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Tuberculin-Purified Protein Derivative-, MPT-64-, and ESAT-6-Stimulated Gamma Interferon Responses in Medical Students before and after *Mycobacterium bovis* BCG Vaccination and in Patients with Tuberculosis

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***Quanti*FERON-*TB* (QIFN) (CSL Limited) is a whole-blood assay for the recognition of infection with *Mycobacterium tuberculosis*. QIFN measures gamma interferon (IFN-γ) production when purified protein derivatives (PPDs) of mycobacteria are incubated with venous blood samples. The specificity of QIFN in medical students before and after BCG immunization was assessed, and sensitivity in patients with tuberculosis was assessed. Antigens were PPD derived from *M. tuberculosis* and two *M. tuberculosis*-specific proteins, ESAT-6 and MPT-64. Of 60 medical students, all of whom had 0-mm tuberculin skin tests (TSTs) at study entry, 58 (97%) were initially classified as negative for *M. tuberculosis* infection by PPD QIFN. Five months after BCG immunization, 7 of 54 students (13%) had a TST result of ≥10 mm and 11 of 54 students (20%) tested positive by PPD QIFN. ESAT-6- and MPT-64-stimulated IFN-γ responses in the medical students were negative prior to and after BCG immunization. For patients with active tuberculosis, 12 of 19 (63%) were positive by PPD QIFN, 11 of 19 (58%) were positive by ESAT-6 QIFN, and 0 of 12 were positive by MPT-64 QIFN. In conclusion, PPD QIFN was negative in 97% of a low-risk population who had not received BCG and who had negative TSTs. The specificities of both the TST and PPD QIFN were reduced following BCG immunization. PPD QIFN and ESAT-6 QIFN were of similar and moderate sensitivity in patients with active tuberculosis, but ESAT-6 QIFN is likely to be more specific because it is not influenced by past BCG exposure.**

The tuberculin skin test (TST) has been used for many years to screen contacts of patients with tuberculosis (TB), to conduct mass screening in schools, and to screen intending migrants from regions where TB prevalence is high. However, despite its widespread use, the TST has well-recognized shortcomings, including the need to recall people to have their responses assessed, subjectivity of interpretation, and reduced specificity in those who have previously received BCG vaccine (8).

Recently, a new test (*Quanti*FERON-*TB* [QIFN]; CSL Limited, Melbourne, Australia) which is designed to screen humans for *M. tuberculosis* infection has been developed. The principle of QIFN is that immune effector cells in whole-blood samples produce gamma interferon (IFN-γ) when stimulated by an antigen (e.g., purified protein derivative [PPD]), which is then measured by enzyme-linked immunosorbent assay (ELISA). QIFN has the potential to overcome some of the difficulties associated with TST, because there is no requirement for patients to return to have their tests read and results are less open to subjective interpretation. Published experience with QIFN is, to date, limited (3, 4, 19). Converse et al. (3) reported that QIFN appeared to be more sensitive than TST in a group of intravenous drug users and had the advantage for this population of not requiring a follow-up visit. Streeton et al. (19) studied 952 individuals who were stratified into several groups with various likelihoods of infection with *M. tuberculosis* on the basis of their TST responses and likelihood of TB exposure. This study reported a specificity for excluding *M. tuberculosis* infection of 98% in unexposed subjects and a sensitivity of 90% for confirming *M. tuberculosis* infection in subjects with significant TST reactions and other risk factors for TB infection.

The standard QIFN kit employs PPDs obtained from *M. tuberculosis* (Human PPD) and *Mycobacterium avium* (avian PPD) as well as negative and positive control antigens (saline and mitogen). However, in this study only results obtained with human PPD and the positive and negative control antigens were considered. The term PPD QIFN therefore refers to QIFN performed with PPD derived from *M. tuberculosis*.

Given the degree of antigenic cross-reactivity of PPDs from different mycobacterial species, it is possible that BCG vaccination could adversely affect the specificity of PPD QIFN just as it does for TST (8, 9, 17). Recently, several new proteins that are encoded by genes present in the *M. tuberculosis* complex but absent from most or all *Mycobacterium bovis* BCG strains have been identified (13). Two such proteins, ESAT-6 and MPT-64, have been shown to have utility for the diagnosis of *M. tuberculosis* infection. ESAT-6 is a low-molecular-weight, secreted protein of *M. tuberculosis* (18). The gene encoding ESAT-6 has not been identified in any strain of BCG, although it is present in some strains of *Mycobacterium kansasii*, *Mycobacterium sulgai*, and *Mycobacterium marinum* (6).

