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Relationship between *NRAMP1* gene polymorphism and efficacy of BCG vaccine in a helminth-infected population

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ABSTRACT. Infection of mothers with schistosomiasis and filariasis has been shown to influence infant responses to neonatal Bacille Calmette-Guérin (BCG) immunization. The genetic makeup of infants is also considered an important determinant for the activity of BCG vaccine. The effect of natural resistance-associated macrophage protein 1 (*NRAMP1*) gene polymorphism on the efficacy of BCG vaccine was examined in neonates with helminth-infected mothers (63 infants) and the results were compared with neonates of uninfected mothers (187 infants). After BCG vaccination, assessment of scar presence, tuberculin test, stool analysis, and IgE level was performed. Polymorphism of the *NRAMP1* gene was investigated by PCR amplification followed by RFLP analysis. We found that patients with heterozygosity of intron 4

(GC) and/or maternal infection with helminth parasites showed reduced efficacy of BCG vaccine against tuberculosis.

Key words: *NRAMP1* gene polymorphism; Tuberculosis; Helminths; BCG vaccine

INTRODUCTION

Mycobacterium tuberculosis is a pathogenic bacterial species in the genus *Mycobacterium* and the causative agent of most cases of tuberculosis (TB). It infects an estimated one-third of the world's population. In 2008, 11 million TB cases were diagnosed (equivalent to 164 cases per 100,000 population) and 1.3 million deaths (WHO, 2010) were reported.

Bacillus Calmette-Guérin (or Bacille Calmette-Guérin, BCG) strain is used as a vaccine against TB. It is often administered at birth in developing countries. BCG vaccine is prepared from an attenuated strain of live *M. bovis*, which has been sub-cultured in an artificial medium for 13 years and has lost its virulence. Immunization against TB is limited to the BCG vaccine. The World Health Organization recommends a single BCG vaccination at birth in countries with a high prevalence of TB disease (Holmes et al., 1998). The scar produced by BCG vaccination has been used as a marker of BCG immunity in retrospective studies where the protective effect of BCG against TB is evaluated (Smith, 1982).

Helminth infection induces T-helper-2 (Th-2) cell responses and production of immunoregulatory cytokines that modulate responses to both helminths and other co-present antigens (Elias et al., 2005). Both Th-1 and cytotoxic responses are required for immunity to viruses, and during infection by bacteria they may be disabled (Maizels and Yazdanbakhsh, 2003). Therefore, helminth infection can contribute to reduced efficacy of the BCG vaccine and increased susceptibility to TB in tropical countries (Bundy et al., 2000).

The observation that sensitization to maternal schistosomiasis or filariasis persists following BCG immunization at birth, is of particular interest (Malhotra et al., 1999). Host genetic factors are also important determinants of TB susceptibility. The solute carrier family 11, member 1 gene (*SLC11A1*) was first cloned as the *Ity*/*Lsh*/*Bcg* locus (Vidal et al., 1993). The human *SLC11A1* gene, also known as natural resistance-associated macrophage protein 1 (*NRAMP1*), is located on chromosome 2q35 and has 15 exons spanning about 14 kb (Marquet et al., 2000). This gene encodes a trans-membrane protein expressed exclusively in macrophages/monocytes and polymorphonuclear leukocytes (Cellier et al., 1994). This protein is believed to act as a transporter of divalent cations, especially Fe^{2+}, across the lysosomal membrane (Barton et al., 1999). The present study aimed to elucidate factors determining BCG vaccine efficacy, particularly the *NRAMP1* gene polymorphism in infants with helminth-infected mothers.

MATERIAL AND METHODS

Neonates selected from Children's Hospital, Mansoura University, Mansoura, Egypt, over a period of 6 months were examined. A written consent was obtained from parents of the infant subjects. The neonates belonged to both infected and uninfected mothers. The efficiency of BCG vaccine was investigated by assessment of the scar, stool analysis, and immunoglobu-

lin E (IgE) levels. The *NRAMP1* gene polymorphism was investigated by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The group with uninfected mothers comprised 187 infants (106 males and 81 females) and the group with infected mothers comprised 63 infants (37 males and 26 females). All of the infants were full term and well nourished. Mothers or infants with any known allergies, human immunodeficiency virus infection, malignancy, or positive sputum for TB were excluded.

