False-positive reactions with enzyme-linked immunosorbent assay of *Mycobacterium tuberculosis* antigens in pleural fluid

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**Summary.** The value of enzyme-linked immunosorbent assay (ELISA) for the diagnosis of tuberculous pleural effusion has not been defined. We performed ELISA by a double antibody sandwich technique with anti-BCG antibody in the solid phase to detect *Mycobacterium tuberculosis* antigen in pleural fluid from 36 patients with pleural effusion (tuberculosis 15, lung cancer 12, miscellaneous 9). Pleural fluids from 12 of the tuberculosis patients, 12 of the cancer patients and one patient in the miscellaneous group had optical densities above the cut-off point. False-positive reactions in patients with lung cancer limit the usefulness of ELISA with conventional anti-BCG antibody for detection of *M. tuberculosis* antigen.

**Introduction**

A reliable and rapid diagnostic test could be extremely useful for patients with tuberculous pleural effusion. Only a few studies have evaluated the role of serological tests for detection of *Mycobacterium tuberculosis* antigen in pleural effusion (Samuel *et al.*, 1985; Choon Taek Lee *et al.*, 1986; Daniel *et al.*, 1986). The number of patients in these studies was small and adequate controls, particularly malignant pleural effusions, were not tested. We evaluated prospectively the diagnostic usefulness of ELISA in patients with pleural effusion presenting over a period of one year.

**Materials and methods**

**Patients**

The 36 patients included in the study (26 males and 10 females) had a mean age of 44.2 (range 19–80) years. All cases had clinical and radiological evidence of pleural effusion; pleural fluid was aspirated and examined for its gross appearance, protein and sugar content, cytology, smear and culture for acid-fast bacilli. A pleural biopsy was taken. Other routine haematological and biochemical tests were done. The patients were followed up and final diagnosis was recorded. Patients on anti-tuberculous chemotherapy were followed up for 9–12 months until drugs were stopped.

**Antiserum**

*M. bovis* strain BCG (obtained from Central Research Institute Kasauli, India) grown in Kirchner’s liquid medium, was harvested by centrifugation, suspended (500 mg wet weight) in 10 ml of NaCl 0.9% and sonicated at 20 kilocycles for 15 min (Somprep 150, MSE Scientific Institute). After centrifugation, 3-ml portions of the supernates were taken for use as antigens in immunodiffusion tests. The protein concentration of the antigen preparation was 1·1 mg/ml, as detected by the method of Lowry *et al.* (1951).

The remaining supernates and deposit were mixed and briefly sonicated (Harboe *et al.*, 1977) to produce the antigen for immunising rabbits. Pre-immune sera were collected from two rabbits (day 1). After mixing the BCG antigen with equal parts of Freund’s incomplete adjuvant, intradermal injections (100 µl) were given at multiple sites in the neck region and above the scapulae on days 2, 16, 30, 44, 72, 100 and 4 weeks after the last injection. Blood was collected from the ear veins every 2 weeks and a positive antibody response was seen as early as day 42. Serum separated from the blood was used as anti-BCG serum after confirmation of antibody production by immunodiffusion.

**ELISA**

The ELISA was performed by the double antibody sandwich method of Harboe *et al.* (1977) in polystyrene flat-bottomed 96-well microtitration plates (Nunc Immunoplate, Denmark). The wells were coated with 100 µl of the anti-BCG serum diluted 1 in 16 with phosphate buffered saline (PBS) (pH 7·2), and incubated overnight at 4°C. Wells were washed three times with PBS.
containing 1 mM Ca\(^{2+}\) and 0·02% Tween 20 (PBSCT) and then incubated with 100-μl samples of pleural fluid, in triplicate, at room temperature for 2·5 h. After three washes with PBSCT the wells were again treated with anti-BCG serum followed by 100 μl of horse-radish peroxidase conjugated with swine anti-rabbit immunoglobulin diluted 1 in 1000 with PBS, and incubated overnight at 4°C. After further washings with PBSCT, 100 μl of freshly prepared substrate (O-toluidine 2·1 mg, n’ dimethyl formamide 1 ml, acetate buffer 100 ml, H\(_2\)O\(_2\) 30%, 50 μl) was added and incubated for 30 min at room temperature; the reaction was stopped by adding 100 μl of 3 N sulphuric acid. Wells with only PBS were negative controls and wells with BCG antigen were positive controls. Absorbance was read at 490 nm in an ELISA reader (Dynatech Laboratories Inc., VA, USA). The mean of three optical density (OD) readings was recorded for each sample.

The clinical diagnosis and the results of the ELISA were recorded separately and not correlated until the end of the study.

