked Immunosorbent Assay. A sensitive ELISA for antibodies against LPS was performed as described by M. Freudenberg [26]. Briefly, 96-well polystyrene plates (Nunc) were coated with purified LPS diluted in chloroform/ethanol (1:9 vol./vol.) and the solvent was evaporated overnight at room temperature. Non-specific binding sites were blocked with blocking buffer (3% bovine serum albumin in PBS). Serum dilutions and supernatants were incubated for 1 h at 37°C. Goat anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM) peroxidase-conjugated antibody was used as a secondary antibody. Negative controls with PBS in place of antigen and MAbs; and ascitic fluid with an irrelevant mAb were used to exclude nonspecific binding.

Passive Hemagglutination (PHA), Passive Hemolysis (PH), and Passive Hemolysis Inhibition (PHI) Tests. The assays were carried out in the microtest version of the tests, described by Mitov et al. [27, 29]. Sheep red blood cells (SRBC) were coated with purified LPS and the reactions were performed in veronal buffer (VB), pH 7.4. Hemolysis titers in PH test (the last dilution at which approximately 50% hemolysis occurred) were determined after incubation for 1 h at 37°C. PHA assay was performed in the same way as PH but without adding complement. The last dilution with hemagglutination was considered as a titer. For PHI, supernatants were preincubated with 10 μg in ml^{-1} of purified LOS from the three serotypes and the reaction was performed as described for the PH test. Negative controls were included in the tests: for PHA, with SRBC, with irrelevant MAb, with PBS in place of MAb218 or 219; for PH, the same controls were used and PBS in place of complement; for PHI, non-coated SRBC were used, with irrelevant MAb, with PBS instead of MAbs specific for *M. catarrhalis*, with PBS instead of C′ (in PH test), and LPS from R-mutants of *S. minnesota* (in PHI test).

Immunofluorescent Test (IFT). The routine indirect immunofluorescent technique was used. The slides were coated with bacteria, air-dried, and fixed in methanol. Non-diluted supernatants were added and incubated at 37°C for 1 h in humid atmosphere. The slides were washed three times in PBS and incubated with FITC-labeled goat anti-mouse antibody for 30 min. Then, the samples were washed again and examined with a fluorescent microscope (CH 30 Olympus).

Test for Bactericidal Activity. Bacteria were grown overnight on tryptic-soy agar (BBL Becton Dickinson), washed three times, and resuspended to 10^4 ml^{-1} in sterile PBS. Ascitic fluid from MAb 219A9 was diluted (10-fold dilutions) in PBS, mixed 1:1 (vol./vol.) with bacteria, and placed in U-bottom 96-well plates (Nunc). Fresh guinea pig complement was added to a 20% final concentration and the plates were incubated for 1 h at 37°C. Heat inactivated (56°C for 30 min) guinea pig serum mixed with MAb and complement without MAb were used as controls. After the incubation, samples were diluted (10-fold dilutions in PBS) and plated on tryptic-soy agar. The colony-forming units per ml (CFU ml^{-1}) were counted after overnight incubation at 37°C.

Results

Production of MAbs Specific for the LOS Antigen of *M. catarrhalis*. Three i.p. applications of heat-killed bacteria resulted in a specific response in all immunized mice detected by ELISA. The mouse with the highest titer in the PHA test was selected and boosted. Fusion gave rise to five hybridomas, strongly reactive in ELISA with *M. catarrhalis* serotype A LOS. Four of the MAbs reacted with the homologous strain in IFT. Only two of them were positive in PHA and PH. Three hybridomas, 217H4, 218A8, and 219A9, were recloned and their antigen-binding properties were studied in detail. Two of the MAbs, clone 217H4 and 218A8, were of IgG3 and clone 219A9 was from IgM isotype.

