Article No. cyto.1999.0491, available online at http://www.idealibrary.com on IDEAL®

INDUCED HETERODUPLEX GENOTYPING OF TNF-α, IL-1β, IL-6 AND IL-10 POLYMORPHISMS ASSOCIATED WITH TRANSCRIPTIONAL REGULATION

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We describe the construction and use of 7 induced heteroduplex generators, reagents for the rapid and unequivocal genotyping of nucleotide sequence polymorphism in TNF-α, IL-1β, IL-6 and IL-10. Polymorphisms detected are those previously associated with regulation of gene transcription: TNF-α positions −308 and −238; IL-1β position +3953; IL-6 position −174; and IL-10 positions −1082, −819 and −592. The reagents were used for analysis of allele and haplotype frequencies in a population of healthy Caucasian volunteer blood donors.

There is now considerable evidence to suggest that a number of cytokine genes are polymorphic, but the complete extent of cytokine gene polymorphism is unknown.[1] Many reports have appeared which describe positive, equivocal or negative associations between cytokine gene polymorphism and human disease, in respect of susceptibility, severity, or clinical outcome.[2] Nevertheless, despite the growing number of cytokine polymorphisms reported, relatively little is known about their functional significance in terms of the effect of the variant on transcriptional activity. Three recent studies have, however, indicated direct associations between polymorphism in cytokine gene promoter sequences and levels of mRNA or protein production. First, a G to A substitution at position −308 in the promoter of the tumour necrosis factor alpha (TNF-α) gene, increases in vitro transcription of TNF-α by approximately 6- to 9-fold.[3] Second, a G to C substitution at position −174 in the promoter of IL-6[4] which is located immediately adjacent to the multiresponsive element (MRE) in the promoter, increases in vitro transcription of IL-6 by twofold (Olomolaiye *et al.*, in preparation). Third, a series of bi-allelic polymorphisms in the promoter of the IL-10 gene have been described at positions −1082,[5,6] −819 and −592.[5,7] Three haplotypes (G/C/C, A/C/C and A/T/A at positions −1082/−819/−592 respectively) have been reported by Turner and colleagues.[5] These authors report that ConA-stimulated cells homozygous for the G/C/C haplotype produce a 1.3-fold higher level of IL-10 than cells possessing all other combinations of haplotype. In addition it has been shown for IL-1β that polymorphism at a site other than the promoter can also influence gene transcription. Thus, Pociot and coworkers[8] showed that a *Taq*I polymorphism within exon 5 of the IL-1β gene at position +3953, could also influence expression levels. This group[9] also described a further polymorphism in the promoter region of TNF-α at −238, resulting in a G to A substitution. Whilst direct correlation of this polymorphism has not yet been clearly associated with subsequent gene expression in vitro, it has been positively correlated with various disease states.[10–12]

To assess the contribution of these and other cytokine gene polymorphisms to human disease, it is desirable to utilize a PCR-based method of population analysis which is simple, rapid, inexpensive, and which provides unequivocal results. We have previously developed an induced heteroduplex method[13,14] for genotyping of mutations, which avoids lengthy post-PCR probe-hybridization manipulations, product digestion with restriction endonucleases, or multiple-primer PCR. Furthermore, it provides unequivocal results in identifying individuals homozygous and heterozygous for a given polymorphism, a feature not always reliable with restriction endonuclease analysis. Here, we have employed induced heteroduplex analysis

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Received 18 September 1998; accepted for publication 11 January 1999

