Does the Difference of the Properties of Trisaccharide-BSA Conjugate (NT-P-BSA) of Mycobacterium leprae Phenolic Glycolipid Influence on its Seroreactivity?

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Since the discovery of the Mycobacterium leprae specific phenolic glycolipid I (PGL I) antigen, several laboratories have synthesized the antigenic sugar part of PGL I, O-(3,6-di-O-methyl-\(\beta\)-D-glucopyranosyl)-(1→4)-O-(2,3-di-O-methyl-\(\alpha\)-L-rhamnopyranosyl)-(1→2)-\(\alpha\)-L-rhamnopyranose, for immunological study of leprosy.\(^1\)\(^,\)\(^2\) The successful synthesis of the haptenic disaccharide- and trisaccharide-protein conjugates and subsequent analysis of immunological properties of these synthetic antigens by enzyme linked immunosorbent assay (ELISA) showed that these conjugates are very specific and sensitive to leprosy sera, especially sera from multibacillary cases.\(^3\)\(^-\)\(^10\) By using these conjugates as antigen, a simple method for the serodiagnosis of leprosy, M. leprae particle agglutination test (MLPA)\(^11\) and some other methods\(^12\) have been developed.

Among these techniques, ELISA has been used as the standard technique to evaluate the sensitivity and specificity of newly developed techniques. Therefore, it is important to standardize the protocols of ELISA and to standardize the synthetic antigen. In general, the concentration of coating antigen is used under total weight basis in ELISA.\(^13\) However, it is difficult to control the sugar contents in the conjugates within a limited range when they were prepared by acyl azide method,\(^14\) which was employed in the most laboratories. During the course of this coupling reaction, the changes in molecular size of carrier protein sometimes took place. These phenomena may influence the sensitivity and specificity of the seroreactivity of the conjugates. Therefore, it is necessary to clarify this point to get reproducible results. In this paper, we deal the influences of the difference of the sugar contents and molecular sizes of carrier protein in the trisaccharide-based conjugate, natural trisaccharide-phenylpropionate-bovine serum albumin (NT-P-BSA).

MATERIALS AND METHODS

Antigen. \(p\)-(2-Methoxycarbonylethyl)phenyl 3-O-methyl-2-O-[2,3-di-O-methyl-4-O-(3,6-di-O-methyl-\(\beta\)-D-glucopyranosyl)-\(\alpha\)-L-rhamnopyranosyl]-\(\alpha\)-L-rhamnopyranoside (NT-P), which was synthesized by the previously reported procedure,\(^4\) was coupled to BSA by acyl azide method\(^14\) under the various sugar to protein ratio, to give NT-P-BSA.
Sera. Tested sera were consisted of 36 leprosy sera (LL 13, BL 10, BB 5, BT 4, TT 4), 20 of tuberculosis sera. Healthy control sera (80) were collected from the sera for general health examination.

ELISA. IgM and IgG anti-PGL I antibodies were quantitated by ELISA and the differences in seroreactivity of five lots of NT-P-BSA were compared. NT-P-BSA (50μl) was coated to flat-bottomed microtiter plate at the sugar concentration of 0.01~10μg/ml and serum dilution was 1:300.

RESULTS

Preparation of NT-P-BSA. NT-P was coupled to BSA under the five different molar ratios of sugar to protein of 25.3, 37.3, 50.1, 65.9 and 82.5 by acyl azide method. In these reactions, the molar contents of the trisaccharide in NT-P-BSA increased with the increase of the ratios used in the coupling reaction. The molar contents of the trisaccharide of NT-P-BSA prepared by this method were 12.4 (lot A), 23.7 (lot B), 36.2 (lot C), 42.7 (lot D) and 51.8 mol (lot E) per one mol of BSA, respectively (Table 1). In contrast to the sugar contents, the recoveries of the trisaccharide incorporated to NT-P-BSA decreased from 35.6% to 15.6% with the increase of the sugar to protein ratios used in the coupling reaction.

