Journal of Epidemiology and Global Health (2014) 4, 43–50

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Comparison of Tuberculin Skin Test result and interferon gamma response to human PPD in BCG scar positive and negative children

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Received 6 May 2013; received in revised form 1 September 2013; accepted 3 September 2013
Available online 9 October 2013

KEYWORDS
BCG vaccine;
Human PPD;
Interferon Gamma Release Assay;
Tuberculin Skin Test;
Vaccine scar

Abstract *Background:* The aim of this study is to compare Tuberculin Skin Test (TST) result and interferon gamma response to human PPD (purified protein derivative), in scar positive and scar negative BCG-vaccinated children.

Methods: Between August 2007 and May 2008 a total of 236 children aged 1–168 months (mean 21 months) admitted to Mofid Children's Hospital, Tehran, Iran, were enrolled in a cross-sectional study. Each patient was examined for BCG vaccine scar and tested with TST and human PPD-based Interferon Gamma Release Assay (IGRA).

Results: Two hundred and twenty one cases out of 236 (44% female, 1–168 months, mean age 21 months) were scar positive of whom 95% TST result was negative. Human PPD-based IGRA was positive in 110 (49.8%), negative in 85 (38.4%) and indeterminate in 26 (11.8%) of scar positive patients.

Fifteen children (40% female, 1–156 months; mean age 42 months) were scar negative. All the scar negative cases were TST negative. Human PPD-based IGRA was positive in 10 (66.7%), negative in 4 (26.7%) and indeterminate in 1 (6.7%) of scar negative patients.

Conclusions: Immune responsiveness to human PPD antigens in scar positive and negative children may not correspond with results of the Tuberculin Skin Test.

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1. Introduction

Tuberculosis (TB) is still one of the most important health problems worldwide with an estimated

http://dx.doi.org/10.1016/j.jegh.2013.09.002
2210-6006/$ - see front matter

prevalence of 12 million (range, 10 million—13 million) cases in 2011 [1]. Since 1928 the BCG vaccine has been used against *Mycobacterium tuberculosis* (MTB) [2].

According to the World Health Organization (WHO) guidelines, a single BCG vaccine should be administered in countries with a high prevalence of active TB disease and now it is mandated in about 64 countries and administered in at least 167 countries [3,4].

Generally, the benefit of the BCG vaccine is seen in the first 5 years of life, but its efficacy might persist for 50—60 years, suggesting that a single dose of an effective BCG vaccine could have a long duration of protection [5].

Currently, it is the only vaccine which causes a characteristic local reaction [6]. A scar occurs in 47.2—100% of cases up to 12 weeks after the BCG vaccination, but may not occur at all in about 10% of infants [6].

The presence or absence of the BCG scar is often used as an indicator of immunization against MTB, and some pediatricians recommend revaccination for Tuberculin Skin Test (TST) negative-scar negative children, but some studies using leukocyte migration inhibition test showed that the absence of a scar may not mean that the child is not immunized [2]. They showed that despite scar failure, the majority of cases could elicit a positive *in vitro* cellular response [2].

Currently, there is no clear guideline for infants who have scar failure. Also, as far as this study is concerned, there is no documentation for using TST by some pediatricians as a good predictor of determining scar negative cases who might need revaccination.

The question still exists if the presence of a BCG vaccine scar corresponds to immunity against MTB and if revaccination is necessary for infants who have scar failure and a negative TST.

New advances in immunology have led to a new *in vitro* test for diagnosing MTB infection named Interferon Gamma Release Assay (IGRA). The mainstay of this test is releasing interferon gamma from T-lymphocytes exposed to mycobacterium antigens [7—9]. Commercially available IGRAs have evolved rapidly over the past decade and have used different antigens. This test was used in this study to indicate immune responsiveness to human purified protein derivative (PPD) antigens in scar negative children, as opposed to its conventional role to diagnose latent tuberculosis infection (LTBI) or TB disease. As far as this research is concerned, IGRA has not been studied yet for evaluating immune responsiveness to human PPD antigens in BCG scar negative cases.

The aim of this study is to compare the interferon gamma response to human PPD and TST result in scar negative and positive children.

