Novel Gelatin Particle Agglutination Test for Serodiagnosis of Leprosy in the Field

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We developed a novel gelatin particle agglutination test (MLPA) for the serodiagnosis of leprosy; this test is especially useful for clinical practice and epidemiological surveys of leprosy in countries in which the disease is endemic. The antigen used in the test is the chemically synthesized trisaccharide moiety of Mycobacterium leprae-specific phenolic glycolipid I. MLPA is a simple and easy technique having sensitivity and specificity comparable to those of the conventional indirect enzyme-linked immunosorbent assay. The new technique was found to be useful for monitoring of chemotherapy and predictive diagnosis of high-risk individuals in contact with persons with leprosy and may be useful for the prediction of relapse. We are now preparing to supply a quality-controlled ready-to-use MLA kit for leprosy control in countries in which leprosy is endemic.

Mycobacterium leprae-specific phenolic glycolipid antigen (PGL-I) was discovered by Brennan and Barrow in 1980 (4). The molecular structure was found to be 3,6-di-O-methyl-β-D-glucopyranosyl(1→4)2,3-di-O-methyl-α-L-rhamnopyranosyl(1→2)3-O-methyl-α-L-rhamnopyranosyl phenyl phthiocerol dimycoloscerate, and the sugar moiety was found to be the antigenic determinant (24).

Soon after the discovery, indirect enzyme-linked immunosorbent assay (ELISA) techniques to detect anti-PGL-I antibody were developed (5, 14, 31) and used as new tools for the serodiagnosis of leprosy. Natural PGL-I, however, is not a convenient antigen for routine serological testing because of its extreme hydrophobicity. This inconvenience was overcome by chemical synthesis of its antigenic disaccharide (11, 19, 23) or trisaccharide (19–21) moiety. The synthesized sugar was conjugated to a carrier protein via various linker arms to make serodiagnostic reagents for indirect ELISA.

Recently we developed a novel gelatin particle agglutination test, named MLPA, by sensitizing gelatin particles with a trisaccharide-based antigen. It was found that this simple test has sensitivity and specificity comparable to those of the conventional indirect ELISA. The new technique was found to be very useful in the field in countries in which leprosy is endemic. In this report, we describe in detail the procedure of the test and discuss the clinical and epidemiological significance of routine measurement of anti-PGL-I antibody.

MATERIALS AND METHODS

Antigen. The antigen used in this test is natural trisaccharide-phenyl propionate-bovine serum albumin (NT-P-BSA). The procedure for synthesis of the antigen was described in detail elsewhere (20, 25). The antigen is a very stable hydrophilic substance with much stronger seroreactivity than that of natural PGL-I.

Sensitization of gelatin particles. Proprietary gelatin particles (2.4 × 10^9/ml) for agglutination tests were activated with 2.5 µg of tannic acid per ml at 37°C for 10 min. After being washed, the particles were mixed with an equal volume of NT-P-BSA (50 µg/ml) and incubated at 37°C for 60 min. The particle suspension was then lyophilized and stored at 4°C until use.

Gelatin particle agglutination test. The lyophilized unsensitized and sensitized particles were reconstituted with 3 ml of reconstituting solution to make a 1% suspension. The rehydrated suspension can be stored at 2 to 8°C for 7 days without significant loss of reactivity.

Three drops (75 µl) of serum diluent were added to the first well and one drop (25 µl) of the same diluent was added to the second and third wells of a 96-well U-type microdilution plate. Test serum (25 µl) was added to the first well, and the same volume of diluted serum was serially transferred from the first to the second well and from the second to the third well. One drop of unsensitized control particles and one drop of sensitized particles were then added to the second and third wells, respectively. After the serum and particles were mixed with a microplate mixer, the plate was incubated at room temperature for 2 h or at 37°C for 1 h. The result was interpreted with the naked eye. Usually, it was easy to distinguish positive from negative results.

