

Sensitivity and Specificity of Enzyme-Linked Immunosorbent Assay in the Detection of Antigen in Tuberculous Meningitis Cerebrospinal Fluids

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A sandwich enzyme-linked immunosorbent assay was developed for its potential utility in the detection of antigen in the cerebrospinal fluid of patients with tuberculous meningitis. Cerebrospinal fluids examined included those from untreated (group Ia) and treated (group Ib) *Mycobacterium tuberculosis* meningitis, nonseptic central nervous conditions (group II) such as epilepsy, viral meningitis, and tetany, and nonmycobacterial septic meningitis (group III). The average levels of antigens determined and percent positive specimens, respectively, for each group were (group): Ia, 1.8 µg/ml and 75% positive; Ib, 0.37 µg/ml and 36% positive; II, 0.036 µg/ml and 100% negative; and III, 0.075 µg/ml and 100% negative. The system developed employed hyperimmune polyclonal antibody raised against *M. tuberculosis* and *Mycobacterium bovis* BCG in burros and rabbits. Cross-reactivity by other mycobacterial species was very low; e.g., 5% for *M. kansasii* and less than 2% for *M. intracellulare*, *M. avium*, *M. vaccae*, and *M. fortuitum*. The test shows promise as a specific adjunct for the early diagnosis of tuberculous meningitis.

Tuberculous meningitis continues to pose a diagnostic problem, especially in children in developing countries. Failure to diagnose the disease promptly in its early stages results in an increase in severity and involvement. Isolation of organisms from cerebrospinal fluid (CSF) has been disappointingly infrequent (4) and time consuming. Sensitive tests such as radioimmunoassays and enzyme-linked immunosorbent assays (ELISAs) for the detection of *Mycobacterium tuberculosis* antigen (8) or antibody (10) appear to be promising procedures for the early diagnosis of tuberculous meningitis.

In the present study, 38 CSF specimens from patients with diagnosed tuberculous meningitis and 52 of nonmycobacterial etiology were examined for the presence of *M. tuberculosis* antigen by a sensitive and specific sandwich ELISA technique.

MATERIALS AND METHODS

Antigens. Antigens were prepared from mycobacteria obtained from the Trudeau Mycobacterial Collection and included the following species and Trudeau Mycobacterial Collection numbers: *M. tuberculosis* H37Rv (102) and Erdman (107), *M. intracellulare* (1403, serovar MAIS 14), *M. avium* (716, serovar MAIS 1), *M. kansasii* (1204), *M. vaccae* (1526), and *M. fortuitum* (1529). The Trudeau collection is currently housed at the American Type Culture Collection in Rockville, Md. Organisms were grown in Long synthetic medium (6) and harvested as young, actively growing cultures either after complete pellicle formation or when growth appeared sufficient. Cultures were collected on a Büchner funnel, washed with distilled water, sonicated as a 50% suspension at 350 W for 15 min, and centrifuged as previously described (2, 3). Protein concentration of the sonic extracts was measured by the method of Lowry et al. (7).

Antisera. A reference anti-*M. tuberculosis* serum prepared

for the World Health Organization was made by immunizing burros with an emulsion containing heat-killed *M. tuberculosis* (Erdman) cells. The emulsion consisted of equal parts of saline and a 35:65 mixture of Arlacel A and Drakeol 6VR. Each milliliter contained 10 mg of dry *M. tuberculosis* cells. Booster injections of cell sonic extract antigens were given weekly until a maximum number of precipitins was produced when examined by rocket immunoelectrophoresis (2). Plasmapheresis was initiated once maximum antibody production was observed. Plasma was treated with 2 M CaCl₂ to obtain serum.

Anti-*Mycobacterium bovis* BCG rabbit serum B-124 was obtained from Dakopatts Inc., Copenhagen, Denmark. Anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase, anti-rabbit IgG-biotin conjugate, avidin-alkaline phosphatase, and *p*-nitrophenyl disodium phosphate were all obtained from Sigma Chemical Co., St. Louis, Mo.

