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Evaluation of Cellular Immune Responses to Mycobacterial Antigens among Immigrants from South-East Asian Countries in South Korea

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Abstract

Healthy immigrant females from Vietnam and Cambodia with their Korean spouses were recruited to measure IFN-γ responses to mycobacterial antigens, using a 6-day whole blood assay. The results showed that there is no significant difference in the proportion of positive IFN-γ responses to mycobacterial antigens, PPD, ESAT-6 and CFP-10, between the Korean males and immigrant females aged 30-<40 years. The whole blood assay using mycobactierial antigens can be a useful tool to detect the prevalence of current and past infection with *M. tuberculosis* as shown in healthy immigrants and their Korean spouses. As also found in this study, if an IFN-γ response to ESAT-6 and CFP-10 is an actual indication of latent infection with *M. tuberculosis*, the chance of past infection in Korean males is as high as in the immigrant females aged 30-<40. Thus, unexpectedly immigrants from Asian countries where TB incidence are higher do not pose a significant health threat to Korean spouses older than 30 year olds.

Keywords: Latent Tuberculosis; Interferon-gamma release tests; Immigrant

Introduction

Tuberculosis remains a major public health problem in South Korea, having an intermediate incidence of 74.3 per 100,000 in 2009 [1]. The results of seven national surveys conducted in South Korea from 1965 to 1995 showed that the prevalence of *M. tuberculosis* (*M. tb*) infection in the population below 30 years of age decreased from 44.6 % in 1965 to 15.5% in 1995 resulting in a 5.5% decline in prevalence each year, and the prevalence of respiratory tuberculosis confirmed by sputum microscopy and culture decreased from 0.94% in 1965 to 0.22% in 1995. Also, the drug resistance rate decreased from 38% in 1965 to 9.9 % in 1995 mainly because of the improvement of cure rate which has increased to 75% [2,3]. In spite of constant improvement in controlling tuberculosis in South Korea, the recent increase in numbers of an older immigrant population could be a risk factor for tuberculosis. Among the various immigrants, women from South-East Asia, who come to Korea to marry Korean men, and who come from countries where there is a high burden of tuberculosis, would have a close relationship with their Korean spouses, and could potentially infect their spouses and children [4,5,6,7]. However, the prevalence of infection with *M. tb* or disease among these immigrant women in South Korea has not been known. Therefore, it is important to evaluate the prevalence of infection with *M. tb* in these groups in order to establish proper health policies and to develop health interventions suited for immigrants and their family members. This group of immigrants and their spouses also provides an opportunity to compare performance of the prolonged whole blood assay in groups of subjects predicted to have greater or lesser exposure to *M. tb*.

The purpose of the study was to evaluate cellular immune responses to mycobacterial antigens in immigrant women from Vietnam or Cambodia, based on the hypothesis that the immigrants would have had greater exposure to M. tb than their Korean spouses.

Materials and Methods

Study population

This study was carried out as part of the project conducted by the KCDC (Korea Centers for Disease Control and Prevention) and Ewha Womens University School of Medicine, Seoul, Korea 'A cohort study for immigrants from South-East Asia, 2006'. Healthy female immigrants from Vietnam or Cambodia who had entered South Korea at least 2 years previously or had at least one child were recruited, with their male Korean spouses. Sample collection from study participants was carried out from November to December 2006. In this cohort, samples from the age-matched individuals aged 30-<40 years were usedto compare cellular immune responses to mycobacterial antigens between female immigrants and their Korean spouses.

Blood collection

The peripheral blood sample (2 ml) was collected in a BD vacutainer tube with sodium heparin at the Ewha Womens University Mok-dong Hospital and delivered to the laboratory at the Korean Institute of Tuberculosis (KIT) within 6 hours. Blood samples from immigrants and their spouses were handled together. The study nurse recorded the time of blood collection and volume of blood. The samples were processed immediately upon arrival in the laboratory.

