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Original Article

Cytokine Gene Polymorphism in BCG Lymphadenopathy

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Abstract

Background: Tumor necrosis factor-beta or lymphotoxin-alpha (LT-α), IL-4 and IL-10 are determining factors in immunity against BCG. Allelic polymorphisms in the regulatory regions of their genes affect the rate of cytokine production and therefore, the host's ability in BCG containment.

Objective: To study the prevalence of –590 (C/T) and –592 (C/A) allelic distribution of IL-4 and IL-10 promoter regions, respectively, and +282 (G/A) polymorphism in the first intron of LT-α in BCG vaccinees with lymphadenopathy comparing to those of controls.

Methods: Polymorphisms in the promoter region of IL-4 and IL-10 were determined by primer induced restriction site PCR and the polymorphism in the first intron of LT-α was investigated using PCR-RFLP method. Forty patients with BCG adenitis and 42 healthy age-matched infants without reactions were included in this study.

Results: No significant differences existed between allele and genotype frequencies of IL-4 or LT-α genes from patients as compared to the controls. A significant difference in genotype distribution of the IL-10 –592 C-to-T polymorphism was observed between patients and controls ($p<0.05$). In this respect, the AA and AC genotypes with lower ability in IL-10 production were found more frequently in the control group.

Conclusion: The lower frequency of AA genotype at position –592 of IL-10 promoter region in patients may have resulted in more IL-10 production leading to weaker immune response that allows bacterial burden and occurrence of lymphadenitis.
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Keywords • BCG • adenitis • IL-10 • IL-4 • LT-alpha • polymorphism

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Introduction

A number of factors have consistently been identified as important in the etiology of BCG-adenitis, including the type and concentration of vaccines, the age of vaccinees, the uses of proper intradermal injection technique and the characteristics of recipient population.[1,2] Genetic factors are also suggested to in-

fluences susceptibility to BCG-adenitis. Inherited genetic polymorphisms at cytokine locus, acting early or late in the immune response, may mediate immune system responsiveness. Tumor necrosis factor-β (TNF-β) or lymphotoxin-alpha (LT-α) is a critical cytokine produced early in the inflammatory reactions. The major sources of this cytokine are T cells. Similar to tumor necrosis factor-alpha, LT-α also uses TNFR I and TNFR II for signaling. Signaling via these pathways can result in different outcomes, such as cell activation, proliferation and apoptosis.[3] LT-α may be involved in dendritic cell activation and maturation and through the role in macrophage activation[4] may be considered as an important factor in the control of BCG-adenitis. In several studies, the important role of this cytokine in murine host defense against BCG has also been reported. In LT-α knockout mice, immune responses to BCG infection were significantly delayed and reduced resulting in immature granulomas allowing uncontrolled infection.[5,6] Furthermore, in response to Mycobacterium tuberculosis, LT-α deficient mice recruited normal numbers of T cells in to their lungs but the lymphocytes were recruited to perivascular and peribronchial areas and were not co-located with macrophages in granulomas.[7] In addition, LT-α can also exist as a membrane-bound heterotrimer complexed with LT-β. This complex through binding to LT-β receptor contributes to the induction of Th1 type of immune response and granuloma formation required for host defense against BCG infection.[8] Interleukin 4 (IL-4) is another cytokine that is produced mainly by T helper cells and early production of this cytokine by other sources, like $CD4^+$ natural killer T cells, mast cells and basophiles have also been reported.[9] Considering adverse effects of IL-4 on IFN-γ production and macrophage activation, an inhibitory effect for this cytokine in BCG containment may be suggested.[10] However, in human IL-4 may also play a protective role through the enhancement of dendritic cells capacity in IL-12 production and thereby deviation of immune system towards the Th1 responses.[11] Interleukin-10 (IL-10) is another cytokine that is produced predominantly by Th2 cells and downregulates macrophage functions.[12] Macrophages can be stimulated to produce IL-10 in response to *Mycobacterium* bovis BCG[13] and some recent studies indicated the enhanced cell mediated immunity and robust granuloma response to mycobacterial infection in IL-10 deficient mice.[14, 15] Therefore, the higher ability of host in IL-10 production might have detrimental effects on mycobacterial (BCG) infections.

