

## Gamma Interferon-Based Immunodiagnosis of Tuberculosis: Comparison between Whole-Blood and Enzyme-Linked Immunospot Methods

Elisabeth Schölvinck,<sup>1,2†</sup> Katalin A. Wilkinson,<sup>2</sup> Adam O. Whelan,<sup>3</sup>  
Adrian R. Martineau,<sup>2</sup> Michael Levin,<sup>1,2</sup> and Robert J. Wilkinson<sup>2,4\*</sup>

Department of Pediatrics<sup>1</sup> and Wellcome Centre for Research in Clinical Tropical Medicine,<sup>2</sup> Faculty of Medicine, Imperial College London, London W2 1PG, Veterinary Laboratories Agency, New Haw Addlestone KT15 3NB,<sup>3</sup> and Tuberculosis Service, Northwick Park Hospital, Harrow HA1 3UJ,<sup>4</sup> United Kingdom

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**The gamma interferon secretion levels of 52 adults whose T cells had been stimulated with the *Mycobacterium tuberculosis* antigens CFP-10 and ESAT-6 were measured by enzyme-linked immunospot methods and a whole-blood-based assay. The test results correlated well ( $r = 0.689$ ,  $P < 0.0001$ ). Its simple format and use of only small volumes of blood make the whole-blood assay suitable for pediatric practice and field trials.**

*Mycobacterium tuberculosis* is a significant threat to global health (11). The skin test reagent used to aid diagnosis of both active and latent tuberculosis, purified protein derivative (PPD), is impaired in specificity and sensitivity (22), and the skin reaction may cause discomfort. Recent interest in the field of immunodiagnosis has therefore concentrated on antigens encoded by the RD1 genomic segment of *M. tuberculosis*, which is deleted from all BCG strains (5, 15, 19). In addition, the idea of replacing the tuberculin skin test (TST) by a method that assays the in vitro production of gamma interferon (IFN- $\gamma$ ) in response to *M. tuberculosis* antigens has gained popularity (2, 6, 9, 17). The most promising approach combines the recognized high sensitivity of ELISPOT analysis with the use of peptides covering the sequence of the RD1-encoded ESAT-6 molecule into a diagnostic test of greater sensitivity and specificity than the TST (4, 13, 18, 21). The enzyme-linked immunospot (ELISPOT) technique requires separation of cells from blood, the use of multiple wells of an expensive precoated plate, and an automated ELISPOT counter. Some have therefore wondered whether and how these advances could be applied to medically underserved environments (3). A kit based on whole-blood production of IFN- $\gamma$  detected by enzyme-linked immunosorbent assay (ELISA) is available and has the advantage of relative simplicity, but being based on PPD, it does not ameliorate the poor specificity of skin testing (6). In addition, this test requires 10 ml of blood, a large amount if one considers its application in pediatric practice.

We were interested to determine, by the use of a sensitive IFN- $\gamma$  ELISA, whether a form of the whole blood stimulation assay that is used by many groups to assay antigen specific responses to RD1 encoded antigens (1, 3, 7, 9, 16) could match

the performance of the ELISPOT assay. Ethical permission for this study was provided by the Harrow local research ethics committee (Harrow LREC 1646). To perform the whole-blood assay, heparinized blood was diluted 1:10 in RPMI and 180  $\mu$ l of the diluted blood was stimulated with 2.5  $\mu$ g/ml ESAT-6 prepared as described previously (20) or 5  $\mu$ g/ml CFP-10 (encoded by the gene adjacent to ESAT-6, obtained commercially from Lionex, Braunschweig, Germany) or no stimulus in duplicate on a 96-well plate. Diluted blood was cultured at 37°C in a CO<sub>2</sub> incubator for 72 h, at which point the supernatants were aspirated for determination of IFN- $\gamma$  level by ELISA (Antibody pair from BD Pharmingen catalogue no. 554548 and 554550). This ELISA has a working sensitivity of <10 pg/ml. Initially we determined the kinetics of antigen specific IFN- $\gamma$  secretion in 4 strongly TST positive (Heaf grade 4) healthy adults (Fig. 1). Responses were heterogenous, but IFN- $\gamma$  secretion into culture supernatants tended to increase with time up to 144 h. By 72 h all 4 donors had shown an appreciable increment over unstimulated levels in response to one or both antigens to offer the prospect that this assay would be positive in *M. tuberculosis* sensitized people. We therefore compared the overnight IFN- $\gamma$  ELISPOT assay as previously described (10) with our 72 h whole-blood assay. IFN- $\gamma$  spot forming cells and the production of IFN- $\gamma$  in whole blood was determined for 52 variously sensitized adults (average age 35.8 years, 18 females, 34 males; 13 with culture or biopsy positive active tuberculosis, 23 strongly TST positive contacts of tuberculosis, 7 weakly TST positive and 9 TST negative). The analysis yielded 104 pairs of data: ESAT-6 and CFP-10 reactivity for each of the 52 subjects. The ELISPOT and ELISA responses correlated well ( $r = 0.689$ , 99% confidence limits 0.524–0.804,  $P < 0.0001$ , Fig. 2). Using cut-offs of 10 pg/ml for the ELISA and 10 spot forming cells/10<sup>6</sup> PBMC for the ELISPOT, the observed agreement between the tests was 81% ( $\kappa = 0.59$ ). Using the 13 patients with active tuberculosis as a “gold standard” for sensitivity, the CFP-10 or ESAT-6 ELISPOT was positive in 100% and the ELISA in 92.3%. The corresponding percentages for strongly TST positive healthy people were 87 and 91%. Interestingly two TST negative infectious diseases

\* Corresponding author. Mailing address: Division of Medicine, Faculty of Medicine, Imperial College London, Room 452, Wright Fleming Institute, St. Mary's Campus, Norfolk Place, Paddington, London W2 1PG, United Kingdom. Phone: 44 (0)2075943810. Fax: 44 (0)2075943894. E-mail: r.j.wilkinson@imperial.ac.uk.

