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# Evaluation of a western blot serum test for the diag-

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*Evaluation of a western blot serum test for the diagnosis of* Mycobacterium tuberculosis *infection. E. Rovatti, M.P. Corradi, M. Amicosante, P.L. Tartoni, W. Panini, A. Ancora, A.M. Cenci, L. Zucchi, L. Monno, G. Angarano, C. Saltini. ©ERS Journals Ltd 1996.*

**ABSTRACT: This study was designed to evaluate the possibility of monitoring *Mycobacterium tuberculosis* infection using a serological assay.**

**A discriminant score comprising antigen fractions of 38, 28, 24 and 19 kDa, identified in western blots using the *Mycobacterium bovis* bacille Calmette-Guérin (BCG) A60 antigen complex was established in a sample of 57 purified protein derivative (PPD)-negative and 47 PPD-positive individuals. It was then tested in a group of 140 subjects undergoing BCG vaccination as a model of tuberculosis complex infection and in a group of human immunodeficiency virus (HIV)-infected individuals as a model of cell-mediated immunodeficiency-related risk of tuberculosis.**

**The discriminant score identified 57 out of 57 (100%) PPD-positives and none (0%) of the 47 PPD-negatives. In the BCG vaccinated subjects, 1.4% tested positive before vaccination and 90% after vaccination. In the HIV-positive subjects, 90% of the PPD-positive and 5% of the PPD-negative subjects had a positive score.**

**This study suggests that the western blot discriminant score is an accurate test to survey *M. tuberculosis* infection in serum samples.**

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Since the recent resurgence of tuberculosis in the industrialized world, due to the human immunodeficiency virus (HIV) epidemic and to increased immigration from areas of higher tuberculosis prevalence [1], tuberculosis control has become a public health priority. The tuberculin purified protein derivative (PPD) skin test, a simple measure of *in vivo* antigen-stimulated delayed type hypersensitivity (DTH), is the standard test to assess the prevalence of latent *Mycobacterium tuberculosis* infection and to identify individuals at risk of developing tuberculosis [2, 3]. The previous finding that antibodies against the *Mycobacterium bovis* antigen complex A60 are found in the serum of PPD-positive subjects [4, 5], suggested that serological tests might be developed to identify PPD-positive individuals. A semiquantitative western blot test using a discriminant score comprising the A60 antigen fractions of 38, 28, 24 and 19 kDa designed to diagnose *M. tuberculosis* infection was evaluated in a group of bacille Calmette Guérin (BCG)-vaccinated subjects as a model of *M. tuberculosis* complex infection.

## Methods

### *Study population*

A sample of 47 PPD-positive nonvaccinated normal subjects (aged 27±11 yrs; 23 females and 24 males; all Caucasians; none with a history of tuberculosis) was identified among nonvaccinated positive reactors evaluated at enrolment into nursing school. They were evaluated as the population with community-acquired latent *M. tuberculosis* infection. A sample of 57 PPD-negative normal subjects (aged 20±6 yrs; 32 females and 25 males; all Caucasians; none with a history of tuberculosis) was identified among prospective hospital workers screened for tuberculin reactivity and evaluated as negative control.

A group of 140 subjects receiving BCG vaccination after enrolling in the Nursing School of USL Modena (aged 23±5 yrs; 89 females and 51 males; all Caucasians; none with a history of tuberculosis) was evaluated as a model of tuberculosis complex latent mycobacterial infection. All subjects were PPD-negative before the intradermal injection of the BCG vaccine (0.1 IU; Imovax BCG, Pasteur Merieux, Lyon, France). Informed consent was obtained from all subjects.

A serum bank from a sample of 102 human immunodeficiency virus (HIV)-positive asymptomatic subjects (48 PPD-positives and 54 PPD-negatives), already enrolled in a study on anti-*M. tuberculosis* antibody responses [5], was used to evaluate the discriminant score in a model of cell-mediated immunodeficiency.