Preparation of mycobacterial antigens

Various mycobacterial antigens were prepared for the whole blood assay. *M. tb* (Purified Protein Derivative (PPD) batch RT49 for in vitro use was obtained from the Statens Serum Institute (SSI), Copenhagen

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Denmark. Phytohaemagglutinin (PHA) was obtained from Sigma, St. Louis, MO and reconstituted to 5 mg/ml in phosphate buffered saline (PBS). The recombinant antigens, Early Secreted Antigenic Target-6 (ESAT-6) and Culture Filtrate Protein-10 (CFP-10) diluted in PBS at 1 mg/ml were obtained from Dr. Taek-Sun Song of the Immunology Department of KIT, Seoul, Korea. Those proteins, ESAT-6 and CFP-10, are expressed by *M. tuberculosis* (but *also M. flavescens, M. kansasii, M. marinum,* and *M. szulgai*), but absent from Bacille Calmette-Guerin (BCG) (and most of the nontuberculous mycobacteria) [8] .

In-house 6-day diluted whole blood assay and IFN-γ ELISA

A 6-day whole blood assay was employed to measure Interferon-gamma (IFN-γ) responses [9,10,11]. Heparinized whole blood was diluted 1 in 5 with RPMI-1640 supplemented with 2 mM L-glutamine (Gibco BRL), and transferred to a 96 well round bottom culture plate (Nunc) at 100 µl per well. To this, a further 100 µl of RPMI/ L-glutamine (Gibco) was added either alone (nil control) or containing antigens including PPD, recombinant ESAT-6 and CFP-10, or PHA as mitogen, to give final concentration of 5 µg/ml for PPD, 2 µg/ml for ESAT-6 and CFP-10 and 5 µg/ml for PHA. Cultures at a final whole blood dilution of 1 in 10 were incubated at 37°C with 5% CO_2. Supernatants were harvested on day 6 and stored at -80°C until assayed for IFN-γ by in-house cytokine Enzyme Linked Immunosorbant Assay (ELISA) as previously described [9]. All ELISA plates were read at 492 nm wavelength using a VersaMax ELISA Microplate Reader and SoftMax® Pro Software.

The IFN-γ response was categorized for analysis as positive or negative for PPD, ESAT-6 and CFP-10 response. Negative control (RPMI) values were subtracted from all IFN-γ ELISA results. The limit of detection was 31 pg/ml, and 125 pg/ml was used as a cut-off for positivity. The cut-off value was decided on the sum of the mean plus two standard deviations derived from the media only wells (negative controls), 109.4 pg/ml.

Data analysis

Data was analyzed using Stata software (version 11.0). Chi-squared tests were used to compare two groups given by the results of IFN-γ response, and Fisher's exact test was used if the count was sparse. A significant p value was taken to be <0.05. The association between different groups was assessed by Spearman's rank correlation coefficient.

Ethics statement

Approval for the study was given by the Institutional Research Board of the International Vaccine Institute, Seoul (protocol # 2006-006). All subjects gave written informed consent prior to their enrolment in the study.

Results

General characteristics

Background information and questionnaires related to health conditions were collected from a total of fifty nine female immigrants and Korean males. For this study, relevant information was extracted as shown in Table 1. The country of origin or nationality of the male and female participants was different: all of the males were Korean while the females were either from Vietnam or Cambodia. The mean age of the females was 32.5 years (range 30-36) and that of the males was 36.7 years (range 33-39). Three of the individuals who responded in the survey had a history of tuberculosis , all of whom were males.

IFN-γ response to PPD, ESAT-6 & CFP-10

Based on the analysis from individuals in both groups aged 30-<40 years (n=59), 89.3% of females and 100% of males gave positive IFN-γ responses (≥125 pg/ml) to PPD, showing that there is no significant difference in positivity between the two groups (p=0.101, Figure 1 a) and b)). The data shows that the proportions of overall positive responders (≥125 pg/ml) to ESAT-6 in immigrant females and the Korean spouses were 21.4% and 25.8% respectively showing that there is no significant difference in the proportion of positive responses between the two groups (p=0.766, Figure 1 c) and d)). The proportions of positive responders (≥125 pg/ml) to CFP-10 in immigrant females and Korean spouses were 17.9% and 32.3% of respectively, but there is no significant difference in the proportion of positive responses (p=0.243, Figure 1 e) and f)).

Correlation between IFN-γ responses to ESAT-6 and CFP-10

As the data on IFN-γ response to ESAT-6 and CFP-10 were not normally distributed, a non-parametric correlation test (Spearman) was used in this analysis. The results show that the IFN-γ response to ESAT-6 is significantly correlated with that of CFP-10 in the immigrant females (n=28, Spearman correlation coefficient= 0.62, p<0.001, Figure 2 a)). In the Korean spouses, the IFN-γ response to ESAT-6 is also significantly correlated with that to CFP-10 (n=31, Spearman correlation coefficient=0.76, p<0.001, Figure 2 b)).

