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Frequency of Cytokine Polymorphisms in Populations From Western Europe, Africa, Asia, the Middle East and South America

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ABSTRACT: PCR-SSOP identification procedures for IL-2, IL-6, IL-10, TNF-α and TNF-β cytokine polymorphisms have been developed. Application of the procedures to a range of diverse geographically distributed populations has identified ethnic differences within the groups studied. Five populations were investigated, Northern Ireland, South African Zulu, Omani, Singapore Chinese and Mexican Mestizos. *Human Immunology 63, 1055–1061 (2002).* © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: cytokine; SNP; PCR-SSOP; populations

INTRODUCTION

The release of cytokines plays a pivotal role in the regulation of the immune response. Polymorphisms in several cytokine genes, mostly single nucleotide polymorphisms (SNPs) or microsatellites, located within the promoter or other regulatory regions, affect gene transcription and cause inter-individual variations in cytokine production. This may arise either directly or indirectly through tight linkage with other, as yet undescribed, nucleotide differences. By allowing differential production of cytokines, these polymorphisms may confer flexibility in the immune response. The presence of certain alleles may influence the outcome of both viral and bacterial infections [1, 2]. In addition, numerous studies have implicated cytokine polymorphisms in susceptibility and resistance to autoimmune diseases [3]. Polymorphism within the cytokine network may provide a clearer insight into the appropriate immunotherapy when dealing with disease, or in the case of post transplant graft survival, immunosuppressive therapy.

There are two main types of immune response that are under the control of cytokines, the cellular (Th1) and the humoral (Th2) responses. The key cytokine in the Th1 response is interleukin (IL)-2, which plays an essential role in the activation and proliferation of T cells during the immune response. The Th2 response involves the activation of B cells and the key cytokine is the pro-inflammatory multifunctional IL-6. Recently, a novel polymorphism (T→G) was reported in the promoter region of the IL-2 gene at nucleotide position -330, with possible influence on IL-2 production levels [4]. A SNP (G→C) in the promoter region of the IL-6 gene at nucleotide position -174 is reported to affect the levels of transcription of the gene product [5, 6].

The pro-humoral, anti-inflammatory IL-10 has been shown to contain three distinct SNPs within its promoter region [7, 8], at nucleotide positions -1082 (G→A), -819 (C→T), and -592 (C→A), with the SNP at position -1082 giving rise to differential IL-10 production. These three polymorphisms have been shown to be in strong linkage disequilibrium, and three main haplotypes (GCC, ACC, and ATA) are known to segre-

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TABLE 1 PCR primer pairs and amplification conditions used for the detection of single nucleotide polymorphisms (SNP) in cytokine genes by PCR-SSOP

Gene	Primer	5′ Sequence 3′	Product size (bp)	Reference
IL-2[a]	AIL-2	TATTCACATGTTCAGTGTAGTTCT	150	(4)
	RIL-2	ACATTAGCCCACACTTAGGT		
IL-6[b]	FIL-6	GAACACAGAAGAACTCAGATGACTGG	627	Adapted from (6)
	RIL-6	AGGAGTTCATAGCTGGGCTCCTGGAG		
IL-10[c]	IL-10F	ATCCAAGACAACACTACTAA	645	This work
	IL-10Rn	GGCAGTCACCTTAGGTCTCTG		
TNF-α[d]	TNF-αF	AGGCAATAGGTTTTGAGGGCCAT	107	Adapted from (9)
	TNF-αR	TCCTCCCTGCTCCGATTCCG		
TNF-β[d]	TNF-βF	CCGTGCTTCGTGCTTTGGACTA	772	Adapted from (9)
	TNF-βR	AGAGGGGTGGATGCTTGGGTTC		

The PCR cycling conditions for these primer pairs are as follows:
[a] 96°C/1 min; 48°C/1 min; 72°C/1 min (35 cycles);
[b] 96°C/1 min; 63°C/1 min; 72°C/1 min (35 cycles);
[c] 96°C/1 min; 56°C/1 min; 72°C/1 min (32 cycles);
[d] 96°C/1 min; 60°C/1 min; 72°C/1 min (40 cycles).
Abbreviations: PCR =polymerase chain reaction; SSOP = sequence-specific oligonucleotide probe; IL = interleukin; TNF = tumor necrosis factor.

gate in most populations [8]. The tumor necrosis factor (TNF) locus consists of two tightly linked genes (α and β) clustered on chromosome 6 in the class III region of the major histocompatibility complex (MHC). TNF-α is a pro-inflammatory mediator of the immune response to allogenic and infectious stimuli. Two polymorphic nucleotides are reported to influence the level of TNF-α production, a G→A transversion in the promoter region (position -308) of the TNF-α gene itself, and another G→A transversion in the first intron (position +252) of the TNF-β [9].