Table 1. Preparation of NT-P-BSA by acyl azide method

<table>
<thead>
<tr>
<th>lot of NT-P-BSA</th>
<th>Molar ratio of the trisaccharide to BSA</th>
<th>Recovery of the trisaccharide (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Used for coupling</td>
<td>Product</td>
</tr>
<tr>
<td>A</td>
<td>25.3</td>
<td>12.4</td>
</tr>
<tr>
<td>B</td>
<td>37.3</td>
<td>23.7</td>
</tr>
<tr>
<td>C</td>
<td>50.1</td>
<td>36.2</td>
</tr>
<tr>
<td>D</td>
<td>65.9</td>
<td>42.7</td>
</tr>
<tr>
<td>E</td>
<td>82.5</td>
<td>51.8</td>
</tr>
</tbody>
</table>

Seroreactivity to NT-P-BSA with various sugar content. In order to study the differences in seroreactivities by the sugar contents of NT-P-BSA, the activities of five different NT-P-BSA preparations (lot A-E) to a lepromatous serum were measured at various sugar concentrations of NT-P-BSA (Fig. 1). All lots showed practically the same reactivity to IgM antibodies at any concentrations and they reached to a plateau at the sugar concentration over 0.1μg/ml. When the activities of IgG antibodies were measured, the titration curves of the reactivities of all lots showed the same pattern and reached to a plateau at 0.1μg/ml, which was the same sugar concentration for IgM antibodies. The difference in the reactivity at a plateau among five lots for IgG antibodies was ranging from 25% to 48%. The variation was larger than that for IgM.

In order to know the differences in both the sensitivity and specificity to individual serum, NT-P-BSA with low sugar content (lot E, 12.4mol/mol of BSA) and lot C (36.2mol/mol of BSA), which is the one widely used for the serodiagnosis, were tested to the sera from leprosy, tuberculosis and healthy controls.
Seroreactivities of NT-P-BSA lot A (■—■), lot B (■—■), lot C (●—●), lot D (○—○) and lot E (□—□) were measured in ELISA for IgM antibodies. Seroreactivities of NT-P-BSA lot A (□—□), lot B (□—□), lot C (○—○), lot D (○—○) and lot E (X—X) were measured by ELISA for IgG antibodies.

(Table 2). In this test, the sugar concentration of 0.1μg/ml was used as the concentration at a plateau. The positive rates of two lots showed excellent concordance (>97%) in all three types of sera in case of IgM. The correlation between the activities of lot C (Y) and lot E (X) to leprosy sera was Y = 1.014X + 0.009 (r = 0.995), which means that there was no difference in sensitivity and specificity between two lots. In case of IgG class antibody, the concordance rates between lot C (Y) and lot E (X) were almost the same as those of IgM. Although the positive rate in IgG class antibody was low (19.4%) for both lot C and E, the correlation of two activities to leprosy sera was Y = 1.011X - 0.009 (r = 0.979), which means again no difference.

**Influence of the molecular size of the carrier protein to the seroreactivity.** The distribution of the molecular size of NT-P-BSA (lot D) prepared by acyl azide method and intensive dialysis was analyzed by a chromatography with Sephadex G-200. Two peaks of sugar-protein conjugates were obtained, the amount of two peaks were almost the same (Fig. 2). The molecular weight (Mw) of the slow moving peak (F-B) was estimated at about 92 KD, which agreed with the value (92,825) calculated from the sugar content (37mol/mol of BSA) assuming that BSA retained an intact monomeric form. The fast moving peak (F-A) had the sugar content of about 45mol/mol. It was eluted at near Vo position and therefore, the Mw could not be estimated from this experiment. Velocity sedimentation analysis of this peak gave the value of Mw = 170-190 KD. This result indicates that the fast moving peak is in a dimeric form. As the BSA used for the coupling reaction did not contain dimeric form, it was produced during the coupling reaction. In order to analyze the influence of molecular size to seroreactivity, the reactivities of these two peaks to IgM, IgG or total Ig antibodies were titrated by using positive sera. Two peak
Fig. 2. Gel filtration chromatography of NT-P-BSA.
NT-P-BSA (lot B) was chromatographed on Sephadex G-200 column (10xX420mm) in a 0.1M NaCl.
Molecular markers (BG: β-galactosidase: Mw 116,000, PB: phosphorylase b: Mw 97,400 and BSA;
bovine serum albumin: Mw 66,000) were chromatographed under the same conditions as those for NTP-BSA.

Fig. 3. Seroreactivities of F-A and F-B.
The seroreactivities of F-A for IgM antibodies (●), F-B for IgG (○), F-A for IgG (●), F-B for IgG (○), F-A for total Ig (○) and F-B for total Ig (○) were measured by ELISA.
fractions showed completely the same reactivity to all classes of antibodies (Fig. 3), suggesting that molecular size has no influence to the seroreactivity of NT-P-BSA.

