Identification of Common Lipooligosaccharide Types in Isolates from Patients with Otitis Media by Monoclonal Antibodies against Nontypeable *Haemophilus influenzae* 9274

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Twenty-one murine monoclonal antibodies (MAbs) were induced by nontypeable *Haemophilus influenzae* (NTHi) 9274. Nineteen MAbs were specific for the lipooligosaccharide (LOS) as determined by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. When the MAbs were assayed with five LOS prototype strains by ELISA, all bound to strain 3198 LOS (type III), while six of the MAbs were also reactive with LOSs from strain 1479 (type I), 5657 (type IV), or 7502 (type V). Ten MAbs had complement-mediated bactericidal activity, and three MAbs were opsonophagocytic against the homologous strain. Five LOS MAbs with different specificities were used to analyze 155 NTHi clinical isolates from the United States and from Japan. These isolates were classified into nine groups by ELISA. Only four isolates (2.6%) were not recognized by any of the five MAbs. Most of the isolates (91.6%) were in four groups which bound three of the five MAbs.

One of three MAbs, 6347C11, had strong activity against the homologous strain and was also bactericidal to 45 clinical isolates (29%) which belonged to the four common patterns (25 belonged to pattern I). These data indicate that these MAbs can be used for LOS typing in which almost all NTHi strains can be typed according to the LOS antigenicity. Among NTHi, at least one conserved LOS epitope which is a target of bactericidal antibodies exists. We conclude that strain 9274 LOS, which is the target for bactericidal antibodies, is a candidate for LOS-based NTHi vaccines.

Nontypeable *Haemophilus influenzae* (NTHi) is an important cause of otitis media (OM) in children and respiratory tract diseases in adults (12, 16, 17). NTHi accounts for 25 to 30% of acute OM and for a larger percentage of cases of chronic OM with effusion (4, 23). This number may underestimate the level because a recent study indicated that live NTHi could be found in a large percentage of culture-negative fluid from OM (20). Since NTHi lacks a capsular polysaccharide for NTHi infection because human antibodies showed bactericidal activity in vitro (1), and a mouse monoclonal antibody (MAb) enhanced opsonization and bacterial clearance in a murine pulmonary challenge model (15). We showed that NTHi LOS-protein conjugates elicited bactericidal antibodies in animals and conferred protection against otitis media in chinchillas (5, 9). The LOS epitopes which elicit these biological active antibodies in the host have not been identified.

NTHi LOS contains an oligosaccharide linked to lipid A without an O-specific polysaccharide (10, 19). One primary oligosaccharide structure of LOS from NTHi strain 2019 has been characterized, and it contains Galβ1-4Glcβ1-(Hepα1-2Hepα1-3)Hepα1-Sanhydro-KDO (19). NTHi LOS is antigenically heterogeneous as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunologic methods. Campagnari et al. (2) reported that about 50% of NTHi strains can be typed into 10 groups on the basis of the antigenic heterogeneity of LOS by rabbit antisera.

There are some anti-LOS MAbs which classified 81% of NTHi strains into eight patterns (18). MAbs are useful in recognizing NTHi strains expressing new LOS types and in identifying conserved and protective epitopes among clinical isolates.

In view of the importance and possibility of NTHi LOS as a vaccine component, we generated MAbs against NTHi 9274 LOS in order to type clinical isolates common to some or most

