Detection of antibodies to *Mycobacterium tuberculosis* plasma membrane antigen by enzyme-linked immunosorbent assay

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Summary. Antibody activity against *Mycobacterium tuberculosis* of sera from an area with a high prevalence of tuberculosis was measured by enzyme-linked immunosorbent assay (ELISA) with a plasma-membrane extract from *M. tuberculosis* strain H₃₇RV. All sera from relapsed tuberculosis patients and 82.5% of sera from new untreated cases gave positive results. The seronegative group of tuberculosis patients gave positive results by direct microscopy and culture. No clear correlation between antibody and delayed hypersensitivity or extent of disease was observed. Chemotherapy was associated with a higher antibody response. Specificity of the test with healthy control subjects from the high prevalence area was 85%. Negative results were obtained with 145 sera from presumed healthy European subjects and with seven sera from BCG-vaccinated subjects.

Introduction

Availability of new, more sensitive antibody assays, particularly enzyme immunoassays, has stimulated a resurgence of interest in the serology of tuberculosis. Several antigens have been evaluated for their serodiagnostic potential in detecting circulating antibodies by enzyme-linked immunosorbent assay (ELISA) (Nassau *et al.*, 1976; Grange *et al.*, 1980; Reggiardo *et al.*, 1980; Daniel *et al.*, 1981; Zeiss *et al.*, 1982; Benjamin and Daniel, 1982; Stroebel *et al.*, 1982; Benjamin *et al.*, 1984). The sensitivities and specificities of these assays vary considerably and interpretation of the reported results is difficult because of limited clinical information.

Recently, antibodies raised against a plasma-membrane antigen from *Mycobacterium tuberculosis* were used to detect antigen in the cerebrospinal fluid of tuberculous meningitis patients (Krambovitis *et al.*, 1984). In the present study, the same antigen was used to develop an ELISA technique to measure antibody responses in tuberculosis. The activity of specific antibody in sera from new and relapsed cases was correlated with extent of disease, delayed hypersensitivity and duration of chemotherapy. The effect of BCG vaccination on the antibody activity and results obtained with sera from healthy subjects are also presented.

Materials and methods

Extraction of antigen.

M. tuberculosis H₃₇RV (NCTC 7416) was grown in Sauton's synthetic medium at 37°C for 4 weeks and killed by the addition of phenol 5% w/v. Phenolised cells were washed three times in 66 mM sodium phosphate buffer pH 7.4 (PB) and extracted with sodium dodecyl sulphate 1% in PB at 80°C for 1 h. After centrifugation at 10 000 *g* for 1 h the supernate was filtered through a 0.22- μ m pore-size membrane (Millipore) and chromatographed with ion retardation resin AG 11A8 (Bio-Rad Laboratories) to remove excess detergent (Kapp and Vinogradov, 1978).

Enzyme-linked immunosorbent assay.

Flat-bottomed microtitration plates (Titertek, Flow Laboratories) were sensitised with a solution of antigen 30–50 μ g/ml in PB at 37°C for 30 min. Excess antigen was washed off with saline containing Tween 20 (Koch-Light Laboratories) 0.05% (TS) for 10 min. Test sera were diluted 1 in 40 in PB containing 1 mM dithiothreitol, 1 mM EDTA and Tween 20 0.05%, and 200- μ l volumes were added to the sensitised wells in quadruplicate. The plate was incubated at room temperature for 1 h after which the wells were washed for 10 min with TS. Peroxidase labelled sheep anti-human IgG (Duncan, *et al.*, 1983) was diluted to working strength with PB containing bovine serum albumin 0.5% and Tween 20 0.05%, and 200 μ l added to each well. The plate was incubated at room temperature for 1 h and washed with eight changes of TS during a

period of 15 min. The substrate-chromogen solution was prepared just before use by dissolving 5-aminosalicylic acid (Cambrian Chemicals) previously purified and recrystallised (Ellens and Gielkens, 1980) 0.8 mg/ml in 0.05 M citrate buffer, pH 6.5, containing 4.5 mM hydrogen peroxide. The substrate-chromogen was added and incubated for 30 min at room temperature. The reaction was stopped by adding 25 μ l of 1M sodium hydroxide to each well and the optical density (OD) was measured at 450 nm (Titertek Multiskan, Flow Laboratories). The results were expressed as "antibody activity". This was the mean OD of each test serum multiplied by 100. To minimise the influence of day-to-day fluctuation of OD on the results, a standard serum was tested alongside the test sera on the same plate and the OD readings were adjusted accordingly. Antibody activity > 50 was considered to represent a positive result.