Discussion

Following recent developments such as the identification of several important protein antigens and cell-mediated mechanisms involved in *M. tb* infection and BCG immunization, there has been much emphasis on the relationship between T cell cytokine responses and protective immunity against tuberculosis [8,12,13,14]. IFN-γ production especially has been measured in *in vitro* assays as a surrogate marker of protection or to indicate the extent of the T cell response induced by vaccines, environmental mycobacteria or infection with *M. tb* [15,16,17,18,19]. In addition, the time of stimulation with mycobacterial antigens has been shown to affect the results of Interferon Gamma Release Assays (IGRA) in some studies [20]. Leyten et al. demonstrated that short-incubation based commercial IGRAs such as QFT-GIT and ELISPOT tests were less sensitive for the detection of past latent TB infection (LTBI) than a long-incubation based lymphocyte stimulation test (LST) using the same *M. tb*-specific peptides from ESAT-6 and CFP-10 [21,22,23]. This indicates that QFT-GIT or ELISPOT might not be an optimal tool to detect individuals with latent infection in high burden areas who have been previously infected with *M. tb*. Therefore, in this

Characteristic	No. (%) of subjects	Age (yr)		No. (%) of subjects with TB history:	
		Mean	Min-Max	Yes	No
Male (Koreans)	31 (52.5)	36.7	33-39	3 (9.7)	28 (90.3)
Female (Cambodians or Vietnamese)	28 (47.5)	32.5	30-36	0 (0.0)	28 (100)
All	59 (100)	34.7	30-39	3 (5.1)	56 (94.9)

Table 1: Characteristics of study participants.

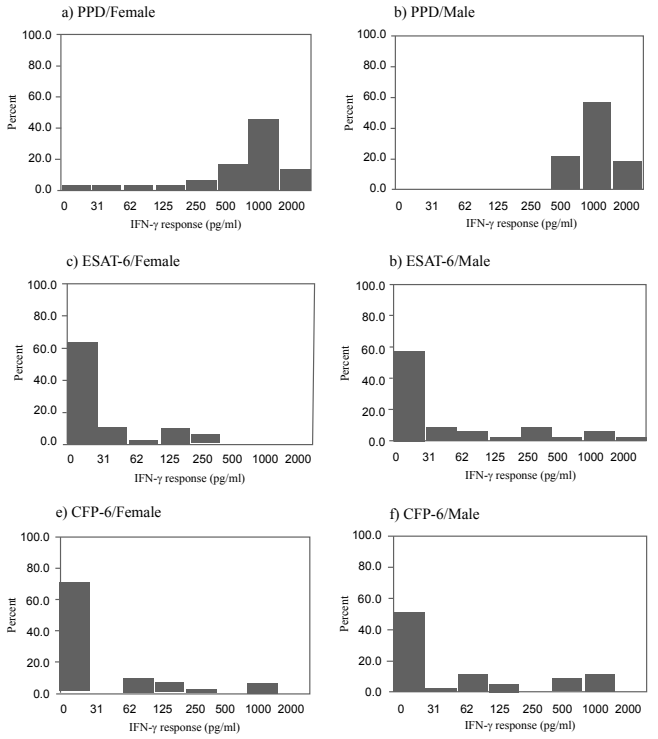

Figure 1: Distribution of IFN-γ responses in immigrant females and their Korean spouses. IFN-γ responses to PPD were measured in immigrant females (a), and in Korean males (b). IFN-γ responses to ESAT-6 were measured in immigrant females (c), and in Korean males (d). IFN-γ responses to CFP-10 were measured in immigrant females (e) and in Korean males (f). IFN-γ responses were defined as negative (<125pg/ml) or positive (≥125 pg/ml).

study, healthy immigrants from Vietnam and Cambodia with their Korean spouses aged 30-<40 years were analyzed to detect participants with latent infection in terms of IFN-γ response using along incubation diluted whole blood assay.

Comparing the proportion of positive responses to PPD between the immigrants and their Korean spouses, it appears that 89.3% of females and 100% of males gave positive IFN-γ responses (≥125 pg/ml) showing there is no significant difference in the proportion of positivity between the two groups (p=0.101).