| Table 1. Characterization of NTHi MAbs |
|--------------------------------------|
| **MAb** | **Specificity** | **Isotype** | **ELISA titer** | **Bactericidal activity** |
|-----------------|----------------|-------------|----------------|--------------------------|
| 6245B4          | LOS            | IgM         | 10,000         |                          |
| 6248F4          | LOS            | IgM         | 24,000         |                          |
| 6249E9          | LOS            | IgM         | 12,000         | 5                       |
| 6253F3          | LOS            | IgG3        | 2,700          |                          |
| 6257C4          | LOS            | IgM         | 11,000         |                          |
| 6259A9          | LOS            | IgM         | 24,000         |                          |
| 6260C2          | LOS            | IgM         | 2,600          |                          |
| 6263F4          | LOS            | IgM         | 8,100          |                          |
| 6267B9          | LOS            | IgM         | 5,000          | 5                       |
| 6340G4          | LOS            | IgM         | 2,700          |                          |
| 6341F5          | OMP            | IgM         | 8,100          |                          |
| 6344C1          | LOS            | IgM         | 1,000          | 300                      |
| 6345G6          | LOS            | IgM         | 2,700          | >900                     |
| 6347C11         | LOS            | IgM         | 8,100          | >900                     |
| 6349E8          | OMP            | IgM         | 8,100          | 5                       |
| 6352H9          | LOS            | IgM         | 8,100          |                          |
| 6353G5          | LOS            | OMP         | 2,700          |                          |
| 6356E5          | LOS            | IgM         | 2,700          | 100                      |
| 6358G13         | LOS            | IgM         | 2,700          | 5                       |
| 6360G12         | LOS            | IgM         | 2,700          |                          |
| 6361A6          | LOS            | IgM         | 2,700          | 300                      |

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* ELISA titers and bactericidal titers are expressed as the reciprocals of the MAb dilution.

* OMP, outer membrane protein.
MATERIALS AND METHODS

Bacterial strains. Ten strains of NTHi, including five prototype strains (1479, 2019, 3198, 5657, and 7502) (2) and strain 9274 (1a), were obtained from M. A. Apicella, University of Iowa. One hundred strains from different areas of the United States were obtained from H. Faden, State University of New York at Buffalo, and another 55 strains were obtained from G. Mogi, Oita Medical University, Japan. All strains were clinical isolates from middle ear fluids or nasal secretions of patients with OM except the prototype strains, which were from patients with chronic bronchitis. Each strain was identified as NTHi by its sender, and its requirement for both growth factors, nicotinamide adenine dinucleotide (NAD) and hemin (Sigma Chemical Co., St. Louis, Mo.). Strain 9274 was typed and its LOS antigens. Strain 9274 was selected for this study because its LOS does not have a terminal lacto-N-neotetraose found in a variety of human cells (14). In addition, this LOS was able to generate bactericidal antibodies against homologous and heterologous strains (9).

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**TABLE 2. Specificity of MAbs directed to prototype LOSs by ELISA**

| NTHi strain (prototype) | MAb(s) |
|------------------------|--------|
| 1479 (I)               | 6248F4, 6249E9, 6257C4, 6259A9, 6260C2, 6267B9, 6340G4, 6344C1, 6353G5, 6356E5, 6358G3, 6360G12, 6361A6 |
| 2019 (II)              | 6254B4, 6263F4, 6347C11, 6352H9 |
| 3198 (III)             | 6253F3, 6345G6 |
| 5657 (IV)              |        |
| 7502 (V)               |        |

* MAb selected for LOS typing.

MONOCLONAL ANTIBODIES TO NTHI LIPOOLIGOSACCHARIDES 97

**Western blot analysis.** Bacteria (10 μg), outer membrane proteins (2 μg), or LOS (0.2 μg) was subjected to SDS-PAGE in a 15% polyacrylamide gel and then transferred onto nitrocellulose membranes at 250 mA for 4 to 6 h (8). After blocking with 3% bovine serum albumin in PBS for 1 h, the membranes were incubated with MAb (about 1:1000) for 3 h followed by goat anti-mouse IgG or IgM labeled with alkaline phosphatase (1:1,000) (Sigma) for 2 h. The membranes were developed using 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium tablets (Sigma). A duplicate gel was silver stained for LOS after SDS-PAGE (22). Western blot analysis of whole cell lysates and LOSs were performed by the method of Kohler and Milstein (13) with modification (8). A whole-cell ELISA was performed as follows. NTHi strains were grown on liquid brain-heart infusion broth with 3% CO2 overnight. The bacteria were fixed with 0.37% formaldehyde and adjusted to an optical density of 0.09 at 4°C overnight. The following day, 100 μl of the incubated solutions in triplicate was transferred to a microtiter plate and the subsequent reactions were performed as described for the ELISA. The percentage of inhibition was calculated as follows: (1 – inhibitor’s mean A405/control’s mean A405) × 100. All these assays were repeated, and the variation was ±15%.

