Use of Serum Antibody and Lysozyme Levels for Diagnosis of Leprosy and Tuberculosis

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Active tuberculosis (TB) and leprosy are difficult to diagnose early because there are few organisms to detect and the specific immune response does not distinguish between active and inactive disease. We developed an immunoassay for leprosy to see whether serum lysozyme levels could be used to identify individuals with clinical leprosy or TB. The immunoassay for lysozyme proved superior to standard enzyme assays that were less sensitive and reliable. The lysozyme assay was compared with assays for antibodies to Mycobacterium tuberculosis lipoarabinomannan (LAM) and M. leprae phenolic glycolipid-1. The sera tested were from Ethiopian leprosy (paucibacillary and multibacillary) and TB patients and from healthy Ethiopian and U.S. controls. The lysozyme assay was able to detect more of the individuals with TB (sensitivity, 100% for 19 patients) or leprosy (sensitivity, 86% for 36 patients) than either antibody assay. In particular, lysozyme levels were raised in a higher proportion of the paucibacillary leprosy patients (83% of 17), for whom the antibody assays were less sensitive; the LAM IgG and the phenolic glycolipid-1 IgM levels were raised in only 62 and 44% of 16 patients, respectively. The data suggest that lysozyme measurements may be useful in the diagnosis of mycobacterial infections and other chronic infectious granulomatoses.

The diagnosis of florid leprosy and tuberculosis (TB) presents few difficulties (6, 16). Multibacillary leprosy (MB-L) can be diagnosed clinically in >90% of cases (16), and extensive pulmonary TB can be easily confirmed by radiological methods, by the detection of acid-fast bacilli in the sputum, and by culture. The diagnosis of paucibacillary leprosy (PB-L), minimal pulmonary TB, and extrapulmonary TB is appreciably more difficult. The clinical features may be ambiguous, and bacteriologic investigations are often negative. Although a biopsy examination may be definitive, facilities for histopathologic studies may not be available in developing countries, in which leprosy and TB are most prevalent. One approach to this problem is to develop increasingly sensitive methods (24, 27) to detect the causative bacilli or their products (19). Another approach, which we have adopted, is to use the response of the host to infer the presence of disease.

Ideally, the host would develop an excellently sensitive response to an antigen that is specific for the infectious agent. Such an ideal has been partly realized in the diagnosis of leprosy, in which antibody responses to the phenolic glycolipid-1 (PGL-1) antigen are highly specific (2). However, departure from the ideal is manifested by a low sensitivity in response to paucibacillary disease. Similarly, the antibody response to the common mycobacterial antigen lipoarabinomannan (LAM) is detected less often in patients with paucibacillary disease (31, 32). These data indicate that the antigen load drives the antibody response and that the detection of paucibacillary disease requires another type of host response marker, one that is less dependent on the antigen load.

Both PB-L and TB are characterized by hypersensitivity granulomas, the predominant components of which are macrophages. We therefore decided to measure a macrophage secretory product, lysozyme, as an indicator of granuloma formation, even though we were fully aware of its lack of specificity; we were hoping for increased sensitivity with paucibacillary disease. Serum lysozyme levels have been measured, and these data have been compared with the results of serum antibody assays to LAM and PGL-1.

MATERIALS AND METHODS

Enzymatic assays for lysozyme. The enzymatic techniques are based on the rate of clearing by lysis of suspensions of killed Micrococcus luteus (M3770; Sigma Chemical Co., St. Louis, Mo.). Two enzymatic methods were used, a liquid phase spectrophotometric technique (22) and a solid phase, agar plate (lysoplate) technique (23). The only modification to the lysoplate method was that 0.1% sodium azide was added to the agar. The zones of lysis were measured from photographic enlargements of the lysoplates.

