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Sensitive Enzyme Immunoassay for Early Diagnosis of Tuberculous Meningitis

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Cerebrospinal fluid from patients with tuberculous, pyogenic, and viral meningitis, as well as from appropriate control individuals, were assayed for immunoglobulin G and immunoglobulin M antibody activity to *Mycobacterium bovis* BCG by an enzyme-linked immunosorbent assay. BCG linked covalently to plastic disks served as the antigen in a classical indirect enzyme-linked immunosorbent assay. A significant difference was found between the tuberculous meningitis group and the nontuberculous meningitis and control groups. All samples from the tuberculous meningitis group gave a positive reaction, and none of the known negative samples gave false-positive reactions. Because of its sensitivity, specificity, and predictive value, this test may be useful in the early diagnosis of tuberculous meningitis.

The diagnosis of tuberculous meningitis, the most common cause of death in tuberculous children, is usually based on clinical, radiological, cytochemical, and bacteriological data, but these procedures are either nonspecific or time consuming. Several laboratory tests have been designed as tools for the early diagnosis of this disease, yet none has been satisfactory (2, 5–7, 10). Early recognition of central nervous system (CNS) tuberculosis therefore remains a challenge for clinicians.

Inflammatory disorders of the CNS are usually accompanied by local immunoglobulin synthesis (4). In tuberculous meningitis there is a vigorous humoral immune response within the CNS characterized by intrathecal synthesis of oligoclonal immunoglobulins. The cerebrospinal fluid (CSF) of these patients contains antibodies directed against *Mycobacterium tuberculosis* that also react against *Mycobacterium bovis* BCG (3). The enzyme-linked immunosorbent assay (ELISA) has proven to be highly sensitive and specific and can be applied to serodiagnosis of numerous infectious and parasitic diseases (9). In this paper we describe an ELISA system which simultaneously detects immunoglobulin G (IgG) and IgM CSF antibodies against BCG linked covalently to special plastic disks. The sensitivity, specificity, and efficiency of this technique may render it a valuable tool for the early diagnosis of tuberculous meningitis.

MATERIALS AND METHODS

Positive samples. Twenty CSF samples were obtained in the second or third weeks of disease from different patients with a bacteriological diagnosis of tuberculous meningitis. The results of the cytochemical analysis suggested tuberculous meningitis in only 75% of the cases. All the patients were under 16 years of age.

Control samples. This group comprised 31 patients with acute pyogenic meningitis, 20 patients with viral meningitis, and 19 children without infection in the CNS, from whom CSF samples had been obtained for diagnostic purposes. The bacteria isolated in the pyogenic meningitis group were *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Proteus mirabilis*, *Streptococcus faecalis*, and *Salmonella reading*.

Reproducibility of the test. Twenty-five CSF samples which had absorbance values between 0.015 and 1.99 were tested in triplicate (replicates R1, R2, and R3) in a double-blind study to establish the intraassay reproducibility of the ELISA method. The data were analyzed by the paired *t*-test.

Antigen. BCG, lot 01-83-16-81, was kindly supplied by P. Atanasiu, Institut Pasteur, Paris. The optimum antigen concentration for coating the solid phase was determined as described by Ruitenberg et al. (8) by resuspending the lyophilized BCG (6 mg [dry weight]) in 0.1 M bicarbonate buffer (pH 9.5). Antigen to a final concentration of 20 μg/ml was added to plastic disks made from a poly(tetrafluoroethylene)styrene graft copolymer substituted with isothiocyanate groups (Cordis Laboratories, Inc., Miami, Fla.) (1 ml per disk). Disks were incubated overnight at 8°C with agitation, after which they were washed with 0.01 M phosphate-buffer saline (pH 7.4) and incubated at 8°C for 60 min in a 0.2% water solution of bovine serum albumin (2 mg/ml) to block any free isothiocyanate groups. Disks were then rinsed with phosphate-buffered saline and lyophilized. The latter dry, coated disks have been stored at 4°C in the presence of a desiccant for up to 6 months without change in activity.