In order to confirm this observation, seroreactivities of F-A and F-B to individual serum (the same sets of sera for Table 2) were tested (Table 3). The results were almost same as those in Table 2 and the correlation between F-A (Y) and F-B (X) was Y = 1.021X + 0.011 (r = 0.973). These results showed that there were no differences in seroreactivity between F-A and F-B.

Table 2. Seroreactivity of NT-P-BSA with high (lot C) and low (lot E) sugar content

<table>
<thead>
<tr>
<th>No of cases</th>
<th>No of positive</th>
<th>Concordant rate(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lot C</td>
<td>Lot E</td>
</tr>
<tr>
<td>Leprosy</td>
<td>20 (55.6)</td>
<td>19 (52.8)</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>20 (5.0)</td>
<td>1 (5.0)</td>
</tr>
<tr>
<td>Healthy</td>
<td>80 (6.3)</td>
<td>4 (5.0)</td>
</tr>
</tbody>
</table>

Sugar content: Lot C: 36.2mol/mol, lot E: 12.4mol/mol. Numbers in ( ) are % positive cases.

Table 3. Seroreactivity of F-A and F-B

<table>
<thead>
<tr>
<th>No of cases</th>
<th>No of positive</th>
<th>Concordant rate(%)</th>
<th>No of positive</th>
<th>Concordant rate(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-A</td>
<td>F-B</td>
<td></td>
<td>F-A</td>
</tr>
<tr>
<td>Leprosy</td>
<td>20 (55.6)</td>
<td>19 (52.8)</td>
<td>97.2</td>
<td>7 (19.4)</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>20 (5.0)</td>
<td>1 (5.0)</td>
<td>100</td>
<td>1 (5.0)</td>
</tr>
<tr>
<td>Healthy</td>
<td>80 (6.3)</td>
<td>4 (5.0)</td>
<td>98.8</td>
<td>8 (10.0)</td>
</tr>
</tbody>
</table>

Numbers in ( ) are % positive cases.

DISCUSSION

The coupling reaction of the synthesized trisaccharide part of PGL I to BSA by acyl azide method is a simple and convenient technique for the preparation of the trisaccharide-BSA conjugate. However, the rate of incorporation of sugar was not reproducible. Serological tests of NT-P-BSA with various sugar contents showed that there was almost no difference for IgM antibodies between the activity of NT-P-BSA with low and high sugar contents, if the antigen concentration is adjusted to the same value based on the sugar concentration for IgM antibody. In the ELISA conducted by Wu for IgM with very low antigen concentrations (0.012μg sugar/ml for lot C and 0.21μg sugar/ml for lot E) for the sera collected in China (48 leprosy, 50 tuberculosis and 50 healthy controls), very good concordant rates (95.9, 100
and 100% for leprosy, tuberculosis and healthy control sera, respectively) were observed. Correlation between lot C (Y) and lot E (X) was Y = 1.3261X − 0.007 (r = 0.879). As shown in Fig. 1, the antigen concentrations used in this experiment was in the proportional region of the activity to concentration, the activity at the antigen concentration of 0.021 μg/ml was higher than at 0.012 μg/ml by 1.2–1.4 times. Therefore, the regression coefficient can be estimated as almost one at the same antigen concentration. These observation supported that there were no differences in seroreactivity of NT-P-BSA with different sugar content. In case of IgG antibody, the difference of the reactivity was found. However, the difference was 25~40%, which does not influence on the positive rate of the sera to NT-P-BSA. And there were no differences between the specificity of the conjugates with low and high sugar contents. Therefore, the difference can be ignored in a practical use. About half of the BSA used in the coupling reaction was converted into a dimeric form. However, there were no differences in the serological activities between NT-P-BSA with dimeric BSA and monomeric BSA. Based on these observations, it was concluded that any lot of NT-P-BSA with variety of sugar content can be used in ELISA without any difference of the seroreactivity as far as the same sugar concentration is used.

SUMMARY

The sugar content of the trisaccharide-BSA conjugate of the phenolic glycolipid I of *Mycobacterium leprae* (NT-P-BSA) increased with the increase of the molar ratio of the trisaccharide to BSA used in the coupling reaction. The difference of the sugar content in NT-P-BSA did not give the influence on the seroreactivity and specificity in ELISA for both IgM and IgG class antibodies. During the course of the coupling reaction, about half amount of BSA was converted to dimeric form. However, there were no differences of the activity and specificity between monomeric form and dimeric form of NT-P-BSA. Based on these results, it was concluded that any lot of NT-P-BSA with variety of sugar content can be used in ELISA without any difference of the seroreactivity.

REFERENCES


