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Enzyme immunoassay using BCG in serodiagnosis of pulmonary tuberculosis

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SUMMARY

Amounts of *Mycobacterium tuberculosis* antibodies were determined in sera from patients with either active or inactive tuberculosis and in healthy subjects by an immunoenzymatic assay in which whole BCG cells attached covalently to polystyrene disks were used as antigen. Statistically significant differences ($P < 0{\cdot}005$) were found both between the active and inactive tuberculosis groups and between the active group and healthy controls. No significant differences were found between the inactive group and controls. Since this procedure is efficient (91 %) and can be used in areas which lack laboratory equipment, it appears promising for individual serodiagnosis and for epidemiological surveys.

INTRODUCTION

Pulmonary tuberculosis is still the major granulomatous disease in developing countries and its diagnosis depends largely on a positive tuberculin test and on clinical, radiological, and bacteriological evidence. However, it is known that the tuberculin test does not become positive in all tuberculosis patients (false negatives) and that a positive dermal test can represent merely the existence of a previous exposure and the consequent sensitization against *Mycobacterium tuberculosis* (false positives). Radiology appears to be more sensitive but less specific than is bacteriology (culture of bacilli and acid fast staining). Hence, there is still a need for an assay which would be capable of differentiating patients with active and inactive tuberculosis from each other and from healthy persons. Since many reports have recently demonstrated the usefulness of detection of anti-myobacterial antibodies in sera for the diagnosis of tuberculosis infection (Nicholls, 1975; Nassau, Parsons & Johnson, 1976; Winters & Cox, 1981) many assays have

been devised and, among these, immuoenzymatic assays have been demonstrated to be sensitive and specific (Kárdjito & Grange, 1980; Grange, Gibson & Nassau, 1980; Tandon, Saxena & Saxena, 1980; Kalish *et al.* 1983, Hernández, Muñoz & Guiscafre, 1984). However the major problem of such assays is the need for certain specialized personnel and equipment which usually are not available in rural areas, especially in developing countries. In this work, we describe an improved immunoassay to detect antibacterial antibodies by using whole BCG cells covalently attached to polystyrene disks which are easily manipulated and give rapid results. The efficiency of the method appears to be sufficiently high to be used for individual diagnosis and in screening studies.

MATERIAL AND METHODS

Samples

Sera were obtained from 40 patients with clinical and radiological evidence of pulmonary tuberculosis and all were smear-positive by auraminerhodamine staining at least 3 months before collection of samples. From these data, this group was considered to have active tuberculosis. Fifteen samples obtained from individuals with inactive tuberculosis as demonstrated by smear-negative tests (patients who have had the disease in the past and only show calcified lesions on X-ray) were also included in this study. Sera from 30 healthy subjects were used as controls. Sera were separated by centrifugation and stored at −40 °C until tested.

Antigens

M. bovis BCG (Danish 1331 strain) was kindly supplied by Dr Jorge Gómez, Instituto Nacional de Higiene, S.S.A. The solid phase was prepared as described previously (Hernández, Muñoz, & Guiscafre, 1984). In brief, BCG (20 μg/ml) suspended in 0·1 M bicarbonate buffer (pH 9·5) was incubated (18 h; 9 °C, gentle shaking) with disks made form a polystyrene copolymer which had been substituted with isothiocyanate groups (Cordis Laboratories Inc. Miami, Florida). The disks were then washed with 0·01 M phosphate-buffered saline (PBS) and then a solution of 2% bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, Mo.) in the same bicarbonate buffer, was added to block free isothiocynate groups. Thereafter, disks were washed in PBS, dried under vacuum and stored at 4 °C in the presence of a desiccant.

Enzyme-anti-immunoglobulin conjugate

Goat anti-human IgG and IgM serum (Helena Labs., Beumont, Texas) was coupled to horseradish peroxidase (Sigma Chemical Co., St Louis, Mo.) by the periodate method (Nakane, 1974) and stored at 4 °C until used. The optium conjugate concentration was determined as described previously (Ruitemberg *et al.* 1976). Working conjugate solution was a mixture of anti-human IgG diluted 1:2000 and anti-human IgM diluted 1:1000 in PBS containing 0·5% BSA and 0·05% Tween 20 (PBS-BSA Tw).