In cattle, ESAT-6-stimulated IFN-γ responses have been shown to distinguish *M. bovis*-infected cattle from noninfected cattle (14). Ravn et al. have shown recently that assays of

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TABLE 1. PPD QIFN and TST results following BCG vaccination for the 54 medical students who completed the study[a]

TST result (mm of transverse induration)	No. of students	No. of students positive by PPD QIFN
0–4	25	1
5	4	4
6	5	0
7	4	1
8	2	1
9	7	1
10	5	2
12	1	1
16	1	0
Total	54	11

[a] Spearman's rho for correlation between TST and human PPD/mitogen percentage = 0.22; $P = 0.17$.

ESAT-6-stimulated IFN-γ may have improved specificity compared with TST in humans with *M. tuberculosis* infection (15).

In guinea pigs infected with *M. tuberculosis* or BCG Danish 1331, MPT-64 has been shown to be a highly specific diagnostic protein (7), and in human studies, it has been shown to stimulate IFN-γ responses in blood from patients with TB (16). However, the gene encoding MPT-64 is absent from some, but not all, strains of BCG (16).

We evaluated QIFN responses in a group of healthy medical students before and after BCG vaccination and in patients with active TB. The test antigens were *M. tuberculosis*-derived PPD (PPD QIFN), ESAT-6 (ESAT-6 QIFN), and MPT-64 (MPT-64 QIFN). Our aims were first to assess the specificity of QIFN in a group of healthy young people who had not been exposed to *M. tuberculosis* and second to assess the influence that BCG vaccination may have on the specificities of both QIFN and TST. Third, we aimed to assess the sensitivities of ESAT-6, MPT-64, and PPD QIFN assays in a group of patients with clinical TB.

MATERIALS AND METHODS

Medical students. Medical students entering the first year of study at Monash University are routinely offered vaccine updates and screening with TST prior to commencing clinical contact. Medical students who were born in Australia or other countries with low TB prevalence and who had no history of prior BCG, no known exposure to a case of TB, and no past history of prolonged overseas travel were approached to enter the study. Consenting students underwent venipuncture for QIFN immediately prior to a 10-IU TST. Those who were TST negative (100%) received a single dose of BCG (Connaught strain; Pasteur Merieux Connaught, Toronto, Canada) 1 month later. Five months after that, these students underwent a second venipuncture for QIFN and then had a second 10-IU TST performed.

The study was approved by the Standing Committee on Ethics in Research on Humans at Monash University.

Patients with TB. All patients with presumed clinical TB admitted to Monash Medical Centre from September 1996 to January 1998 were eligible for this arm of the study and were enrolled provided that they consented, had received fewer than 12 days of anti-TB therapy at the time of venipuncture for QIFN, and had not undergone a TST or BCG vaccination within the previous 2 months. TSTs were not performed on patients with active TB.

This arm of the study was approved by the Human Research and Ethics Committee of Monash Medical Centre.

TST. In Australia, a standard TST consists of the intradermal injection of 0.1 ml of a 100-IU/ml solution of PPD (CSL tuberculin; CSL Limited). Under Australian guidelines, the TST is defined as positive at ≥10 mm, or if BCG has been administered in the past, the result is defined as positive at ≥15 mm (1, 10, 12).

QIFN. QIFN was performed according to the manufacturer's instructions (4, 19). For the whole-blood stimulation step of the assay, 1-ml aliquots of heparinized blood were incubated with antigens provided in the kit (nil control [saline], human PPD [*M. tuberculosis*-derived PPD], and mitogen [phytohemagglutinin]). Following stimulation, levels of IFN-γ in the whole blood supernatant were estimated by ELISA and obtained in international units per milliliter by reference to a standard curve. For PPD QIFN, a positive result was defined as a PPD-stimulated IFN-γ level/mitogen-stimulated IFN-γ level of greater than 15%, as specified by the manufacturer. PPD supplied with the QIFN kit is obtained from the same manufacturer as the PPD used for the medical student TSTs (CSL tuberculin; CSL Limited).

Recombinant ESAT-6 and MPT-64. Recombinant ESAT-6, produced in *Escherichia coli*, and recombinant MPT-64, produced in *Mycobacterium smegmatis*, were prepared as previously described (2, 14, 16). Both proteins were greater than 95% pure, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining, and were sterilized by filtration with 0.2-μm-pore-size filters before use in whole-blood incubations. ESAT-6 and MPT-64 were used at a previously determined optimal concentration of 5 μg/ml (data not shown). For ESAT-6 and MPT-64 QIFNs, for which few previous data were available, we expected to observe low levels of IFN-γ production, and we arbitrarily defined positive as a result higher than the mean plus 5 standard deviations of results obtained prior to BCG vaccination for the medical students.