It seems that the proportion of positive IFN-γ responses to PPD is very high in all individuals, which might be induced by infection with *M. tb*, BCG vaccination or infection with nontuberculous mycobacteira (NTM).

Based on a World Health Organization (WHO) country profile in 2009, Cambodia and Vietnam are known to have high burdens of tuberculosis having an incidence rate of 442 per 100,000 population and 200 per 100,000 population respectively [24]. Taking this into account, it was assumed that many of the adult immigrants before coming to live in Korea might have had more chances to be exposed to *M. tb*. With this difference in burden of tuberculosis between Korea and the countries from which the immigrants had migrated, a higher proportion of positive IFN-γ responses (≥125 pg/ml) to ESAT-6 and CFP-10 were expected in the immigrant group. However, the data shows that the proportions of positive responders to ESAT-6 and CFP-10 in immigrant females and Korean spouses aged 30-<40 years were not significantly different. This might be because the Korean spouses as well as the immigrant wives have had high TB infection risks since childhood, which is different from the initial hypothesis that the immigrants from Vietnam and Cambodia would have had greater exposure to *M. tb* than their Korean spouses. For example, the rates of positive IFN-γ responses to one or other of ESAT-6 or CFP-10 in a group of Korean military officers aged over 30 years (48.0%) were similar to that in the Korean spouses aged 30-<40 years (51.6%) (Lee, unpublished data).

Interpretation of the results presented in this study is complicated by differences in the groups in terms of sex, country of origin, and region (ie, rural or urban). All the immigrants were female and their Korean spouses were male. This could be a confounding factor but cannot be avoided in a study of this type. The study by Gallant et al., found no impact of sex on Interferon Gamma Release Assay (IGRA) or TST results (also, a sex effect was not detected in any of the other related studies carried out in Korea) [25].

As also noted in the study by Gallant et al., long term whole blood assays may be confounded by BCG vaccination [25]. However, the BCG vaccination status of the this cohort was not available in the survey. Instead, the BCG vaccination ruling in the country relevant to 30-40 year olds should be examined as a reference. Based on a WHO 'Immunization Profile' for Cambodia and Vietnam, the reported immunization coverage of BCG vaccine was not more than 50 % prior to 1980 in both countries. In case of South Korea, the national tuberculosis prevalence survey conducted in 1995 reported that the BCG vaccination scar prevalences by age groups of 20-24 years and 25-29 years were 96.9% and 97.4% respectively [2]. Taking into consideration of these references, we assumed that not more than 50% of immigrant females were vaccinated with BCG, and about 95% of Korean males might have been vaccinated with BCG in the past. This difference in BCG coverage may possibly affect the results on the positivity for mycobacterial antigens (such as PPD) among Korean males compared to immigrant females.

In conclusion, the finding that sensitive recognition of the ESAT-6 and CFP-10 antigens was detected in this study group suggests that these assays can be used to monitor exposure to, and infection with *M. tb* in South Korea and elsewhere. As also found in this study, if an IFN-γ response to the ESAT-6 and CFP-10 is an actual indication of latent infection with *M. tuberculosis*, then the chance of past infection

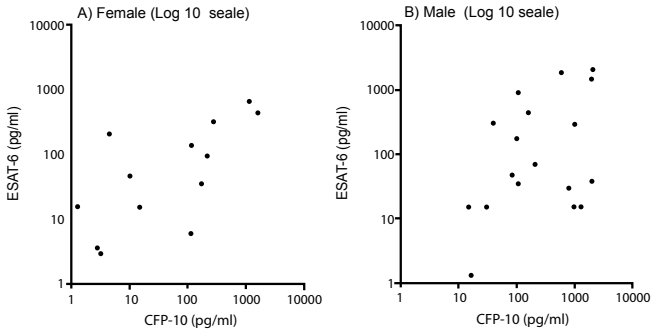

Figure 2: Scatter plot of IFN-γ responses to ESAT-6 and CFP-10. The relationship between IFN-γ response to ESAT-6 and CFP-10 in immigrant females (A), and in Korean males (B) aged 30-40 years is shown (Log 10 scale).

in Korean males is as high as in immigrant females. This may lead to the conclusion that immigrants from East-Asian countries where TB incidence are higher do not pose a significant health threat to older Korean males above 30 years of age.

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