

## The humoral immune response after BCG vaccination in humans: consequences for the serodiagnosis of tuberculosis

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*The humoral immune response after BCG vaccination in humans: consequences for the serodiagnosis of tuberculosis. M. Turneer, J.P. Van Vooren, J. Nyabenda, F. Legros, A. Lecomte, J. Thiriaux, E. Serruys, J.C. Yernault.*

**ABSTRACT:** The IgM and IgG response to BCG vaccination was investigated in 75 adults, tuberculin negative before vaccination, using an enzyme-linked immunosorbent assay with purified protein derivative (PPD) as antigen. The mean optical density (OD) increased significantly ( $p < 0.001$ ) in both immunoglobulin classes. Increase in at least one class was significant in 89% of the subjects. The observed increase in anti-PPD IgG was rather small but comparable to that seen in 17 newly diagnosed tuberculosis patients with negative direct smear [mean OD (SD): 0.59 (0.38) in vaccinated and 0.70 (0.48) in patients] but significantly lower ( $p < 0.001$ ) than that seen in 31 newly diagnosed patients with positive direct smear [mean OD (SD): 1.07 (0.67)]. With 55% of sera above the upper normal limit, smear positive patients differentiated ( $p < 0.001$ ) from vaccinated subjects (20% of positive sera) whilst smear negative patients (29% of positive sera) did not. We conclude that BCG vaccination induces a definite but small increase in anti-PPD serum IgM and IgG, which is likely to interfere when interpreting serological tests for the diagnosis of tuberculosis, especially in those patients who would most benefit from an early and fast diagnosis.

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Several immunological techniques have been applied to detect serum antibodies against mycobacterial antigens in tuberculosis (Tb) patients [1]. The enzyme-linked immunosorbent assay (ELISA) is now widely used for the diagnosis of active Tb [2]. It permits evaluation of the response in the different immunoglobulin classes, IgG being the most discriminatory when comparing Tb patients with healthy control subjects [3-5].

Whether previous BCG administration can be a confounding variable is not well established, since attempts to study the humoral response after vaccination have yielded controversial results. Most of the studies were conducted with techniques of relatively low sensitivity and on a limited number of subjects. Sera were obtained at very different periods of time (up to 40 yrs) after vaccination and samples before vaccination were not always available for comparison [1]. Moreover, in none of these investigations were IgM antibodies studied, although they are classically considered to be the best indicator of a recent infection [6].

In view of this incomplete information, we decided to investigate the serum IgM and IgG response following BCG vaccination in 75 young adults with negative tuberculin skin-test at the time of vaccination, using an ELISA with two purified protein derivatives of

tuberculin (PPD) as antigens. We also measured concomitantly the serum anti-PPD IgG and IgM levels in 48 newly diagnosed cases of Tb before treatment.

### Patients and methods

#### Study population

a) *Vaccinated subjects.* Seventy-five skin-test negative members of the Erasmus Hospital personnel (51 females, 24 males), attending for annual medical examination, agreed to participate in this study. Their mean age was 28 yrs (range 21-49). According to national regulations they had to receive a BCG vaccine; some of them might have been in contact with Tb patients professionally.

The skin-tests consisted of the intradermal injection of two tuberculin units of purified protein derivative ("PPD 2 UT", Institut Pasteur du Brabant); they were repeated three months after the BCG vaccination. An induration diameter of at least 6 mm was considered to be a positive reaction.

Vaccination was performed by intradermal injection of lyophilized BCG vaccine (Institut Pasteur du

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Brabant). One dose of vaccine (0.1 ml) contained  $1-4 \times 10^8$  colony forming units.

The control serum samples were collected before the first skin-test with a second sample taken 18-78 days (mean: 47) after vaccination.

b) *Tuberculosis patients*. The serum of 48 patients with active Tb was taken before any specific treatment had been given. Of the patients, 44 had pulmonary Tb, 2 had pleural Tb and 2 had urinary Tb. The diagnosis rested on at least one culture of sputum, bronchial lavage, gastric tubage, pleural fluid or urine positive for *M. tuberculosis*; a direct smear was positive in 31 patients (65%). The patients ranged in age from 19-88 yrs (mean: 48) and consisted of 30% women and 70% men.

### Antigens

ELISA was performed using three antigens:

1) A PPD prepared from seven different strains selected from the species *M. tuberculosis* and *M. bovis* (PPD-7S). The same batch was used for laboratory experiments and for manufacturing the "PPD 2 UT" used for clinical skin-testing.