Parasitological examination

Stool examination was performed for mothers and infants using direct smear (Kato thick smear, Katz et al., 1972), trichrome staining (Bukusuba et al., 2004), blood film for *Wuchereria bancrofti* (Garcia and Bruckner, 1993), rapid ELISA test for filarial antibody (CTK Biotech Inc.) (Fayez et al., 2010), and Fumouze Diagnostics IHA for the detection of *Schistosoma mansoni* (Van Gool et al., 2002); and Ziehl Neelsen stain was used for sputum analysis for TB bacilli (Ellis and Zabrowarny, 1993).

Vaccination of subjects

Infants were injected with 0.1 mL BCG vaccine (Pasteur-Mérieux-Connaught, Lyons, France) intradermally in the deltoid region of the left arm at birth. Six months after vaccination, monthly follow-up visits were used to assess the presence of scar. Scar size was measured in both transverse and vertical planes and the average was recorded in millimeters. "Visible scar" was defined as a scar measuring 2 mm or more (Santiago et al., 2003).

Tuberculin test

Six months after vaccination, all 250 participants received a tuberculin skin test (TST) using 0.1 mL purified protein derivative containing 5 TU Tuberculin PPD (VACSERA, Egypt). The TST was administered to the volar surface of either forearm using the Mantoux technique. Indurations were measured 48 to 72 h later. The average of the transverse and vertical plane measurements was recorded as the induration size in mm: 1-4 mm was negative, >5 mm was positive (Chadha, 2001).

Determination of IgE level

IgE assessment was performed using the ELISA kit KP21 IW IgE total I IEMA WELL, Radim, Italy (Matta et al., 2007).

DNA extraction and investigation of polymorphism (469 + 14 G/C) in intron 4 of the *NRAMP1* gene

Genomic DNA was extracted from peripheral blood using a Gentra genomic DNA purification kit. The region containing RFLP within the *NRAMP1* gene was amplified with *Taq* DNA polymerase. A set of primers were designed to amplify a 624-bp fragment including 469 + 14 G/C of intron 4 of *NRAMP1* for polymorphism (Liu et al., 1995). The forward

primer used was: 5'-TCTCTGGCTGAAGGCTCTCC-3'. The reverse primer used was: 5'-TGTGCTATCAGTTGAGCCTC-3'.

Each PCR cycle used 300 ng DNA, 200 mM dNTP, 500 nM primer, and 2.5 U Taq DNA polymerase (Amplitaq Gold, Perkin-Elmer, Norwalk, CT, USA). DNA was initially denatured for 3 min at 94°C, and then PCR amplification was performed via 30 cycles using the following temperature program: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The PCR amplification was completed by a final extension at 72°C for 7 min. The amplification yielded a product of 624 bp.

Upon cleavage with 5 U *Apa*I (Boehringer-Mannheim) for 16 h at 37°C, the DNA fragments were detected using 3% agarose gel stained with ethidium bromide and visualized under UV light. Allele C produced 2 bands of 455 and 169 bp, whereas the G allele remained intact. Agarose gel electrophoresis revealed 3 patterns: normal genotype GG with 624-bp fragments, heterozygous mutated genotype GC with 624-, 455-, and 169-bp fragments, and homozygous mutated genotype CC with 455- and 169-bp fragments.

Statistical analysis

Data were analyzed using the SPSS statistical package version 17. Variables are reported as number and percent. The χ^2 test was used for comparison between groups. Logistic regression analysis was used to predict the independent predictors of TB susceptibility and a 95% confidence interval was calculated. P value of ≤ 0.05 was considered to be statistically significant.

RESULTS

Among the infected mothers, 43 were found to possess *S. mansoni* antibodies by the indirect hemagglutination test and only 2 were positive for *S. mansoni* by Kato Katz smear. In addition, there were 18 mothers who were found to have filarial antibodies by the rapid ELISA test. Among those with filarial diagnoses, 6 mothers with negative blood film had unilateral limb edema. The remaining 12 mothers had equal normal limb circumferences (Table 1). Mothers in the uninfected group and the infants belonging to both groups tested negative by stool examination, blood film, and indirect hemagglutination test for *S. mansoni*, and by rapid ELISA for filaria.