Table 1. Reaction of Mabs 218A8 and 219A9 in ELISA and immunofluorescent test with the three *M. catarrhalis* serotypes

	ELISA with 0.5 μg/well LOS of serotype[b]			IFT with serotype[c]		
MAb[a]	A[d]	B[e]	C[f]	A[d]	B[e]	C[f]
218A8	0.665 ± 0.085	—	0.228 ± 0.037	++	−	++
219A9	1.200 ± 0.114	0.909 ± 0.101	0.783 ± 0.066	+++	++	+++

[a] MAbs from culture supernatants.
[b] All of the tests of ELISA were considerate negative controls with saline buffer pH 7.4 instead of antigen.
[c] The three-grade systems were used for detection of results of immunofluorescent test: +++, when all of the bacterial cells were located in the pile with strong intensity of bright equal to positive control intensity; ++, when the 50–70% of bacterial cells were located in the pile and a few of them were along with intensity of fluorescence bright lower than positive control intensity; +, when the small parts of bacterial cells were in the pile, very often bacterial cells were along with very lower bright than positive control intensity; negative, absence of fluorescence.
[d] Serotype A *M. catarrhalis:* strain 353 CCUG, clinical strains: 50, 108, 1027, 10838.
[e] Serotype B *M. catarrhalis:* strain 26400CCUG.
[f] Serotype C *M. catarrhalis:* strain 26391CCUG, clinical strain 1055.

Table 2. Reaction of MAbs 218A8 and 219A9 in tests with LOS coated erythrocytes

	PHA[b]			PH[b]			PHI[c]		
MAb[a]	A	B	C	A	B	C	A	B	C
218A8	16[d]	—	—	16	—	—	NT	NT	NT
219A9	32	—	2	512	—	128	—	64	—

NT, not tested; (−)1/T < 2.
[a] MAbs from culture supernatants.
[b] SRBC coated with LOS from serotypes A, B, and C.
[c] Inhibition of PH with A, B, and C LOS of SRBC coated with serotype A LOS and MAb 219A9.
[d] Reciprocal (1/T) endpoint titer.

Characterization of the MAbs Antigen Specificity. ELISA results showed strong specificity of the MAbs to the homologous *M. catarrhalis* LOS antigen. The alkaline extraction of LOS had no effect on the MAbs reaction. Clone 217H4 reacted only with *M. catarrhalis* serotype A, while clone 218A8 reacted with two serotypes A and C. MAb 219A9 recognized a common epitope on the LOS of the three *M. catarrhalis* serotypes (Table 1). The tests were performed with 0.5 μg LOS purified by the phenol-water method. IFT with the three serotypes confirmed the ELISA results. All of the ELISA tests were considered negative controls with saline buffer (pH 7.4) in place of antigen and MAbs. Although clone 218A8 bound with a lower affinity to C LOS in ELISA, the intensity in IFT was equal, ++ in the three-grade system for A and C strains. MAb 219A9 demonstrated approximately identical affinity to the different serotypes in ELISA and IFT, with five clinical strains of *M. catarrhalis* that were tested (Table 1). ELISA experiments showed that supernatants from clone 219A9 reacted in dilutions up to 1:500 with 0.5 μg LOS of the three serotypes. Under the same conditions (0.5 μg LPS per well), MAb 219A9 cross-reacted also with *H. influenzae* and *H. parainfluenzae* LPS in dilutions 1:50 and 1:250, respectively. In a parallel series, wells coated with *H. influenzae* LPS were treated with periodic acid (PJA) to determine the carbohydrate nature of the MAb specific epitope (Fig. 1). These results were confirmed by IFT with eight different *H. influenzae* and *H. parainfluenzae* clinical isolates. Reactions with LPS isolated from other Gram-negative bacteria including R-mutants of *S. minnesota* and purified lipid A were not observed. For complete characterization of their biological properties and functional characteristics, the antibodies were tested in PHA and PH. In both assays, MAb 217H4 did not react. Only the cross-reactive clones were able to agglutinate LOS-coated erythrocytes and expressed complement-fixing activity in PH (Table 2). MAb 218A8 reacted with serotype A LOS but not with serotype C LOS. The antibody titer of supernatants was equal in both reactions. In contrast to the results in ELISA and IFT, MAb 219A9 did not react with serotype B in PHA and PH. The titers with both serotypes A and C in PH were higher than in PHA (Table 2). The PHI test was performed to demonstrate the specificity.