Competitive inhibition immunoassay to detect lysozyme. The competitive inhibition immunoassay is based on the dot enzyme-linked immunosorbent assay (ELISA). Lysozyme (100-ng dots; 3 mm, diameter) was immobilized on 0.45-μm-pore-size nitrocellulose (NC) paper (162-0015 Trans-Blot Transfer Medium; Bio-Rad Laboratories, Richmond, Calif.) by dipping a solution of the protein through a 96-sample manifold device (Hybri*Dot System 1050MM; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The NC paper was cut into strips, each having four dots, and immersed in a blocking agent (1% bovine serum albumin [BSA]-0.5% gelatin in Tris-buffered saline, pH 7.6). The strips were agitated at room temperature overnight. All incubation periods were at room temperature on a platform rocker. Polyclonal antiserum to human lysozyme was prepared by immunizing rabbits with human lysozyme (GCC-4025m; Alpha Therapeutic Corp., Los Angeles, Calif.) in Freund’s incomplete adjuvant. Either a lysozyme standard or 10 μl of human serum was added to a tube containing 1.5 ml of rabbit anti-human lysozyme at a dilution of 1:7,500. After 1 h, an NC strip with immobilized lysozyme was added
to each tube and incubated for another 2 h. The NC strips were washed with 1% BSA in Tris-buffered saline and incubated in a 1:1,000 goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase conjugate (dilution, 1:1,000; 170-6515; Bio-Rad) for 2 h. The NC strips were washed again with 1% BSA in Tris-buffered saline and developed with a solution of 4-chloro-1-naphthol and hydrogen peroxide. The NC strips that had incubated with the test serum were evaluated by comparing their color intensity to the strips that had incubated with standard lysozyme solutions. These standards (0, 15, 30, and 60 ng of lysozyme) yielded dots of decreasing color intensity that were assigned values of 0, 1, 2, and 3, respectively. Test serum strips were assigned a corresponding value by matching color intensities by eye.

Figure 1 is a photograph of representative results from a competitive inhibition immunoassay for lysozyme.

ELISAs for antibodies to PGL-1 and LAM. The ELISA for antibodies to PGL-1 and LAM is an adaptation of a standard technique described by Johnstone and Thorpe (12). Polystyrene plates (96 well, flat bottom; Immulon 2; Dynatech Laboratories, Inc., Chantilly, Va.) were coated with antigen in a bicarbonate buffer, pH 9.6 (100 μl per well), and stored overnight at 4°C. The antigens, PGL-1 and LAM, were provided by P. J. Brennan, Colorado State University, Fort Collins, through National Institutes of Health contract AI-52882, as lyophilized material, dissolved in phosphate-buffered saline (PBS) to a convenient concentration, e.g., 1 or 2 mg/ml, and stored at −20°C. To coat the plates, PGL-1 was diluted to 250 ng/ml and LAM was diluted to 500 ng/ml in the bicarbonate buffer. The wells were washed with PBS containing 0.05% Tween-20 (PBS-Tween), blocked with 0.5% gelatin in PBS-Tween for 30 min, and then washed again. One-hundred microliters of human serum test samples, diluted 1:300, was added to the wells. The plates were incubated at 37°C for 2 h and then washed with PBS-Tween. One-hundred microliters of goat antiserum, diluted 1:1,000, to either human IgG or IgM conjugated with alkaline phosphatase (A-3150 or A-3275; Sigma) was added to the wells and incubated for 2 h at 37°C. At the end of the incubation period, the plates were washed with PBS-Tween. Disodium p-nitrophenyl phosphate substrate tablets (104-105; Sigma) were dissolved in diethanolamine buffer, pH 9.8, and added to each well. The color was allowed to develop for 30 min at room temperature at which time the reaction was stopped with 3 M NaOH. The optical density (OD) of the solution in the wells was measured by using an ELISA reader with a 405-nm filter after blanking against an antigen control. Each plate comprised 24 sets of four wells that were used for reagent controls, positive and negative control sera, and test sera from patients. The positive control for PGL-1 antibodies was a pool of sera from lepromatous leprosy patients, and the positive control for LAM antibodies was a pool of sera from TB patients. The negative controls were serum samples from healthy volunteers in the United States.

Description of patients and controls. The TB patients had been admitted to the Black Lion Hospital, Addis Ababa, Ethiopia, and none had received anti-TB chemotherapy for longer than 1 month. A diagnosis of TB was based upon a clinical evaluation, sputum smear, and/or chest X-ray. The leprosy patients had active disease of variable duration and were treated at the All African Leprosy Education and Rehabilitation Training Centre (ALER, Addis Ababa, Ridley-Joloping criteria were used for disease classification (30). The groupings of MB-L and PB-L were based on the following criteria: MB-L included lepromatous leprosy and borderline lepromatous leprosy patients; PB-L included tuberculous leprosy, borderline tuberculoid, indeterminate leprosy, and neural leprosy patients. No borderline leprosy patients were available for the study.