LT-α gene is located on chromosome 6 within the class III region of the MHC locus.[16] The IL-4 and IL-10 genes are located, in the Th2 cytokine locus on chromosome 5[17] and chromosome 1, respectively.[18] A functional polymorphism of the C-to-T type at the position–590 in the promoter region of the IL-4 gene[19] and three single nucleotide polymorphisms at positions –1082(G/A), -819(C/T) and –592(C/A) in the promoter region of IL-10 gene have been reported.[20] In 1991, Messer et al. also described a polymorphic site in the first intron of the LT-α gene.[21] In this regards, a single base substitution at position +252 of LT-α gene results in two allelic forms, in which, the presence of G or A nucleotides defines the low and high producer alleles [21], respectively. The polymorphism within the IL-4 gene also results from a functional single base substitution in which cytosine is replaced by thymidine at the position of –590 of the promoter region.[19] This point mutation was reported to be associated with increased IL-4 gene promoter activity.[19] From the three polymorphisms described in IL-10 promoter region, the presence of A nucleotide at position –592 is usually associated with a low IL-10 producing genotype.[22, 23]

Immunity to *Mycobacterium tuberculosis* and BCG induced protection is essentially elicited through the generation of appropriate cell mediated immunity or Th1 response. In this respect, host ability in production of pro-inflammatory (LT-α) or anti-inflammatory (IL-4 and IL-10) cytokines may play an important role. Therefore, we asked whether a di-allelic polymorphisms within the genes of these cytokines might play a role in lymphadenitis after BCG vaccination. We respectively investigated the –590 (C-to-T) and –592 (C-to-A) allelic distribution of IL-4 and IL-10 promoter genes and a G-to-A transition at position of +252 in the first intron of LT-α in a collection of 40 patients with BCG adenitis.

Materials and Methods

Patients and Controls

Forty infants with BCG adenitis (24 males and 16 females) along with 42 normal infants (22 males and 20 females), from our previous study population[29], were investigated in this study. All patients and controls were vaccinated with BCG during the first postnatal week.

Determination of cytokine genotypes

Genomic DNA was extracted from peripheral blood leukocytes by a salting out procedure. The genotype of LT-α was determined by PCR-RLFP method.[24] Allelic forms of IL-4 and IL-10 genes were studied using primer induced restriction site.[25, 26] For LT-α genotyping 10 µl of PCR reaction mixture were comprised of: genomic DNA samples (125 ng), 200 µmol/L dNTPs, 2 mM MgCl2, 1X Taq DNA polymerase buffer, two units of Taq DNA

Table 1a: *Rsa*I RFLP of the IL-10 promoter region in BCG adenitis

	Genotype frequency (%)			Allele frequency (%)	
	TNFB*1/1 GG	**TNFB*1/2 AG**	**TNFB*2/2 AA**	**TNFB*1 G**	**TNFB*2 A**
BCG adenitis (n=40)	4(10%)	11(27.5%)	25(62.5%)	19(23.7%)	61(76.3%)
Control (n=42)	0(0%)	18(42.8%)	24(57.2%)	18(21.4%)	66(78.6%)

Table 1b: *Rsa*I RFLP of LT-α gene in BCG adenitis

	Genotype frequency (%)			Allele frequency (%)	
	CC	**AC**	**AA**	**A**	**C**
BCG adenitis (n=40)	20 (50)	18 (45)	2 (5)	22 (27.5)	58 (72.5)
Control (n=42)	13(30.9)	20 (47.6)	9 (21.5)	38(45.2)	46 (54.8)

Table 1c: *Ava*II RFLP of the IL-4 promoter region in BCG adenitis

	Genotype frequency (%)			Allele frequency (%)	
	CC	**CT**	**TT**	**C**	**T**
BCG adenitis (n=40)	30(75%)	10(25%)	0(0%)	70(87.5%)	10(12.5%)
Control (n=42)	26(61.9%)	13(30.9%)	3(7.2%)	65(77.4%)	19(22.6%)

polymerase (Bohringer Manheim, Germany) and 10 pmol of each test primer. Cycling conditions were as follows: one cycle of 95 °C for 5 minutes followed by 31 cycles of 94 °C for 30 seconds, 61°C for 150 seconds, and 72 ºC for 30 seconds; and finally one cycle of 72 °C for 10 minutes. Subsequent to amplification, the PCR product (10µL) was digested with 5 units of *NcoI* at 37°C for >16 hours. TNFB*1 allele gave 2 fragments of 586 and 196 bp, and TNFB*2 allele a single 782-bp fragment (Figure 1). Determination of IL-4 and IL-10 promoter gene polymorphisms were carried out under the conditions previously described[25, 26] except that the $MgCl_2$ concentration were 2.2 mM and 2.7 mM, and the annealing temperature was set up on 53°C for

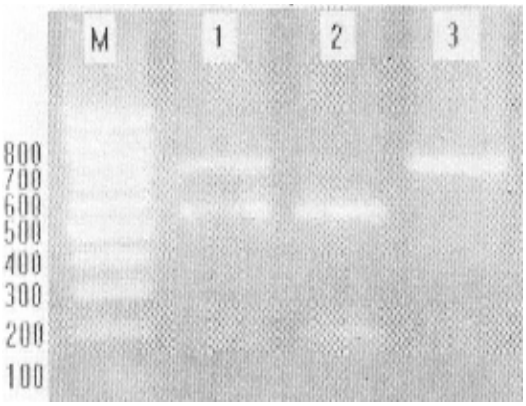

Figure 1. *NcoI* restriction endonuclease digestion of PCR products amplified from the first intronic region of the LT-α gene. M=DNA size marker, 1=heterozygous for the TNFB1/2 genotype, 2=homozygous TNFB*1 allele, and 3=homozygous for the TNFB*2 allele.

