Western Blot Analysis of Neisserial Lipooligosaccharide at the Lower Femtomole Level with Normal Human Sera

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Lipooligosaccharide (LOS) is a major immunogenic component of pathogenic Neisseria species such as Neisseria meningitidis and N. gonorrhoeae. Recent immunochromatography studies have found that normal human sera (NHS) contain bactericidal anti-LOS antibodies that bind to the oligosaccharide (OS) moiety of neisserial LOS. Although affinity-purified anti-LOS antibodies can be characterized using 10–100 ng of LOS samples (up to a few tens of pmoles), a more sensitive immunoblotting assay must be established in order to analyze NHS directly and characterize anti-LOS antibodies without affinity purification. We examined analytical PAGE/blot conditions using a 15-well mini gel. For the first time, Western blot detection of LOS at the lower femtomole level was accomplished by both chromogenic and chemiluminescent detection. A model LOS, 15253 LOS, was detected in a low femtomole range (62.5–500 pg, 16–125 femtomole) even with 10 pM LOS, 15253 LOS, was detected in a low femtomole level was accomplished by both chromogenic and chemiluminescent detection. A model LOS, 15253 LOS, was detected in a low femtomole range (62.5–500 pg, 16–125 femtomole) even with 10 pM LOS.

Key words: lipooligosaccharide; lipopolysaccharide; Western blotting; PAGE; Neisseria

Lipooligosaccharide (LOS) is a major immunogenic component of pathogenic Neisseria species such as Neisseria meningitidis and N. gonorrhoeae. LOS is composed of an oligosaccharide (OS) and lipid A,1 and the OS moiety consists of a conserved core and a structurally variable region. The variable region differs both in size and in structure and can be linked to more than one site of a core OS. The core OS of LOS from Neisseria bacteria is a pentasaccharide. N-acetyl-D-glucosamine (GlcNAc), two L-glycerol-D-manno-heptose (Hep), and two 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) residues are sequentially linked to form GlcNAc1–2Hep1–3Hep1–5KDOα2–4KDO. The variable OS is linked to Hep1 or to both Hep1 and Hep1 of the core OS, resulting in the production of two major branched OS structures. Thus, 3,4-branched and 2,3,4-dibranched LOSs are produced by the bacteria.1–3

In a previous study,3 we purified a bactericidal IgG from normal human sera (NHS) using an affinity column of 2,3,4-dibranched 15253 LOS produced by N. gonorrhoeae strain 15253. This anti-15253 LOS IgG contained at least three species of IgG2, and each of the three IgG2s was found to recognize a different OS structure expressed in the 2,3,3,4-dibranched 15253 LOS. Other investigators5,6 have also purified anti-LOS human IgGs using N. meningitidis LOSs. Their immunochromatography data also indicate that affinity-purified IgGs are bactericidal and bind to the OS moieties of the 3,4-branched LOSs. These recent data by us3,7 and other investigators5,6 show that NHS contains anti-LOS Abs that are specific for the OS of LOS. They also suggest that NHS may contain numerous unidentified anti-LOS antibodies that recognize the OS moiety of LOS. Humans are the only natural host of pathogenic Neisseria bacteria, and therefore the molecular basis of the recognition specificity of human antibodies to LOS produced by these bacteria is important in order to understand the immune responses of the host.

Anti-LOS antibodies in NHS are minute in amount, and their affinities tend to be much lower than those of murine monoclonal antibodies (MAbs). One of the major potential problems in analyzing the binding characteristics of these antibodies in NHS and other body fluids is the detection level of LOS by Western blot. Although affinity-purified anti-LOS antibodies can be analyzed using 10–100 ng of LOS samples (up to a few tens of pmoles),3,7 a more sensitive Western blotting assay must be established in order to analyze NHS directly and characterize anti-LOS antibodies without affinity purification. For this purpose, we examined PAGE/Western blot analytical conditions to detect LOS at the lower femtomole level by NHS.