CSF samples were obtained from 90 subjects from India and classified as follows. Group I consisted of 38 samples from subjects diagnosed as having tuberculous meningitis. Four of these infections were proven bacteriologically by showing the presence of acid-fast bacilli on microscopy, and the others were diagnosed on the basis of clinical presentation and CSF changes showing a moderate protein rise, lymphocyte cellular infiltration, and reduced sugar and chlorides. Along with CSF findings, there was supporting evidence of a strongly positive Mantoux test, X-ray evidence of pulmonary tuberculosis, a strong family history of exposure to *M. tuberculosis*, failure to grow bacterial cultures, response to antituberculosis treatment, and nonresponsiveness to other routine antibiotics. Group II consisted of 22 control samples from patients with febrile convulsions, epilepsy, viral encephalitis, and tetany. None of these had evidence of primary tuberculosis. Group III consisted of 30 samples from patients with symptomatic septic nonmycobacterial meningitis (provided by Carl Frasch, Office of Biologics, Research and Review, U.S. Food and Drug

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Administration, Bethesda, Md.). All of the group III samples were purulent, with high protein and cell concentrations, and most were from patients with proven bacterial meningitis.

ELISA procedures. Two types of double antibody sandwich ELISA procedure were developed. The first procedure was carried out as follows. Dynatech Immulon I plates were coated with 100 μ l of a 1:1,000 dilution of anti-*M. tuberculosis* burro serum in Tris hydrochloride buffer (pH 8.5) overnight at 28°C. After three washes with saline-Brij 35, the plates were incubated with 100 μ l of *M. tuberculosis* sonicate antigen. To study cross-reactivity with other mycobacterial species, at this step we used sonicate antigens of *M. intracellulare*, *M. avium*, *M. kansasii*, *M. vaccae*, or *M. fortuitum* instead of *M. tuberculosis*. Phosphate-buffered saline with 1% bovine serum albumin and 0.1% Brij 35 was used to make the dilutions. Incubation was carried out at 28°C for 2 h. After three saline washes, anti-BCG rabbit serum at 1:1,000 dilution was added and incubated for 2 h at 28°C. This was followed by sheep anti-rabbit IgG alkaline phosphatase conjugate and incubated for another 2 h at 28°C. Absorbance at 410 nm was read 30 min after the addition of *p*-nitrophenyl disodium phosphate substrate in an ELISA reader (Dynatech Industries, Inc., McLean, Va.). This procedure is referred to below as sandwich ELISA.

The biotin-avidin procedure, referred to as biotin-avidin ELISA, was used to improve sensitivity (1). The procedure is essentially as described above except that anti-rabbit IgG-biotin conjugate was added after incubation with anti-BCG antibody. This was followed by avidin alkaline phosphatase and substrate. Dose response curves were constructed with each of these antigens and compared with *M. tuberculosis* standard curves. For the test to detect antigens, 1:2 dilutions of CSF were used. Standard curves with *M. tuberculosis* antigen concentrations ranging from 4.5 ng/ml to 5.0 μ g/ml were generated. Concentrations of antigen in CSF were determined in micrograms per milliliter by referring to the standard curve (11).

RESULTS

Figure 1 shows the dose response curves with *M. tuberculosis* sonicate antigen. The sensitivity of detection was calculated as follows. The mean and standard deviation of optical densities observed with a zero antigen dose were calculated. The lowest dose showing an optical density above the mean of the zero dose plus three standard deviations was considered the sensitivity of the assay. This was 3 ng/ml for the biotin-avidin ELISA and 15 ng/ml for the sandwich ELISA.

