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DIAGNOSTICS

Serodiagnostic markers for the prediction of the outcome of intensive phase tuberculosis therapy

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SUMMARY

Treatment failure and relapse may affect many tuberculosis (TB) patients who undergo standard anti-TB therapy. Several independent studies suggested unsuccessful sputum culture conversion at month 2 of treatment (slow response) as risk factor for treatment failure and relapse. However, earlier than month 2 identification of patients with a high risk for poor treatment outcome would offer significant clinical trial and individual patient care benefits.

The sensitivity and specificity of serological IgG and IgA responses against four recombinant mycobacterial antigens (ABC transporter PstS3, secreted L-alanine dehydrogenase, culture filtrate protein Tpx and 6 kDa early secretory antigenic target esxa (ESAT-6)) were evaluated separately in a blinded fashion in 21 smear-positive pulmonary TB patient sera taken at diagnosis before commencement of directly observed anti-TB treatment short course comprising 13 slow responder and eight fast responder subjects.

We observed a general pattern of higher antibody levels in sera of slow responders. Most pronounced were high levels of anti-alanine dehydrogenase IgG, anti-Tpx IgG, anti-ESAT-6 IgG and anti-ESAT-6 IgA antibodies at diagnosis being associated with slow response with 100% specificity each and 46.2, 53.8, 53.8 or 53.8% sensitivity, respectively, when compared to fast response ($P = 0.020$, 0.021, 0.040 and 0.011, respectively). Discriminant analysis showed that the combined use of anti-Tpx IgG and anti-ESAT-6 IgA antibody titers before treatment predicted slow responders with 90.5% accuracy.

These preliminary results suggest that combinations of serodiagnostic markers measured prior to initiation of treatment may be suitable for the prediction of early treatment response. This approach holds promise and requires further evaluation for its utility in the prediction of treatment failure and relapse, the evaluation of new TB therapeutics, as well as in the care of individual patients.

1. Introduction

The currently most effective known standard treatment strategy against TB is “Directly observed treatment short course” (DOTS) and consists of a four-drug intensive phase of two months, followed by a two-drug continuation phase of four months.[1–4] The International Union Against Tuberculosis and Lung Disease (IUATLD) recommends that sputum smear or culture status are assessed at the end of the 2-month intensive phase of treatment in order to monitor patient progress.[5] Failure of sputum conversion at this time point implies the need for a continuation of the intensive phase of anti-TB treatment with four drugs and sputum culture with drug sensitivity re-testing to exclude drug resistance. However, a time period of more than two months before the first indications of treatment efficacy is long and has implications for individual patients and control programs. In addition, the bacteria have time to adapt, frequently creating drug tolerant[6] and drug resistant[7,8] strains during ineffective therapy. Furthermore, month

Abbreviations: AlaDH, L-alanine dehydrogenase; ESAT-6, 6 kDa early secretory antigenic target; PstS3, phosphate-specific transporter S3.

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2 culture-positivity of sputum (slow intensive treatment response) has been shown to correlate with treatment failure and/or the relapse rate after termination of therapy.[9–12] The timely identification of those patients who show a slow intensive treatment response may therefore be of great benefit for the control of TB if this is also associated with adverse treatment outcomes like failed treatment and relapse.

Serodiagnostic data, which compare specific antibody titers at diagnosis between fast and slow intensive phase TB treatment responders or subsequent treatment outcome are rare,[13,14] although it is tempting to hypothesize that differences between these two groups might also be reflected on the level of humoral response at the time of diagnosis or during early treatment. If so, serodiagnosis may be an easy and useful supplementary method for the early identification of those patients who are at greater risk for treatment failure and relapse and who require more efficient treatment regimens and monitoring.

The main purpose of the present study was to provide proof-of-concept data and to find an optimal combination of serodiagnostic variables at diagnosis of TB that may have potential for the prediction of early treatment response, as defined by the sputum culture status at month 2. We show that the individual and/or combined use of certain serodiagnostic markers indeed reflect significant baseline differences between fast and slow early TB treatment response groups.