Materials and Methods

Lipooligosaccharide (LOS), lipopolysaccharide (LPS), and antibodies. The neisserial LOS samples used in this study are as follows: 15253 LOS,10 JW31R LOS,30 WG LOS,3 F62 LOS,9 PID-2 LOS.10

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Abbreviations: AF, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Hep, L-glycerol-D-manno-heptose; KDO, 2-keto-3-deoxy-D-manno-octulosonic acid; LOS, lipooligosaccharide; LPS, lipopolysaccharide; MAb, monoclonal antibody; NBT, nitroblue tetrazolium chloride; NHS, normal human sera; OS, oligosaccharide; PBS, phosphate buffered saline; POD, peroxidase; PVDF, polyvinylidene fluoride
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302 LOS, 220 LOS, and MS11mkA LOS. The following Salmonella rough mutant LPSs were purchased from Sigma Chemical (St. Louis, MO): Ra (Salmonella typhimurium TV119), Re (S. minnesota R5), Rd (S. minnesota R7), and Re (S. minnesota Re 595). The Rb mutant LPS (S. minnesota R345) was from List Biological Laboratories (Campbell, CA). Stock solutions (μg/mL) of LPS and LPS samples were prepared by dissolving them in 50 mM Tris-HCl buffer (pH 6.80) containing 1 mM EDTA and 2% SDS. LOS and LPS samples were diluted (100 μg–1 ng) with 34 mM Tris-HCl buffer (pH 6.80) containing 39% v/v glycerol and 3.9% saturated bromophenol blue in water before applying them to PAGE gels. Based on reference standard LPS migration and/or previous structural studies of LOS, a value of 4,000 Da was used to quantify gonococcal LOSs and the truncated LPSs. Mouse monoclonal antibody (Mab) 2C7 was provided by Dr. Sunita Gulati (University of Massachusetts Medical School, Worcester). It was purified from ascitic fluid by chromatography using a Hi-Trap Protein G column (1 mL, GE Healthcare, Buckinghamshire, UK). The washing and elution buffers used in chromatography were 0.1 mM Tris–HCl (pH 8.00) and 0.1 M Gly–HCl (pH 2.70), respectively. The protein concentration was measured with a BCA Protein Assay kit (Pierce, Rockford, IL) as described previously. A commercially available NHS (pooled male serum, lot number 113K0485) was purchased from Sigma-Aldrich (Tokyo). Marine anti-lipid A antibody was from Sanbio (Uden, Netherlands), and a 1:1,000-diluted solution in PBS (pH 7.42) was used. Goat anti-mouse IgG, alkaline phosphatase (AP) or peroxidase (POD) conjugates, and goat anti-human IgG (γ-chain specific, AP or POD conjugates) was from Sigma-Aldrich.

**Dot blotting and PAGE.** For dot blotting, a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) was used. Both 10-well and 15-well gels of 0.75 mm thickness (Protean II and Protean III/Tetra Cells (Bio-Rad Laboratories, Hercules, CA) was used. Both 10-well and 15-well gels of 0.75 mm thickness (Protean II and Protean III/Tetra Cells (Bio-Rad)) using Gel Drying Solution and Cellophane Support (both from Bio-Rad). The washing and elution buffers used in chromatography were 0.1 mM Tris–HCl (pH 8.00) and 0.1 M Gly–HCl (pH 2.70), respectively. The protein concentration was measured with a BCA Protein Assay kit (Pierce, Rockford, IL) as described previously. A commercially available NHS (pooled male serum, lot number 113K0485) was purchased from Sigma-Aldrich (Tokyo). Marine anti-lipid A antibody was from Sanbio (Uden, Netherlands), and a 1:1,000-diluted solution in PBS (pH 7.42) was used. Goat anti-mouse IgG, alkaline phosphatase (AP) or peroxidase (POD) conjugates, and goat anti-human IgG (γ-chain specific, AP or POD conjugates) was from Sigma-Aldrich.