Methods employing the polymerase chain reaction (PCR) and sequence-specific oligonucleotide probes (SSOP) have been developed to detect known polymorphisms within IL-2, IL-6, IL-10, TNF-α, and TNF-β. The frequency of the different genotypes for these cytokines was investigated in populations from five different continents to evaluate global frequencies for the variant alleles that may cause cytokine dysfunction and establish a databank of information for future disease studies.

MATERIALS AND METHODS

Population Samples

DNA from unrelated individuals from five geographically diverse populations were selected for study as follows.

Northern Ireland: This group consisted of Western European Caucasian blood donors (n = 100).

Zulu: The Zulu-speaking people belong to the Natal Nguni linguistic division of the central Bantu. Blood donors (n = 86) were randomly selected. All individuals were of pure descent, residing in Kwa-Zulu, Natal, South Africa.

Omani: Oman, situated on the south east coast of the Arabian peninsula, borders the United Arab Emirates, Saudi Arabia, and Yemen. The Omani population is predominantly Arab (74%), whereas a proportion of the remaining fraction has connections with the Indian subcontinent and Africa. The Omani group in this study (n = 80) was healthy individuals comprising blood donors and potential bone marrow donors. They resided in various areas in the country and were considered to be representative of a normal population for this region.

Singapore Chinese: Blood donors from Singapore, of Chinese origin (n = 83).

Mexican: Individuals (n = 40) of Mexican Mestizo origin. Mexican Mestizos are described as individuals born in Mexico descended from the original inhabitants of the region and from individuals of Caucasian (mainly Spaniard) and African (from slaves brought from western Africa) origin [10].

HLA class I alleles had previously been determined in all samples [11–14]. The populations tested were chosen to cover a range of geographic locations.

PCR Amplification

DNA extracted by the salting out method [15] was subjected to PCR amplification in 50 μl reaction volumes containing 67 mM Tris-HCL pH 8.8; 16 mM $(NH4)_2SO_4$; 2.0 mM $MgCl_2$ (IL-2, IL-6), 1.0 mM $MgCl_2$ (TNF-α, TNF-β) and 1.5 mM $MgCl_2$ (IL-10); 0.01% (w/v) Tween; 200 μM of each dNTP; 0.2 μM of each primer; 1.5 units of *Taq* polymerase; and 0.1-0.5 μg DNA. Primer pairs either designed in this laboratory or adapted from previous publications were used to achieve specific products for each cytokine, under the specified cycling conditions (Table 1). Each PCR in-

TABLE 2 Probes and conditions used for PCR-SSOP analysis of cytokine polymorphisms

Probe	5′ Sequence 3′	Wash temperature/ probe concentration	SNP	SNP position
IL-2G	GTTCTAGGACAAAGAA	44°C/20 pmoles	G	−330
IL-2T	GTTCTATGACAAAGAA	42°C/20 pmoles	T	
IL-6G	GTCTTGCGATGCTAAAG	50°C/40 pmoles	G	−174
IL-6C	GTCTTGCCATGCTAAAG	50°C/40 pmoles	C	
IL-10/1A	CTTTGGGAAGGGGAAGT	52°C/50 pmoles	A	−1082
IL-10/1G	CTTTGGGAGGGGGAAGT	54°C/40 pmoles	G	
IL-10/2C	TGTAACATCTCTGTGCC	50°C/20 pmoles	C	−819
IL-10/2T	GATGTAAATATCTCTGTGCC	58°C/40 pmoles	T	
IL-10/3C	GCCTGTCCTGTAGGAAG	54°C/20 pmoles	C	−592
IL-10/3A	GCCTGTACTGTAGGAAG	54°C/20 pmoles	A	
TNF-αA	GCCATGAGGACGGGGT	54°C/30 pmoles	A	−308
TNF-αG	GCCATGGGGACGGGGT	58°C/20 pmoles	G	
TNF-βA	CTGCCATGATTCCTCTC	52°C/20 pmoles	A	+252
TNF-βG	CTGCCATGGTTCCTCTC	56°C/20 pmoles	G	

Abbreviations: PCR = polymerase chain reaction; SSOP = sequence-specific oligonucleotide probe; SNP = single nucleotide polymorphism; IL = interleukin; TNF = tumor necrosis factor.

volved an initial denaturation step of 96°C for 5 minutes and a final extension step of 72°C for 5 minutes. Amplified product was verified after electrophoresis by visualization on a 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml).