Patients

Sera and case histories from 100 bacteriologically proven tuberculosis patients and 41 "healthy" subjects were collected at the T.B. Research Institute, Pretoria, South Africa during a period of 2 months. The tuberculosis group consisted of 48 African and 52 Asian patients, of whom 87 were male and 13 were female; their mean ($\pm$ SD) age was 40 ($\pm$ 13). The control group consisted entirely of African subjects, of whom 35 (85%) were male and 6 (15%) female; their mean age was 33 ($\pm$ 13). The sera were tested blind. Sera from a group of five tuberculosis negative adult subjects were studied before BCG vaccination and again 2 months after vaccination. Two other subjects were followed for 2 years after vaccination.

Control sera were collected from 95 healthy company employees in Britain and 50 sera were obtained from the National Blood Transfusion Service (Britain). No medical information was obtained on these samples; all were presumed to be from healthy individuals.

Results

New and relapsed infections

All sera with the exception of one from a case of lymphatic tuberculosis were from patients with pulmonary tuberculosis, 19% of whom had relapsed infections. Sera from 92.5% of the new cases and from all relapsed cases gave a positive antibody test (fig. 1). However, there was no difference in mean antibody activity between sera from the new cases (235 ± 140) and from relapsed cases (255 ± 133).

Effect of chemotherapy

The mean antibody activity of the 11 sera from untreated tuberculosis patients was 137 ($\pm$ 80); 82.5% of such sera gave positive results (table I). Of the patients studied, 89 were on anti-tuberculous

Fig. 1. Distribution of serum antibody activity in relapsed and new pulmonary tuberculosis patients (bar represents the cut-off point). Each point represents one patient's serum sample.

therapy consisting mainly of isoniazid, streptomycin and pyrazinamide, supplemented with rifampicin and ethambutol, and 85 had responded well to treatment; they had gained, on average, 3 kg/month in weight and their chest radiographs had improved considerably. The proportion of positive tests and the mean antibody activity increased considerably during the first 3 months of therapy (table I). The four patients who were not responding to therapy had high antibody activity.

Extent of disease

Antibody activity was assessed in relation to severity of disease classified, from the extent of cavitation, as minimal, moderately advanced and far-advanced. No marked differences were noted between the three groups (table II), although the trend was towards higher antibody activities in sera

Table I. Effect of chemotherapy on *M. tuberculosis* antibody

Duration of therapy (months)	Number of sera	Mean antibody activity (SD) and range	Percentage of sera that gave positive results
0	11	137 (80) 29-280	82.5
1	24	217 (133) 37-560	91.5
2	28	241 (142) 33-450	96.5
3	23	300 (111) 93-432	95.0
> 3	14	262 (123) 45-450	94.0

SD = Standard Deviation

Table II. Comparison of antibody activity with severity of disease

Duration of therapy (months)	Number of sera	Mean antibody activity ($\pm$ SD) and range in sera from patients with		
		Minimal cavitation	Moderately advanced cavitation	Far advanced cavitation
0	11	106 $\pm$ 6 (2)* 110 - 112	130 $\pm$ 63 (4) 29 - 200	155 $\pm$ 100 (5) 43 - 281
1	24	196 $\pm$ 107 (6) 49 - 350	219 $\pm$ 152 (9) 67 - 560	228 $\pm$ 114 (9) 37 - 323
2	28	120 $\pm$ 58 (4) 73 - 219	216 $\pm$ 121 (11) 33 - 330	300 $\pm$ 127 (13) 75 - 450
3	23	290 $\pm$ 94 (3) 180 - 410	274 $\pm$ 132 (8) 93 - 424	320 $\pm$ 94 (12) 175 - 432
> 3	14	282 $\pm$ 75 (3) 176 - 346	221 $\pm$ 120 (5) 45 - 380	220 $\pm$ 127 (6) 66 - 410

* Numbers of sera tested in each category are given in parentheses.

from far-advanced cases, particularly before, and during the early stages of, treatment.

