Enzyme Immunoassay of Teichoic Acids from *Listeria monocytogenes*

KEI-ICHI KAMISANGO,† MASATO NAGAOKA, HIDEJI FUJI, AND ICHIRO AZUMA*

Section of Chemistry, Institute of Immunological Science, Hokkaido University, Kita-ku, Sapporo, 060 Japan

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Amino groups were introduced into *Listeria monocytogenes* teichoic acids by reductive amination, and the product was coupled to biotin. Teichoic acids were assayed by their binding to specific antibody adsorbed to a solid phase, followed by detection of the antigen-antibody complex by horseradish peroxidase-avidin. Less than 20 ng of teichoic acid was detectable.

The enzyme immunoassay has been widely used for microanalyses of protein and other natural products (3, 10); however, an enzyme immunoassay of bacterial polysaccharide rarely has been reported. With respect to oligosaccharides, Himmelspach et al. (5) reported that amino groups were introduced into various oligosaccharides by reductive amination reaction and converted into $^{131}$I-labeled derivatives with the Bolton-Hunter reagent, and the immunoreactivity of the labeled oligosaccharide derivatives was analyzed by the equilibrium dialysis and the Farr assay.

In the present work, for a new method of performing an immune microassay of bacterial polysaccharides, an amino group was introduced into the reducing terminal of teichoic acids of *Listeria monocytogenes* by reductive amination reaction by the method of Himmelspach et al., and amino groups, thus introduced, were biotin labeled (Fig. 1). Then, micro-binding assays were carried out with specific antibodies and biotin-labeled teichoic acid on the solid phase. In addition, some antigenicities of the teichoic acids of *L. monocytogenes* will be discussed.

The cell walls of *L. monocytogenes* were obtained by a method similar to that described earlier (6). The four strains of *L. monocytogenes*, NTCC 2459 (serotype 1a), NTCC 5105 (serotype 3a), NTCC 5214 (serotype 4a), and NTCC 21 (serotype 4d), kindly supplied by T. Nagai, Sapporo Medical College, were used in this study. Briefly, the heat-killed cells were disrupted with a Dynomill, and crude cell wall fragments were collected by centrifugation at 10,000 × g for 30 min. The fractions thus obtained were treated repeatedly with pronase, trypsin, and $\alpha$-chymotrypsin, washed with water, and lyophilized. No protein component was detected in these cell walls by amino acid analyses. Reference samples of 1-amino-1-deoxyglucitol and 2-acetoamido-1-amino-1,2-dideoxyglucitol were prepared from glucose and $N$-acetylglucosamine, respectively, by a method similar to that described by Wiegang and Ziegler (11). Teichoic acids were extracted from the cell wall with 10 mM glycine-hydrochloride buffer (pH 2.5) (12.5 mg/ml) at 100°C for 20 min and purified by column chromatographies on DEAE-cellulose and Sephacryl S-200 columns in manners similar to that described previously (6). Reductive amination of the teichoic acids and dextran T-10 was carried out by the method of Himmelspach et al. (5) with some modifications. The polysaccharide (4 mg), NaCNBH$_3$ (2 mg), and CH$_3$COONH$_4$ (154 mg) were dissolved in 1 ml of water and stirred at 37°C for 14 days. The mixtures were sufficiently dialyzed against distilled water, and the nondializable fraction was lyophilized. Biotin labeling of polysaccharide was carried out by a method similar to that described by Guesdon et al. (4).