2) A PPD prepared from *M. bovis* BCG (PPD-BCG).

Both PPD's (Institut Pasteur du Brabant) were produced following the now classical method described by MAGNUSSON and BENTZON [7].

3) Tetanus toxoid (Institut Pasteur du Brabant), 2600 fl-mg<sup>-1</sup> total nitrogen.

### ELISA procedure

It was based on the method of ENGVALL and PERLMANN [8]. Immulon Microelisa<sup>®</sup> plates (Dynatech, Kloten, Switzerland) were coated by adding 2 µg of PPD-BCG or PPD-7S in 100 µl of 0.05 M Tris-HCl buffer (pH 8.2) to each well. After incubation in a moist chamber for 2 h at 27 °C, the plates were kept overnight at 4°C. They were then washed four times with 0.01 M phosphate buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 (T) using a Titertek<sup>®</sup> microplate washer (Flow Laboratories, Brussels). Blocking was carried out with 0.5% gelatine in 0.06 M carbonate buffer (pH 9.6) for 1 h. Wells were washed as before and 100 µl of serum diluted in PBS-T containing 0.5% gelatine (G) was added. Each dilution was run in duplicate. After 2 h incubation and washing, the wells were filled with 100 µl of peroxidase-conjugated rabbit immunoglobulins directed against human IgG or IgM (Dakopatts, Copenhagen, Denmark) diluted 1:400 in PBS-T-G and incubated for 90 min. After washing the amount of peroxidase bound to the wells was quantified using a freshly prepared solution of *o*-phenylenediamine (10 mg per 100 ml) and hydrogen peroxide (8 µl 30% H<sub>2</sub>O<sub>2</sub> per 100 ml) in 0.15 M citrate buffer (pH 5.0) as a substrate. The enzymatic reaction was stopped with 8 N H<sub>2</sub>SO<sub>4</sub> after 15 min incubation. The optical density was read at 492 nm with a Titertek<sup>®</sup> Multiskan pho-

tometer.

Wells without sera were used as controls for the conjugate. In order to reduce the effect of inter-assay variations, the pre- and post-vaccination sera obtained from the same individual were tested in the same plate. To assume the reproducibility of the assay, one negative and two positive (with medium and low activity) reference sera were included in each plate. The antibody concentrations were expressed as the optical density values (OD) obtained after correction of the readings according to the mean variations of the reference sera.

This procedure was followed, with slight modifications, for tetanus antibody determination. Tetanus toxoid was used at 0.1 µg per well.

Total IgM and IgG were determined by radial immunodiffusion using commercial kits (Hoechst-Behring, Brussels).

### Statistical analysis

The Wilcoxon test for paired data was used to compare OD values before and after vaccination; the Mann-Whitney U test was used for comparisons between Tb and non-Tb groups. Correlation between ELISA activity to both antigens was calculated by using Spearman's correlation coefficient.  $\chi^2$ -test or McNemar's test (paired samples) were applied to evaluate the significance of differences in positivity frequencies between groups.

## Results

### BCG vaccination

The dilutions giving the best discrimination between pre- and post-vaccination samples were determined in preliminary experiments by testing two-fold serial dilutions of sera. For IgM, the working dilution was set at 1:20. For IgG, the comparison was best performed at 1:80. In these conditions, the OD variation of one negative and one positive sera tested separately ten times in the same plate was of the same order of magnitude for both immunoglobulin classes. A maximum difference of 8.3% was noted between the lowest and the highest values found for the positive serum, with a variation coefficient (VC) of 2.7%. For the negative serum, the values were 14.4 and 6.3%, respectively. A variation  $\geq 19\%$  (3 VC) between pre- and post-sera was therefore considered to be a significant change.

The OD values obtained for IgM and IgG to PPD-7S in paired sera taken before and after BCG vaccination are shown in figure 1. For IgM, the mean (standard deviation) optical density increased from 0.37 (0.22) to 0.52 (0.26). For IgG, the mean values changed from 0.37 (0.25) to 0.59 (0.38). Anti-PPD-BCG IgM increased from 0.30 (0.17) to 0.42 (0.21) and IgG from 0.28 (0.22) to 0.43 (0.30). A significant increase ( $p < 0.001$ ) was found in the IgM as well as in the IgG classes with both antigens.



