A Rapid, Simple Enzyme Immunoassay for Detection of Antibody to Individual Epitopes in the Serodiagnosis of Tuberculosis

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The antibody response to individual epitopes has previously been analysed by competition assay using ¹²⁵I- or enzyme labelled monoclonal antibodies. A modification of the test is described in which competition of human sera with unlabelled mouse monoclonal antibodies at the limiting dilution is revealed by peroxidase labelled anti-mouse IgG conjugate. Analysis of 54 sera from patients with pulmonary (36) and extrapulmonary (18) tuberculosis and 31 controls indicated that the modified test compares favourably with the test based upon directly labelled antibodies. Diagnostic sensitivity for five monoclonal antibodies evaluated was 11.1 % (TB78), 35.2 % (TB23 and TB68), 37.0 % (TB71) and 61.1 % (TB72) at 97.5 % specificity. For TB72, sensitivity was highest for pulmonary disease (69.7 %). The modified assay is also easier to standardise for screening new monoclonal antibodies using a single enzyme-labelled conjugate.

Tuberculosis accounts for greater morbidity and mortality than any other infection with 8 million new cases per annum, half of whom have smear-negative or extrapulmonary disease (1). Despite this, the laboratory tests available for its diagnosis are far from satisfactory, microscopy being rapid but insensitive and culture being sensitive but slow. Several alternative techniques are under evaluation including the use of nucleic acid probes and the polymerase chain reaction for detecting mycobacterial DNA, immunoassays for identifying antigen, and gas liquid chromatography and mass spectrometry for detecting specific mycobacterial lipids. The technical problems associated with the analysis of sputum and tissue samples are great, however, and these methods cannot yet be applied to the clinical diagnosis of tuberculosis (2).

Serological techniques, by contrast, are simple, inexpensive and readily applicable as rapid diagnostic tests. The specificity of antibody responses to crude preparations of Mycobacterium tuberculosis has been poor (3–5). Sensitisation by environmental mycobacteria or BCG vaccination with antigens cross-reacting with Mycobacterium tuberculosis may be responsible for this low specificity. In an attempt to avoid this problem, several researchers have used purified fractions of mycobacterial antigens in enzyme immunoassays (EIAs). However, several of these purified antigens contain multiple and potentially cross-reactive epitopes (2–8).

Tests which measure the serological response to individual epitopes by competitive inhibition of the binding of murine monoclonal antibodies (MABs) have been useful in the serodiagnosis of tuberculosis and bypass the requirement for purified antigens (9–10). Because the labelling of MABs may interfere with their epitope-specific binding sites, an improved rate of antibody detection might be achieved by using a sandwich EIA technique based on unlabelled MABs and peroxidase conjugated anti-mouse IgG.

The purpose of this study was to develop a new immunoassay which dispensed with the requirement for radioactive isotopes and improved the diagnostic characteristics of the solid-phase antibody competition test (SACT). A sandwich EIA using unlabelled MABs and peroxidase conjugated goat anti-mouse IgG (SACT-SE) was compared with tests based on either radioactive iodine (SACT-R) or peroxidase labelled (SACT-E) MABs.
Materials and Methods

Patients. Sera were collected from 54 adult patients at Northwick Park Hospital and the London Chest Hospital prior to starting treatment for suspected tuberculosis. All patients were subsequently confirmed as having tuberculosis on the basis of positive cultures (n = 41), the presence of caseating granulomata on tissue biopsy (n = 2), and/or the resolution of disease clinically and radiographically with treatment (n = 11). Twenty-nine patients had smear positive disease, 22 of whom had pulmonary and 7 extra-pulmonary infection. The remaining 25 patients had smear negative disease (14 pulmonary and 11 extra-pulmonary). Sera were also collected from 9 patients with self-healed tuberculosis, 23 patient controls with a variety of diseases which simulate tuberculosis and 8 healthy residents of the UK. All sera were stored at -20 °C until testing.

Reagents. Mycobacterium tuberculosis soluble extract (MTSE) was prepared from Mycobacterium tuberculosis H37Rv as described previously (11). Five murine MABs specific for the tubercle bacillus were used (10). These were coded as TB23 (19kDa), TB68 (14kDa), TB71 & TB72 (38kDa), and TB78 (65kDa), the molecular weight of the corresponding antigens being given in parentheses. MABs were conjugated to horse-radish peroxidase as described by Rylatt et al. (12). Radiolabelling of the MABs with 125Iodine was performed using the iodogen technique (13).