**ELISA inhibition test.** LOSs were used to inhibit the reactions between MAb 6347C11 and the coating LOS antigen of strain 9274 (8). Briefly, LOSs of strains 9274, 3198, 5657, 7502, and 9274 underwent a serial twofold dilution with PBS, and then 200 μl of each dilution or PBS was incubated with 200 μl of the appropriately diluted MAb (A405, about 1.0) at 4°C overnight. The following day, 100 μl of the incubated solutions in triplicate was transferred to a microtiter plate coated with 9274 LOS, and the subsequent reactions were performed as described for the ELISA. The percentage of inhibition was calculated as follows: (1 – inhibitor’s mean A405/control’s mean A405) × 100. All these assays were repeated, and the variation was ±15%.

**Opsonophagocytic assay.** Human peripheral polymorphonuclear leukocytes (PMNs) were separated from 50 ml of heparinized whole blood from normal adults. Briefly, blood cells containing leukocytes were sedimented by using Histopaque (Sigma), and the upper layer of erythrocytes rich in PMNs was transferred into four 50-ml tubes. After washing with Hanks’ balanced salt solution...
Components of the assay included 0.1 ml of a log-phase bacterial suspension (1 × 10^6 CFU/ml in HBSS), 0.1 ml of complement-inactivated (preincubated at 56°C for 30 min) MAb (1:5), 0.1 ml of 20% human AB serum as a complement source (Sigma), and 0.1 ml of PMNs (3 × 10^6/ml in HBSS with 0.05% gelatin). Samples were incubated at 37°C for 30 min, diluted, and plated on chocolate agar within 1 min, PMNs were stabilized by adding 8 ml of 3.5% sodium chloride and resuspended in a minimal volume of HBSS, and counted with a hemocytometer.

Within 1 min, PMNs were stabilized by adding 8 ml of 3.5% sodium chloride and resuspended in a minimal volume of HBSS, and counted with a hemocytometer. Erythrocytes in each tube were lysed by adding 24 ml of sterile water. The cells were washed twice, resuspended in a minimal volume of HBSS, and counted with a hemocytometer.

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**RESULTS**

**Screening and isotyping MAbs.** Twenty-one hybridoma cell lines were selected on the basis of their reactivity and specificity by ELISA and Western blotting (Table 1). Two of these were IgG3 (6253F3 and 6358G3) and the others were IgM. One MAb, 6352H9, had lambda light chains, while the others had kappa light chains. Nineteen MAbs recognized both the purified LOS and whole cells of strain 9274, indicating that they are LOS specific while the other two MAbs, 6341F5 and 6347C11, reacted strongly with LOSs of strains 3198 and 9274, and weakly with LOS of strain 1479. **Bactericidal and opsonophagocytic activities of MAbs.** All MAbs were tested for complement-mediated bactericidal activity in vitro. Nine MAbs showed bactericidal activity against the homologous strain, and five of them showed high bactericidal activity; however, no correlation between their bactericidal activity and ELISA titers was seen (Table 1). MAbs were also assayed for opsonophagocytic activity using the homologous strain 9274. Only three showed opsonophagocytic activity (Table 3).

**Typing of clinical isolates with selected MAbs.** Five LOS typing MAbs (6253F3, 6263F4, 6344C1, 6345G6, and 6347C11) were selected for the typing of 155 NTHi clinical isolates on the basis of different LOS antigenic determinants, isotypes, and biological activities (Tables 2 and 3). Three MAbs (IgM) showed bactericidal activity against the homologous strain 9274. One IgG and two IgM MAbs showed opsonophagocytic activity against strain 9274. The bactericidal or opsonophagocytic activity of the MAbs showed no correlation with their ELISA titers.