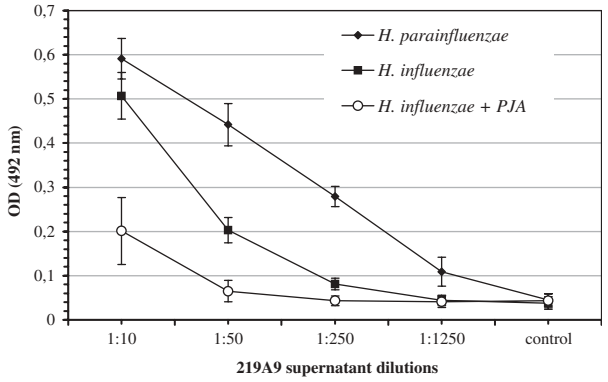

Fig. 1. Cross-reaction of MAb 219A9 with LPS from *H. influenzae* and *H. parainfluenzae*. ELISA plates were coated with 0.5 μg/well LPS. In parallel series, wells coated with *H. influenzae* LPS were treated with periodic acid (PJA) to determine the carbohydrate nature of the MAb specific epitope. Each sample was run in six wells. The results are in OD_{492nm} from one of the repeated experiments.

Preincubation of MAb 219A9 with A and C LOS completely inhibited the reaction, while serotype B LOS had no effect.

Test for Bactericidal Activity. MAb 219A9 from ascitic fluid significantly reduced the number of viable bacteria in the presence of active complement. Incubation with ascitic fluid diluted 1:10 led to a 5-fold decrease of *M. catarrhalis* serotype A CFU ml^{-1} (Fig. 2A). The effect of more than 50% bacterial killing of the homologous serotype was observed in dilutions up to 1:80, $P < 0.02$ (Student t-test). In 10-fold dilution, MAb 219A9 showed comparable bactericidal activity against the three serotypes (Fig. 2B). The CFU ml^{-1} of *M. catarrhalis* serotype B and C were also reduced by approximately 80% in comparison with the controls without antibody or MAb in the presence of heat inactivated complement, $P < 0.001$ (Student t-test). The MAb 219A9 showed comparable bactericidal activity against five clinical strains *M. catarrhalis* in the same reaction.

Discussion

The LPS is the major surface antigen and virulence factor of Gram-negative bacteria including moraxellae [9, 29]. LPS-specific IgM and IgG antibodies are effective agglutinins that can facilitate the clearance of pathogens and could be protective against the endotoxin shock in generalized Gram-negative infection [30, 31]. The protective efficacy of MAb cross-reactive with A and C, but not with B strains, has been studied [15]. It was established also that *M. catarrhalis* LOS-specific antibodies could react with *N. meningitidis* type IV pili [32]. The

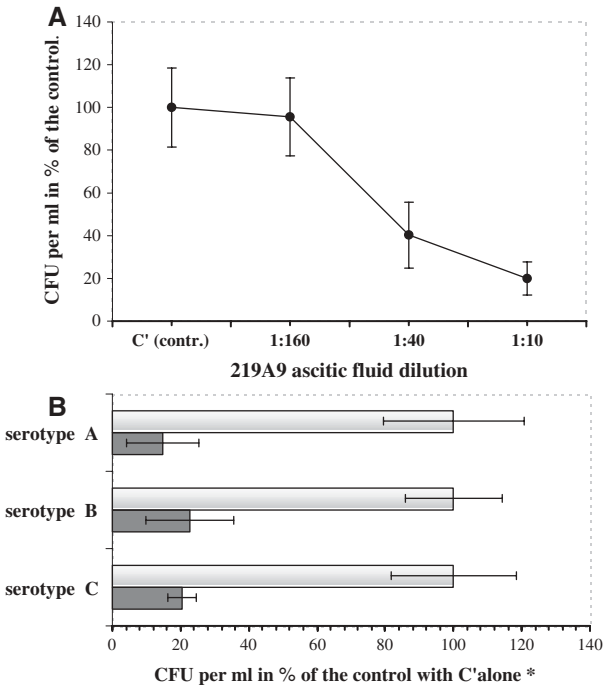

Fig. 2. (**A**) Bactericidal activity of MAb 219A9 against the three *M. catarrhalis* serotypes A, B, and C. The test was performed in the presence of fresh guinea pig complement. Heat-inactivated serum was used in the control wells. The results are presented in CFU ml^{-1} as a % of the control without MAb. Serial dilutions of ascitic fluid were incubated in five wells with live homologous (serotype A) *M. catarrhalis.* (**B**) Bactericidal activity of the ascitic fluid diluted 1:10 was compared to the referent strains and for four clinical isolates of serotypes A and one of serotype C. *C′, complement.

major OMPs, UspA1, UspA2, Hag, TbpB, and OMP CD of *M. catarrhalis* were identified as targets of antibodies to surface epitopes in the majority of adults with COPD [33]. However, the immunogenicity of the different LOS epitopes and their importance for the host immune defense are not well understood and require further investigations.