RESULTS

Medical students. Sixty medical students were initially recruited and underwent TST and venipuncture for QIFN. All 60 students had negative TST results at study entry (0 mm of transverse induration) and were subsequently vaccinated with a single batch of BCG. Five months after vaccination, QIFN and TSTs were repeated for 54 (90%) of the students; 6 students failed to complete the study. Of these 54 students, 25 (46%) had TST responses of 0 to 4 mm and 29 (54%) had responses of ≥5 mm (Table 1). Seven students (13%) had TST results of ≥10 mm. Under current Australian guidelines (12), one student with a 16-mm result was defined as having a TST result suggestive of *M. tuberculosis* infection. On review, this individual was clinically well and had a normal chest X-ray.

Prior to BCG vaccination, 2 of 60 students (3%) had a positive PPD QIFN response. Five months after BCG vaccination, 11 of 54 students (20%) had a positive PPD QIFN response. One of these 11 students was also positive by PPD QIFN on her initial assay. On review, this student was clinically well and had a normal chest X-ray.

Five months after BCG vaccination, increases in both the diameter of TST induration and the human PPD/mitogen percent response were observed for the majority of students compared with their initial results (Table 1 and Fig. 1). However, no significant association was observed between the TST and human PPD/mitogen percent responses (Spearman's rho = 0.17; $P = 0.22$).

ESAT-6 QIFN and MPT-64 QIFN assay results were obtained for 54 of the 60 students initially and for all 54 students

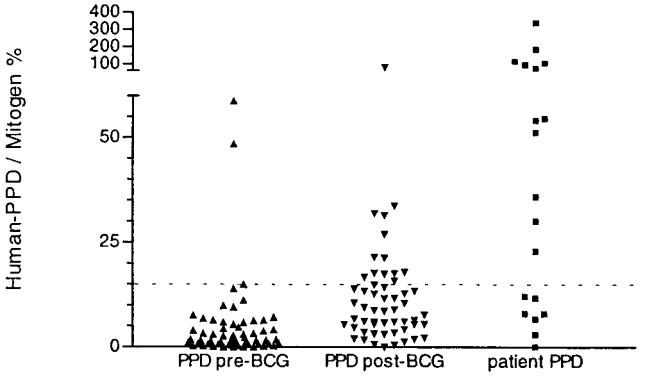

FIG. 1. PPD QIFN results for medical students before and after BCG vaccination and for patients with TB. The vertical axis shows human PPD/mitogen percent IFN-γ responses. The broken line shows the >15% cutoff recommended by the manufacturer to separate positive from negative results. The scale on the vertical axis is interrupted.

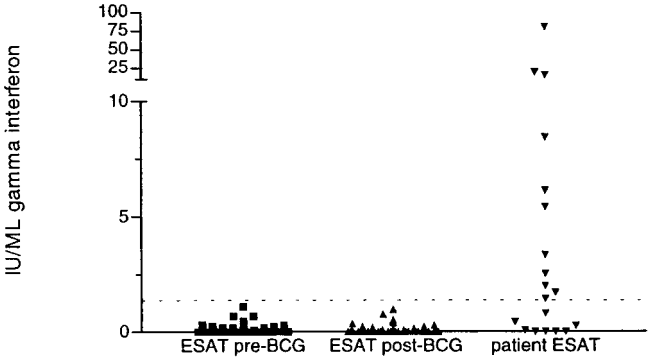

FIG. 2. ESAT-6 QIFN results for medical students before and after BCG vaccination and for patients with TB. The vertical axis shows the IFN-γ level. The broken line shows the mean plus 5 standard deviations of the pre-BCG results for the medical students, used to classify patients with TB as positive or negative. The scale on the vertical axis is interrupted.

who completed the study. For technical reasons, evaluable ESAT-6 QIFN results for six students and MPT-64 QIFN results for five students were not available from the initial venipuncture. At the completion of the study, paired results were therefore obtained for 50 students for ESAT-6 QIFN and 51 students for MPT-64 QIFN. ESAT-6-stimulated IFN-γ levels were very low or undetectable in all students both before and after BCG vaccination (Fig. 2). Results for MPT-64 QIFN were more widely distributed than those for ESAT-6 QIFN, but for consistency we adopted the same definition of positivity (mean plus 5 standard deviations). By this definition, MPT-64 QIFN was positive for one student prior to BCG vaccination but negative for all students following BCG vaccination (Fig. 3).