(i) **Whole-cell ELISA.** A total of 155 NTHi clinical isolates from the United States and Japan were typed by whole-cell ELISA using five selected LOS MAbs, and 10 typing patterns were obtained (Table 4). Overall, 97.4% (151) of the isolates reacted with at least one MAb, accounting for eight groups. Thirty-seven percent of the isolates belonged to pattern 1. Patterns 1 through 4 comprised 91.6% (142) of the total isolates. It is interesting that all isolates belonging to these four patterns reacted with three of five MAbs (6263F4, 6344C1, and 6347C11). Isolates from both the United States and Japan showed a similar pattern of reactivity.

The reaction patterns of five prototype strains were also determined by ELISA with purified LOSs or whole cells as a coating antigen (Table 5). Type III and V prototype strains showed agreement by both ELISAs while the other three prototype strains were different. In the LOS ELISA, the five MAbs recognized four prototype LOSs but not type II (2019 LOS). While type III LOS was associated with pattern 1, all of the other four LOSs were grouped in patterns 5 to 10, which accounted for only 9% of the 155 clinical isolates. In a whole-cell ELISA, the MAbs recognized all prototype strains, and both 1479 (type I) and 5657 (type IV) belonged to pattern 2.

(ii) **Western blot analysis.** MAb 6347C11 was selected for Western blot analysis because of its high ELISA titer, high biological activities (Tables 2 and 3). Three MAbs (IgM) reacted strongly with LOSs of strains 3198 and 9274, and weakly with LOS of strain 1479.

**TABLE 3. Summary of five selected LOS MAbs for LOS typing**

| MAb (class) | ELISA titer | Bactericidal titer | Opsonophagocytic activity (%) |
|-------------|-------------|--------------------|-------------------------------|
| 6253F3 (IgG3) | 2,700 | 24 |
| 6263F4 (IgM) | 8,100 | 0 |
| 6344C1 (IgM) | 1,000 | 300 | 68 |
| 6345G6 (IgM) | 2,700 | >900 | 0 |
| 6347C11 (IgM) | 8,100 | >900 | 70 |

**TABLE 4. LOS typing of NTHi clinical isolates with five LOS-specific MAbs by whole-cell ELISA**

| Typing pattern | No. of isolates (no. from United States/Japan) | ELISA reaction with MAbs | % of isolates (no. from United States/Japan) |
|----------------|-----------------------------------------------|--------------------------|---------------------------------------------|
| 1              | 58 (35/23)                                    | +                        | 37 (35/42)                                  |
| 2              | 28 (21/7)                                     | +                        | 19 (18/20)                                  |
| 3              | 29 (18/11)                                    | +                        | 17 (18/16)                                  |
| 4              | 27 (18/9)                                     | +                        | 37 (21/3)                                   |
| 5              | 6 (3/3)                                       | +                        | 19 (18/20)                                  |
| 6              | 1 (1/0)                                       | +                        | 17 (18/16)                                  |
| 7              | 1 (1/0)                                       | +                        | 4 (3/5)                                     |
| 8              | 1 (0/1)                                       | +                        | 6.0 (1/0)                                   |
| 9              | 4 (3/1)                                       | +                        | 3 (2/3)                                     |
| 10*            | 0 (0/0)                                       | +                        | 0                                           |

Total (no. from United States/Japan): 155 (100/55) 88 (53/35) 145 (94/51) 143 (93/50) 93 (57/36) 143 (92/51) 100

% of total: 100 57 94 92 60 92

* Prototype strain 1479 LOS (I) belongs to the pattern 10 (as determined by LOS ELISA).
bactericidal and phagocytic activities, and high positive rate of ELISA reaction with 143 clinical isolates. A total of 139 isolates (90%) showed a positive reaction and 16 isolates showed a negative reaction in Western blot analysis. These results were consistent with those of the ELISA except that four isolates were positive in whole-cell ELISA and negative in Western blot studies.