Delayed hypersensitivity

A series of 78 patients, mostly with minimal and moderately advanced pulmonary disease were tuberculin tested for delayed hypersensitivity by the Heaf test. Skin reactions were classified from anergic (grade 0) to strongly positive (grade IV). There were no marked differences in antibody activity between the groups (table III) although the group with strong skin reactions (grade IV) gave lower antibody results. Five sera were from patients before treatment; four of these patients had grade I

reactions and their sera gave a mean antibody activity of 150 (SD 90) and the fifth had a grade II reaction and an antibody activity of 112.

Control sera

The mean antibody activity of 145 sera collected mainly from European subjects was 23 (SD 8.5). None of these exceeded the cut-off point though three samples were borderline (fig. 2). Samples were also collected at the hospital from 41 "healthy" African volunteers. Distribution of the antibody activity of individual sera is shown in fig. 3. Specificity of the test with this control group was 85%.

Table III. Comparison of the antibody test results with delayed hypersensitivity

Skin test grade	Number of sera	Mean antibody activity (SD) and range	Percentage of sera that gave positive results
0	4	308 (57) 238-380	100
I	32	264 (137) 29-560	94
II	15	270 (121) 43-432	93
III	22	266 (139) 37-450	95.6
IV	5	141 (140) 33-400	60

Fig. 2. Distribution of serum antibody activity in healthy European subjects (bar represents the cut-off point). Each point represents one subject's serum.

Fig. 3. Distribution of serum antibody activity in healthy subjects living in a high prevalence area (bar represents the cut-off point). Each point represents one subject's serum.

Effect of BCG vaccination

Sera from a selected group of five healthy adults with tuberculin-negative skin tests were collected before BCG vaccination and 2 months after vaccination. The results showed no marked changes in their antibody activities (fig. 4). Sequential samples from two other individuals also showed no significant changes in antibody activity during 2 years after BCG vaccination (fig. 5).

Discussion

It is generally accepted that infection with *M. tuberculosis* often induces a humoral response

Fig. 4. Antibody activity of sera from five subjects before and after vaccination with *M. bovis* strain BCG.

which can be detected and measured with mycobacterial antigens (Grange, 1984). The role of these antibodies is not clear, particularly as there is no apparent involvement in the expression of acquired immunity (Suter, 1956) nor in the process of phagocytosis (Mackness, 1954; Reggiardo and

Middlebrook, 1974). A better understanding of the humoral mechanism, particularly in relation to other clinical manifestations, may lead to a more rational design of diagnostic tests based, at least partially, on the detection of immune responses. Many recent serological studies utilised enzyme immunoassay techniques because of the sensitivity and relative simplicity of such assays. In the present study an ELISA based on a heat-stable plasma-membrane antigen was developed to compare immune responses with other parameters of clinical significance.

In ELISA systems for measuring circulating antibodies, results have been expressed in three main ways: end-point antibody titre, untransformed OD readings at a single sample dilution, and antibody-activity units in relation to reference sera. Single OD readings are inconsistent because of systematic and random variability and titrations suffer from poor reliability and relatively higher reagent costs (Malvano *et al.*, 1982). The third option was preferred for our studies. A reference serum selected for activity close to the cut-off point which would reflect linearly any between-run variation of test samples was introduced to the assay. "Antibody activity" was derived by dividing the OD value of the test sample by the OD value of the reference serum and multiplying by 40 (during

Fig. 5. Antibody activity of sequential serum samples from two individuals vaccinated with *M. bovis* strain BCG.

initial experiments the OD readings of the reference serum were 0.4). A similar reference system was used by Kalish *et al.* (1983) to measure circulating antibodies to purified protein derivative.

In the early stages of the assay's development, specific serum IgG, IgM and IgA antibodies were measured. The most pronounced specific antibody which correlated with active disease was IgG which is in agreement with previous reports (Grange *et al.*, 1980; Benjamin and Daniel, 1982; Zeiss *et al.*, 1982; Kalish *et al.*, 1983; Freedman *et al.*, 1966). Anti-tuberculous IgA activity was almost undetectable, and IgM activity was far less discriminatory than that of IgG. To reduce IgM interference, samples were diluted in dithio threitol before testing, and the tracer used in the assay was anti-IgG. The most suitable dilution for testing samples was 1 in 40, determined from the sigmoidal regressions of 42 titrated samples. Similar optimum dilution levels have been described in other ELISA tests for tuberculosis (Reggiardo *et al.*, 1980; Straus and Wu, 1980; Benjamin and Daniel, 1982; Stroebel *et al.*, 1982).