Table 1. Different parasites found among mothers of the infected group.

Parasites	Method of detection	No. of subjects
Schistosoma mansoni	Kato Katz Smear test	2
Wuchereria bancrofti	IHA	43
Unilateral limb edema	ELISA	6
Equal normal limb circumference	ELISA	12

IHA = indirect hemagglutination test.

PCR determination of polymorphism (469 + 14 G/C) in intron 4 of *NRAMP1* (Figure 1) revealed that there is significant association between extent of scar, elevated serum IgE level, helminth infection, and presence of GC genotype versus GG genotype (Table 2). There

was also significant association between extent of scar, elevated serum IgE level, GC genotype, and maternal infection status (infected and non-infected groups) (Table 3).

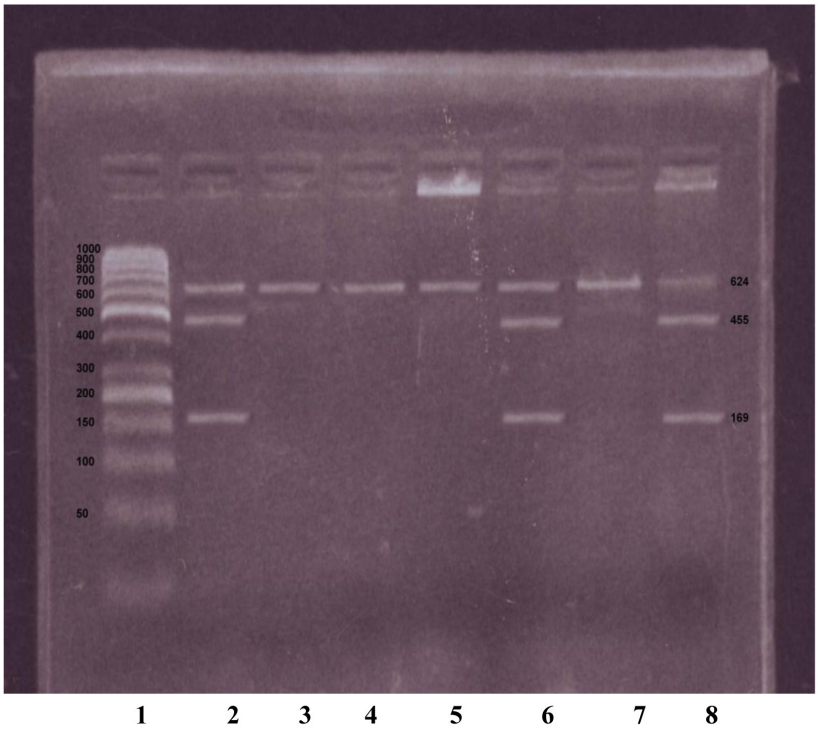

Figure 1. Fragments from polymorphic intron 4 sites after cleavage are shown as follows. *Lane 1* = DNA size marker (50 bp), *lanes 3*, *4*, *5*, and *7* = homozygotes G/G (624 bp), and *lanes 2*, *6*, and *8* = heterozygotes G/C (624, 455, 169 bp).

Table 2. Scar presence, tuberculin test, and IgE level among *NRAMP1* genotypes.

	Intron 4: G/G (149) [N (%)]	Intron 4: G/C (101) [N (%)]	Significance test
Infant gender			
Male (141)	86 (61)	55 (39)	$\chi^2 = 0.261$
Female (109)	63 (57.8)	46 (42.2)	P = 0.610
Presence of scar			
Present (186)	125 (67.2)	61 (32.8)	$\chi^2 = 17.449$
Absent (64)	24 (37.5)	40 (62.5)	P = 0.000
Tuberculin test			
<5 mm (55)	16 (29)	39 (71)	$\chi^2 = 27.3$
5-10 mm (195)	133 (68.2)	62 (31.8)	P = 0.000
IgE value			
Normal (191)	133 (69.6)	58 (30.4)	$\chi^2 = 33.838$
Elevated (59)	16 (27.1)	43 (72.9)	P = 0.000
Maternal infection			
Uninfected (187)	125 (66.8)	62 (33.2)	$\chi^2 = 16.2$
Infected (63)	24 (38)	39 (62)	P = 0.000

Table 3. Comparison between infants belonging to infected and uninfected mothers for the presence of scar, tuberculin test result, serum IgE level, and polymorphism (469 + 14 G/C) in intron 4 of the *NRAMP1* gene.