Specificity studies were done by substituting *M. tuberculosis* sonicate with sonicates from other mycobacteria, i.e., *M. intracellulare*, *M. avium*, *M. kansasii*, *M. fortuitum*, and *M. vaccae*. These species were chosen to include a broad group, of which *M. kansasii* and *M. intracellulare* are known to cross-react with *M. tuberculosis* to a much higher degree than the Runyon group 4 species of *M. vaccae* and *M. fortuitum*. Figures 2 and 3 show the cross-reactivity patterns obtained with the sandwich and biotin-avidin ELISAs, respectively. Percent cross-reactivity was calculated as follows. The amount of cross-reacting antigen was estimated from the *M. tuberculosis* curve by extrapolation. This was divided by the determined amount of total protein added and multiplied by 100. The sandwich ELISA showed a maximum cross-reactivity of 5% with *M. kansasii* and only 2% with other mycobacteria. With the biotin-avidin ELISA, however, there was 22% cross-reactivity with *M. intracellulare* and up to 10% with other antigens. Although the biotin-

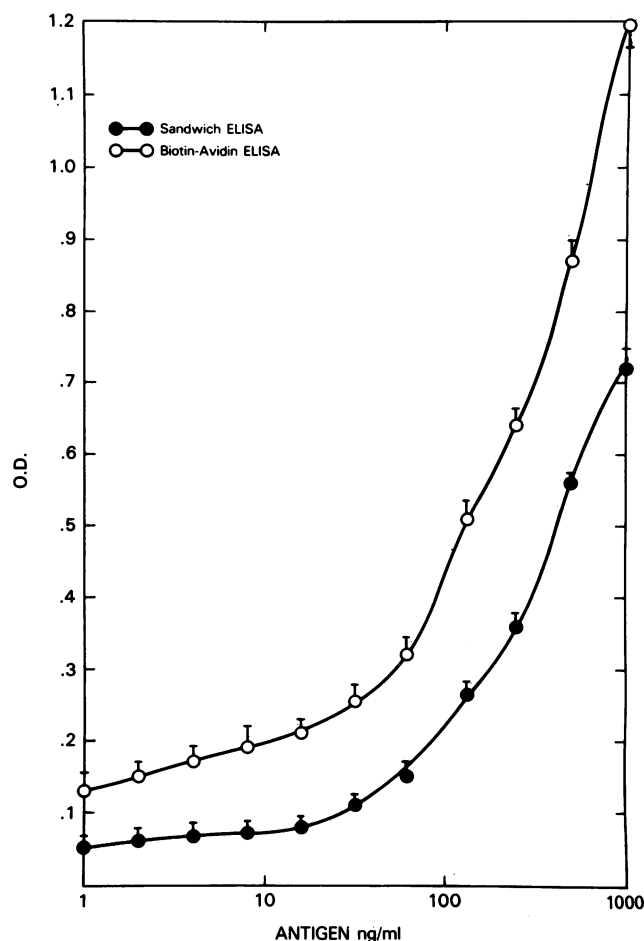


FIG. 1. Comparison of dose response curves between sandwich ELISA and biotin-avidin ELISA. O.D., Optical density.

avidin procedure was five times more sensitive, it was also considerably less specific. Because of its greater specificity, the sandwich ELISA was used to detect tubercular antigens in CSF samples (Fig. 4).

Table 1 shows that the 22 nontuberculous, nonbacterial meningitis CSF control samples (group II) showed a mean antigen concentration of 0.036 ± 0.037 μ g/ml, whereas 30 symptomatic cases of nonmycobacterial septic meningitis (group III) showed an average antigen concentration of 0.075 ± 0.060 μ g/ml. We used the mean plus three standard deviations of group III as the upper limit of negativity. Values up to 0.25 μ g/ml were considered negative, and levels above this were considered positive for antigen. Of the 38 samples obtained from those meeting the diagnostic criteria for tuberculous meningitis, 23 were above the 0.25- μ g/ml level for positivity, whereas the remaining 15 were negative.