### *PPD skin test*

Skin test reactions to PPD (5 IU (Biocine Test PPD, Sclavo, Siena, Italy) bioequivalent to 5 IU of US Standard

PPD (PPD-S) [6]), were evaluated 48 h after injection, according to American Thoracic Society (ATS) recommendations [7]. Skin reactions were scored as positive if the induration was 10 mm in diameter in the HIV-negative nonvaccinated subjects and 5 mm in diameter in BCG-vaccinated subjects and in HIV-positive individuals.

*Interferon-γ (IFN-γ ) release*

Since it is known that the PPD skin test is a poor indicator of BCG immunization, *in vitro* release of IFN-γ by T-cells in response to PPD was used as an indicator of delayed type hypersensitivity (DTH) [8]. IFN-γ release was analyzed in a subgroup of PPD-positive nonvaccinated subjects (n=12), in a subgroup of BCG-vaccinated subjects (n=24), and in a subgroup of PPD-negative control subjects (n=18). PPD-stimulated IFN-γ release was quantified in cryopreserved supernatants (-20°C) from 5 day cultures using an enzyme immunoassay (IFN-γ EIA, Technogenetics, Milano, Italy). IFN-γ responses were scored as positive if higher than the 97th percentile of the unstimulated release levels.

*Serologic testing*

Western blot analysis using the *M. bovis* BCG antigen complex A60 (a gift from C. Cocito, University of Louvain, Bruxelles, Belgium) was used to assess the antibody response to BCG as described previously [4]. Monoclonal antibodies recognizing HSP65 (TB78), p38 (TB72), p19 (TB23) and p14 kDa (TB68) *M. tuberculosis* complex proteins (kindly provided by J. Ivanyi, MRC, Hammersmith Hospital, London, UK) were used as controls.

*Semiquantitative western blot score*

In order to discriminate BCG-immunized from non-immunized subjects, the western blot result was analyzed

Table 1. – Identification by log-linear analysis of antibody specificities discriminating PPD-negative from PPD-positive nonvaccinated normal subjects