Enzyme-conjugated anti-immunoglobulin. A mixture of goat antibodies to human IgG and IgM labeled with alkaline phosphatase was obtained from Cordis Laboratories. The optimum conjugate concentration was determined by standard titration methods (8).

ELISA test procedure. For each assay 100 μl of the sample was diluted in 400 μl of diluent (6% bovine serum albumin, 0.05% Tween 20 in phosphate-buffered saline). A disk coated with BCG was added to each sample and incubated with continuous shaking for 60 min at 37°C. Disks were then washed six times with 2.5 ml of phosphate-buffered saline by using a Cordis disk washer (Cordis Laboratories). Disks were then incubated for 60 min at 37°C with 0.5 ml of the enzyme-conjugated immunoglobulins. The disks were again washed as described above and transferred to new vials to reduce background from nonspecific binding of enzyme conjugate to the surface of the glass vials. The enzyme reaction was started by the addition of 0.5 ml of *p*-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine buffer (pH 9.8) containing 0.001 M $MgCl_2$. After incubation for 15 min at 37°C the reaction was stopped by the addition of 0.1 ml of 3 M NaOH, and the absorbance was read at 405 nm

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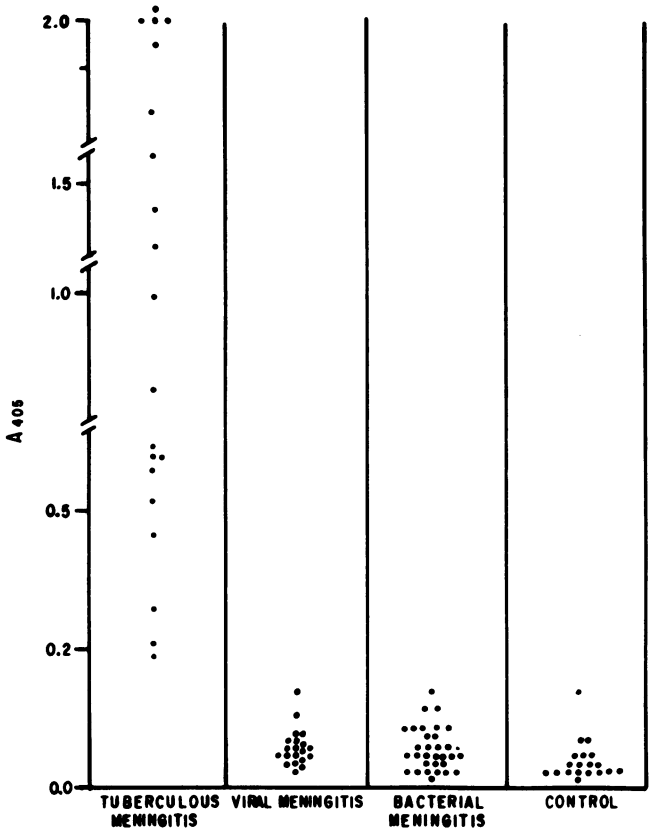

FIG. 1. Distribution of IgG and IgM antibody levels to *M. tuberculosis* in CSF from tuberculous meningitis and control groups.

against a blank of substrate. The entire procedure takes ca. 3 h.

Statistical analysis. Sensitivity, specificity, efficiency, and predictive value were evaluated as recommended by Galen and Gambino (1).

RESULTS

The distribution of the individual values for optical density at 405 nm for the different groups studied is presented in Fig. 1 and Table 1. Absorbances for the tuberculous meningitis group (20 cases) ranged from 0.233 to 2.00 with a mean value of 1.108 (Table 1). For the viral meningitis (20 cases), bacterial meningitis (31 cases), and control (19 cases) groups, optical densities were all below 0.200, and the means ranged from 0.046 to 0.076 (Fig. 1; Table 1).

Standard errors, confidence limits, and variation coefficients for these groups are presented in Table 1. The confidence limits showed that for the positive samples the probability of the absorbance at 405 nm being lower than 0.643 was less than 0.003; likewise, for the bacterial meningitis, viral meningitis, and control groups, the probability of the absorbance readings being greater than 0.101, 0.09, and 0.073, respectively, was lower than 0.003 (Table 1).