The control groups were healthy volunteers from Ethiopia (ET-C) and the United States (US-C). The Ethiopian control groups were hospital and laboratory personnel from the Black Lion Hospital, ALER, or the Armauer Hansen Research Institute (AHRI) in Addis Ababa. The US-Cs were medical students from Wayne State University, Detroit, Mich. Statistical analysis. In order to determine whether there were significant differences in serum lysozyme concentrations in any of the diagnostic groups, a one-way analysis of variance was calculated by using the results of the lysoplate enzyme assay and the immunoassay. Tukey's test for a multiple comparison of means was used to determine whether there was a significant difference among any of the diagnostic groups. In addition, a chi-square test with Yates' correction was applied to the data from the lysoplate enzyme assay and the immunoassay to determine whether there was a significant difference between these assays. The methods used for these calculations were described previously by Snedecor and Cochran (34).

RESULTS

Inadequacy of the liquid enzyme assay. The spectrophotometric method (22) for determining lysozyme concentrations yielded incongruous results. Some sera, instead of clearing the bacterial suspension and decreasing the OD, actually had an increase in OD. A similar phenomenon was reported by Harrison and Swingler (9), who attributed the effect to unidentified serum macromolecules that could either activate or inhibit the action of lysozyme. Since some of the sera were visibly lipemic, it was thought that these lipids might be interfering with the assay. However, the removal of lipids with chloroform did not improve matters.

Assays for lysozyme differentiated between the controls and patient groups. Serum constituents did not interfere with the lysoplate assay, the results of which are reported in Table 1. The one-way analysis of variance revealed significant variations (P < 0.01) among the groups. Tukey's test showed that there were statistically significant differences in the serum concentrations of lysozyme between ET-Cs and leprosy patients.
patients \((P < 0.05)\), ET-Cs and TB patients \((P < 0.01)\), and between leprosy and TB patients \((P < 0.01)\). There was no significant difference between the serum concentrations of lysozyme for the ET-Cs and US-Cs, even though the mean for the US-Cs was lower. The sera that were assayed by the lysoplate method were also tested by using the immunoassay for lysozyme (Table 1). The one-way analysis of variance revealed significant variation between the groups \((P < 0.01)\), and Tukey’s test yielded results similar to those obtained from the lysoplate assay.

**Comparison of the lysoplate assay with the immunoassay for lysozyme.** Since both the lysoplate assay and the lysozyme immunoassay differentiated between the control and patient groups, it was necessary to decide which assay was more sensitive. Accordingly, the number of patients with a positive serum lysozyme test, defined as >2 SDs above the mean for the US-Cs, was determined and a chi-square test was applied to the data. Of the patients with either TB or leprosy, 40 of 54 had elevated serum lysozyme levels as determined by the lysoplate method compared with 49 of 54 patients as determined by the immunoassay (Fig. 2). These data demonstrated the superior sensitivity of the immunoassay (chi-square, 4.26; \(P < 0.05\)).

**Absolute lysozyme concentration in serum.** The mean values for serum lysozyme in the ET-Cs were 5.31 \(\mu g/ml\) and a dot score of 1.00 for the lysoplate and immunoassay, respectively (Table 1). This dot score is equivalent to 1.5 \(\mu g/ml\), approximately 30% of the enzyme value. However, the two assays were proportionate over the measured range of lysozyme levels. The cause of the discrepancy between the absolute lysozyme concentrations generated by enzyme and immunoassays is unclear but has been observed by other investigators, who estimated the mean serum lysozyme concentration at 1.6 \(\mu g/ml\) in normal adults (28).

**Antibodies to LAM are present in TB and leprosy patients.** As shown in previous studies (5, 17, 33), serum IgG antibodies to LAM were increased in patients with leprosy or TB (Fig. 3B). The means for all the leprosy patients, the MB-L and PB-L subgroups, and TB patients were raised and remarkably similar (Table 2). The background level of LAM-specific IgM in control subjects was somewhat higher than the corresponding IgG values. Raised levels of IgM antibodies were observed in leprosy patients, particularly those with multibacillary disease (Fig. 3A), but remarkably, the anti-LAM IgM levels in TB patients were indistinguishable from those of controls. In contrast to the LAM data, serum levels of IgM antibodies to PGL-1 were raised only in leprosy patients, particularly the MB-L group (Fig. 4; Table 2). The IgG antibody titrations yielded analogous results (data not shown).