(iii) Bactericidal assay. MAb 6347C11 was bactericidal for 30 of 100 isolates from the United States (30%) and 15 of 55 isolates from Japan (27%) (Fig. 2). All of these isolates belonged to one of the four major patterns and 25 of them (55%) belonged to pattern 1. The percentage of the bactericidal activity of MAb 6347C11 was 51, 29, 28, and 6% in the U.S. strains and 30, 29, 45, and 11% in Japanese strains for patterns 1, 2, 3, and 4, respectively.

ELISA inhibition by NTHi LOSs. ELISA inhibition testing to further characterize the specificity of MAb 6347C11 was performed. The activity of the MAb was strongly inhibited by the LOSs from the homologous strain and strain 3198 (type III). However, it was not inhibited significantly by LOSs from strains 2019 (type II) and 7501 (type V) at a concentration up to 1.0 mg/ml (Fig. 3).

Inhibition by NTHi LOSs of bactericidal activity. The bactericidal activity of MAb 6347C11 was inhibited 100% by the LOS from strains 9274 and 3198 at a concentration of 10 μg/ml but not by LOS from strain 2019 or 7501.

DISCUSSION

Previous studies showed that NTHi LOSs are antigenically heterogeneous. Campagnari et al. (2) established an LOS-based serogrouping system for NTHi by using rabbit sera generated by different NTHi strains. Only 50% of their 72 strains could be typed into 10 groups (2). Five prototype strains representing 78% of the 36 typed strains were used in our study as references. Patrick et al. (18) produced four MAbs directed against LOSs by immunizing mice with six NTHi strains and assayed LOS. A total of 69 isolates were typed into nine patterns and 19% of the isolates could not be typed because they did not react to any of these MAbs. These studies indicated that a more comprehensive LOS typing system is required because of antigenic diversity among NTHi strains.

We generated 21 MAbs from hybridomas after a single fusion with the spleens of two mice immunized with LOS and whole bacteria from NTHi strain 9274. The resulting MAbs showed different specificities by LOS ELISA with five prototype strains (2). All MAbs bound to strain 3198 LOS (type III) but not strain 2019 (type II), while some MAbs also bound to other LOSs. We examined 155 clinical isolates from the United States and Japan by using five selected LOS-specific MAbs. The results showed that these strains could be typed into nine groups by whole-cell ELISA. Only four isolates (2.6%) did not react with any of the five MAbs, but reacted with MAb 6341F5, which is directed against whole-cell or outer membrane proteins but not to LOS. These results indicate that almost all NTHi isolates could be typed according to LOS by these MAbs.

The majority (91.6%) of the clinical isolates were identified as patterns 1 through 4, with the highest percentage in pattern 1 from both geographic sources. In addition, the isolates matching these four patterns all reacted with three (6263F4, 6344C1, and 6347C11) of the five LOS typing MAbs. These data suggest that patterns 1 through 4 are common LOS types for the clinical isolates, and some of the LOS epitopes identified by these MAbs are common to the majority of the clinical strains.

Since about 50% of the MAbs showed complement-mediat-
ated bactericidal activity against the homologous strain, and some of them showed opsonophagocytosis against the same strain. Further studies were performed to identify common functional epitopes among the clinical isolates. MAB 6347C11 with both functional activities was selected for testing all the clinical isolates by bactericidal assay in which about 30% of the strains from either source could be killed by the MAB. In addition, all the bactericidal isolates belonged to one of the four major groups and 55% of them belonged to pattern 1. These results indicate that the target of the bactericidal MAB is relatively conserved among the clinical isolates.

In summary, the LOS MAbs are useful for typing clinical isolates, and may be potentially useful for treatment of patients with NTHi infections. Strain 9274 LOS, which contains common epitopes with functional targets, is a candidate for preparing LOS-based conjugate vaccines.

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