**Optimization of analytical conditions for chemiluminescence detection of LOS.** As a model LOS for this analysis, we used 2,3,4-dibranched 15253 LOS and Mab 2C7, which binds to 15253 LOS. First, BSA and casein were examined as blocking reagents by dot blot analysis using a nitrocellulose membrane and a fixed concentration of Mab 2C7 (150 μL), and then treated with the same secondary Ab at two different dilutions (1:25,000 and 1:50,000), as described in A above. Although a commercially available thick pad (Bio-Rad) was used several times for blotting, the LOS samples were not transferred as well as when the stack of filter papers was used. Electroblotting was conducted for 90 min at 15 V.

**Western blot analysis.** A separate gel was electroblotted as follows: Nitrocellulose or polyvinylidene fluoride (PVDF) membranes (both from Bio-Rad, 0.2 μm pore size) and eight sheets of filter paper (No. 2, 90 min at 15 V. For chromatographic detection, the electroblotted nitrocellulose or PVDF membranes were sequentially incubated in containers made from a Parafilm sheet for 1 h at room temperature, unless otherwise stated, with the following: 1% casein (Nacalai Tesque) in PBS, PBS (3 x 10 min), MAB 2C7, or NHS (37 °C) diluted in PBS, PBS (3 x 10 min), and the corresponding AP conjugated secondary antibody (anti-mouse IgG for MAB 2C7 or anti-human IgG for NHS). The following substrates were used: Western Blue Stabilized Substrate (Promega, Madison, WI) and 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP)/nitro blue tetrazolium chloride (NBT) (both from Wako). A working solution of BCIP/NBT was prepared by mixing the following: 1% BCP in H2O (240 μL), 1.5% NBT in 70% DMF (240 μL), 50 mM of levamisole hydrochloride (Wako) in H2O (160 μL), and 10 mL of AP substrate buffer (100 mM Tris–HCl, 100 mM NaCl, 5 mM MgCl2, 0.05% Tween 20 in H2O, pH 9.50), treated with 2 h in a heating block (100 °C). After centrifugation of the mixture (10,000 rpm for 15 min at 4 °C), the precipitates obtained were washed with water (5 mL x 2) and then dried. The lipid A preparation (200 ng and 200 μg) was analyzed by TLC immunostaining using aluminum sheets (5 x 7.5 cm, Merck Kieselgel 60 F254, Germany), as described previously. Commercially available NHS was absorbed with JW31R LOS as follows: each U16 Maxisorp well (Nunc, Roskilde, Denmark) was coated with JW31R LOS (5 or 25 ng/μL, 200 μL), and after blocking with 1% BSA in PBS containing 10 mM MgCl2, 1 h at room temperature, each coated well was incubated with the 1:20 diluted NHS (100 μL) for 1 h at 37 °C. Then, this pre-absorbed NHS (20 wells) was diluted in PBS (2.5-fold) and used in Western blotting.

**Results and Discussion.**

**Optimization of analytical conditions for chemiluminescence detection of LOS.** As a model LOS for this analysis, we used 2,3,4-dibranched 15253 LOS and Mab 2C7, which binds to 15253 LOS. First, BSA and casein were examined as blocking reagents by dot blot analysis using a nitrocellulose membrane and a fixed concentration of Mab 2C7 (150 μL), and then treated sequentially with MAb 2C7 (150 μL), and another gonococcal LOS, F62 LOS, was selected as negative control for the Mab. As shown in Fig. 1A, 10 ng (2.5 pmole) of 15253 LOS was visualized after blocking with 1% casein in PBS.
containing Tween 20 (0.05%) and subsequent treatment with a 1:25,000 dilution of anti-mouse IgG (POD conjugate) and SuperSignal West Dura Substrate. The presence of Tween 20 in the blocking solution was necessary for better detection of the LOS. In contrast to casein, blocking with 1% BSA in the presence of the same detergent gave a much higher background than the 1% casein solution (Fig. 1A).

Using the low femtomole level of 15253 LOS (500–1,000 pg, 125 to 250 femtomole), dilution of the secondary Ab was also re-examined. As shown in Fig. 1B, 500 pg (125 femtomole, lane 2) of the LOS was detected with a 1:25,000 dilution of the anti-mouse IgG, whereas 750 pg of the LOS was barely detected with 1:50,000 dilution. In addition, the nitrocellulose and PVDF membranes were examined for more sensitive detection of LOS, and this comparative analysis using a 10-well gel indicated that the PVDF membrane gave several-fold better detection than the nitrocellulose membrane (data not shown). Hence, the PVDF membrane and 1:25,000 diluted secondary Ab were chosen for further analysis.