**Lysozyme assay is more sensitive than mycobacterial anti-**

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**TABLE 1.** Mean serum lysozyme concentrations determined by the lysoplate enzyme assay and by the competitive inhibition immunoassay

<table>
<thead>
<tr>
<th>Diagnostic group (n)</th>
<th>Lysozyme concn ((\mu g/ml)) in lysoplate assay (mean ± SD)</th>
<th>Dot score in immunoassay (mean ± SD)</th>
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<tbody>
<tr>
<td>US-C (27)</td>
<td>4.07 ± 1.36</td>
<td>1.07 ± 0.38</td>
</tr>
<tr>
<td>ET-C (14)</td>
<td>5.31 ± 1.42</td>
<td>1.00 ± 0.55</td>
</tr>
<tr>
<td>Leprosy (36)</td>
<td>7.80 ± 2.33</td>
<td>2.22 ± 0.68</td>
</tr>
<tr>
<td>PB-L (17)</td>
<td>7.85 ± 2.16</td>
<td>2.12 ± 0.70</td>
</tr>
<tr>
<td>MB-L (19)</td>
<td>7.76 ± 2.56</td>
<td>2.32 ± 0.67</td>
</tr>
<tr>
<td>TB (19)</td>
<td>12.78 ± 4.53</td>
<td>2.84 ± 0.37</td>
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</tbody>
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**FIG. 3.** IgM and IgG antibodies to LAM. The dotted line represents an OD 2 SDs above the mean of the US-Cs. Open circles represent individual OD readings from the immunoassay.

**FIG. 2.** Comparison of the lysoplate enzyme assay with the competitive inhibition immunoassay for lysozyme. The patients were all Ethiopian. Data are expressed in terms of the percent of individuals in each diagnostic group with serum lysozyme levels 2 SDs above the mean of the US-C group. Open bars represent results from the immunoassay, and closed bars represent results from the lysoplate assay.
body assays but is less specific. The preceding data were summarized in terms of the percentage of individuals with increased (positive) levels of lysozyme, IgG antibodies to LAM, and IgM antibodies to PGL-1 (Fig. 5). It is evident that the lysozyme assay was the most sensitive indicator of mycobacterial infection, being positive in 100% of TB patients and more than 80% of MB-L and PB-L patients. The specificity was low, as expected. By comparison, the anti-LAM IgG titers were significantly raised in approximately 60% of the TB and leprosy patients. As shown in other studies (2), the PGL-1 IgM levels were raised only in leprosy patients; 79% of MB-L patients and 44% of PB-L patients.

**DISCUSSION**

There is a pressing need for improved diagnostic tests for leprosy and TB, especially among patients with PB-L, minimal pulmonary TB, and extrapulmonary TB. The tuberculin test is specific for the genus *Mycobacterium*, but its usefulness is vitiated by the fact that positive results are associated with non progressive subclinical infection, previous *Mycobacterium bovis* BCG vaccination, and cured TB, as well as active disease. Moreover, the test is negative in approximately 20% of patients with active TB (21). Serological tests have fared no better. Historically, reproducibility has been a problem due in part to the use of crude preparations. The recent availability of purified antigens such as LAM (11) and PGL-1 (10) has resulted in reproducible assays. However, both suffer from insensitivity in paucibacillary patients. In this study, the PGL-1 IgM antibody assay was positive in 79% of the MB-L patients but only 44% of the PB-L patients. The LAM IgG antibody assay was positive in approximately 60% of the PB-L, MB-L, and TB patients. Although no clinical details for the TB patients are available, it is probable that most, if not all, had extensive disease.

Although the IgG anti-LAM levels in leprosy and TB patients were closely similar, the IgM levels were not. The mean IgM anti-LAM levels in TB patients did not differ from those of healthy controls, but the corresponding values in leprosy patients were substantially raised (Table 2). The last finding is reminiscent of the response of leprosy patients to PGL-1, in which IgM antibodies predominate (2, 15), and suggests that in leprosy, but not TB, the switch from IgM to IgG, in response to glycolipid antigens, may be defective. Possibly this phenomenon reflects a preponderance of Th1 cells in leprosy (35), but at a practical level, the presence of both IgM and IgG antibodies to LAM is strongly indicative of leprosy.