2. Materials and methods

2.1. Participants

2.1.1. Study population

We enrolled 21 individuals with new smear-positive pulmonary TB and 15 healthy control individuals with no clinical signs of TB or other diseases in an epidemiological field site in metropolitan Cape Town in South Africa with a population of whom 99.7% are of mixed race. The incidence of new smear-positive TB in this community was 341/100,000 population in 2002.[15]

2.1.2. TB patients

The 21 patients with active smear-positive pulmonary TB were part of a larger study of which results have been published recently[16–18] and fulfilled the following inclusion criteria: sputum smear and culture-positivity for *Mycobacterium (M.) tuberculosis*, no multi-drug resistant (MDR) *M. tuberculosis* strains, HIV-negativity, taking at least 80% of prescribed doses during the intensive phase of treatment, no pregnancy and first-time TB. In accordance with the South African National Tuberculosis Program [based on World Health Organization (WHO) guidelines], the patients received a fixed drug combination (depending on body weight) under direct observation. Chest radiography, Ziehl–Neelsen sputum smears as well as BACTEC sputum cultures were performed as previously described.[16–19] Blood samples were taken at diagnosis prior to initiation of treatment and at week 26 after start of treatment (the last blood sample being taken on the last day of chemotherapy). Serum was separated by centrifugation (1250× *g*) for 7 min and stored in aliquots at −80 °C.

2.1.3. Healthy community controls

The 15 healthy community controls fulfilled the following inclusion criteria: residence in the same community, absence of clinical signs of TB or other diseases, absence of prior TB, a Mantoux skin test (TST) induration of 0 mm, HIV negativity and no pregnancy. The TST was performed as previously described.[20] Blood samples were taken directly before the TST. Serum was separated by centrifugation (1250 × g) for 7 min and stored in aliquots at −80 °C.

2.2. Antigen cloning and purification

Four cloned and purified recombinant proteins of *M. tuberculosis* (Table 1) were used for the detection of human IgG and/or IgA antibodies in the sera of the 15 healthy, *M. tuberculosis* uninfected control individuals and 21 TB patients via enzyme-linked immunosorbent assay (ELISA). The production of the functional *M. tuberculosis* L-alanine dehydrogenase (AlaDH) in the heat-induced strain *Escherichia (E.) coli* CAG629 (pMSK12) has been described previously.[21,22] *M. tuberculosis* 6 kDa early secretory antigenic target (ESAT-6), phosphate-specific transporter S3 (PstS3) and Tpx were each expressed in *E. coli* strains using standard expression systems (IPTG induced). The antigens were purified using standard chromatographic methods (affinity chromatography, ion exchange chromatography, size exclusion chromatography). Insoluble antigens were solubilized (refolded) from denaturating conditions (8 M urea) into buffers free of choatropic reagents.

2.3. Enzyme-linked immunosorbent assay

Polystyrene 96-well Maxisorb microtiter plates (Greiner) were coated overnight at 2–8 °C with 0.1 μg/well of highly purified specific antigen in PBS pH 7.5. After three times washing with 300 μl/well PBS-T (0.15 M PBS, pH 7.5, 0.05% Tween-20), the plates were blocked with 300 μl of blocking solution/well (1% BSA in PBS pH 7.5) for two hours at 37 °C and washed three times with PBS-T. Human serum was diluted 1:200 in PBS pH 7.5/0.5% BSA buffer and 0.1 ml of the diluted sera were pipetted into the antigen-coated wells of the microtiter plate in duplicate. All serum samples were measured in duplicate apart from the baseline sample of one TB patient who had insufficient sample volume, whose anti-IgG response to PstS3 was measured as single sample. After 45 min incubation at 37 °C the washing step was repeated. Anti-human-IgG (1:40,000), anti-human-IgA (1:12,000) or anti-human-IgM (1:12,000) antibodies labeled with horseradish peroxidase (HRP) (Pierce) in blocking solution were added and the plate was incubated for 30 min at 37 °C. After a further washing step, the enzyme activity was assayed by incubation with substrate tetramethylbenzidine (TMB) for 10 min at 37 °C. The color development was terminated by adding 0.2 M H_2SO_4 stop solution and quantified by measuring the optical density at the wavelength of 450 nm (OD_{450}) by an automatic microplate reader (MR7000; Dynatech Deutschland GmbH, 73274 Notzingen, Germany). The mean OD of the blank wells was subtracted from sample values. For each antigen, all data were measured simultaneously. If duplicate absorbency values varied >15% the measurement was repeated. Laboratory personnel performing the serodiagnosis assays were blinded to the clinical status of the patients or the controls. Subsequent record reviews were done by clinical staff to classify the individuals into clinical groups without knowledge of the serologic response phenotype.

2.4. Statistical analysis

In case of independent data sets with normal distributions and equal or unequal variances, *P* values were determined by Student's

Table 1
Recombinant antigens of *M. tuberculosis* used in this study.

Protein name(s)	Rv no. of gene	Mol. mass (kDa)	References	Ig class
PstS3	Rv0928	37.9	46–49	IgA, IgG
Tpx; CFP20	Rv1932	16.9	39–45	IgA, IgG
ESAT-6	Rv3875	9.9	50,51	IgA, IgG
AlaDH	Rv2780	38.7	14,21,22	IgA, IgG