Antisera against *L. monocytogenes* types 1a, 3a, 4a, and 4d were obtained by a method similar to that described earlier (7). The whole immunoglobulin fractions were prepared by precipitation at a 50% saturation of ammonium sulfate from the whole antisera (2). The antibodies specific to each type of *L. monocytogenes* were obtained as follows. The cell wall of *L. monocytogenes* (200 mg), previously washed twice with 0.1 M NaHCO$_3$ solution containing 0.5 M NaCl (solution A), and 2 ml of anti-1a whole immunoglobulin fraction (50 mg/ml of protein) were incubated at room temperature for 1 h. The cell wall was washed with solution A three times and then extracted twice with 1 ml of 0.2 M glycine-hydrochloride buffer (pH 2.3). The extract was immediately neutralized with NaHCO$_3$ and dialyzed against phosphate-buffered saline (PBS) (fraction 1; total amount of protein, 50 μg). The cell wall then was washed twice with solution A and extracted twice with 1 ml of 4 M guanidine-hydrochloride. The extract was dialyzed against PBS (fraction 2; total amount of protein, 750 μg). Fraction 2 was used as the specific antibody to *L. monocytogenes*.

The binding assay of the specific antibody with biotinyl teichoic acid was carried out on the solid phase in triplicate. Polystyrene microtiter plate wells (Nunc-Immuno Plate II) were coated with 50 μl of antibody solution dialuted serially (twofold) with PBS by incubation at 37°C for 2 h. The wells were washed with PBS three times and treated with 0.5% bovine serum albumin-PBS for 1 h at room temperature. After washing with PBS three times, 50 μl of biotinyl teichoic acid was added, and the solution was incubated at 4°C overnight. The wells were washed with Tris-hydrochloride buffer saline three times and treated with 50 μl of horseradish peroxidase-Avidin D in Tris-hydrochloride buffer saline (10 μg/ml) at room temperature for 10 min. The wells were washed with Tris-hydrochloride buffer saline three times, and peroxidase activity in the wells was mea-

* Corresponding author.
† Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

FIG. 1. Biotin labeling of the teichoic acids from *L. monocytogenes* types 1a, 3a, 4a, and 4d and dextran T-10.
TABLE 1. Free amino groups and reducing groups of teichoic acids before and after reductive amination reaction

<table>
<thead>
<tr>
<th>Teichoic acids and dextran</th>
<th>Before reductive amination</th>
<th>After reductive amination</th>
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</thead>
<tbody>
<tr>
<td>T-10</td>
<td></td>
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<tr>
<td></td>
<td>Free amino group</td>
<td>Reducing group</td>
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<tr>
<td></td>
<td>1a</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3a</td>
<td>15</td>
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<tr>
<td></td>
<td>4a</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>4d</td>
<td>18</td>
</tr>
<tr>
<td>Dextran T-10</td>
<td>0</td>
<td>303</td>
</tr>
</tbody>
</table>

Measured by the method of Maiolini and Masseyeff with small modification (7). Inhibition studies were carried out with various inhibitors in the presence of 25 μg of biotinyl teichoic acid at a 25-μg/ml concentration of antibody solution. The results are expressed as percent inhibition by the following formula: % inhibition = [1 - (absorbance with inhibitor-blank)/(absorbance without inhibitor-blank)] × 100.

Analytical methods were described earlier (6).

Table 1 shows the contents of free amino groups and reducing groups of the teichoic acids of L. monocytogenes types 1a, 3a, 4a, and 4d, and dextran T-10. It has been shown that several teichoic acids extracted from cell walls by mild acid treatment have linkage regions to cell walls containing N-acetylglucosamine as the reducing terminal (1, 8). However, the teichoic acids of L. monocytogenes types 1a, 3a, 4a, and 4d have ca. 50 nmol of reducing groups per mg, and the reduction of each teichoic acid with NaBH₄, followed by acid hydrolysis, gave glucosaminol. Therefore, the teichoic acids of L. monocytogenes seem to have linkage regions containing N-acetylglucosamine as the reducing terminal. After reductive amination, the contents of free amino groups of teichoic acids and dextran T-10 were found to have increased, and those of reducing groups were found to have decreased. Furthermore, the acid hydrolysates of teichoic acids and dextran T-10 gave 1,2-diamino-1,2-dideoxyglucitol and 1-amino-1-deoxyglucitol on the amino sugar analysis, respectively. These data suggested that parts of the reducing