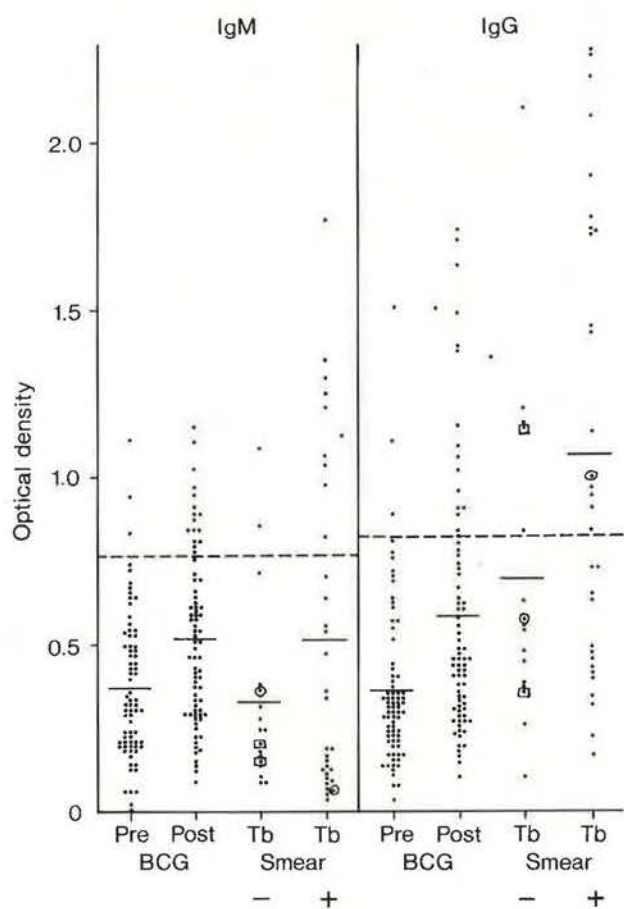


Fig. 1. - IgM and IgG levels to PPS-7S determined by ELISA in 75 healthy subjects before and after BCG vaccination and in patients with bacteriologically proven active tuberculosis. Patients are subdivided into smear negative (17) and smear positive (31) and consisted of 44 pulmonary Tb, 2 pleural Tb (□) and 2 urinary Tb (○). Sera were diluted 1:20 for IgM and 1:80 for IgG determinations. Optical densities were corrected according to reference sera. The mean antibody levels (—) and the normal upper limits (---) stated at an OD corresponding to the 95th percentile of the subjects before vaccination are indicated.

With PPD-7S as antigen, an increase in IgM was observed for 50 (67%) and in IgG for 55 (73%) paired sera. No significant correlation was found between variations in IgM or IgG and a significant rise in levels in at least one of them appeared in 67 (89%) vaccinated subjects. With PPD-BCG, increase in IgM, IgG or at least one of them was shown in 69, 68 and 88% of subjects, respectively.

Taking as a cut-off point an OD value corresponding to the 95th percentile of the control subjects before vaccination, 3 (4%) subjects had positive IgM or IgG levels before while 16 (21%) had positive IgM ( $p < 0.001$ ) and 15 (20%) had positive IgG ( $p < 0.01$ ) levels after vaccination. As many as 36% of the vaccinated subjects were positive in one of the immunoglobulin classes, compared to 7% before vaccination (table 1).

No correlation was found between the variations in anti-PPD and anti-tetanus toxoid antibodies. More-

over, no significant difference appeared in mean anti-tetanus antibody levels between sera collected before and after BCG vaccination, whilst for the same paired sera ( $n=12$ ) the difference in mean levels to PPD-7S was significant (IgM:  $p=0.001$ ; IgG:  $p=0.004$ ). Total IgM and IgG levels determined on paired sera from twelve subjects were not affected by BCG vaccination.

Table 1. - Comparison of IgM and IgG positivity frequencies in control and BCG vaccinated healthy subjects (75 paired sera) and in newly diagnosed tuberculosis patients (48 sera)

Group	Number of positive sera†/total (%)		
	IgM	IgG	IgM or IgG
Healthy subjects			
Controls‡	3/75 (4)	3/75 (4)	5/75 (7)
Vaccinated §	16/75 (21)***	15/75 (20)**	27/75 (36)***
Tuberculosis patients			
Direct smear -	2/17 (12)	5/17 (29)**	6/17 (35)**
Direct smear +	9/31 (29)***	17/31 (55)***,***	20/31 (65)***,*

†: The cut-off was stated at an OD value corresponding to the 95th percentile of the controls; ‡: sera taken before tuberculin skin-test and BCG vaccination; §: sera taken 18-78 days after BCG vaccination (mean: 47).  $\chi_2$  test or MacNemar's test (paired samples) for significance of differences; comparison with controls: \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; comparison with vaccinated: \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ .

Women and men responded alike. The response to vaccination was assessed in relation to the time elapsed between BCG administration and blood sampling. The vaccinated subjects were therefore subdivided into four groups each comprising all subjects tested during a period of two weeks, i.e. 13 for the 3rd and 4th week, 19 for the 5th and 6th week, 31 for the 7th and 8th week and 9 for the 9th and 10th week. No significant difference in either the mean variations between pre- and post-sera or the percentages of individual significant increases were observed. The proportions of positive sera in each group were comparable.

Three months after vaccination, 67 (89%) individuals were skin-test positive. Significant increases in mean levels between pre- and post-sera were found in both tuberculin positive ( $p < 0.001$ ,  $n=67$ ) and tuberculin negative ( $p < 0.017$ ,  $n=8$ ) subjects. No significant difference was noted in mean antibody levels or individual variations between skin-test positive and negative subjects. The numbers of positive sera were comparable in both groups.

Whatever the immunoglobulin class (G or M) a strong correlation ( $R \geq 0.90$ ;  $p < 0.001$ ) was found between the levels of antibodies directed against PPD-7S and PPD-BCG. The PPD-BCG response, however, was systematically and significantly ( $p < 0.001$ ) lower



than the PPD-7S response. Therefore, antibodies to PPD-7S only were sought in Tb patients.

#### Active tuberculosis

As seen in figure 1 anti-PPD-7S IgM levels were similar in healthy subjects and in Tb patients, whereas the latter group had higher IgG levels, the difference being more marked in smear positive patients. Mean (SD) IgG levels were equal to 0.70 (0.48) and 1.07 (0.67) in smear negative and smear positive patients, respectively. As indicated in table 1, 17/31 smear positive Tb patients had an IgG level above the normal upper limit (OD value corresponding to the 95th percentile of the control subjects before vaccination) compared to 5/17 smear negative Tb patients and 15/75 vaccinated subjects. There was no significant difference between the latter two groups. Of the smear positive patients 20/31 (65%) were positive in at least one immunoglobulin class, whilst smear negative patients were positive no more often than vaccinated subjects (35 vs 36%).

#### Discussion

The main purpose of this study was to examine the humoral IgM and IgG response to BCG vaccination in humans using an ELISA with purified protein derivatives as antigens, and to compare the antibody levels after vaccination with those observed in newly diagnosed cases of active tuberculosis.

The choice of PPD's as antigens was due to a number of factors. Firstly, these antigens are readily available and have probably been those most tested in ELISA for serodiagnosis of Tb [2]. Secondly, the specificity attained with them is generally better than that obtained with still more complex mixtures such as culture filtrates [9]. Certainly, one could expect to obtain more accurate results with purified antigens specific for infecting human strains, however, in spite of an increasing number of investigations, no one purified specific antigen has yet become extensively available. Thirdly, PPD's were used in order to assess the diagnostic advantages likely to result from a modern serological methodology over older ones, and to relate the humoral response to the cellular one measured by the cutaneous reaction where PPD's remain until now the main antigens in use.

Two different PPD's were used, PPD-7S prepared from seven human and bovine strains (also used for skin-testing) and PPD-BCG prepared from the BCG strain used for vaccination, with the hope of detecting a higher response to the latter antigens. Since STANFORD [10] reported that PPD made from *M. tuberculosis* possesses antigens of groups i and ii in large amounts and relatively little species- and sub-species-specific group iv antigens, an underestimation of antibodies against poorly represented antigens (*i.e.* the strain-specific ones) has to be borne in mind [11] and would explain a lack of differentiation

between the results with both PPD's. In fact PPD-BCG yielded systematically lower antibody levels. However, whatever the PPD used, similar conclusions can be drawn from the present study, so that the observed differences have no clinical relevance and probably only reflect the well known variability observed in potency of PPD preparations [12].