† Present address: Academisch Ziekenhuis Groningen, 9700RB Groningen, The Netherlands

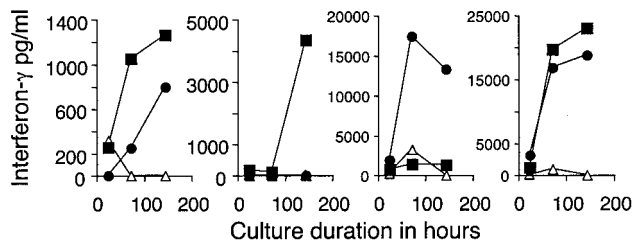


FIG. 1. Kinetics of IFN- $\gamma$  secretion in the whole blood assay. Heparinized whole blood was diluted 1/10 in RPMI and cultured in the presence or absence of antigen for varying periods. Supernatants were aspirated and the IFN- $\gamma$  content determined by ELISA. The response of 4 different healthy donors with strong TST reactions is shown. By 72 h all 4 donors had shown an appreciable increment over unstimulated levels in response to one or both antigens. Symbols: ■, ESAT-6; ●, CFP-10; △, no stimulus.

clinical staff, with known repeated exposure to infectious tuberculosis, had weakly positive reactions in both the ELISPOT and ELISA. Whether these results are true false positives, or indicate that these people had actually become infected without developing skin test positivity, is not clear. In their recent ELISPOT-based analysis of a school outbreak of tuberculosis, Ewer et al. detected similar individuals (13). A large prospective trial in a high incidence environment is required to determine whether the IFN- $\gamma$  production in response to RD1 encoded antigens better predicts, as suggested (12), the risk of subsequent active tuberculosis than the TST.

In low incidence environments, the IFN- $\gamma$  response to RD1 encoded antigens clearly differentiates people sensitized by *M. tuberculosis* from those who are BCG vaccinated (18). However, the genes for ESAT-6 and CFP-10 are present in *M. marinum* and *M. kansasii* and the potential confounding effect of these organisms on specific diagnosis would best be determined prospectively (3, 14). It is interesting to note that while the correlation between the overnight ELISPOT assay and the

72-h whole-blood assay was generally good there were some discordant results (Fig. 2). The fundamental difference between the assays is timing, and it is feasible that the ELISA fails to detect IFN- $\gamma$  production by short-lived effector cells that are particularly well detected by ELISPOT analysis. Conversely, cells that require to differentiate before producing IFN- $\gamma$  may not be detected by the overnight ELISPOT method. From a practical perspective, as little as 250  $\mu$ l of blood used in this whole-blood assay provides ostensibly similar sensitivity to ELISPOT analysis. The requirement for such a small sample may be of particular value when considering the potential to extend RD1 based immunodiagnosis to pediatric practice. In addition the simpler format would be more readily adaptable to medically underserved environments where the research application of such whole-blood assays to populations has been shown to be entirely feasible (8). The difficult clinical scenario presented by HIV coinfection should also be directly addressed, as skin testing performs worst in these people at very high risk of tuberculosis (11).

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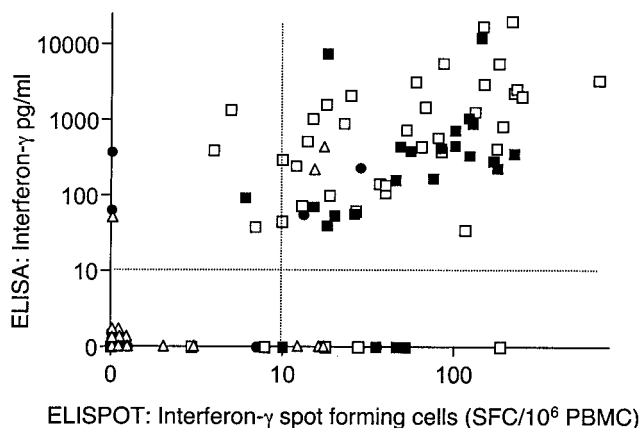


FIG. 2. Correlation between the overnight ELISPOT assay and 72 h whole blood assay. An overnight IFN- $\gamma$  ELISPOT assay and 72 h whole blood was performed in 52 variously sensitized adults. The horizontal and vertical dotted lines represent the ELISA and ELISPOT thresholds respectively. Symbols: ■, active tuberculosis; ●, Heaf grades 1 to 2; □, Heaf grades 3 to 4; △, Heaf grade 0. PBMC, peripheral blood mononuclear cells.

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