The overall sensitivity of the assay was 94% but with untreated cases was only 82.5%. These figures compare favourably with results of other studies with different antigens (Nassau *et al.*, 1976; Grange *et al.*, 1980; Reggiardo *et al.*, 1980; Daniel *et al.*, 1981; Reggiardo *et al.*, 1981; Stroebel *et al.*, 1982; Ziess *et al.*, 1982; Benjamin *et al.*, 1984). We conclude from the consistent sensitivity figures of these serodiagnostic tests that tuberculosis evokes a specific humoral response against a wide range of antigens, but a proportion of patients, estimated to be *c.* 20%, fail to produce specific antibodies at a significantly high level before therapy. Because of these patients, sensitivity of a test for active disease becomes low.

In the present study, most of the patients whose sera were non-reactive in the test had far-advanced pulmonary tuberculosis with heavy infections and smear-positive sputa. This group did not present problems in clinical diagnosis. In contrast, microscopy-negative, culture-positive cases had a high antibody activity. A possible explanation for this observation may be related to pathogenesis. Constant release of organisms or antigens from the site of infection may lead to (i) chronic stimulation of antibody synthesis resulting in progressive production of low avidity antibody, and (ii) formation of circulating immune complexes of the more reactive antibodies with the released antigen resulting in a considerable reduction of detectable antibody. This hypothesis is consistent with two observations: (i) tuberculosis causes an increase in the overall con-

centrations of circulating IgG and IgA (Faulkner *et al.*, 1967; Nash, 1979; Skvor *et al.*, 1979), not reflected in the specific antibody as measured by ELISA with mycobacterial antigens (Kaplan and Chase, 1980; Krambovitis, 1985); and (ii) the presence of circulating immune complexes has been demonstrated (Carr *et al.*, 1980; Johnson *et al.*, 1981) and there is an inverse relationship between the concentration of immune complexes and anti-mycobacterial antibody (Carr *et al.*, 1980). An alternative explanation which has been proposed is the existence of an immunologic spectrum (Daniel *et al.*, 1981). This concept suggests that at one extreme of the spectrum tuberculosis is localised to confined foci with well developed cell-mediated immunity (CMI) but little or no detectable antibody; and at the other end of the scale there is disseminated disease with poor CMI but with high antibody levels (Daniel, 1980). The present study demonstrated the existence of a wide pathogenic and immunologic spectrum but no clear distinction between antibody and delayed hypersensitivity, which is an index of CMI. The mean antibody activity of the tuberculin-anegetic group was slightly higher than that of the tuberculin-highly reactive group. Neither was there any correlation between extent of disease and antibody, although antibody activity was marginally higher in far-advanced cases.

Chemotherapy was associated with increased antibody activity as shown by groups of patients treated for approximately the same period of time. Similar responses have been reported in tests with different mycobacterial antigens (Janicki *et al.*, 1971; Kaplan and Chase, 1980; Daniel *et al.*, 1981) and these may be associated with increasing levels of antigen release as suggested by Kaplan and Chase (1980).

Antibody activity of control subjects from the high prevalence area was higher than that of the European controls, and sera from the high prevalence area gave a false-positive rate of 15%. Similar results were reported by Benjamin and Daniel (1982) who used a cytoplasmic protein as antigen. Several reasons for false-positive reactions have been suggested (Grange, 1984) but in the present study the higher probability of exposure to infection was considered to be the main factor for these results. No attempt was made to exclude active tuberculosis by accepted criteria when the samples were taken and infection cannot be excluded.

Vaccination with BCG did not elevate antibody activity in seven adults studied. Similar results have been reported with routine BCG vaccinations (Fusillo and Weiss, 1958; Kalish *et al.*, 1983).

although large doses or repeated injections of BCG for treatment of neoplastic disease can induce a high antibody response at least in other test systems (Wile *et al.*, 1977; Kaplan *et al.*, 1980; Winters and Lamm, 1981).

The results of the present study demonstrated a spectrum of humoral responses in active tuberculosis which did not depend on the clinical status of the patient. As the overall positive and negative predictive values were 94% and 85%, respectively, with samples from a high prevalence area, the assay may

have applications in the serodiagnosis of tuberculosis. The results of such a test, however, must be interpreted with supporting evidence from other accepted criteria.

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