50 seconds and 63°C for 70 seconds, respectively. Following the amplification of the desired IL-4 and IL-10 promoter regions, the RFLP assay was performed using *AvaII* and *RsaI* restriction enzymes, respectively. After digestion of PCR products from IL-4 promoter region, the T allele gave a product of 195 bp while two fragments of 177 and 18 bp were produced as a result of C allele digestion (Figure 2). Presence of cytosine at -592 position of IL-10 promoter region gave a product of 412 bp but presence of adenine at this site resulted in 176 and 236 bp bands. Reaction products were separated on a 2-2.5% agarose gel and stained with ethidium bromide (Figure 3).

Statistical tests

The χ^2 test was applied for statistical analysis and Fisher's exact test and the Yate's correction were used for small group of patients, when necessary.

Results

In patients suffering from BCG lymphadenopathy, genotype and allele frequency of LT-α promoter polymorphism (+252) and IL-4 promoter RFLP with *Ava* II polymorphism (–590) did not differ significantly from those of healthy unrelated controls. However, comparison of the +252 LT-α genotype distribution between patients and controls were approximated to statistically significant level (p=0.058) shown in table 1b. In fact, the frequency of TNFB1/1 genotype was 10 times more in BCG adenitis patients compared to those of controls. In respect to IL-4 –590 C-to-T polymorphism, a non-significant increase in frequency of CT and TT genotypes was observed in controls (30.9% and 7.2%) compared to BCG-adenitis patients (25% and 0%) as given in table 1c. These two geno-

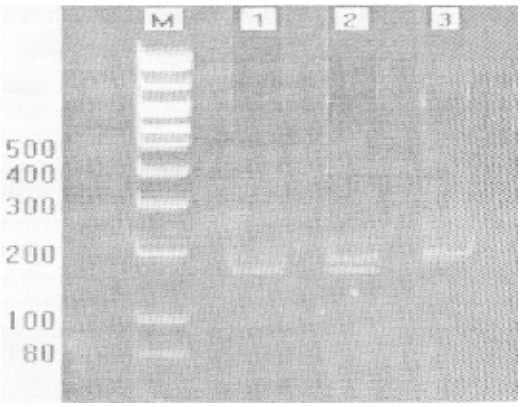

Figure 2. *AvaII* restriction endonuclease digestion of PCR products amplified from –562 nucleotide to –756 nucleotide of the IL-4 promoter sequence. M=DNA molecular weight marker, 1=homozygous for the wild type allele (C allele), 2=heterozygous for the wild type allele and the –590 C→T allele, 3=homozygous for the –590 C→T allele.

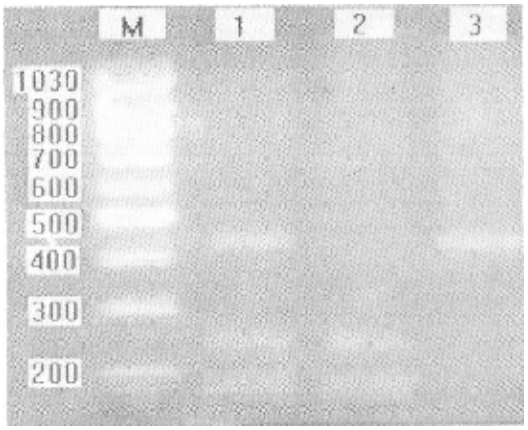

Figure 3. *RsaI* restriction endonuclease digestion of PCR products amplified from the IL-10 promoter sequence. M=DNA size marker, 1=heterozygous for the wild type allele and the –592 C→A allele, 2=homozygous for the –592 C→A allele, 3=homozygous for the wild type C allele.

types represent the potential to produce high levels of IL-4.