Hybridization and Chemiluminescent Detection

Oligonucleotide probes were designed from regions of IL-2, IL-6, IL-10, TNF-α, and TNF-β to identify the SNPs of interest within each cytokine (Table 2). Amplified DNA (2 μl) was applied to a charged nylon membrane, as previously reported [16]. Hybridization with digoxigenin-labeled probes and chemiluminescent detection procedures were as previously described [11]. Hybridization was performed at 45°C except in the case of IL-2, in which the hybridization temperature was reduced from 45°C to 40°C because of the low melting temperature of both probes. Probe sequences, hybridization conditions, and stringency wash temperatures are given in Table 2.

STATISTICAL METHODS

Allele and genotype frequencies were compared between the five populations using the chi-squared test for contingency tables. Checks for departures from Hardy-Weinberg equilibrium were performed using the chi-squared goodness of fit test. Haplotype frequencies were estimated by the expectation-maximization (EM) algorithm using the Arlequin software package [17] and compared using a likelihood-ratio chi-squared test obtained by extending a method for the comparison of two groups (cases and controls) to the comparison of five populations [18].

RESULTS

The findings of the tests for departure from Hardy-Weinberg equilibrium showed that 3 of the 35 tests were significant ($p = 0.005$ in the Omani population for the IL-2 -330 polymorphism, and $p = 0.02$ in the Zulu population for the IL-10 -819 and -592 polymorphisms, which were in perfect linkage disequilibrium). This is no larger a number of significant results that might have been expected to occur by chance alone. Genotype and allele frequencies are displayed in Table 3 and all showed significant differences between the five populations ($p < 0.001$).

The T/G allele distribution of the IL-2 -330 polymorphism is similar for four of the groups studied, with the two alleles occurring at an approximate ratio of 2:1 in these populations. In contrast, within the Zulu group the frequency of the T polymorphism is greatly increased with respect to G, where it is present as 95.9%. The most noticeable feature detected within the IL-6 analysis was the complete absence of the C polymorphism within the Singapore Chinese group and the presence in only one individual in the Zulu population. Although this allele occurs at low frequencies in most of the populations, it exists in almost equal proportions with the G polymorphism within the Northern Ireland group. As a result, the non-Caucasian populations predominate in presentation of the high producer GG genotype.

The amplified IL-10 region permitted the analysis of SNPs at three sites, -1082, -819, and -592. Within the IL-10 -1082 site the G allele frequency was dramatically reduced in the Singapore Chinese samples compared to the other populations, leading to an absence of the GG genotype in this population. The IL-10 -819 and IL-10

TABLE 3 Percentage genotype and allele frequencies of IL-2, IL-6, IL-10, TNF-α, and TNF-β polymorphisms in various populations