2. Materials and methods

2.1. Study design and population

This cross-sectional study was conducted between August 2007 and May 2008 at Mofid Children's Hospital, Tehran, Iran, after receiving approval from the ethics review committee of the Pediatric Infections Research Center (PIRC).

Patients between 1 month and 14 years old admitted to Mofid Children's Hospital that had received the BCG vaccine at birth and met the inclusion criteria were enrolled in this study in a sequential manner. All cases had an infectious illness.

Informed consent was taken from their parents before enrollment. The inclusion and exclusion criteria are depicted in Table 1. Each vaccinated patient was examined for the BCG vaccine scar by a pediatrician and then tested with both TST and human PPD-based IGRA.

2.2. Blood collection procedures

Before performing TST, 4 mL of venous blood was collected in a sodium heparinized tube and transferred to the laboratory where four tubes for every patient were prepared containing 150 μg (3 drops) normal saline (negative control), phytohemagglutinin (a mitogen used as a positive control), human PPD (from MTB), and avian PPD (from *Mycobacterium avium*) respectively; 1 mL heparinized whole blood was added to each tube within six hours of collection.

Aliquots of heparinized whole blood were incubated with the test antigens at 37 °C and in a humidified atmosphere for 16—24 h. The tubes were centrifuged and plasma supernatants were collected, frozen and stored at −70 °C. Enzyme-linked immunosorbent assay (ELISA) test was performed within three months of blood collection to determine the level of interferon-gamma (IFN-γ) produced in each tube.

2.3. Interferon gamma assays

IGRA was performed according to the manufacturer's recommendations (R&D, America). A mitogen and saline solution were used as positive and negative controls, respectively. The amount of INFγ produced in response to the human PPD in ex-

Table 1 Inclusion and exclusion criteria for participant selection.

Inclusion criteria
1. BCG vaccinated at birth
2. Child aged between 1 month and 14 years
3. Admitted in the pediatric ward
4. Parent willing to provide informed consent
Exclusion criteria
1. congenital or acquired immune deficiency disorders
2. history of allergy to PPD
3. history of active tuberculosis disease or close contact to documented tuberculosis case
4. Unstable cardiopulmonary condition
5. malignancy

cess of the saline control (human PPD—negative control) was calculated, as was the amount of INF-γ produced in excess of saline control by the avian PPD (avian PPD − negative control) and also in excess of mitogen (positive control − negative control). A positive test result indicating immune responsiveness to human PPD antigens was defined by the following two criteria:

1. [(human PPD − negative control)/(positive control − negative control) $\geqslant$ 0.15] and
2. [(human PPD − negative control) − (avian PPD − negative control)/(human PPD − negative control) $\geqslant -0.1$]

These two criteria are summarized as below:

$$\frac{\text{human PPD} - \text{negative control}}{\text{positive control} - \text{negative control}} \geqslant 0.15$$

and

$$\frac{\text{human PPD} - \text{avian PPD}}{\text{human PPD} - \text{negative control}} \geqslant -0.1$$

An IGRA result indicating reactivity to *M. avium* complex was defined by the following two criteria:

1. [(avian PPD − negative control)/(positive control − negative control)] $\geqslant$ 0.2 and
2. [(human PPD − negative control) − (avian PPD − negative control)/(human PPD − negative control) < -0.1]

These two criteria are summarized as below:

$$\frac{\text{avian PPD} - \text{negative control}}{\text{positive control} - \text{negative control}} \geqslant 0.2$$

and

$$\frac{\text{human PPD} - \text{avian PPD}}{\text{human PPD} - \text{negative control}} < -0.1$$

The IGRA was considered to be indeterminate if the positive control minus negative control result was less than 0.5 IU. All other IGRA result profiles were considered negative.

2.4. Tuberculin Skin Test

Purified protein derivative 0.1 m (5 IU) (manufactured by Razi Institute of Iran) was injected intradermally at the junction of 1/3 proximal to 2/3 distal of the volar side of the forearm. The induration at the injection site was measured by a pediatrician and recorded in millimeters 48—72 h after the injection. The interpretation of the results (i.e., positive or negative TST) was performed by the pediatrician for each case according to the recommendation of the American Academy of Pediatrics (AAP) [10].

2.5. Statistical analysis

Quantitative data were summarized as median and minimum—maximum and categorical data as raw numbers and percentages. To compare frequency of positive results between the two study groups, chi square and Fisher exact tests were used.