TABLE 2. Confidence intervals for the mean differences among three absorbance values for 25 randomly selected CSF samples tested in triplicate for intra-assay reproducibility (two-tailed *t* test)

Sample[a]	Mean ± SEM	Confidence interval (0.01)	Range
D12	0.014 ± 0.023	0–0.04	−0.45–0.27
D31	0.023 ± 0.016	0–0.06	−0.13–0.28
D32	0.013 ± 0.019	0–0.06	−0.20–0.36

[a] D12 is R1 minus R2; D31 is R3 minus R1; D32 is R3 minus R2. R1, R2, and R3 are the replicates of each sample.

For the intra-assay reproducibility test the three possible mean differences (i.e., D12, D31, and D32) were calculated, together with their standard errors and confidence intervals (Table 2). These results indicate no significant differences among replicates. Interassay reproducibility evaluated by running three tests on different days with CSF samples from eight patients with tuberculous meningitis and 10 patients randomly selected from the control group showed an average coefficient of variation of 6.6%.

DISCUSSION

Simultaneous detection of IgG and IgM antibodies against BCG with the use of covalently attached antigen gave a sensitivity of 100% in our ELISA. We believe this is due to simultaneously decreasing nonspecific binding of antibodies and increasing sensitivity. The first effect would be achieved by covalent binding of antigen to the activated solid phase and then blocking of the remaining active groups. The second effect would result from an increase in the actual number of antibody molecules bound in the reaction.

No overlapping of values from positive and negative samples was observed (Fig. 1). A mean of 0.058 and a standard deviation of 0.037 were obtained for all of the control groups, and none of the positive samples gave absorbance values below 0.233. Therefore, absorbance values above 0.2 can be considered as ELISA positive since this value is significantly higher (>2 standard deviations) than the control mean values. Selecting such an absorbance value as the cutoff point allows detection of positive samples over the wide range of antibody responses seen in these patients (Fig. 1).

Although it was not possible to include a BCG-vaccinated CSF control group, we estimated that about 30% of the patients from the various control groups had been vaccinated with BCG. Moreover, in our country 30% of children above 5 years of age and 60% of the adult population are positive for purified protein derivative as a result of primary infection. The marked difference in antibody activity between samples from tuberculous meningitis and the other groups studied may indicate, as proposed by Kinkman et al. (3), that antibodies present in CSF are synthesized within the

TABLE 1. Results of ELISA assays used to detect IgG and IgM antibody activity to *M. tuberculosis in* CSF samples

Group (no. of samples)	Mean ± SE	Two-tailed confidence limit (0.997)	Range	Coefficient of variation
Tuberculous meningitis (20)	1.108 ± 0.155	0.643–1.751	0.233–2.0	61.3
Bacterial meningitis (31)	0.076 ± 0.010	0.051–0.101	0.026–0.172	52.6
Viral meningitis (20)	0.066 ± 0.008	0.042–0.09	0.028–0.172	54.5
Control (19)	0.046 ± 0.009	0.019–0.073	0.013–0.188	85.2

CNS, precluding interference of serum antibodies present in patients with previous exposure to *M. tuberculosis*.

Based on the predictive value model, the analysis of our data showed that sensitivity and specificity were 100% (1). Accordingly, if sensitivity equals specificity, test efficiency is independent of prevalence and equal to sensitivity. Also, when the specificity of a test is 100%, the predictive value is 100%.

Concerning the cost-effectiveness of the test, it must be pointed out that preliminary experiments with polystyrene microtiter plates as an alternative to the disk method indicated poor absorption characteristics for BCG and also that desorption may occur. In addition the plate method requires expensive readers and washing devices and is best suited for processing large numbers of samples. The disk method requires less expensive equipment, is more suitable for low-volume applications, and, above all, offers the advantages of a covalent method of solid-phase attachment.

In conclusion, the ELISA procedure described here for the simultaneous detection of IgG and IgM antibodies to *M. tuberculosis* in CSF appears to be very sensitive and highly specific with a high predictive value. Anti-tuberculous IgG and IgM CSF determination by ELISA may be a useful method for the early diagnosis of tuberculous meningitis.

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