Results

The pleural effusion was right-sided in 21 patients, left-sided in 13 and bilateral in 2. Fluid was serous in 24, haemorrhagic in 8, purulent in 3, and chylous in one patient. The pleural fluid protein concentration ranged from 2·0 to 6·4 g%, the fluid was exudative (pleural fluid protein/serum protein >0.5) in 33 patients. The fluid cell content was predominantly lymphocytic in 29 patients. Effusions with predominance of neutrophils were associated with bacterial pneumonia in 5 patients, amoebic liver abscess in one, and anaplastic carcinoma in another patient.

A final diagnosis of tuberculosis was made in 15 patients (Group I). Three were culture positive and 6 had characteristic histopathological features on biopsy. Each of the remaining 7 patients in this group had clinical features suggestive of tuberculosis as well as an exudative, predominantly lymphocytic, pleural effusion, and a positive tuberculin test. Pleural biopsy in these cases showed chronic non-specific inflammation but no granuloma. All these patients responded to antituberculous chemotherapy without recurrence of effusion. In 12 patients (Group II) a diagnosis of pleural effusion due to primary lung cancer was made. The diagnosis of lung cancer was proved by cytology (6 patients), pleural biopsy (6), supravacular lymmph node biopsy (1) and necropsy (1). In 2 patients both pleural biopsy and pleural fluid cytology were positive. The various cell types recorded were: adenocarcinoma (5), anaplastic carcinoma (6) and undifferentiated carcinoma (1). Of the remaining cases (Group III), 6 were infectious in origin, 2 were due to pleural metastases and one to non-Hodgkin’s lymphoma.

Optical densities (OD) recorded at 490 nm with pleural fluid from each of these patients are shown in the figure. Patients in Group I had values from 0·11 to 0·87 (mean 0·40 SEM 0·07); in Group II they were 0·22 to 1·10 (mean 0·51 SEM 0·07) and in Group III 0·13 to 0·30 (mean 0·18 SEM 0·02). Taking an OD value of 0·20 as an arbitrary cut-off point, 12 of 15 patients in Group I, all 12 patients in Group II and one of 9 patients in Group III had a positive result. Thus the overall sensitivity was 80% and overall specificity 38%. False-positive reactions were specially marked in patients with primary lung cancer and pleural involvement. The positive result in a patient in Group III was in a 70-year-old woman with ovarian carcinoma and metastasis to the peritoneum and pleura.

Discussion

The mean concentration of M. tuberculosis antigen in pleural fluid has been shown to be 75·25 SEM 20·5 ng/ml in patients with pleural effusion (Samuel et al., 1985). This is sufficient for detection by a sensitive assay like radioimmunoassay (RIA) or ELISA. In our patients with tuberculous pleural effusion, ELISA gave positive results in 12 (80%) of the 15 studied. This compares favourably with results reported by Samuel et al. (1985), who used RIA in patients with pleural and peritoneal tuberculosis. Of the 7 patients proved to have tuberculosis in that study, 5 had positive results, and 12 of the total group of 41 patients (including probable cases of tuberculosis) had positive results. With ELISA, 65% positivity was reported from Korea (Choon Taek Lee et al., 1986) and 4 of the 7 patients evaluated in a field study in Bolivia were reported to be positive (Daniel et al., 1986).

Figure. OD 490nm values in pleural fluids from 36 patients. Points represent the means of three values; bars represent SEM.
The disquieting finding in our study was that all patients with pleural effusion secondary to lung malignancy had high levels of absorbance of IgG to BCG. Sharing of antigens between tumour cells and BCG has been described (Minden et al., 1976) and it is interesting to note that BCG has been used for immunotherapy of many kinds of cancer, including lung cancer. The intra-pleural administration of BCG after surgery has been shown to benefit patients with stage I lung cancer in long term studies at one center (Maver et al., 1982). This beneficial effect has been attributed to non-specific potentiation of immunological mechanisms, but it is possible that there are common antigens in BCG and lung cancer cells, as have been described for BCG and malignant melanoma cells (Minden et al., 1976).

Therefore, there is likely to be considerable difficulty in differentiating tuberculous pleural effusions from pleural effusions in patients with primary lung cancer on the basis of values obtained with ELISA. Whether using antibodies to specific M. tuberculosis antigens, as suggested by some workers (Benjamin and Daniel, 1982; Krambovitis, 1986), can obviate the problem requires further study. However, in a recent trial of ELISA with M. tuberculosis antigen 5 for the diagnosis of pulmonary tuberculosis in China it was reported that patients with lung cancer were responsible for most of the false-positive results (Yu Ma et al., 1986).

REFERENCES


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