Patients with BCG adenitis had a decrease in A nucleotide and an elevation in C nucleotide at position –592 of IL-10 promoter, comparing to controls ($p<0.03$). Moreover, there was a significant difference in genotype distribution of the IL-10 –592 polymorphism between patients and controls ($p<0.05$) shown in table 1a. Of interest was the finding that AA and AC genotypes which were found more frequently in controls remained associated with a lower ability in IL-10 production.[22, 23]

No clear associations were found between these gene polymorphisms and the clinical data, such as the size and the number of enlarged lymph nodes.

Discussion

In immunocompromised individuals, BCG vaccination results in disseminated Mycobacterial infection.[27] Furthermore, the use of BCG vaccine in apparently normal individuals may be associated with a significant number of adverse reactions; among them, lymphadenitis is the most frequent one.[28] In a previous study[29] we reported that the TNF-α polymorphism was not statistically related to the presence of BCG adenitis and we suggested that in susceptibility to BCG lymphadenopathy, other alleles that lie in the same or other chromosomal regions might be important.

By considering the important role of LT-α, IL-4 and IL-10 in mycobacterial infections[5-8, 14] and the presence of gene polymorphism that affect their production,[19-21] we hypothesis that the genetic ability of host in production of these cytokines may be a major determinant in bacterial containment and susceptibility to BCG adenitis. Therefore, we used association-based case-control study to compare the allele frequency of these three candidate genes in individuals who are affected by BCG-adenitis, compared to healthy controls.

LT-α is an important cytokine in defense against intracellular bacteria and its role in BCG eradication has been shown in several studies.[5-8] Within the LT-α gene, a G-to-A substitution at position +252 of the first intron of the gene was suspected to be associated with less strong LT-α response.[21] In our present study, the difference in genotype frequency of LT-α between patient and control groups approximated to a significant level ($p=0.058$). In fact, there is a definite trend indicating that more of the patients have the genetic ability to produce high levels of LT-α (GG genotype). This pattern is not in agreement with previous reports correlating the presence of this cytokine to resistance to Mycobacterial infections.[5-8] One explanation for this quite puzzling finding is that LT- α may be a minor disease associated allele relative to alleles that lie in the same haplotype.

The finding that controls carry more frequently a promoter polymorphism predisposing them to high production of IL-4 was also unexpected. Nonetheless, supportive data were obtained by Ebner et al., who reported that production of IL-12 by human monocyte-derived dendritic cells is further enhanced by the presence of IL-4 during stimula-

tion.[11] Furthermore, while IL-4 may individually suppress the Th1 immune responses, when presented simultaneously with TGF-β, can regulate the differentiation of CD8 cells towards the Th1 responses. That is the addition of both IL-4 and TGF-β was reported to induce IFN-γ secretion by CD8+ cells and enhance their cytotoxicity.[30] Therefore, the in situ combination of different cytokines may determine the ability of IL-4 in diverting immunity towards Th1 or Th2 responses. Further studies should clarify the true nature of IL-4 polymorphism effects with regard to BCG adenitis.

It is well established that IL-10 is an important immunosuppressive cytokine.[12] [31] In animal models, the increased resistance to Mycobacterial infection in the absence of IL-10 has also been reported.[14,15] It is therefore reasonable to assume that individuals with the ability to produce only low levels of IL-10 are more potent in BCG controls and therefore will be resistant to BCG-lymph–adeno–pathy. In this regard, IL-10 promoter polymorphisms have been implicated in determining the severity of several inflammatory diseases. Presence of cytosine at position –592 of IL-10 promoter is associated with systemic lupus erythematosus and is consistent with the high IL-10 concentrations seen in these patients.[22] Presence of A nucleotide at this position determines the low IL-10 production capability and is associated with a more severe form of rheumatoid arthritis.[23] In our study the frequency of high producing allele of IL-10 (C nucleotide at position –592) was higher in patients as shown in Table 1a ($p<0.03$). That the genetic ability to produce low levels of IL-10 is more prevalent in controls is not surprising. In fact normal individuals with low secretion of IL-10 should have more MHC class II molecules on their antigen presenting cells and thereby should have better capabilities to present bacterial peptides to T cells, leading to improved elimination of BCG and prevent the occurrence of BCG-adenitis.

In summary, the multi-factorial nature of post BCG immune responses, complicate the ability to differentiate the role and contribution of individual parameters for the development of BCG adenitis. However, our results demonstrate an additional factor that should be considered in BCG-adenitis; the genetic make-up of vaccinees. We therefore, propose that a prospective larger case-control study can decipher the true role of cytokine gene polymorphism in BCG-adenitis. Nonetheless, our data strongly suggest an association between IL-10 cytokine gene polymorphism and occurrence of BCG-lymphadenopathy. Furthermore, with respect to the importance of two other polymorphisms in the IL-10 promoter region, it is apparent that analysis of three different IL-10 polymorphisms as a haplotype will be more informative.

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