P-values less than 0.05 were considered as statistical significant. Agreement (actual agreement) was calculated by dividing concordant results (those cases with similar results of TST and IGRA tests) by total of cases. The Kappa statistics were calculated to test the difference of actual agreement to chance agreement and interpreted according to Landis and Koch suggestions.

Values of <0.4, 0.4—0.75 and >0.75 were consistent with poor, good and excellent concordance, respectively.

3. Results

Out of 236 patients enrolled in this study, 103 (46.6%) were female. The mean age of the patients was 21 months (from 1 month to 14 years). The number of under-five-year group was 180 (74%). Out of a total of 236 patients, 221 (93.6%) with a median age of 21 months (1—168 months) were scar positive and 15 (6.4%) cases with a median age of 42 months (1—

156 months) did not have a scar. However, scar negative rate in children under and above 60 months was 5.7% and 8.3%, respectively, which was not statistically significant (p-value = 0.54). The characteristics of these two groups are shown in Table 2. Comparison of the response to human PPD in scar positive and negative groups (not considering indeterminate results) is shown in Table 3. All TST positive cases were IGRA positive. There was no statistically significant distinction between results of TST and IGRA in scar positive and negative groups. Mean difference between two study groups evaluated by both t-independent test and non-parametric Mann–Whitney test was not significant (p-values 0.15 and 0.14 respectively).

The agreement between TST and IGRA results was poor (actual agreement = 43.5%, Kappa = 0.004, p-value = 0.87).

4. Discussion

Currently, BCG vaccine is injected at birth to nearly all newborns in Iran under the expanded program of immunization, but does not always result in scar formation.

A sequence of changes develops that result in a BCG vaccine scar. Two to three weeks after an appropriately administered injection of a potent dose of BCG vaccine, a papule develops at the site of injection which slowly increases in size, and at 5 weeks reaches between 4 and 8 mm. Then it ulcerates and within 6–12 weeks heals spontaneously and leaves a permanent scar [6].

The onset and completion of scar formation occurs after 12 weeks in 47.2–100% of BCG vaccinated newborns, but can be delayed for six months or longer [2,6–12]. In this study 6.4% of cases were scar negative. The rate of scar failure in other studies was wide and ranged from 5% to 16% when the BCG vaccine was administered at birth [2,11–14].

There is a difference between scar failure and abortive reaction. Scar failure means that scar formation does not occur after BCG vaccination, which happens in about 10% of vaccinated cases; but abortive reaction means that the BCG scar may disappear after the passage of time [6]. In a prospective study, abortive reactions occurred in 9.9% of infants [15]. Different reasons such as weak vaccine, lost potency and wrong technique are considered responsible for scar failure [12].

This study was cross-sectional, so it could not differentiate between these two groups. Scar failure causes clinical concern in some physicians who believe that the absence of scar formation at the site of inoculation may be indicative of unsuccessful BCG vaccination in an individual and some recommend performing TST for such cases and if negative consider revaccination [16,17].

From 2001 IGRA has been used as an aid for detecting MTB infection. The test is based on the measurement of IFN-γ released from sensitized lymphocytes exposed with *Mycobacterium* antigen. IFN-γ is a cytokine mainly released by T-lymphocytes after antigenic stimulation. It plays a major role in immune responsiveness to *M. tuberculosis* as the major macrophage activating factor [18].

Many commercial and research versions of IGRA that use different types of *Mycobacterium* antigens are available; some are based on MTB specific antigens, such as early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP10), but the human PPD-based IGRA was chosen so that the antigens used to compare responsiveness were similar to those present in the TST [19].

In this study 71.4% of scar negative-TST negative cases had positive human PPD-based IGRA results.

Table 2 The characteristics of scar negative and positive groups.

Characteristic	Scar negative	Scar positive
Number	15 (6.4%)	221 (93.6%)
Age (months)		
Median	4	21
Range	1–156	1–168
<5 years	10 (67%)	166 (75%)
Male	9 (60%)	124 (56%)
TST		
Negative	15 (100%)	210 (95%)
Positive	0%	11 (5%)
Human PPD based IGRA		
Indeterminate	1 (6.6%)	26 (11.7 %)
Positive	10 (66.7%)	110 (49.8%)
Negative	4 (26.7%)	85 (38.5%)

Table 3 Comparison of interferon gamma response to human PPD, in scar positive and negative groups (not considering indeterminate results).