Patients with TB. Nineteen patients with TB were recruited for this study (16 who were culture positive, 2 who were histology positive, and 1 with a clinical diagnosis). There were nine cases of nodal TB, seven of pulmonary TB, and one each of meningeal-pulmonary TB, septic arthritis, and hip-pelvis osteomyelitis. Of the three patients without confirmatory cultures, two had histologically typical nodal disease and one had a clinical diagnosis of pulmonary disease and responded well to empirical therapy. One patient had Crohn's disease and was receiving immunosuppressive therapy at the time of diagnosis of pulmonary TB. All were human immunodeficiency virus

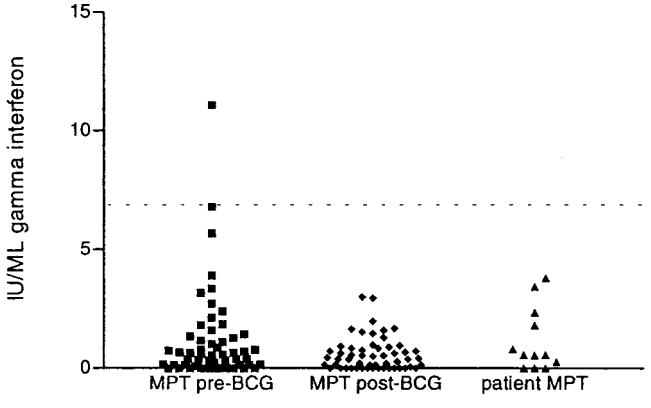

FIG. 3. MPT-64 QIFN results for medical students before and after BCG vaccination and for patients with TB. The vertical axis shows the IFN-γ level. The broken line shows the mean plus 5 standard deviations of the pre-BCG results for the medical students, used to classify patients with TB as positive or negative.

TABLE 2. Full data set for patients with TB

Patient no.	IFN-γ (IU/ml) by ELISA with stimulating antigen:		Human PPD/mitogen %	IFN-γ (IU/ml) by ELISA with stimulating antigen:	
	Human PPD	Mitogen		ESAT-6	MPT-64
1	63.5	63.4	100.1	0.3	3.43
2	25.0	35.1	71.4	3.3	0.27
3	60.2	168.0	35.8	5.4	3.80
4	71.2	130.5	54.6	2.5	1.81
5	22.1	43.2	51.0	2.0	2.35
6	9.9	18.3	54.2	1.7	0.54
7	3.9	136.2	2.8	0.0	0.56
8	1.8	22.5	7.8	0.0	0.00
9	5.7	47.9	11.8	0.0	0.56
10	18.7	153.7	12.2	0.8	0.81
11	0.0	65.3	0.0	0.0	0.00
12	11.4	49.9	22.9	0.1	0.00
13	59.2	62.8	94.2	6.1	—[a]
14	1.4	21.7	6.6	1.4	—
15	35.4	31.6	112.0	15.7	—
16	5.7	74.1	7.8	0.4	—
17	135.6	72.3	187.7	80.8	—
18	70.5	21.2	332.2	8.4	—
19	48.4	161.0	30.1	19.7	—

[a] —, not tested.

antibody negative, and all except the patient with Crohn's disease were clinically immunologically normal. Blood samples were collected from all patients within 12 days of the commencement of anti-TB therapy; the majority (16 of 19) were collected within 1 day of commencement. PPD and ESAT-6 QIFNs were carried out for all 19 patients, and MPT-64 QIFN was carried out for 12.

By PPD QIFN, 12 of the 19 patients had positive results, giving a sensitivity of 63% (Table 2; Fig. 1). Results for ESAT-6 QIFN were distributed over a wide range (Table 2; Fig. 2), and 11 of the 19 patients tested by ESAT-6 QIFN were positive (sensitivity, 58%). For MPT-64 QIFN there was a narrower range of responses, and all 12 patients tested negative (Table 2; Fig. 3).

For the 19 patients tested by both PPD QIFN and ESAT-6 QIFN, 10 were positive by both assays, 2 were positive by PPD QIFN but negative by ESAT-6 QIFN, and 1 (the patient with pulmonary TB who also had Crohn's disease) was positive by ESAT-6 QIFN but negative by PPD QIFN. Six patients were negative by both assays. Since no patients underwent TST, we were unable to compare TST with QIFN in this group.