Further analysis of these 38 samples showed the effect of chemotherapy on antigen levels. At the time the samples were collected, 24 untreated subjects had signs typical of tuberculous meningitis, and 75% of these CSF samples were positive, ranging from 0.3 to 10 μ g/ml. Fourteen had been treated and were free of signs of meningitis, and only 5 of these 14 treated patients had positive antigen levels (Table 1).

DISCUSSION

The detection and identification of antigens of *M. tuberculosis* in pathologic specimens such as CSF adds a powerful

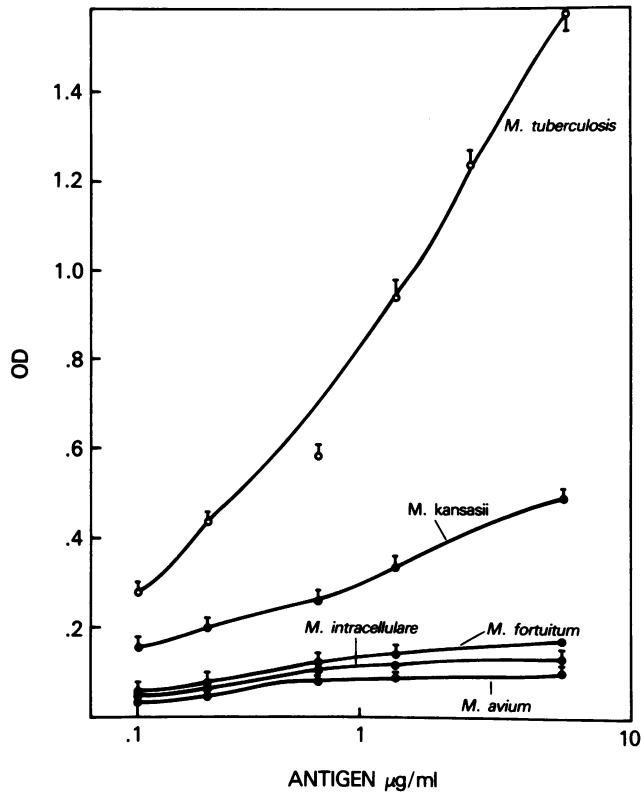


FIG. 2. Cross-reactivity between *M. tuberculosis* and other mycobacterial antigens by sandwich ELISA. OD, Optical density.

criterion for the diagnosis of tuberculosis. In this study, we describe an ELISA procedure which has a high degree of sensitivity and specificity. Two groups of controls were studied, one with encephalitis, tetany, viral meningitis, etc.,

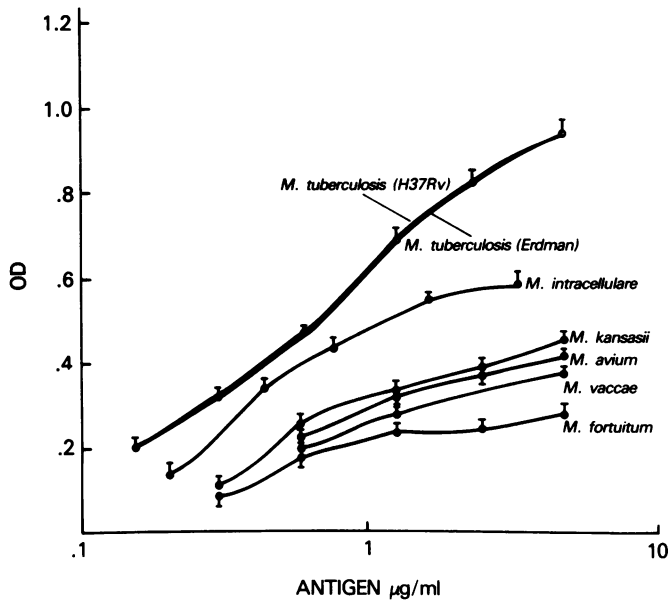


FIG. 3. Cross-reactivity between *M. tuberculosis* and other mycobacterial antigens by biotin-avidin ELISA. OD, Optical density.