SACT-Sandwich Enzyme Immunoassay (SACT-SE). Polyvinyl microtitre plates (Dynatech, UK) were coated with 50 µl (10 µg/ml) of MTSE diluted in PBS and incubated for 20 h at 4 °C in a humidified atmosphere. After a single wash with PBS-Tween 20 (0.05 % w/v) (PBST), wells were blocked with 200 µl of dried milk diluted 1 % w/v in PBST (PBSTM) and incubated for 1 h at 37 °C. The PBSTM was then tipped off and the plates patted dry. Four five-fold dilutions of human sera (1/5-1/625; 25 µl per well) were added to duplicate wells and incubated at 37 °C for 1 h. Without washing, 25 µl per well of murine MAB diluted in PBSTM to give 90 % maximal binding in wells without competing serum (high control) was added. Dilution of neat MAB solution (5 mg/ml) ranged from 1:5000 to 1:100,000. Plates were shaken on a Dynatech Microtitre Varishaker for 30 see and incubated at 37 °C for 2 h. Subsequently, wells were washed five times with PBST and patted dry. Fifty µl per well of an affinity purified goat anti-mouse IgG horse-radish peroxidase conjugate diluted in PBSTM to 1/10,000 was added to each well; the reaction was halted after 10 min at room temperature with 50 µl of TMB/H2O2 added; the reaction was halted with 75 µl of TMB/H2O2 added; the reaction was halted with 75 µl of 0.5 M sulphuric acid. The absorbance was subsequently read as previously described.

SACT-Radioimmunoassay (SACT-R). For the SACT-R, the method previously described was used (13). Polyvinyl microtitre plates (Dynatech) were coated with 50 µl of MTSE (30 µg/ml) per well and incubated at 4 °C for 20 h. After washing once with PBS, the wells were blocked with 3 % bovine serum albumin (BSA) for 1 h at 20 °C and then washed twice with PBS. Four five-fold serum dilutions in 3 % BSA (1/5-1/625; 25 µl per well) were added to duplicate wells and incubated for 4 h at 20 °C. Without washing, 25 µl per well of 125I-monoconal antibody diluted in 3 % BSA was added to give binding between 1000-2000 cpm in wells without competing serum (high control). Plates were shaken as previously described and incubated overnight at 4 °C. Subsequently, the wells were washed five times with PBST, dried, and counted in an LKB 1260 Multigamma II counter (LKB instruments, USA).

Calculation of Data. Wells without competing serum represented high controls (optical density always ≥ 0.8) and wells without MTSE coating were taken as low controls. Individual antibody titres were expressed as the dilution of serum which inhibited binding of the MAB to MTSE by 50 % (ID50). This was derived by interpolating from the straight line between the two serum dilutions which were either side of the 50 % inhibition value. Results calculated to be less than the first serum dilution (ID50 < 5) were only regarded as significant if there was > 30 % reduction in the optical density of the high control. Discrimination between positive and negative sera was based on a cut-off value represented by two standard deviations above the log mean antibody titre in the control group. The minimum and maximum titres in calculations were 1 and 1000 respectively. Correlation between MAB probes was performed using a modification of Spearman's rank method for correlating more than two parameters, where the coefficient of concordance is represented as 'W' (14).

Results

A good correlation was observed between the results obtained for all three assays for TB23 (W = 0.64; P < 0.05), TB68 (W = 0.60; P < 0.05), TB71 (W = 0.75; P < 0.01), TB72 (W = 0.80; P < 0.01) and TB78 (W = 0.68; P < 0.01) (Figures 1 and 2). For each MAB probe, the antibody titres obtained for individual sera were similar whichever test method was used. Antibody titres to the TB72 epitope were consistently higher than titres to the other four MAB probes. At 97.5 % specificity, the single most discriminating MAB for the SACT-SE and SACT-R tests was TB72 (61.1 % and 38.9 % sensitivity respectively), whereas for the SACT-E assay it was TB68 (55.6 %) (Table 1). For TB72 using the SACT-SE