| Antibody specificities* | | | | | PPD-ve ** | | PPD+ve# | | | Classification$ | | Odds ratio | | Proportion$$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| p19 | p24 | p28 | p38 | p45 | Obs. | Exp. | Obs. | Exp. | Logit## | Correct | Incorr. | PPD- | PPD+ | PPD- | PPD+ |
| - | - | - | - | - | 54 | 42.4 | | 1 | 3.78 | 54 | | 43.77 | 0.02 | 0.98 | 0.02 |
| + | - | - | - | - | | 4.1 | | 1 | 1.46 | 0 | | 4.31 | 0.23 | 0.81 | 0.19 |
| - | + | - | - | - | | 5.2 | | 1.3 | 1.39 | 0 | | 4.03 | 0.25 | 0.8 | 0.2 |
| + | + | - | - | - | | 0.5 | | 1.3 | -0.92 | 0 | | 0.4 | 2.52 | 0.28 | 0.72 |
| - | - | + | - | - | 1 | 3.6 | | 0.8 | 1.47 | 1 | | 4.35 | 0.23 | 0.81 | 0.19 |
| + | - | + | - | - | | 0.3 | | 0.8 | -0.84 | 0 | | 0.43 | 2.33 | 0.3 | 0.7 |
| - | + | + | - | - | | 0.4 | | 1.1 | -0.91 | 0 | | 0.4 | 2.49 | 0.29 | 0.71 |
| + | + | + | - | - | | 0.04 | 2 | 1.1 | -3.23 | 2 | | 0.04 | 25.3 | 0.04 | 0.96 |
| - | - | - | + | - | 2 | 4.8 | | 1.4 | 1.27 | 2 | | 3.58 | 0.28 | 0.78 | 0.22 |
| + | - | - | + | - | | 0.5 | 1 | 1.3 | -1.04 | 1 | | 0.35 | 2.84 | 0.26 | 0.74 |
| - | + | - | + | - | | 0.6 | 2 | 1.8 | -1.11 | 2 | | 0.33 | 3.03 | 0.25 | 0.75 |
| + | + | - | + | - | | 0.1 | 2 | 1.8 | -3.43 | 2 | | 0.03 | 30.78 | 0.03 | 0.97 |
| - | - | + | + | - | | 1.1 | 1 | 3.1 | -1.03 | 1 | | 0.35 | 2.81 | 0.26 | 0.74 |
| + | - | + | + | - | | 0.1 | 6 | 3.1 | -3.35 | 6 | | 0.03 | 28.53 | 0.03 | 0.97 |
| - | + | + | + | - | | 0.1 | 5 | 4.2 | -3.42 | 5 | | 0.03 | 30.48 | 0.03 | 0.97 |
| + | + | + | + | - | | 0.04 | 1 | 4.1 | -5.73 | 1 | | 0.01 | 309.5 | 0.01 | 0.99 |
| - | - | - | - | + | | 2.9 | | 0.1 | 3.78 | 0 | | 43.77 | 0.02 | 0.98 | 0.02 |
| + | - | - | - | + | | 1 | | 0.2 | 1.46 | 0 | | 4.31 | 0.23 | 0.81 | 0.19 |
| - | + | - | - | + | | 0.4 | | 0.1 | 1.39 | 0 | | 4.03 | 0.25 | 0.8 | 0.2 |
| + | + | - | - | + | | 0.1 | | 0.3 | -0.92 | 0 | | 0.4 | 2.52 | 0.28 | 0.72 |
| - | - | + | - | + | | 1.2 | | 0.3 | 1.47 | 0 | | 4.35 | 0.23 | 0.81 | 0.19 |
| + | - | + | - | + | | 0.4 | | 1 | -0.85 | 0 | | 0.43 | 2.33 | 0.3 | 0.7 |
| - | + | + | - | + | | 0.1 | | 0.4 | -0.91 | 0 | | 0.4 | 2.49 | 0.29 | 0.71 |
| + | + | + | - | + | | 0.1 | 2 | 1.3 | -3.23 | 2 | | 0.04 | 25.3 | 0.04 | 0.96 |
| - | - | - | + | + | | 0.8 | | 0.2 | 1.27 | 0 | | 3.58 | 0.28 | 0.78 | 0.22 |
| + | - | - | + | + | | 0.3 | | 0.8 | -1.04 | 0 | | 0.35 | 2.84 | 0.26 | 0.74 |
| - | + | - | + | + | | 0.1 | | 0.3 | -1.11 | 0 | | 0.33 | 3.03 | 0.25 | 0.75 |
| + | + | - | + | + | | 0.04 | 1 | 1.1 | -3.43 | 1 | | 0.03 | 30.78 | 0.03 | 0.97 |
| - | - | + | + | + | | 0.9 | 1 | 2.6 | -1.03 | 1 | | 0.36 | 2.81 | 0.26 | 0.74 |
| + | - | + | + | + | | 0.3 | 10 | 9.3 | -3.35 | 10 | | 0.03 | 28.53 | 0.03 | 0.97 |
| - | + | + | + | + | | 0.1 | 5 | 3.4 | -3.42 | 5 | | 0.03 | 30.48 | 0.03 | 0.97 |
| + | + | + | + | + | | 0.04 | 8 | 12.4 | -5.73 | 8 | | 0.01 | 309.5 | 0.01 | 0.99 |

*: combinations of A60 antigen fractions recognized by serum antibodies in the western blot; **: PPD-negative nonvaccinated normal controls; #: PPD-positive nonvaccinated normal controls; ##: logit model coefficients obtained with each combination; $: PPD-negative and PPD-positive subjects retroclassification using log-linear analysis; $$: probability values associated with each combination. Obs: observed; Exp: expected. PPD: purified protein derivative (tuberculin).

in a semiquantitative fashion to obtain positive or negative scores; correspondence analysis was applied to the western blot responses of the sample of PPD-positive (n=47) and PPD-negative (n=57) nonvaccinated normal controls (see above). Antigen fractions significantly associated with the response to the PPD skin test were those of 19, 24, 28, 38 and 45 kDa. Log-linear analysis [9, 10] was used to identify the most parsimonious model (PPD×p19, PPD×p24, PPD×p28, PPD×p38, p45×p19, p45×p28, p38×p28, p38×p45) and the corresponding logit model (logit (PPD-/PPD+)=W+ $W_{PPD\times p19}$ + $W_{PPD\times p24}$ + $W_{PPD\times p28}$ + $W_{PPD\times p38}$). The 58–65 kDa A60 fractions corresponding to cross-reactive heat shock proteins [4] were not included in the model (table 1).