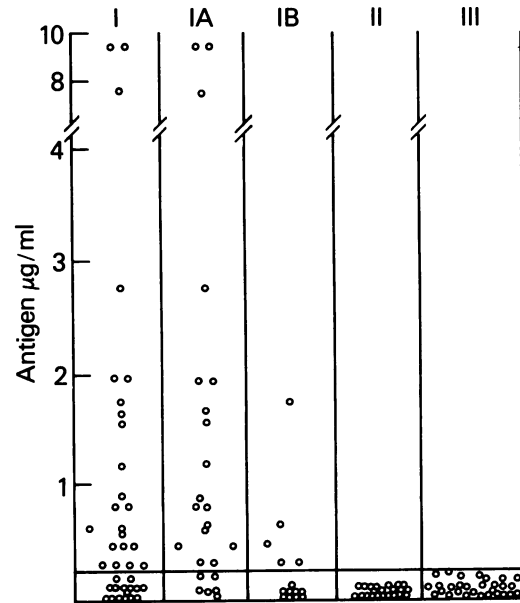


FIG. 4. Antigen levels in CSF samples. Columns: I, Tuberculous meningitis; Ia, untreated tuberculous meningitis; Ib, treated tuberculous meningitis; II, nonseptic meningitis; III, septic meningitis.

in which the IgG content in CSF was lower, and the other with symptomatic septic meningitis, in which the IgG concentration was known to be higher. Both groups showed low reactions in the ELISA, indicating that there was no appreciable cross-reactivity due to increased immunoglobulins in CSF. By using the mean of the septic meningitis control group plus three standard deviations as the upper limit of negativity, we saw no false positivity.

ELISA readings reflecting positive tuberculous antigen values greater than 0.25 µg/ml could be detected in 75% of the 24 samples of untreated tuberculous meningitis. With treatment, however, the level of antigen detection decreased to 35.7%. It appears that ELISA may be helpful in charting the effectiveness of treatment and the prognosis. It is highly likely that all the positive reactions with high values were due to *M. tuberculosis*. That the reactions were specific for *M. tuberculosis* is supported by a comparison of dose response curves with those of several other species of mycobacteria. *M. kansasii* cross-reacted to a level of only 5%, and *M. intracellulare*, *M. avium*, *M. fortuitum*, and *M. vaccae* cross-reacted only 2%. However, since nontuberculous mycobacterial meningitis is rare, we were not able to determine this in a population of patients.

Sensitivity could be increased about fivefold to a capability of 3 ng of protein per ml by the use of a biotin-avidin

TABLE 1. *M. tuberculosis* antigen levels in CSF samples

Group or subgroup	No. of samples	Mean antigen Conc (µg/ml ± SD)
I Tuberculous meningitis	38	1.5 ± 2.4 ^a
Ia untreated	24	1.8 ± 2.9 ^a
Ib treated	14	0.37 ± 0.54 ^a
II Nonseptic meningitis	22	0.036 ± 0.037
III Septic meningitis	30	0.075 ± 0.060

^a P < 0.01 compared with group III.

procedure, but this method was considerably less specific. At a sensitivity of 15 ng/ml, the sandwich ELISA proved to be highly useful and accurate.

Kadival et al. (5) and Samuel et al. (9, 10) have shown that antigen or antibody detection is useful in the diagnosis of pulmonary, meningeal, pleural, and abdominal tuberculosis by radioimmunoassay. In a limited study of 10 CSF samples from patients with tuberculous meningitis, Sada et al. (8) showed the potential usefulness of ELISA in the detection of antigen.

The retrospectively determined high correlation between the demonstration of tuberculous antigen in CSF and active tuberculous meningitis warrants the application of the ELISA technique as early as possible for the diagnosis of suspected cases of the disease.

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