	Infected (63) [N (%)]	Uninfected (187) [N (%)]	Significance test
Infant gender			
Male	37 (26.2)	104 (73.8)	$\chi^2 = 0.186$
Female	26 (23.9)	83 (76.1)	P = 0.777
Scar			
Present	27 (14.5)	159 (85.5)	$\chi^2 = 43.998$
Absent	36 (56.3)	28 (43.7)	P = 0.000
Tuberculin			
<5 mm	33 (60)	22 (40)	$\chi^2 = 45.303$
5-10 mm	30 (15.4)	165 (84.6)	P = 0.000
IgE			
Normal	29 (15.2)	162 (84.8)	$\chi^2 = 43.08$
Elevated	34 (57.6)	25 (42.4)	P = 0.000
Intron 4			
G/G	24 (16.1)	125 (83.9)	$\chi^2 = 16.176$
G/C	39 (38.6)	62 (61.4)	P = 0.000

Table 4 shows that infants with high IgE levels, a tuberculin test of less than 5 mm, heterozygosity of intron 4 (GC), and/or maternal infection were at greater risk of BCG vaccine failure than those with normal IgE values, a tuberculin test of 5-10 mm, homozygosity of intron 4 (GG), and uninfected mothers.

Table 4. Regression analysis of predictors of efficiency of BCG vaccine among infants.

	B	P	OR (95%CI)
IgE value			
High	-	0.000	2.1 (1.2-10.1)
Normal	1.4		1 (r)
Tuberculin test			
<5 mm	-	0.000	6.3 (2.3-21.8)
5-10 mm	2.7		1 (r)
Intron 4			
GC	-	0.000	4.1 (2.7-11.2)
GG	0.436		1 (r)
Type of group			
Infected	-	0.000	11.2 (8.1-31.2)
Uninfected	1.6		1 (r)
Constant model			
χ^2, P	1.1, 37.7, P = 0.000		
% correctly predicted	33.1, 84.0		

OR = odds ratio; 95%CI = 95% confidence interval; r = reference group.

DISCUSSION

Although BCG vaccine has been in use since 1921 and approximately 3 billion doses have been dispensed, the efficacy of the vaccine continues to be debated (Colditz et al., 1994). In the present study, it was found that there was significant association between scar existence, elevated serum IgE level, maternal helminth infection, and reduced efficacy of the vaccine in infants belonging to the GC genotype as compared to infants belonging to the GG genotype. Simultaneous infection with helminthic parasites might prevent the development of protective

responses following vaccination (Lipner et al., 2006). There may be a negative correlation between the dose of infectious helminth and the magnitude of the immune response (Steel and Nutman, 2003). The *NRAMP1* gene polymorphism is another factor affecting BCG efficacy (Nikonenko et al., 1996).

Vaccination card checking, BCG scar rate, and percentage of TST conversion can track the degree of coverage, vaccine efficacy, and exposure to *Mycobacterium* in the community (Ismaiel et al., 2008). Usually, BCG vaccination almost invariably results in tuberculin conversion, with a positive tuberculin skin test developing 4-8 weeks after vaccination (Menzies, 2000). TST applied after BCG vaccination usually produces a reaction of <10 mm; there is an association between tuberculin reaction (5 to 9 mm) and the presence of a BCG scar (Kebede, 1993).