*Statistical analysis*

The McNemar test was used to assess concordance between assays. The Kruskal-Wallis one-way analysis of variance (ANOVA) and the Mann-Whitney U-test were used with the Ryan correction on multiple comparisons. The discriminant model obtained in the nonvaccinated PPD-positive and PPD-negative population samples (see above) was used for the identification of individuals immunized by BCG, and to compare the performance of the test with the PPD skin test in the group of HIV-seropositive subjects. Data are presented as the median and interquartile range (IQR) or as the mode. An IBM-compatible 486 computer was used with the BMDP (BMDP Statistical Software Inc., Los Angeles, CA, USA) and Statistical Analysis System (SAS) (SAS Institute Inc., Cary, NC, USA) programs.

## Results

Table 1 shows the identification by log-linear analysis of antibody specificities discriminating PPD-negative from PPD-positive subjects. The 57 PPD-negative nonvaccinated normal controls and the 47 PPD-positive nonvaccinated normal controls were classified based upon five antibody specificity combinations (table 1, columns 1–5; in columns 6 and 7 the observed and expected values are shown). All of the PPD-negative and PPD-positive subjects were correctly retroclassified (100%) using log-linear analysis.

The measure of PPD-stimulated release of IFN-γ by T-cells *in vitro* showed that T-cells from BCG-vaccinated subjects (n=24; 87(87) median (interquartile range) IU·mL$^{-1}$) and nonvaccinated PPD-positive individuals (n=12; 84 (401) IU·mL$^{-1}$; p>0.8 compared to BCG-vaccinated subjects) released IFN-γ in significantly higher concentrations than non-BCG-vaccinated PPD-negative controls ((n=18); 2 (5) IU·mL$^{-1}$; p<0.01 compared to BCG-vaccinated subjects) (fig. 1), indicating immunization-specific DTH.

The analysis of serum antibodies against the A60 antigen fractions before and after BCG vaccination showed that vaccination induced a specific antibody response (fig. 2). Strikingly, whilst only two of the BCG-vaccinated subjects had a positive western blot score before vaccination, 126 (90%) had a positive score after vaccination.

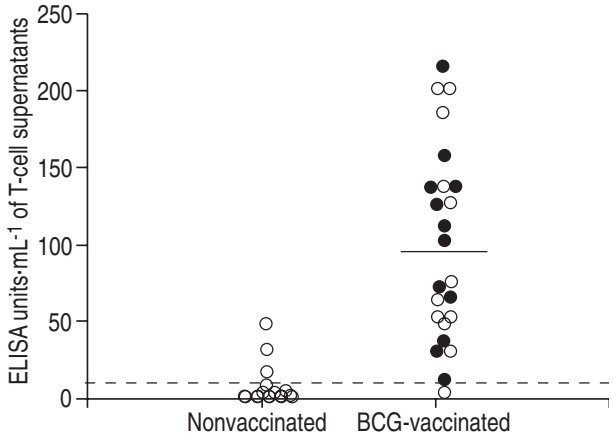


Fig. 1. – PPD-stimulated IFN-γ release by T-cells from nonvaccinated and BCG-vaccinated individuals. IFN-γ levels were measured in PPD-stimulated T-cell supernatants from PPD-negative nonvaccinated normal controls and BCG-vaccinated subjects. The unstimulated IFN-γ release threshold (97th percentile of IFN-γ unstimulated release levels) is shown as a dashed line. ●: BCG-vaccinated subjects with a positive PPD skin test after vaccination; ○: BCG-vaccinated subjects with a negative PPD skin test. PPD: purified protein derivative; IFN-γ: interferon-γ; BCG: bacille Calmette-Guérin; ELISA: enzyme-linked immunosorbent assay.

