Enzyme-Linked Immunosorbent Assay Using Mycobacterium tuberculosis Antigen 5 and PPD for the Serodiagnosis of Tuberculosis*

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Enzyme-linked immunosorbent assays (ELISA) of immunoglobulin G antibody to Mycobacterium tuberculosis antigen 5 and tuberculin purified protein derivative (PPD) were assessed for the serodiagnosis of tuberculosis in 41 patients with active tuberculosis, 19 patients with inactive tuberculosis, and 59 healthy control subjects. Patients with active tuberculosis were studied serially at monthly intervals following the initiation of therapy. When contrasted with our earlier studies of sera from patients in Bolivia and Argentina, serum titers in Cleveland patients with active tuberculosis were somewhat lower. Geometric mean titer in patients with active tuberculosis was 1:68 with antigen 5 and 1:46 with PPD. Titer was correlated with patient age, male sex, extent of tuberculosis, and history of prior tuberculosis. However, these associations were not statistically significant. During monthly follow-up for 16 months after the initiation of therapy, ELISA titers remained essentially stable. Thus, no convincing evidence was acquired to support the hypothesis that higher titers in sera from South American patients related to more chronic or more extensive disease. Receiver operating characteristics of ELISA with antigen 5 were better than those obtained using PPD and were similar to those reported by others for sputum smear. In a situation where tuberculosis screening is warranted, ELISA with antigen 5 might have a place if it recognizes a different population than does sputum smear.

Many years of pessimism and negative experience with serologic tests for the diagnosis of tuberculosis have given way to cautious optimism on the part of investigators in several laboratories who are now exploring the use of enzyme-linked immunosorbent assays (ELISA) for the serodiagnosis of tuberculosis.\(^1\)\(^2\) Sensitivities of greater than 60 percent and specificities of greater than 90 percent have been reported, and projections of data to hypothetical populations of high prevalence, either representing those of developing countries or those of patients with disease processes suspected of being tuberculosis, indicate that accuracies of both positive and negative prediction are high.

In our experience, sera from patients with tuberculosis in Bolivia and Argentina have had higher geometric mean titers than sera from patients in the United States.\(^3\) Control subjects have similar low titers in all groups studied. The result of this difference is that ELISA provides a more accurate diagnostic test using sera from Bolivian and Argentinian patients than from North American patients. We hypothesized that this difference might be due to longer standing and more extensive disease in the patients whom we studied from South America. Since data were not available to classify the extent of disease in our previous studies, and since duration of tuberculosis is difficult to estimate with confidence, we undertook the present study in order to attempt to relate ELISA titers to patient characteristics including extent of pulmonary disease and duration of disease after initiation of therapy.

Most investigators have used tuberculin purified protein derivative (PPD) as the antigen for ELISA. We have previously found Mycobacterium tuberculosis antigen 5 to be a more satisfactory ELISA antigen,\(^7\) but this antigen is not readily available. In order to confirm our earlier observations, we included titrations using both antigens in this study.

Materials and Methods

Study Population

The 41 patients with active tuberculosis, 19 persons with inactive tuberculosis, and 59 control subjects entered into this study were enlisted prospectively as volunteers from among persons presenting during an 18-month period to the East Side Cuyahoga County Tuberculosis Clinic for the treatment of tuberculosis, for diagnostic study as cases of suspected tuberculosis, or for screening to establish the absence of tuberculosis. This clinic is located in an urban, impoverished neighborhood of Cleveland on an arterial road linking downtown Cleveland with major eastern suburbs. Services are provided for the entire county and include tuberculosis diagnosis and treatment, investigation of tuberculosis contacts, and screening.

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Serodiagnosis of Tuberculosis (Daniel, Debanne, van der Kuyp)
of such individuals as school teachers who are required to present evidence of freedom from tuberculosis for employment. The patient population was weighted both towards those persons most likely to contract tuberculosis and towards the medically indigent. The control population was more representative of the adult population of Cuyahoga County, but also included a substantial number of persons who were family contacts of patients with tuberculosis. Data collected at this clinic have demonstrated that approximately 15 percent of those who are seen for the evaluation of suspected tuberculosis ultimately are found to have active tuberculosis (van der Kuyp F; unpublished data).