In recent studies, immunization with heat-killed bacteria gave rise to serotype-specific and cross-reactive hybridoma clones producing anti-LOS MAbs. The antibodies did not react with LPS antigens extracted from a variety of Gram-negative bacteria including R-mutants of *Enterobacteriaceae*. In addition, a large number of clinical isolates were tested in IFT with the panel of three MAbs: 217H4, 218A8, and 219A9 (not presented). Clone 219A9 was broadly cross-reactive and bound the LOS of the three serotypes in ELISA and reacted with all *M. catarrhalis* strains in IFT. The common for all three LOS serotype structures is 6-linked branch alpha-D-Galpara-(1 → 4)-beta-D-Galpara-(1 → 4)-alpha- D-Glc-para-(1 → 2)-beta-D-lcpara of the oligosaccharide part of LOS [2, 13]. Probably, the epitope recognized by

MAb 219A9 is localized on this branch of the molecule. The antibody binds with equal affinity to A, B, and C antigens, which also supports this hypothesis. The cross-reactions with *H. influenzae* and *H. parainfluenzae* LPS in ELISA and with whole bacteria in IFT demonstrate that the MAb-specific LOS epitope is common for *M. catarrhalis* and different species of *Haemophilus*. The presence of conserved oligosaccharide structures among *Haemophilus* and *Moraxella* located in the common branch of the *M. catarrhalis* LOS molecule has been reported [9]. In contrast, clone 218A8 reacts only with a common for A and C LOS epitope, which is not present in serotype B. There are no O-acetylated sugars in *M. catarrhalis* LOS [2]. Thus, the alkaline treatment did not affect the antigen-binding properties of both MAbs. The results show that MAbs 218A8 and 219A9, specific for common epitopes, could be useful for the detection of *M. catarrhalis* and its LOS antigen in clinical samples by IFT and ELISA. IFT could be used in the serotyping of clinical isolates. In contrast to the ELISA results, in PHA, PH, and PHI, MAb 219A9 reacted with A and C, but not with serotype B LOS-coated erythrocytes. The experiment was repeated several times with the same results. However, the MAb showed strong complement-fixing activity against the strains of all three serotypes in the bactericidal tests with live bacteria, which is typical for IgM antibodies. These results illustrate the protective properties of the common LOS epitope for the three serotypes. The common branch in the LOS molecule has been established as an important factor involved in complement resistance of *M. catarrhalis* [21]. The mutant strains that do not express the P^k epitope alpha-D-Galpara-(1 → 4)-beta-D-Galpara-(1 → 4)-alpha-D-Glcpara are more sensitive to the bactericidal effect of normal human serum. Thus, our results support the data about the protective capacity of antibodies directed to the common epitopes and confirm the important role of humoral immune response to *M. catarrhalis* LOS antigens [31]. This also confirms that LOS-based vaccines using single synthetic oligosaccharide epitopes conjugated to protein carriers are a promising strategy in the immunoprophylaxis of *Moraxella* infections [2, 10, 34]. Immunization with LPS-based conjugate from non-typeable *H. influenzae* enhances bacterial clearance from the nasopharynx in the mouse experimental model [35]. However, there is no evidence concerning the protective efficacy of cross-reactive immunity to LOS determinants, conserved among moraxellae and other bacteria. Our report is the first demonstrating a MAb specific for a common *M. catarrhalis* LOS epitope that is cross-reactive with *Haemophilus spp.* LPS antigen. This clearly shows that LOS-based vaccines against serotypes A, B, and C *M. catarrhalis* may also induce a broad spectrum immune response to other respiratory pathogens, which is very attractive with respect to the development of efficient immunoprophylaxis for bacterial respiratory tract infections.

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