The serum lysozyme immunoassay was positive in 86% of the leprosy patients, both MB-L and PB-L, and 100% of the TB patients. The lysozyme immunoassay, therefore, is a sensitive diagnostic test. Even 83% of the PB-L patients, who are more difficult to identify, were detected by the serum lysozyme immunoassay. The similarly raised serum lysozyme level in both PB-L and MB-L patients is enigmatic. PB-L is characterized by relatively few localized lesions containing highly activated macrophages, while MB-L is noted for numerous and extensive lesions of virtually anergic macrophages (3, 13, 14). We hypothesize that in PB-L, the granuloma macrophages secrete increased amounts of lysozyme per cell, whereas the macrophages of MB granulomas secrete normal or subnormal amounts of lysozyme per cell, but total secretion from the excessive number of macrophages leads to raised serum lysozyme levels, nonetheless. In either case, a raised blood lysozyme level is indicative of active granulomatosis (1, 18, 20), rather than quiescent or cured disease or post-BCG vaccination.

When comparing the different methods of measuring lysozyme, we found that the immunoassay was the most satisfactory. The liquid phase spectrophotometric method did not always work with serum because of interfering substances that gave anomalous results. The lysoplate method is not subject to this problem, but the zones of lysis are difficult to measure accurately. In a typical experiment, the zone diameter ranged from 8 mm (5 μg of lysozyme per ml) to 12 mm

**TABLE 2. Mean ELISA OD readings of antibodies to LAM and PGL-1**

<table>
<thead>
<tr>
<th>Diagnostic group (n)</th>
<th>OD (mean ± SD)</th>
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<tbody>
<tr>
<td>ET-C (14)</td>
<td>0.156 ± 0.116</td>
<td>0.164 ± 0.164</td>
<td>0.124 ± 0.091</td>
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<tr>
<td>Leprosy (35)</td>
<td>0.501 ± 0.366</td>
<td>0.461 ± 0.309</td>
<td>0.352 ± 0.421</td>
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<tr>
<td>PB-L (16)</td>
<td>0.463 ± 0.299</td>
<td>0.345 ± 0.309</td>
<td>0.296 ± 0.162</td>
<td></td>
</tr>
<tr>
<td>MB-L (19)</td>
<td>0.533 ± 0.420</td>
<td>0.558 ± 0.296</td>
<td>0.731 ± 0.471</td>
<td></td>
</tr>
<tr>
<td>TB (18)</td>
<td>0.519 ± 0.357</td>
<td>0.193 ± 0.114</td>
<td>0.154 ± 0.106</td>
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</table>

**FIG. 4. IgM antibodies to PGL-1.** The dotted line represent an OD 3 SD above the mean of the US-Cs. Open circles represent individual OD readings.
(40 μg of lysozyme per ml). Consequently, a minor error of even 0.5-mm in measurement produced a major (100%) error in the estimate of the lysozyme concentration. Such errors were minimized by photographing the plates and using enlarged prints for measurements, a rather clumsy procedure.

Elevated serum and urine lysozyme levels have been reported in many diseases, e.g., in monocytic and mononuclear leukemias (23), in renal dysfunctions (36), and in granulomatous diseases such as sarcoidosis (25), Crohn’s disease (4), leprosy (29), and pulmonary TB (26). In developing countries, the prevalence of infectious granulomatous diseases (e.g., in TB, leprosy, schistosomiasis, or leishmaniasis) greatly exceeds the prevalence of leukemias or nephrotic syndromes. Raised serum lysozyme levels have also been observed in subjects with AIDS and human immunodeficiency virus (HIV) lymphadenopathy syndrome (7), but it is unclear whether HIV antibody positivity alone increases the serum lysozyme concentration. The prevalence of HIV infection in developing countries varies, but it happens to be very low in Ethiopia (8), the source of the sera that we examined. We envisage the use of the lysozyme immunoassay as a screening test for infectious granulomas that include TB and leprosy. In certain populations, subjects with clinically overt HIV infection may also be identified. This screening process would identify individuals with active infections, who would be investigated further by using more specific tests (microbiological, serological, or histopathological) to determine the causative agent.

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