When the serum yielded a positive result in the qualitative test, a semiquantitative test was performed by further serial twofold dilution to find the endpoint of positive reaction. The antibody titer was expressed as the highest dilution giving complete agglutination.

Indirect ELISA. The antibody titer in the test serum was simultaneously measured by conventional indirect ELISA to test the reliability of MLPA.

An ELISA plate (NUNC-ImmuNoPlate Maxisorp F96; Nunc A/S, Kamstrup, Roskilde, Denmark) was coated with 50 µl of NT-P-BSA (0.431 nmol of sugar per ml), and then the free surface of the well was blocked by incubation with 1% BSA at 37°C for 60 min. The test serum, diluted 1:300 with dilution buffer (phosphate-buffered saline with 20% fetal calf serum and 0.5% Tween 20), was added to the well and incubated at 37°C for 60 min. After the plate was washed with washing buffer (phosphate-buffered saline with 0.05% Tween 20), peroxidase-conjugated anti-human immunoglobulin G (IgG) or IgM antiserum (Dako Immunoglobulins A/S, Copenhagen, Denmark) was added to the well and incubated

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at 37°C for 60 min. After the excess conjugate was removed with washing buffer, substrate solution (0.4 mg of o-phenylenediamine per ml and 0.4 µl of 30% hydrogen peroxide per ml in 0.1 M citrate-phosphate buffer, pH 5.0) was added and the plates were developed in the dark for 15 to 30 min. The reaction was stopped with 1.25 M sulfuric acid. The \( A_{592} \) and \( A_{610} \) were then measured with a two-wavelength microplate photometer (MTP-22; Corona Electric, Katsuta, Japan).

**Sera.** A total of 1,466 serum specimens from various sources was used in this study. They were composed of 532 leprosy serum specimens (69 active lepromatous, 333 inactive lepromatous, 9 active borderline, 35 inactive borderline, 3 active tuberculoid, and 83 inactive tuberculoid specimens), 149 tuberculosis specimens, 70 household contact specimens, 167 occupational contact specimens, 120 normal pregnancy specimens, and 428 noncontact control specimens.

**RESULTS**

Since the indirect ELISA has been used to measure the anti-PGL-I antibody, we evaluated MLPA by comparing the results with those of the conventional indirect ELISA. MLPA showed an excellent positive correlation with IgM antibody, but there was no such correlation with IgG antibody (Fig. 1).

The concordance in positivity between MLPA and ELISA in IgM anti-PGL-I antibody testing is summarized in Table 1. The concordance rates were between 88.9 and 100%, showing excellent agreement between the two methods.

The antibody titers in the test sera are shown in Table 2. The positivity rates in active lepromatous and borderline leprosy serum specimens were 79.7 and 66.9%, respectively, whereas the positivity rates in inactive lepromatous, borderline, and tuberculoid specimens were 42.0, 22.9, and 0.0%, respectively. The highest positivity rates for antibody were found in lepromatous and borderline sera. The positive rates in nonleprotic sera were between 2.7 and 10%. The antibody titers of positive nonleprotic sera were, however, lower than or equal to 1:64 except for one household contact. The sensitivities of MLPA and ELISA were 76.5 and 69.1%, respectively, and the specificities of MLPA and ELISA were 92.3 and 95.1%, respectively. There were no statistically significant differences in sensitivity and specificity between the two methods.

We collected serial blood samples from 40 patients to analyze the changes in antibody titer during chemotherapy. The antibody titers in 38 of 40 patients (95%) declined in parallel with clinical improvement, showing the usefulness of routine titration of anti-PGL-I antibody for monitoring of chemotherapy.

Of 70 household contacts, 5 (7.1%) tested positive by MLPA (Table 2). Among these five, we found a young female contact with a high antibody titer and a low lepromin reaction.