In the sample of BCG-vaccinated subjects tested for IFN-γ release, 22 of the 23 subjects who released IFN-γ above background levels had a positive western blot score (p=1.0; McNemar test, for concordance between IFN-γ release and the western blot score). Similarly, 8 of the 9 non-BCG-vaccinated PPD-positive individuals who released IFN-γ above background levels had a positive western blot score (p=0.62; McNemar test). In contrast, of the 18 non-BCG-vaccinated PPD-negative controls,

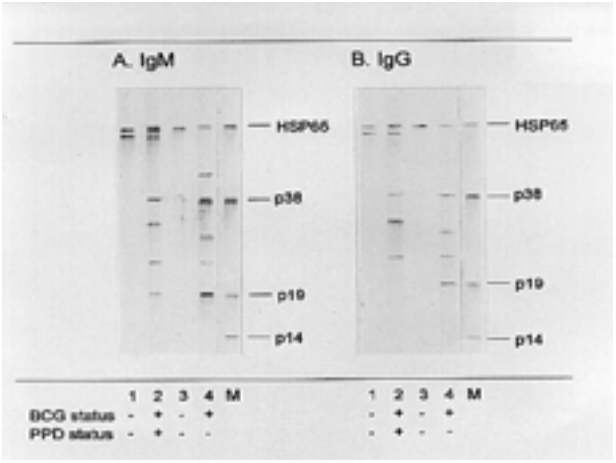


Fig. 2. – Antibody response against the *M. bovis* BCG A60 antigen complex after BCG vaccination. The figure shows examples of western blot analysis of the IgM (panel A) and IgG (panel B) of serum antibody responses to BCG vaccination. Vaccine status and PPD skin test response status are indicated below. The analyses of sera drawn before (lanes 1 and 3) and after BCG vaccination (lanes 2 and 4) are shown. An example of western blot analysis of the serum of a subject with a positive PPD skin test after vaccination is shown in lanes 1–2; an example of an individual with a negative PPD skin test in lanes 3–4. The A60 fractions recognized by monoclonal antibodies TB78 (HSP 65), TB72 (p38), TB23 (p19) and TB68 (p14) are shown in lane M. Ig: immunoglobulin. For further abbreviations see legend to figure 1.

only three were IFN-γ positive. None had positive western blot scores (p=0.25; McNemar test), thus indicating a significant level of concordance between the *in vitro* DTH measure and the western blot test.

The evaluation of a group of 102 HIV-infected subjects showed a significant degree of concordance between PPD skin testing and the western blot score. Out of the 48 PPD-positive subjects, 43 had a positive and five a negative western blot score. Out of the 54 PPD-negative subjects, only three had a positive western blot score and 51 were negative in both tests (p=0.24; McNemar test).

## Discussion

This study shows that a semiquantitative western blot test against the 38, 28, 24 and 19 kDa protein fractions of the A60 antigen complex can identify individuals with latent mycobacterial infection.

Current concepts of *M. tuberculosis* contagion and latent infection are that the mycobacterial infection maintains a DTH reaction, dominated by CD4+ Th1 IFN-γ releasing lymphocytes [8], that is reflected by the reaction of the skin to the intradermal injection of PPD [2], the standard indicator of *M. tuberculosis* infection. The antibody response to *M. tuberculosis* is thought not to reflect antituberculosis cell-mediated immunity [11, 12]. Furthermore, it may be nonspecific because of cross-reactivity with atypical mycobacterial proteins [13]. Although, with these limitations, the study shows that the semiquantitative measure of the antibody response to four tuberculosis complex-specific proteins in the Western blot test is significantly correlated with the DTH response to tuberculosis antigens, as measured by *in vitro* IFN-γ release.

Could serological testing be more sensitive than skin testing? The finding that as many as 30% of tuberculosis patients are PPD-negative [14] suggests that a number of false negative reactions may be expected in healthy subjects as well. This study was not designed to compare the sensitivity of this new test with that of the PPD skin test. Since all individuals in the BCG infection model as well as those in the control group were young healthy individuals, the ability of the serum test to identify individuals with false negative skin reactions could not be established since false negative reactions to PPD are infrequent at the age of the study subjects [15, 16].

Interestingly, when the serum test was used to identify individuals with *M. tuberculosis* infection in a group of HIV-infected persons as a model of deficient cell-mediated immunity [17], the western blot showed a significant degree of concordance with the PPD skin test scored using 5 mm induration as the threshold, suggesting that this test may attain the same sensitivity as the skin test when it is used in its most sensitive mode.