Detection of the model LOS at lower femtomole level using a 15-well gel

Enhancement of LOS detection by PAGE/Western blot was examined using a 15-well gel format. The concentrations of separated LOS samples on this format were expected to be higher than those on the 10-well gel due to the narrower lane width of the former gel. By comparatively analyzing 15253 LOS using the two gels, we found that PAGE using the 15-well gel provided several-fold better detection of both LOS and immunostaining. Only the results obtained with the 15-well gel are shown in Fig. 2. The detection limit of silver staining on the 10-well was several ng, whereas even 500 pg (125 femtomole) of LOS was silver stained on the 15-well gel (Fig. 2A). Two hundred and fifty pg (62.5 femtomole) of LOS was immunostained using Western Blue Stabilized Substrate (Fig. 2B), whereas the chemiluminescent substrate provided a slightly improved detection level, visualizing 62.5 pg (16 femtomole) of the LOS (Fig. 2C).

We also examined to determine whether femtomole detection of LOS would be possible when the binding affinity of the MAb was adjusted to a level similar to those of the oligocolonal anti-LOS antibodies present in NHS. For this purpose, we used anti-15253 LOS IgG2.3) We confirmed by ELISA that the binding affinity of 10 pm of the MAb to 15253 LOS was slightly lower than that of anti-15253 LOS IgG2. Similar to the results shown in Fig. 2C, 62.5 pg of LOS was also detected with 10 pm of the MAb (data not shown), although the detection sensitivity decreased slightly. Thus we accomplished lower femt mole detection of the model LOS with murine MAb 2C7.

Detection of binding of IgG in NHS to neisserial LOSs and Salmonella truncated LPSs at the lower femtomole level

Finally, we analyzed anti-LOS IgG in a commercially available NHS, and examined whether low-femt mole amounts of LOS and LPS samples can also be detected with IgG in NHS (NHS-IgG) without affinity purification. The LOSs and LPSs used in a previous characterization of the anti-15253 LOS IgG23) were also analyzed in this study, and the binding patterns of NHS-IgG to the antigens were compared with those of anti-15253 LOS IgG2.

As shown in Fig. 3A, 10 ng of each antigen preparation was silver stained, although the highest molecular...
The above results indicate that the NHS used in this study also contained IgG that recognized 2,3,3,4-dibranched and 3,4-dibranched neisserial LOSs. The distinct difference between NHS-IgG and the anti-15253 LOS IgG2 lay in binding capability toward 302 and 220 LOSs. NHS-IgG bound to their major components, whereas anti-15253 LOS IgG2 bound to their minor components that were not silver stained.\(^3\) This difference indicates that the IgG populations bound to the 302 and 220 major LOS components are different from the anti-15253 IgG2.

Chromogenic detection with BCIP/NBT (Fig. 3B) was found to be slightly better than chemiluminescent detection using SuperSignal West Dura Substrate (Fig. 3C). However, the level of chemiluminescence might be enhanced either by re-examining the dilution of the secondary POD conjugated Ab or by using a more sensitive substrate. Detection of similar amounts of LOS samples by both chromogenic and chemiluminescent detection should make it possible to determine binding characteristics of anti-LOS antibodies by double stain experiments.

\textit{NHS-IgG bound to the OS moiety of neisserial LOSs and Salmonella truncated LPS samples}

To determine whether the binding of NHS-IgG to the LOS and LPS samples was due to the carbohydrate moiety, the electroblotted antigens were treated with anti-lipid A MAb before incubation with a 1:50 dilution of NHS (Fig. 3D). The IgG binding patterns to the LOS and LPS samples remained almost unchanged even after this treatment. Furthermore, TLC immunostaining of lipid A prepared from F62 LOS with the same dilution of NHS showed that NHS-IgG bound to none of the lipid A components (data not shown), as had been observed for anti-15253 LOS IgG2.\(^3\) These two results indicate that NHS-IgG probably bound to the OS moiety but not to the hydrophobic portion of the LOSs and the LPSs, as confirmed by the results obtained with NHS pre-absorbed with JW31R LOS, as described below.