DISCUSSION

In this study we aimed to assess the effect of recent BCG vaccination on the specificity of the QIFN assay and to evaluate the diagnostic utility of *M. tuberculosis*-specific recombinant antigens in the assay.

We assumed that the medical students were a cohort of uninfected healthy individuals, since they were all born in Australia or other countries with a low TB prevalence (10), had no history of significant overseas travel, had no known exposure to cases of TB, had not previously received BCG vaccination, and were TST negative at the beginning of the study. PPD QIFN was negative in 97% of these low-risk students with negative TSTs. Similarly, Streeton et al. (19) reported a specificity for the QIFN assay of 97.6%. Two students whom we regarded as uninfected were positive for *M. tuberculosis* infection by QIFN PPD prior to BCG vaccination. We are uncertain as to whether

the discordant results arose from false-negative TST or false-positive PPD QIFN responses, although the latter seems most likely.

Five months after BCG vaccination, approximately half of the students had a reactive TST of ≥5 mm, seven had a TST response of ≥10 mm, and one had a TST result of ≥15 mm, which is suggestive of *M. tuberculosis* infection by Australian criteria for positivity. In comparison, 20% of these students had positive PPD QIFNs following BCG vaccination. There was no statistically significant correlation between the magnitudes of TST and human PPD/mitogen percent IFN-γ responses after BCG vaccination, suggesting that the two tests may be measuring independent parameters. This finding supports previous studies (5) which have demonstrated that there is little correlation between TST and in vitro IFN-γ responses in BCG vaccinees. Moreover, these studies have shown that while protection from active TB is not correlated with the TST response, a strong IFN-γ response may be a marker of host resistance (5). This suggests that the PPD QIFN results in the present study would be a more accurate marker for BCG protective efficacy than those of the TST. The present study clearly demonstrates that both the TST and PPD QIFN are affected by recent BCG vaccination, although the duration of this effect is presently unknown.

In contrast to the TST and PPD QIFN assays, positive IFN-γ responses to ESAT-6 were not detected either before or after BCG vaccination for any of the students tested. For MPT-64, there were measurable responses, but, as for ESAT-6, results were not influenced by BCG vaccination in the students. This was the anticipated result, as the genes encoding both ESAT-6 and MPT-64 are absent from the Connaught strain of BCG used in this study.

When assessing the sensitivity of the QIFN assay, we were aware that the ideal group to study would have been healthy individuals known to be *M. tuberculosis* infected. However, because of problems with specificity of TSTs in those with prior BCG vaccination (BCG has been widely used in Australia), it is very difficult to identify a group of healthy individuals, without overt disease, who are definitely *M. tuberculosis* infected. Thus, we chose to assess QIFN in patients with active TB, even though it would be expected that some may be anergic at the time of testing. Of 19 patients with confirmed TB, 63% were positive by PPD QIFN. Although we did not perform TSTs on the patients, it is likely that some would also have had negative TST results (11).

When ESAT-6 was used instead of PPD in QIFN assays, 58% of patients were classified as positive, using a cutoff derived from the medical student arm of the study. While levels of IFN-γ induced by ESAT-6 were approximately fivefold less than those induced by PPD, the sensitivity of the test in this study for correctly identifying patients with active TB was reduced only marginally compared with PPD QIFN. Studies with cattle using the bovine equivalent of the QIFN assay have demonstrated that ESAT-6 correctly distinguishes *M. bovis*-infected animals from those exposed to other, nontuberculous mycobacteria (14). However, the cattle used in these studies were classified as infected but healthy and were not suffering from active TB. It is well recognized that a proportion of human patients with active TB may be anergic at the time of diagnosis (11), which may explain the reduced sensitivity of ESAT-6 QIFN in this study. A wider range of results were obtained with MPT-64, but all patients with TB were classified as negative by using our arbitrary cutoff. This finding is in contrast to those of Roche et al., who found that some patients with active TB had significant responses to MPT-64 in the QIFN assay (16).

In this study, PPD QIFN was negative in 97% of healthy individuals who had not received BCG, but as with TST, specificity was reduced following BCG vaccination. In contrast, ESAT-6 and MPT-64 QIFN results were not affected by BCG. In addition to its excellent specificity, ESAT-6 QIFN showed sensitivity similar to that of PPD QIFN for detecting patients with clinical TB. New diagnostic tests for *M. tuberculosis* such as the PPD and ESAT-6 QIFN assays hold considerable promise and deserve further study.

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