Demographic data and clinical status data were collected on all patients on special sheets. Each subject was identified by a randomly assigned eight-digit number, and the serum from this person was identified only by this number and the date. Data sheets were delivered to the Department of Biometry, Case Western Reserve University School of Medicine, and demographic and clinical data entered into a computer file; no information on patient status was available in the laboratory performing the ELISA titrations.

Determination of the activity of tuberculosis and extent of pulmonary tuberculosis was performed at the time of entry into the study according to the 1969 diagnostic standards of the National Tuberculosis and Respiratory Disease Association.\(^6\) Among the 41 study subjects with active tuberculosis, four had minimal disease, 16 had moderately advanced disease, and 17 had far advanced disease. Two patients had disseminated tuberculosis, and one had extrapulmonary tuberculosis. One patient had silicotuberculosis. All diagnoses of tuberculosis were confirmed bacteriologically.

**ELISA Titrations**

Serum was separated from clotted blood promptly after collection and stored at −70°F until titered. The *M. tuberculosis* antigen\(^5\) and PPD\(^11\) prepared in the investigators' laboratory were used in the ELISA assay as described previously.\(^3,7\) ELISA titrations were performed as previously described,\(^3,7\) with endpoints being read using an automated plate reader. In brief, polystyrene microtiter plates were sensitized 48 hours or longer with antigen 5.5 µg/ml or PPD, 10 µg/ml in the presence of 1 percent glacial acetic acid and then quenched for one hour with 0.1 percent bovine serum albumin. Patient sera were then serially diluted in duplicate in the plate wells through the range of 1:20 to 1:12,800 in phosphate buffered saline (1 part .15 molar sodium phosphate buffer pH 7.2 and 9 parts .15 molar sodium chloride) containing 0.05 percent Tween-80 (polyoxyethylene 20 sorbitan monooleate), with final volumes of 50 µl using microtiter loops. After one hour of incubation, plates were washed three times with phosphate buffered saline containing Tween-90 (Tween-PBS), and 50 µl of 1.200 alkaline phosphatase-conjugated goat antihuman immunoglobulin G was added to each plate well. After one hour of incubation, plates were again washed three times with Tween-PBS, and finally 50 µl of disodium p-nitrophenyl phosphate substrate, 1 mg/ml in 0.05 molar sodium carbonate buffer pH 9.8 containing 0.02 percent magnesium chloride were added. Controls included on each microtiter plate included a standard color solution with an optical density of 0.24 and a 1:50 dilution of a positive reference serum. When the color developed by the reference serum was equal to the color standard, the plate was read on an automated plate reader at the ratio of 405/630 nm.

Results were expressed as the greatest serum dilution giving a color greater than that of the 1:500 reference serum. In order to permit calculation of geometric mean titers, sera which were negative at 1:20 were assigned an arbitrary end-point of 1. For statistical analysis, serial dilution data were normalized by log-arithmetic transformation using the formula \(x = \log_2(\text{reciprocal titer})/10\). Calculation of standard deviations, standard errors, correlation coefficients, and Student's t-test for nonpaired data or analysis of variance (ANOVA) were performed on the transformed data.

### Table I—Characteristics of Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Active Tuberculosis (n = 41)</th>
<th>Inactive Tuberculosis (n = 19)</th>
<th>Normal Controls (n = 59)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs ± SD</td>
<td>51.6 ± 18.8</td>
<td>53.9 ± 14.8</td>
<td>38.1 ± 14.8</td>
</tr>
<tr>
<td>Sex, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>61.0</td>
<td>73.7</td>
<td>42.4</td>
</tr>
<tr>
<td>Female</td>
<td>39.0</td>
<td>26.3</td>
<td>57.6</td>
</tr>
<tr>
<td>Race, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>19.5</td>
<td>68.4</td>
<td>37.3</td>
</tr>
<tr>
<td>Black</td>
<td>75.6</td>
<td>31.6</td>
<td>59.3</td>
</tr>
<tr>
<td>Hispanic</td>
<td>4.9</td>
<td>0</td>
<td>3.4</td>
</tr>
</tbody>
</table>

### Results

The age, sex, and race characteristics of the study population are presented in Table 1. Tuberculosis patients, active and inactive, were older than control subjects and more likely to be men, both characteristics probably reflecting the distribution of tuberculosis in the North American population. Blacks predominated among subjects with active tuberculosis. Patients with inactive tuberculosis were predominantly male, were older than the other subjects, and were more frequently white. This group included persons followed-up for many years who had developed tuberculosis at an earlier time. The apparent racial shift in tuberculosis probably reflects the changing demography of urban Cleveland.