	Scar positive	Scar negative	*p*-Value
Human PPD based IGRA	110 (56.4%)	10 (71.4%)	0.27

It means that despite negative TST, IFN-γ response to human PPD antigens might exist.

Surprisingly, the number of scar negative-IGRA positive cases was more than scar positive-IGRA positive cases and this may be contrary to the results of a study performed by Anuradha et al. [20]. Host genetic factors of the vaccinated children such as polymorphism in IFN-γ (_874T/A) gene may play an important role in IFN-γ response to mycobacterium antigens. Children with TT genotype produce higher levels of IFN-γ compared with the other genotypes AT and AA [20]. A genetic study to determine A and T alleles in scar positive and negative groups was not performed. In addition, the difference in numbers between these two groups might have some influence on the results.

In this study the indeterminate result of IGRA in the scar negative and positive groups was 6.6% and 11.7% respectively. In adult studies, indeterminate results were 0.1—11% in different studies and in one of the recent studies, 21.4% of cases had indeterminate results [21,22]. In one pediatric study, Connell et al. reported 17% indeterminate results [21]. Indeterminate result means that the positive control (mitogen) fails to react. This may be owing to several factors, such as lymphocytopenia, very young or very old age, congenital or acquired immune deficiencies and usage of steroid or other immune suppressant agents, or lymphocyte damage following freezing of the blood sample during transport [23]. The reason for this problem in this study is not very obvious because none of the participants were immune-deficient, and there was no error in transport, handling and processing, which may destroy the lymphocytes in the blood samples. In pediatric studies indeterminate results are more than adults, so the indeterminate results of this study might be a result of the age of cases [21].

In this study the agreement between TST and IGRA results was poor (actual agreement = 43.5%, kappa = 0.004, *p*-value = 0.87). Kang et al. also found a poor correlation between TST and IGRA among healthy volunteers [24]. It seems that concordance between TST and IGRA is related to studied population characteristics, including BCG vaccination status, risk of exposure to MTB and also the type of antigens used for IGRA. The studied cases had a low risk of exposure to MTB because one of our most important exclusion criteria was any history of active TB disease or close contact with a patient with TB disease. In addition, all of them were administered the BCG vaccine at birth and there might be some cross-reaction between human PPD as the main Ag used in human PPD-based IGRA and the BCG vaccine. These reasons might explain the discrepancy found between TST and IGRA results in the study cases. In contrary, Okada et al. and Tsiouris et al. found agreement between these two tests (Kappa = 0.63 and 0.56, respectively) [25,26]. In both studies the participants were at a high risk for MTB Infection, and the aim of IGRA usage was the evaluation of MTB infection in their studied population. In addition, antigen-specific based IGRA was used that contained antigens, such as ESAT6 and CFP10, which had no cross-reaction with BCG vaccine antigens that might influence the agreement.

The CDC recommends that when the probability of latent TB infection (LTBI) is low, treatment is not suggested for persons at a low risk who are IGRA positive but TST negative. In addition, the CDC discourages the use of diagnostic tests for LTBI among populations at a low risk for infection with MTB because of the high rates of false positive results. Also, positive human PPD-based IGRA results in BCG vaccinated children may relate to a cross-reaction between BCG vaccine antigens and human PPD rather than infection with MTB, therefore, it is believed that these cases did not need treatment and/or follow-up [27].

The study had some limitations:

(1) The number of scar negative cases was few, so it is suggested to repeat this investigation with more cases.
(2) The number of abortive reaction cases could not be determined, so it is suggested that an investigation comparing the gamma interferon response between cases with scar failure and those in whom the scar fades with time be designed.

5. Conclusion

To conclude, it seems that immune responsiveness to human PPD in scar negative and positive children

may not correspond to TST results, and scar negative children may be better evaluated with a more accurate tool, such as IGRA than contenting with TST alone. In addition, apparent discordance between TST and IGRA that results in scar negative cases would argue against the practice of using TST in scar negative children.

Conflict of interest

None declared.

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