**DISCUSSION**

During the early stage of serological study of PGL-I, natural PGL-I or decapoylated PGL-I was used as the coating antigen for indirect ELISA (5, 12, 31). These antigens were later replaced by synthetic glycoconjugates with terminal disaccharide-based (6, 23) or trisaccharide-based (20) antigen. Comparative studies of these glycoconjugates proved that their seroreactivities are nearly identical (30) but that those of the trisaccharide-based antigens are superior (10, 22). We compared the agglutination capacities of a trisaccharide-based antigen (NT-P-BSA) and two disaccharide-based antigens with linker arms of phenyl propionate (ND-P-BSA) and an allyl group (ND-A-BSA). The last was kindly supplied by Immunology of Leprosy, World Health Organization. It was found that ND-P-BSA has lower sensitivity and ND-A-BSA has lower specificity than particle agglutination (data not shown). Only NT-P-BSA showed sensitivity and specificity comparable to those of indirect ELISA.

The comparative study of MLPA and indirect ELISA for determining IgM antibody titers proved that both methods have basically identical sensitivities and specificities. There is a significant positive correlation in antibody titers and a very good concordance in positivity between the two methods. MLPA, however, cannot detect IgG antibody because of the poor agglutination capacity of IgG anti-PGL-I antibody.

We have clear evidence that IgG antibody definitely binds
TABLE 2. Distribution of anti-PGL-I antibody measured by MLPA

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of serum specimens</th>
<th>No. of positive tests</th>
<th>% positive</th>
<th>No. of positive tests at antibody titer</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1:32</td>
<td>1:64</td>
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<tr>
<td>Active lepromatous</td>
<td>69</td>
<td>55</td>
<td>79.7</td>
<td>20</td>
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<tr>
<td>Inactive lepromatous</td>
<td>333</td>
<td>140</td>
<td>42.0</td>
<td>70</td>
</tr>
<tr>
<td>Active borderline</td>
<td>9</td>
<td>8</td>
<td>67.7</td>
<td>1</td>
</tr>
<tr>
<td>Inactive borderline</td>
<td>35</td>
<td>8</td>
<td>22.9</td>
<td>4</td>
</tr>
<tr>
<td>Active tuberculoid</td>
<td>3</td>
<td>1</td>
<td>33.3</td>
<td>1</td>
</tr>
<tr>
<td>Inactive tuberculoid</td>
<td>83</td>
<td>0</td>
<td>0.0</td>
<td>1</td>
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<tr>
<td>Household contact</td>
<td>70</td>
<td>5</td>
<td>7.1</td>
<td>2</td>
</tr>
<tr>
<td>Occupational contact</td>
<td>167</td>
<td>14</td>
<td>8.4</td>
<td>12</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>149</td>
<td>4</td>
<td>2.7</td>
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</tr>
<tr>
<td>Pregnancy</td>
<td>120</td>
<td>12</td>
<td>10.0</td>
<td>12</td>
</tr>
<tr>
<td>Noncontact</td>
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<td>33</td>
<td>7.7</td>
<td>27</td>
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* Antibody belonging to IgG class.

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to NT-P-BSA on the surface of gelatin particles (data not shown), but this binding does not result in agglutination of the particles. This immunochemical characteristic is unique to IgG anti-PGL-I antibody because IgG antibodies to other protein antigens usually agglutinate sensitized gelatin particles. The reasons for the low agglutinating capacity of IgG antibody to PGL-I remain obscure.

Since the initial stage of serological study of PGL-I, it has been said that the main immunoglobulin class in anti-PGL-I antibody is IgM (32). We found high levels of IgG in leprosy patients treated over a long term in Japan. Britton et al. (7) and Lyons et al. (26) reported almost the same results. IgG antibody, however, looks clinically less important than IgM, because only IgM antibody seems to reflect the status of the disease. The fact that MLPA cannot detect the IgG class of anti-PGL-I antibody, therefore, does not affect the practical utility of MLPA.

On the basis of our comparative study, we concluded that although MLPA is simple and easy to perform in the field in countries where leprosy is endemic, the new test has sensitivity, specificity, and practical utility comparable to those of the conventional indirect ELISA.