The end-point titration data for all subjects are summarized in Table 2. Data obtained using both antigen 5 and PPD as antigens are presented. With both antigens, sera from persons with inactive tuberculosis had titers which did not differ significantly from those obtained in sera from control subjects. Patients with active tuberculosis had higher titers than did those with inactive tuberculosis and control subjects. This difference was greater using antigen 5 than using PPD.

Group averages of titers obtained at study entry of patients with active tuberculosis using both antigen 5 and PPD are presented in Table 3 for groups defined in terms of age, race, sex, extent of disease, and prior antituberculosis therapy. Although higher geometric mean titers were observed in older persons, men, persons with far advanced disease, and persons with a history of prior episodes of tuberculosis, none of the differences observed was found to be significant at the \(p = 0.05\) level using analysis of variance (ANOVA) procedures.

The ANOVA utilized patient based data; that is, the laboratory results at each patient's study entry were used to ensure that there would be data for each patient. Since study entry occurred at different lengths of time after the initiation of therapy for different patients, a graph of summary longitudinal data obtained on patients during the course of treatment for
tuberculosis is presented in Figure 1 to illustrate potential time dependency. The remarkable feature of these data is that with both antigen 5 and PPD, the geometric mean titers of patients under treatment remained essentially constant throughout the entire period of observation.

Since the sample of patients yielding data for any given number of months after the initiation of treatment is different for each month, an investigation of the potential interaction of time dependency and patient type was conducted by focusing on the average geometric mean titers of patients during the ninth to eleventh months after initiation of their therapy, or thereabouts (i.e., for patients with no visits in this period, their closest visit was used if it was within a month of the desired period and was consistent with their other visits). No evidence of interactional effects was detected here, as t-tests failed to indicate significant differences in average geometric mean titer of patients with active tuberculosis as a function of age, race, sex, or extent of disease, precisely the same result as that focusing on study entry regardless of when study entry occurred.

The study included eight patients with active tuberculosis who were followed-up for more than 18 months after the initiation of therapy. Of these, two had titers of 1:40 or less throughout the period of observation. Of the six remaining patients, four had a significant fall in titer occurring between 18 and 20 months. The remaining two patients had high and unchanged titers when last seen at 19 months after the initiation of therapy.

Tuberculin skin testing data were available for 57 of the 59 control subjects, and these data allowed examination of the relationship of ELISA result to the tuberculin reaction. In 23 subjects with a skin test reaction, \( \geq 10 \) mm, the geometric mean ELISA titer with antigen 5 was 1:13.1; in 23 subjects with skin test reactions \( \leq 9 \) mm, the geometric mean ELISA titer with antigen 5 was 1:13.0. For ELISA using PPD, these figures were 1:20.0 and 1:21.9, respectively. Thus, in healthy control subjects, there was no relationship between ELISA titer and tuberculin skin test reactivity. Skin test data were available for only five of the 19 subjects with inactive tuberculosis. Among the 41 patients with active tuberculosis, skin testing data were available for 18. All but one had positive reactions with all of these reactions \( \geq 10 \) mm. The mean reaction diameter was 17.3 mm. The correlation coefficient between skin test reaction diameter and ELISA titer was .31 with antigen 5 and .29 with PPD.

In order to provide an assessment of the reproducibility of the ELISA method, 234 of the study sera were titered on two occasions separated by one year, different technicians performing the two series of titrations. On each occasion, each serum was titered in duplicate against both antigen 5 and PPD. There was concordance within a range of ± one dilution for both the simultaneous duplicates and the two titrations at a
one-year interval for 219 (94 percent) of the sera tested. For six of the 15 discordant instances, the disagree-
ment was limited to one of the two antigens. For purposes of the present study, all of the sera with
discordant results were retitered, and the results in
agreement with the repeat titration used.

DISCUSSION

The ELISA titers to antigen 5 which were obtained
were very similar to those which we previously ob-
tained in sera from patients in Cleveland which had
been stored for many years. In the current study, the
geometric mean titer was 1:68; in the earlier study, it
was 1:64. In the earlier study, the sensitivity at a
dilution of 1:40 was 84 percent; in the present study, it
was 63 percent. This consistency of results occurred
despite the passage of two years, changes in personnel,
and minor changes in technique (principally including
the use of an automated plate reader) which occurred
between the two studies.