Pre-absorbed NHS was prepared by incubating a 1:20 dilution of NHS (200 \(\mu L\)) in each ELISA well coated with JW31R LOS (1 or 5 \(\mu g\)) and then diluting this absorbed NHS to 1:50 with PBS. Figure 3E shows the results obtained after treatment of NHS with 5 \(\mu g\) of the LOS. Almost identical results were obtained even with 1 \(\mu g\) of the LOS. The most striking difference before and after absorption was the binding capability of NHS-IgG to JW31R LOS and its truncated form, 15253 LOS (Fig. 3B and E, lanes 1 and 2). The binding to these LOSs was almost abolished. If the IgG binding to the two LOSs was due to the lipid A moiety, binding to other neisserial LOSs and the truncated LPSs would also have decreased drastically. However, NHS-IgG remained to bind to these LOSs and LPSs, which indicates, at the least, that the NHS-IgG binding to JW31R LOS and 15253 LOS was due to the OS epitopes shared by the two 2,3;3,4-dibranched LOSs.

The presence of common epitopes among the 2,3;3,4-dibranched LOSs has been demonstrated in our previous studies, which characterized MAb 2C7,\(^2\) anti-JW31R LOS IgG,\(^7\) and anti-15253 LOS IgG2.\(^3\) For example, anti-JW31R LOS IgG2 bound to both JW31R LOS and 15253 LOS, but not to WG LOS (Fig. 4A),\(^7\) The...
observed binding decrease of NHS-IgG to WG LOS (Fig. 3B and E, lane 3), as well as to the 3,4-branched LOSs and the truncated LPSs (Fig. 3B and E, lanes 4–13) was probably due to a loss of NHS-IgG during the extra incubation steps which included washing with PBS. The results obtained in the above three separate experiments, treatment with anti-lipid A MAb prior to NHS, TLC immunostaining of the lipid A with NHS, and absorption of NHS with JW31R LOS, indicate that the binding of NHS-IgG to the LOS and LPS samples used in this study, at this level of dilution, was due to their carbohydrate moieties.

In this study, we focused our efforts on improving analytical conditions for PAGE/Western blot analysis and accomplishing detection of the binding of anti-LOS IgG in NHS to selected neisserial LOSs at the lower femtomole level. To gain further insight into the prevalence of anti-LOS antibodies in NHS, we intend to examine other preparations of NHS and randomly selected serum samples by the established analytical method. The results will be published elsewhere. As described earlier, recent studies have shown that NHS contains bactericidal anti-LOS IgGs, but the specificities of these anti-LOS IgGs have not yet been fully characterized. Characterization of such antibodies should help not only to understand the immune responses of the host against pathogenic Neisseria species, but also to develop effective carbohydrate-based vaccines against those bacteria.

**Conclusions**

We established improved analytical conditions for PAGE/Western blot detection of LOS using a 15-well mini gel. One ng (250 femtomole) of 15253 LOS was silver stained, and this showed that several ng (~1 pmole) of LOS preparations can be analyzed by silver staining. For the first time, Western blot detection of LOS at the lower femtomole level was accomplished by both chromatographic and chemiluminescent detection. Model LOS, 15253 LOS (62.5 pg, 16 femtomole), was detected even with 10 pm of MAb 2C7. Furthermore, the binding of NHS-IgG to similar amounts (50–250 femtomole) of neisserial LOSs and Salmonella truncated LPSs was detected with 1:50 and with 1:100 diluted NHS. The results obtained here indicate that the binding of NHS-IgG to the LOS and LPS samples, at this level of dilution, was probably due to their carbohydrate moieties. They also indicate that NHS contains numerous anti-LOS antibodies that recognize specific OS structures expressed in neisserial LOSs. The detection level accomplished in this study should help not only to analyze microbial glycolipids on the outer membrane but also to characterize antibodies or proteins that bind to those antigens.

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**References**