Cytokine	N. Ireland (n = 100)	Zulu (n = 86)	Omani (n = 80)	Singapore Chinese (n = 83)	Mexican Mestizos (n = 40)
IL-2 (T330G)					
T	62.0	95.9	56.9	68.1	70.0
G	38.0	4.1	43.1	31.9	30.0
Genotype					
TT	38.0	91.9	40.0	43.4	50.0
TG	48.0	8.1	33.8	49.4	40.0
GG	14.0	0	26.2	7.2	10.0
IL-6 (G174C)					
G	54.0	99.4	87.5	100.0	93.8
C	46.0	0.6	12.5	0	6.2
Genotype					
GG	30.0	98.8	76.2	100.0	90.0
GC	48.0	1.2	22.5	0	7.5
CC	22.0	0	1.3	0	2.5
IL-10 (G1082A)[a]					
G	57.5	38.1	34.8	2.5	35.9
A	42.5	61.9	65.2	97.5	64.1
Genotype					
GG	34.4	9.5	13.9	0	17.9
GA	46.2	57.1	41.8	4.9	35.9
AA	19.4	33.4	44.3	95.1	46.2
IL-10 (C819T)[a]					
C	81.7	66.1	75.3	32.7	59.0
T	18.3	33.9	24.7	67.3	41.0
Genotype					
CC	64.5	38.0	57.0	11.1	35.9
CT	34.4	56.0	36.7	43.2	46.2
TT	1.1	6.0	6.3	45.7	17.9
IL-10 (C592A)[a]					
C	81.7	66.1	75.3	32.7	59.0
A	18.3	33.9	24.7	67.3	41.0
Genotype					
CC	64.5	38.0	57.0	11.1	35.9
CA	34.4	56.0	36.7	43.2	46.2
AA	1.1	6.0	6.3	45.7	17.9
TNF-α (G308A)					
G	77.0	77.9	91.9	88.0	97.5
A	23.0	22.1	8.1	12.0	2.5
Genotype					
GG	61.0	62.8	83.8	77.1	95.0
GA	32.0	30.2	16.2	21.7	5.0
AA	7.0	7.0	0	1.2	0
TNF-β (G252A)					
G	38.5	62.8	40.0	51.2	36.3
A	61.5	37.2	60.0	48.8	63.7
Genotype					
GG	13.0	37.2	17.5	30.1	12.5
GA	51.0	51.2	45.0	42.2	47.5
AA	36.0	11.6	37.5	27.7	40.0

[a] Sample numbers reduced for IL-10 analysis (N. Ireland, n = 93; Zulu, n = 84; Omani, n = 79; Singapore Chinese, n = 81; Mexican Mestizos, n = 39).
Abbreviations: IL = interleukin; TNF = tumor necrosis factor.

-592 allele and genotype frequencies obtained were identical, with respect to each population. The Singapore Chinese group also showed the greatest difference in allele distribution from the other populations within both the -819 and -592 IL-10 sites. As shown in Table 4, only three IL-10 haplotypes (GCC, ACC, and ATA) were identified, and their frequencies differed significantly between the populations ($p < 0.001$). Considering

TABLE 4 IL-10 haplotype frequencies identified in various populations

IL-10 haplotype (-1082/-819/-592)	N. Ireland (n = 93)	Zulu (n = 84)	Omani (n = 79)	Singapore Chinese (n = 81)	Mexican Mestizos (n = 39)
GCC	57.5	38.1	34.8	2.5	35.9
ACC	24.2	28.0	40.5	30.2	23.1
ATA	18.3	33.9	24.7	67.3	41.0

Abbreviation: IL = interleukin.

these haplotypes (Table 4), the haplotype associated with high production of IL-10 (GCC) occurred most frequently in the Northern Ireland Caucasian group. The IL-10 low producer type (ACC) was found to predominate in the Omani group. The low IL-10 -1082 G frequency in the Singapore Chinese (2.5%) results in a lower frequency of the high producer haplotype GCC and a higher ATA frequency compared to the other populations. A fourth haplotype (GTA), identified in a Southern Chinese study [19] and a Dutch Caucasian population [20], was not identified within the present study.

With respect to TNF-α, the G allele predominates in all groups, occurring with frequencies >75%. The Northern Ireland and South African Zulu groups produced similar TNF-α genotype frequencies. Most noticeable was the complete absence of the TNF-α AA genotype in the Omani and Mexican Mestizos. A predominance of the +252 A allele was observed within three of the populations for the TNF-β gene; this predominance switches to the G allele in the remaining groups. Comparing the TNF-β genotype frequencies within the populations, the intermediate producing GA genotype occurs at an almost constant level throughout.

DISCUSSION

This study of the frequencies of cytokine polymorphisms in IL-2, IL-6, IL-10, TNF-α, and TNF-β genes within geographically diverse populations has proven both effective and extensive in highlighting the ethnic genetic variation within these gene frequencies. Allele frequency variation was such that, within each cytokine, there was a tendency for at least one population to present itself as "different" from the other groups; for example, the South African Zulu's with a high IL-2 -330 T allele frequency (95.9%) or the Caucasian Northern Ireland group with a lower IL-6 -174 G allele frequency, relative to the other populations. Only in the case of the TNF-α -308 polymorphism was the allele distribution relatively conserved throughout all five populations. Within the IL-10 gene, strong linkage is known to exist between the three polymorphisms, as reflected in the identical IL-10 -819 and -592 frequencies within each population.