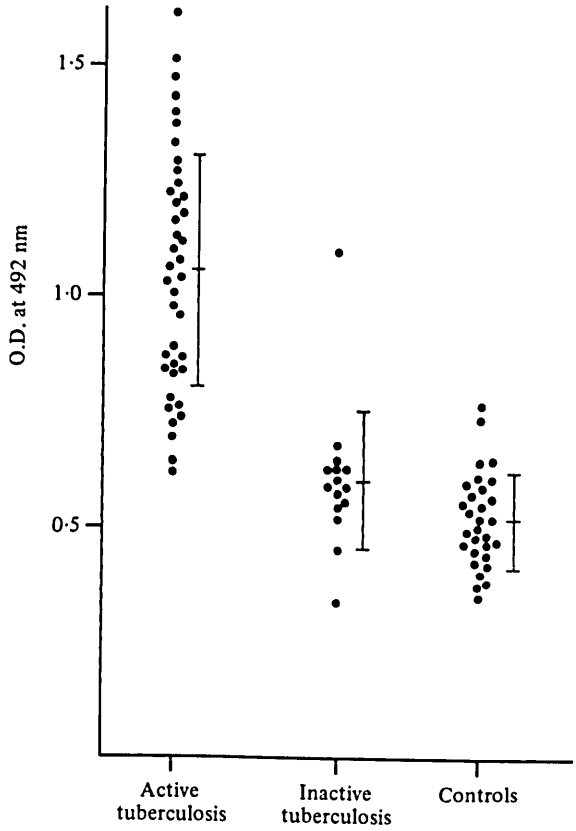

Fig. 1. Distribution of individual mycobacterial antibody values of each group, expressed as optical density. The mean and the S.D. are indicated. The statistical differences between the groups are indicated in the text.

ELISA test procedure

Five microlitres of each serum sample was diluted in 495 μl of PBS-BSA Tw, in a glass vial. A BCG-coated disk was added to each vial and incubated (60 min; 37 °C; with shaking). The disks were then washed with PBS and incubated with 0·5 ml of the conjugate for 30 min, as above. The disks were again washed and transferred to clean vials to eliminate nonspecific conjugate binding to the surface of the glass vial. Ten microlitres of substrate (0·05 % *o*-phenylenediamine (OPD) and 0·05 % H_2O_2 in 0·1 M citrate-phosphate buffer, pH 5·0) was added to each disk and incubated for 30 min at 37 °C. The reaction was stopped by addition of 100 ml of 6 M-H_2SO_4 and the optical density was read at 492 nm with substrate used as a blank. The optical density (O.D.) values were considered to be representative of the relative antibody amounts (Kalish *et al.* 1983; Hernández, Muñoz, & Guiscafre, 1984).

Statistical analysis

The statistical significance of the differences between groups was determined by Student's *t* test. Analysis of the specificity, sensitivity, positive predictive value, negative predictive value and efficiency of the method was done with the use of predictive value theory developed by Galen & Gambino (1975) for clinical surveys.

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RESULTS

Serum from patients with active (smear-positive) pulmonary infection due to *M. tuberculosis* had greater mean antibody levels to BCG than did healthy subjects ($P \leqslant 0{\cdot}005$). The active group also showed statistically significant differences compared with the group of patients with inactive tuberculosis. The latter group, although having higher mean antibodies levels than did the healthy controls, did not differ significantly from the control group. The distribution for optical density for the different groups studied is shown in Fig. 1.

The anti-mycobacterial activity of the sera could not be associated with the clinical or radiological findings or with the time or type of treatment or the evolution of the disease.

Analysis of the efficiency of this method of serodiagnosis showed a sensitivity of 100 % and a specificity of 80 %. The positive predictive was 80 %, the negative predictive value was 100 %. Therefore, the efficiency of the method for this study was 91 %. All data were obtained by using an O.D. of 0·60 as the cut-off point (see Fig. 1).

DISCUSSION

The presence of *M. tuberculosis* antibodies in body fluids of tuberculosis patients has been considered to be a possible basis for diagnosis because it correlates better with the active infection than does the cellular response detected by tuberculin reaction (Kardjito & Grange, 1980). Many assays have been proposed and those of greater sensitivity have shown to be more specific. However, such methods require technical and laboratory resources such as plastic plaques, a special spectrophotometer, etc. Hence, the feasibility of applying this method in endemic rural areas appear to be minimal. Results of the present work showed that disks to which BCG had been covalently attached can be used for the detection of serum antibodies with an efficiency similar to that of the plaque method. Due to the fact that the coated disks, after being dried, did not show changes in their activity and efficiency for up to 6 months (Hernández, Muñoz, & Guiscafre, 1984), that the intra-assay reproducibility has been demonstrated (Hernández, Muñoz & Guiscafre, 1984), and that the ease of manipulation permits utilization of each disk separately for individual tests, we propose that this improved method can be used in areas which lack optimal laboratory conditions.

Data concerning the predictive values and the statistical differences between groups confirmed that the mycobacterial antibody titres in serum correlated with the existence of the active infection, as was reported elsewhere (Kalish *et al.* 1984). Recent reports have shown that, in a group of serum samples, the antibody titres determined by using p.p.d., *M. tuberculosis* (H37Rv), BCG cell walls, or BCG whole cells, did not vary significantly (Winters & Cox, 1981). This implies that our results are as valid as those from assays in which tubercle bacilli, or their products, were used. Additionally, the use of whole BCG as antigen eliminates several steps, including that of the manipulation of virulent *M. tuberculosis*.

An interesting observation in our results was that, in the groups of active infection patients, the distribution of antibody titres showed a bimodal frequency curve. This

result could be correlated with neither clinical nor laboratory finding. The possibility that this event may be related to the absence of anti-mycobacterial cellular immunity, detected by methods *in vitro*, is currently being studied in our laboratory.

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