![FIG. 2. Procedures of the binding assay of the specific antibody with the biotinyl teichoic acid. Symbols: Y, specific antibody; ↓, biotinyl teichoic acid; ↓, horseradish peroxidase-avidin D.](image)

![FIG. 3. Binding curves of the specific antibodies with the biotinyl teichoic acids on solid phase. (a) anti-1a specific antibody and biotinyl teichoic acid from type 1a; (b) anti-3a specific antibody and biotinyl teichoic acid from type 3a; (c) anti-4a specific antibody and biotinyl teichoic acid from type 4a; (d) anti-4d specific antibody and biotinyl teichoic acid from type 4d. Amounts of the biotinyl teichoic acids were 25 ng (●) or 5 ng (○). Experimental procedures are described in the text.](image)

![FIG. 4. Inhibition of the binding of the specific antibodies and the biotinyl teichoic acids with intact teichoic acids from L. monocytogenes types 1a (●), 3a (○), 4a (■), and 4d (□). (a) anti-1a specific antibody and biotinyl teichoic acid from type 1a; (b) anti-3a specific antibody and biotinyl teichoic acid from type 3a; (c) anti-4a specific antibody and biotinyl teichoic acid from type 4a; (d) anti-4d specific antibody and biotinyl teichoic acid from type 4d.](image)
The reductive-aminated teichoic acids were biotin labeled with N-hydroxysuccinimide biotin, and then the binding assays of the specific antibodies with biotinyl teichoic acid were carried out on the solid phase (Fig. 2). The specific antibodies were obtained from the whole immunoglobulin fractions with each type of cell wall as the immunoadsorbent. Figure 3 shows the binding curves of the specific antibodies and the biotinyl teichoic acids. In all cases, the maximal bindings were observed at the concentration of ca. 50 μg of the specific antibodies per ml (2-fold dilution in Fig. 3) and the minimum binding at 6.25 μg/ml (16-fold dilution in Fig. 3). At the concentration of 50 μg of the specific antibodies per ml, it was possible to detect less than 20 ng of the biotinyl teichoic acid. Since the quantitative precipitin reactions have been found to require at least several micrograms of teichoic acid (6, 9), the sensitivity of this method was thought to be much higher than those of the quantitative precipitin reactions. Inhibition testing in the binding assay was carried out with intact teichoic acids as the inhibitor in the presence of 25 ng of biotinyl teichoic acid at the concentration of 25 μg of the specific antibodies per ml (Fig. 4). The binding of the biotinyl teichoic acids from types 1a, 3a, 4a, and 4d with the anti-1a, 3a, 4a, and 4d specific antibodies were strongly inhibited with the intact teichoic acids from types 1a, 3a, 4a, and 4d, respectively. These results suggested that any significant change of the antigenicities of the teichoic acids hardly occurred during the biotin labeling. It was also found that the binding of biotinyl teichoic acid from types 1a and 3a with the anti-1a and -3a specific antibodies were inhibited with the intact teichoic acid from types 3a and 1a, respectively. This result suggested that there were some cross-reactivities between the antigenicities of 1a and 3a because of the common component of N-acetylg glucosamine. Although the components of the teichoic acid from type 3a were similar to those from type 4a, any strong cross-reactivity between 3a and 4a was not observed. It was presumed that the different phosphate linkages between the teichoic acids gave some different antigenicities on each teichoic acid (Fig. 5). The structures of teichoic acids will be described in detail elsewhere (H. Fujii et al., submitted for publication.) Cross-reactivities of teichoic acids between L. monocytogenes and other bacteria will be studied in our laboratory.

The method described is suitable for the immunomicroassay of the teichoic acid of L. monocytogenes with the specific antibodies. However, this method seems to be applicable for not only the teichoic acid of L. monocytogenes but also for any bacterial polysaccharide because dextran T-10 was reductive aminated by a method similar to that used for the teichoic acid of L. monocytogenes.

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