There are many reports of decreases in antibody titer by effective chemotherapy (2, 7, 12, 14, 15, 18, 28). The results of MLPA presented here agree with those of earlier reports. The titration of anti-PGL-I antibody is a new routine laboratory test for monitoring of the effect of chemotherapy (17).

The prediction of relapse after multidrug therapy is one of the most important problems in leprosy control today because relapsed patients with high levels of bacilli will be new sources of infection. There are no reports on the changes in anti-PGL-I antibody titer during relapse yet. We found a relapsed patient with borderline lepromatous leprosy who showed an elevation in antibody titer at least 10 months before the clinical signs of a relapse appeared. It is possible that the elevation of antibody titer is the first sign of relapse. Regular measurement of anti-PGL-I antibody after multidrug therapy will be useful for predictive diagnosis of relapse.

We have increasing evidence that anti-PGL-I antibody titer is a good indicator for detecting high-risk people among contacts (10, 15, 27). We found a female household contact from a family with multiple cases of leprosy who showed a continuously high IgM antibody titer. On the basis of a 2-year observation of 724 household contacts in French Polynesia, Chanteau et al. (9) reported that 1 of 8 (12.5%) highly seropositive household contacts developed lepromatous leprosy, whereas 3 of 631 (0.23%) seronegative contacts developed leprosy. This result means that the attack rate of highly seropositive contacts is 40 times greater than that of seronegative contacts. Douglas et al. (16) also reported that the attack rate in high-antibody contacts was 20 times greater than that in low-antibody or negative contacts in Cebu, Philippines. Thus, the serological study of Mycobacterium leprae-specific PGL-I resulted in a new possibility for diagnosing high-risk contacts before they develop overt clinical disease; consequently, we will be able to concentrate our preventive effort on people at high risk.

Japanese household contacts did not show a statistically significant increase in positivity in tests of anti-PGL-I antibody. It is not reasonable to expect higher positivity in household contacts in countries such as Japan in which leprosy is not endemic, because only high-risk contacts show the elevated antibody titer and most of the household contacts in these countries do not develop leprosy.

Seropositivity in household contacts in countries in which leprosy is endemic is, however, considerably different. Agis et al. studied 109 intradomiciliary contacts in Guadeloupe and reported that the positivity rates in paucibacillary and multibacillary patients were 25 and 43%, respectively, and that antibody titers were higher in contacts with high levels of bacilli (1). Buchanan et al. reported that the seropositivity rates of contacts in Sri Lanka and Mexico were 36 and 21%, respectively (8). Baumgart and colleagues conducted a population survey in a village in Papua New Guinea in which leprosy is highly endemic and reported that the seropositivity rate in the population was 32% (3). These observations suggest that there is a positive correlation between seropositivity in contacts and the prevalence rate of leprosy and that serological tools such as MLPA will be useful for epidemiological surveys in the near future.

It is important for us to understand the reliability of MLPA before applying it in the field. Figure 2 shows the relationship between the reliability of MLPA and the prevalence rate of leprosy in a given population. We can estimate the reliability of the test if we know the prevalence rate in the population. Some epidemiologists pointed out that the usefulness of serological tests for screening high-risk groups for leprosy by general population surveys is limited by the low reliability of positive in low-prevalence populations (3, 10). It is obvious that the reliability of positive is low in low-prevalence populations if we use 1:32 or 1:64 as a cutoff value (2). In such circumstances, we can use 1:128 as the cutoff and classify 1:32 and 1:64 as "under observation." We have to use different cutoff values for different prevalence groups.

After 10 years of evolution of the serodiagnosis of leprosy, we have reached a turning point, moving from a laboratory
phase to a practical application phase. At this stage of research, it is important to develop simple and reliable techniques and supply quality-controlled, inexpensive diagnostic reagents, preferably as a kit, for field workers and epidemiological investigators. There are several sophisticated techniques for detecting anti-PGL-1 antibody (13, 29, 33), but none of them fulfills this requirement. We are now preparing to supply MLPA as a ready-to-use kit for field application which will be available in the near future.

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LITERATURE CITED