Before vaccination relatively high serum levels of antibodies to mycobacterial antigens were detected in tuberculin negative healthy young adults as already reported by BARDANA *et al.* [13]. BCG vaccination resulted in a further low, but significant, increase in serum anti-PPD IgM and IgG levels, that was already apparent on day 18 and was maintained up to 12 weeks after vaccination. MAUCH and BREHMER [14], measuring IgG by a solid-phase radioimmunoassay, reported a low increase of antibody levels against purified tuberculin and whole BCG cells, but not against mycobacterial cytoplasmic antigen, in only two of six BCG vaccinated adults. More recently, KRAMBOVITIS [15], measuring IgG activity against *M. tuberculosis* plasma membrane antigen, found no marked changes in seven adults two months after BCG vaccination. In both studies, however, all IgG levels remained in the normal range after vaccination whereas 20% of ours were above it. We could find no relationship between the IgM and the IgG response. Also there was no increase in the total IgM or IgG levels, a common finding in Tb patients [16-18], and no increase in antibodies against tetanus toxoid, a totally unrelated antigen, so that a non-specific adjuvant-like effect of BCG can be ruled out.

Three months after vaccination 89% of tuberculin skin-tests were positive. However, there was no difference in antibody levels between converters and non-converters. Our results agree with those of NEVEU *et al.* [19] who found no difference in antibody levels between skin-test positive and negative vaccinated subjects, and with those of GUPTA *et al.* [20] who were unable to find any difference in ELISA titres between control subjects with positive and negative Mantoux test. These authors, however, did not measure pre- and post-vaccination levels in the same subjects.

Although the increase in antibody levels after vaccination was rather small, it may have implications for the serodiagnosis of active tuberculosis, at least when PPD antigens are used. Even if we consider only IgG, which is more discriminating than IgM [3-5], we found that smear negative Tb patients do not differentiate well from recently vaccinated subjects, whereas smear positive patients did better. In other words, patients who offer diagnostic problems with bacteriological methods also fail to differentiate from vaccinated subjects and differentiate poorly from healthy controls.

Another factor must be considered, *i.e.* the time elapsed between vaccination and the measure of the humoral immune response. In our series, the vaccinated subjects were not systematically tested at fixed times. The second blood samples were taken



in an almost random manner between 18 and 78 days after BCG administration, so that the time course of the response could not be inferred from our results. Nevertheless, this reflects the actual situation well, where individuals at risk need rapid diagnosis of Tb after having had BCG vaccination on a previous occasion some time in the past. At three months post-vaccination, higher levels of antibodies are maintained compared to the pre-vaccination period. It may well be that they will progressively subside over the years and come back to the baseline levels observed in unvaccinated control subjects. A more careful study of the time course is needed to uncover additional information. We think that the only result on this topic is reported by KRAMBOVITIS [15], who found no difference in IgG activity in sequential serum samples taken during a period of thirty months from two BCG vaccinated individuals, but the levels never exceeded the cut-off point and the first post-vaccination samples were taken after two months.

In conclusion, we found that the small increase in anti-PPD IgG levels observed soon after BCG vaccination in humans makes the serodiagnosis of tuberculosis (using these PPD antigens) a rather insensitive procedure in those patients (direct smear negative) who would most benefit from an early and fast diagnosis.

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**RÉSUMÉ:** Nous avons investigué les réponses IgM et IgG après vaccination au BCG chez 75 adultes, tuberculino-négatifs avant la vaccination, en utilisant un test d'Elisa dont l'antigène était la PPD. La densité optique moyenne a augmenté significativement dans les deux classes d'immuno-globulines ( $p < 0.001$ ): l'augmentation, dans au moins une de ces classes, s'avère significative chez 89% des sujets. L'augmentation observée dans le taux d'IgG anti-PPD est assez faible, mais comparable à celle vue chez 17 nouveaux cas de tuberculose dont l'expectoration est négative à l'examen direct (densité optique moyenne de  $0.59 \pm 0.38$  chez les vaccinés et de  $0.70 \pm 0.48$  chez les patients). Elle est toutefois significativement plus faible ( $p < 0.001$ ) que celle observée dans 31 nouveaux cas avec expectoration positive à l'examen direct (densité optique moyenne de  $1.07 \pm 0.67$ ). Par une fréquence élevée (55%) de sera au-dessus de la limite supérieure de la normale 55% les patients BK positifs se différencient ( $p < 0.001$ ) des sujets vaccinés (20% de sera positifs), tandis que les patients BK négatifs (29% de sera positifs) ne le font pas. Nous concluons que la vaccination par le BCG induit une augmentation nette mais limitée des IgM et IgG anti-PPD, qui est susceptible d'interférer lorsque l'on doit interpréter un test sérologique du diagnostic de tuberculose, spécialement chez les patients qui bénéficieraient le plus d'un diagnostic précoce et rapide.