The lack of accepted microbiological methods sensitive enough to identify individuals with latent *M. tuberculosis* infection [18], prevents comparison of the true sensitivity and specificity of the PPD skin test and the serum test. However, the skin test and the western blot score have different merits. The skin test is inexpensive, it does not require laboratory facilities and it may be accurately administered by trained nurses. The antibody test requires rather simple laboratory techniques, although more complex and expensive than the skin test, and may reduce operator's contact with contaminated needles. This test may be used as part of serum test panels to screen populations, such as health workers, prisoners and HIV-seropositive individuals, who have not received BCG vaccination. Furthermore, since it may be performed on stocked sera, this test may be retrospectively used on serum banks in epidemiological studies of the prevalence of *M. tuberculosis* infection.

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## References

1. Bloom BR, Murray CJL. Tuberculosis: commentary on a re-emergent killer. *Science* 1992; 257: 1055–1064.
2. Huebner RE, Schein MF, Bass JB Jr. The tuberculin skin test. *Clin Infect Dis* 1993; 17: 968–975.
3. Rosenberg T, Manfreda J, Hershfield ES. Two-step tuberculin testing in staff and residents of a nursing home. *Am Rev Respir Dis* 1993; 148: 1537–1540.
4. Amicosante M, Paone G, Ameglio F, *et al*. Antibody repertoire against the A60 antigen complex during the course of pulmonary tuberculosis. *Eur Respir J* 1993; 6: 816–822.
5. Saltini C, Amicosante M, Girardi E, *et al*. Early abnormalities of the antibody response against *Mycobacterium tuberculosis* in human immunodeficiency virus infection. *J Infect Dis* 1993; 168: 1409–1414.
6. US Code of Federal Regulations. Title 21. Chapter 1. Food and Drug Administration. Subchapter F Biologics.
7. American Thoracic Society. The tuberculin skin test. *Am Rev Respir Dis* 1981; 124: 356–363.
8. Orme IM, Andersen P, Boom WH. T-cell response to *Mycobacterium tuberculosis*. *J Infect Dis* 1993; 167: 1481–1497.
9. Choulakian V. Exploratory analysis of contingency tables by log-linear formulation and generalizations of correspondence analysis. *Psycometrika* 1988; 53: 287–291.
10. Goldstein M, Dillon WR. Discrete discriminant analysis. New York; Wiley, 1978.
11. Havlir DV, Wallis RS, Boom H, Daniel TM, Chervenak K, Ellner JJ. Human immune response to *Mycobacterium tuberculosis* antigens. *Infect Immun* 1991; 59: 665–670.
12. Carlucci S, Beschin A, Tuosto L, *et al*. Mycobacterial antigen complex A60-specific T-cell repertoire during the course of pulmonary tuberculosis. *Infect Immun* 1993; 61: 439–447.
13. Kalish SB, Radin RC, Phair JP, Levitz D, Zeiss CR, Metzger E. Use of an enzyme-linked immunosorbent assay technique in the differential diagnosis of active pulmonary tuberculosis in humans. *J Infect Dis* 1983; 147: 523–530.

14. Maher J, Kelly P, Hughes P, Clancy L. Skin anergy and tuberculosis. *Respir Med* 1992; 86: 481–484.
15. Stead WW, To T. The significance of the tuberculin skin test in elderly persons. *Ann Int Med* 1987; 107: 837–842.
16. Thompson NJ, Glassroth JL, Snider DE Jr, Farer LS. The booster phenomenon in serial tuberculin testing. *Am Rev Respir Dis* 1979; 119: 587–597.
17. Johnson MP, Coberly JS, Clermont HC, *et al*. Tuberculin skin test among adults infected with human immunodeficiency virus. *J Infect Dis* 1992; 166: 194–198.
18. Walker DA, Taylor IK, Mitchell DM, Shaw RJ. Comparison of polymerase chain reaction amplification of two mycobacterial DNA sequences, IS6110 and the 65 kDa antigen gene, in the diagnosis of tuberculosis. *Thorax* 1992; 47: 690–694.