The findings of this study correlate with other available population data. The complete absence of genotypes in IL-2 (GG) and IL-6 (CC) in the African group has been previously noted [21], albeit in an African-American study. In terms of the Caucasian population, a low occurrence of the high producer IL-6 -174 genotype (GG) has been previously reported [21–23]. When comparing the Northern Ireland IL-2 data to other European Caucasian findings [22, 24] a shift from a 3:2 to a 3:1 ratio, respectively, of the T/G allele frequencies of the IL-2 polymorphism was observed. As a result the IL-2 GG genotype was increased in the Northern Ireland population (14%) in comparison to the German (7.2%) and South East England (3.9%) populations. This variation may point to the limited infusion of outside genetic influence in the Northern Ireland population or may simply be due to an anomaly in the sample selection for this population.

The high IL-10 -1082 A allele frequency in the Singapore Chinese group correlates with other reports [19, 25]. This increased A allele frequency in Asians, relative to Caucasian frequencies, was also noted in the comparison of another Western European population and a Southern Chinese group [24]. In addition, a recent comparison study involving Swedish Caucasian and Hong Kong Chinese groups [25] identified a lower IL-10 AA frequency in Caucasians (18%) compared to the Asian group (89%). Although the Caucasian frequencies reported in these studies [24, 25] agree with the Northern Ireland data, a Greek study [26] has recently indicated a lower frequency of the high producer genotype (GG) when the data was compared to German and British population information. The low producing IL-10 genotype has been correlated with long-term graft acceptance during a recent renal allograft study [27]. In contrast, a liver transplant study found a higher percentage of patients with rejected transplants to be low IL-10 producers, compared to nonrejecting patients [28].

In the case of TNF-α, the similarities between the Northern Ireland and Zulu populations in this study are analogous to data from an American Caucasian and African-American study [21]. The TNF-α genotype frequencies identified within the Singapore Chinese group are consistent with reported data for a Taiwanese population [29]. The exact role the A allele plays in the

transcription of the TNF-α gene remains controversial, with several studies reporting high transcriptional activity and other groups not able to replicate these findings [30]. TNF-α -308 polymorphisms have been associated with autoimmune diseases [31] and transplant rejection episodes [27, 31], although it is still unclear whether these associations are in fact the result of linkage disequilibrium with other TNF-α polymorphisms or the HLA system. From the HLA types of the Caucasian group it was apparent that the TNF-α -308 A allele is likely to be present on a haplotype with HLA-A*01, -B*08, -Cw*07, and DRB1*03, a haplotype identified previously [32]. Due to the close proximity of the TNF genes, the TNF-β +252 polymorphism was also investigated. As this polymorphism has also been implicated in high production of TNF-α [9], this could effectively identify the degree of linkage between the polymorphisms within the two genes. With respect to the Northern Ireland population, eight different TNF-α/TNF-β haplotypes were observed, with GG/AA (35%), GA/GA (26%), and GG/GA (23%) predominating.

The study has shown the benefits of using PCR-SSOP as a reliable typing procedure for cytokine polymorphism detection. PCR-SSOP, a technique already proven reliable for HLA assignment, is ideally suited for the analysis of the large sample numbers beneficial for population studies. In addition the systems are easily adapted into laboratories already performing similar techniques for HLA typing. Prior to the development of the SSOP techniques employed in this study, the technique of PCR-restriction fragment length polymorphism was evaluated as an identification method for the cytokine polymorphisms. However, digest results proved difficult to reproduce especially for IL-2 using the *Mae* I restriction enzyme and TNF-α and TNF-β with the *Nco* I restriction enzyme in some PCR applications. In addition to the improved accuracy of the SSOP techniques there is the added advantage of cost efficiency, with the elimination of expensive restriction enzymes. Interestingly, a recent study of polymorphisms in mannose binding lectin, TNF and lymphotoxin α genes, in relation to recurrent miscarriage [33], identified SSOP typing as the preferred method of analysis, highlighting discrepancies with restriction enzyme-based methodologies.

On examination of the extensive, continuing work in the area of cytokine polymorphism, it is becoming increasingly evident that the associations of these point mutations with overall immune function are widely varied and extend into almost every area of immunologic investigation [3]. However, much conflicting evidence exists as to the exact role of many cytokines, and it may be some time before clarity in defining these functions can be attained. The results gained from this work may go some way to reinforcing the work of others on variation in ethnic immune responses, in areas such as transplantation [21, 34, 35].

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