ELISA titers could not be associated with race. Titer
did not appear to be associated with age, sex, extent
of disease, and with a history of prior treatment for
tuberculosis (Table 3). However, none of these associa-
tions was statistically significant at the p = 0.05 level.
The constancy of titers during the first 16 months after
the initiation of therapy (Fig 1) was striking. Thus, we
found no conclusive evidence to support the hypo-
thesis that greater extent or greater chronicity of disease
in the patients whom we studied from South America
were responsible for higher ELISA titers. In our
earlier studies, Bolivian patients had a geometric mean
titer of 1:143 and Argentinian patients a geometric
mean titer of 1:75.7

Receiver operating characteristics derived from
projecting our present data to a population with a 15
percent tuberculosis prevalence (the prevalence
among tuberculosis suspects investigated at the East
Side Cuyahoga County Tuberculosis Clinic) are pre-
sent in Table 4. Results with both antigen 5 and PPD
are shown, and results based on dilution end-points of
both 1:40 and 1:80 are included. At both dilutions,
ELISA with antigen 5 provides a diagnostic test which
is more sensitive, more specific, and more accurate
than ELISA with PPD. In our prospective study in
Argentina,7 we concluded that ELISA with antigen 5 at
a serum dilution of 1:80 provided the best diagnostic
test because of its high specificity and consequently
small error of prediction. This remains true in the
present study, but sensitivity is only 49 percent,
reflecting the lower titers in Cleveland patients with
tuberculosis. At a dilution of 1:40, sensitivity is 63
percent, but specificity is lower and the accuracy of
positive prediction is only 57 percent.

Considering that ELISA can be done rapidly,
ELISA with antigen 5 might have a place in case
finding among tuberculosis suspects. Its accuracy and
sensitivity should be compared with those of the sputum
smear. Kim and his colleagues8 found that

Table 4—Receiver Operating Characteristics of ELISA
IgG Titers Projected to a Population with a 15% Prevalence
of Active Tuberculosis

<table>
<thead>
<tr>
<th>Antigen 5</th>
<th>PPD</th>
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<tbody>
<tr>
<td>1:80</td>
<td>1:40</td>
</tr>
<tr>
<td>1:80</td>
<td>1:40</td>
</tr>
</tbody>
</table>

Sensitivity, % | 48.5 | 63.4 | 31.7 | 56.1 |
Specificity, %  | 98.3 | 91.5 | 93.2 | 83.1 |
Accuracy of positive prediction, % | 83.5 | 56.9 | 45.2 | 36.9 |
Accuracy of negative prediction, % | 91.6 | 93.4 | 88.6 | 91.5 |
Error of prediction, % | 9.1 | 12.7 | 16.0 | 21.0 |

CHEST  /  88  /  3  /  SEPTEMBER, 1985  391
one-fourth of all culture-positive tuberculous patients had negative sputum smears. Lipsky and his colleagues found that the smear was positive in 65 percent of sputum samples which were subsequently positive on culture. In a study in India which considered many operational aspects, Bailey et al. found that sputum smear-based diagnosis was positive in 65 percent of cases.

In Kenya, Aluoch and his colleagues identified 601 persons with chronic cough or hemoptysis as tuberculosis suspects among 20,756 general medical clinical attendees. Among the suspects, 43 were found to have tuberculosis by all available diagnostic modalities, including one with a positive sputum smear but negative culture and normal x-ray film. If this latter case is considered a false positive, then the 13 smear positive cases provided a sensitivity of 31 percent among all cases of tuberculosis or of 65 percent among the 20 culture positive cases of tuberculosis. Specificity of sputum smear was 99.8 percent. If ELISA recognizes a different population of patients than does the sputum smear, then it could complement sputum examination in making early clinical decisions before culture results are available. The work of Zeiss and his colleagues suggests that this, indeed, may be the case.

In this study, we found antigen 5 to be a better antigen for use in ELISA than PPD. This repeats our earlier observations. We again included PPD in our study because of the more ready availability of PPD. Other workers, notably Kalish, Radin, Zeiss and their colleagues, have reported favorable experiences with PPD.

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