In this study it was found that the gender of the infant played no role among infected and uninfected groups while there was significant effect of scar existence, IgE level, and intron 4 polymorphism. This result is in accordance with Zwingenberger et al. (1991), who stated that helminthic infections in both animals and humans predispose the host to respond with a Th2-like immune profile, in which levels of interleukins (IL)-4, IL-5, and IL-10 are much higher than levels of IFN-γ and IL-2. Also, cytokines derived from Th1 and Th2 immune responses cross-regulate one another.

The *NRAMP1* gene is the human equivalent of the murine *NRAMP1* gene for resistance to intracellular parasites including BCG, *Leishmania*, and *Salmonella* (Buschman and Skamene, 2001). Innate immunity to TB is under the control of a single gene, which is designated as *NRAMP1*, also known as the *SLC11A1* gene (Blackwell et al., 2003). A study in Gambia found that heterozygotes for intron 4 of *NRAMP1* were at increased risk for TB, whereas in mice, only homozygotes for the *NRAMP1* D169 variant have been found to be susceptible to intracellular pathogens (Bellamy et al., 1998). The small number of homozygotes in that study precludes a definite analysis of their susceptibility to TB. However, homozygotes do not appear to be more susceptible than heterozygotes. These data suggest that in human *NRAMP1* gene variants, the TB susceptibility allele is dominant, whereas in the murine *NRAMP1* D169 variant, the resistance gene is dominant.

Kim et al. (2003) found that the frequency of mutant genotypes of intron 4 was significantly higher in tuberculous pleurisy ($P = 0.01$). Bellamy et al. (1998) found that heterozygous intron 4 polymorphism was particularly overrepresented among those with TB in West Africa ($P = 0.006$).

Koh et al. (2005) stated that heterozygous intron 4 (469 + 14 G/C) of *NRAMP1* is observed with significantly greater frequency in patients with non-TB mycobacterial lung infections than in control subjects. On the other hand, Nikonenko et al. (1996) found that the efficiency of BCG vaccine is not dependent on the *NRAMP1* gene polymorphism. They ascribed their negative result to the fact that TB susceptibility measured by mortality does not necessarily correspond to TB susceptibility measured by parasite growth in organs. Also, although the *NRAMP1* gene is able to regulate bacterial growth in organs after a high level of infection, this finding has been documented only for intravenous injection of BCG vaccine. However, in the experiments by Nikonenko et al. (1996) mice were vaccinated subcutaneously.

Alm et al. (2002) found no statistically significant interaction using logarithmic regression between BCG vaccination and the *NRAMP1* gene polymorphism in relation to atopy. Atopy induces Th-2-like immune response. This imbalance is thought to be a result of both

constitutional and environmental impacts (Romagnani, 2000). It has been proposed that the *NRAMP1*-encoded protein transports metal ions from the phagosomal lumen into the cytoplasm. Thus, metal-ion depletion in the phagosomal lumen becomes a rate-limiting step in the functioning of the metalloenzymes of the phagocytosed bacteria. This restricts the ability of the bacteria to produce and activate enzymes such as superoxide dismutase and prevents the propagation of the ingested microorganisms. On the other hand, an increased concentration of metal-ions in the phagosome produced by a defective *NRAMP1*-encoded transport protein may promote the growth of the mycobacteria and render the invaded organism sensitive to the pathogen (Bellamy et al., 1998). The same author also stated that the heterozygous genotype of the *NRAMP1* gene might lead to a divalent cation concentration in human lysosomes more conductive to mycobacterial growth. Finally, it is also possible that *NRAMP1* gene-variant homozygotes that are infected with *M. tuberculosis* in childhood succumb to the disease rapidly and are therefore not overrepresented among adults with TB. The discovery of *NRAMP1*-related genes in several bacteria suggests that the pathogens use the same strategy in competition for the limited amounts of metal-ions inside the lysosomes (Makui et al., 2000).

CONCLUSION

The study found that maternal infection by helminth parasites can reduce the efficacy of BCG vaccine administered to infants against TB. Heterozygosity of intron 4 of the *NRAMP1* gene can also affect the efficacy of the vaccine, especially among infants belonging to infected mothers. Further studies are recommended for characterizing the molecular mechanism by which the *NRAMP1* gene influences the efficiency of BCG vaccine. These studies may lead to new therapies for the prevention of TB.

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