1043–4666/99/100789+07 $30.00/0
KEY WORDS: polymorphism/genotyping/heteroduplex

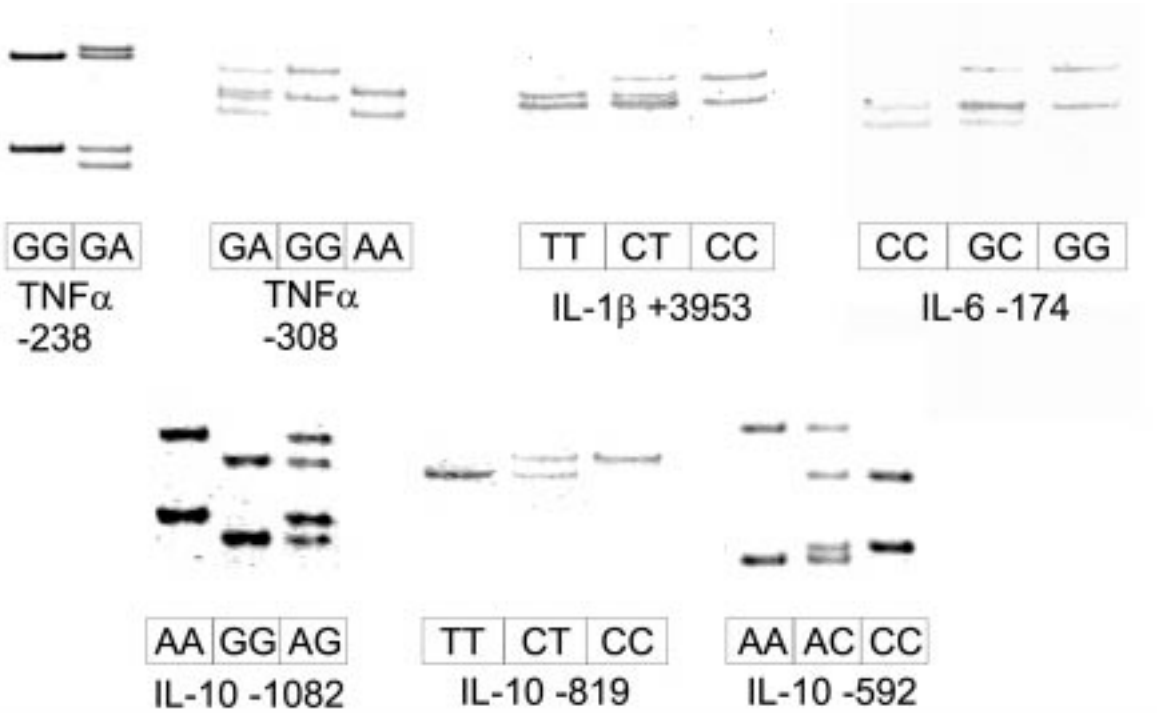

Figure 1. IHG-based genotyping of cytokine gene polymorphisms.

Only the heteroduplex bands are shown. Genotypes are given beneath each lane.

for genotyping of the TNF-α, IL-1β, IL-6 and IL-10 polymorphisms described above. Polymorphisms in PCR products are identified by induction of conformational changes in heteroduplexed DNA following interstrand hybridization of amplicons from genomic DNA with an induced heteroduplex generator (IHG), and subsequent visualization of mobility shifts using nondenaturing polyacrylamide electrophoresis.[13] In the present report, induction of the requisite mismatches is achieved by construction of appropriate IHGs containing small polypurine insertions adjacent to sites of polymorphism.[15,16]

RESULTS

Typical gel patterns resulting from the induced heteroduplex analyses for the cytokine gene polymorphisms are shown in Figure 1. In all cases, unequivocal identification of homozygosity and heterozygosity for the allelic variants was observed. Allelic specificity of heteroduplex patterns observed for the TNF-α −308 and the IL-10 −1082, −819 and −592 polymorphisms, was established by analysis of DNAs from a panel of 58 cell lines which had previously been genotyped using sequence-specific oligonucleotide probing (SSOP). Allelic specificity of heteroduplex patterns for the IL-6 −174 polymorphism was established by comparison with nucleotide sequencing results of cloned alleles, with single stranded conformational polymorphism (SSCP) analysis, and with restriction endonuclease analysis using *Nla*III digestion.[4] Allelic specificity of heteroduplex patterns for the IL-1β +3953 polymorphism was established using restriction endonuclease analysis using *Taq*I digestion[8] Polymorphic variants of the TNF-α −238 alleles were established by comparison of determined allele frequencies with published data.[17]

Allele frequencies for each polymorphism, and haplotype frequencies for the IL-10 polymorphisms are shown in Tables 1 and 2, respectively. Our observed frequencies did not differ statistically from those reported for TNF-α −308,[17–19], TNF-α −238,[17] IL-1β,[8] IL-6,[4] or IL-10.[20] Repeat analysis of the DNA from the healthy blood donors, by IHG typing was completely consistent in all cases.

DISCUSSION

The intrinsic role of cytokines as modulators of the immune response, and their subsequent effects in various immunological disorders, has been the focus of much recent interest. It is clear that the critical balance between the pro- and anti-inflammatory cytokines can influence the outcome of an immunological challenge. Thus, specific cytokine gene polymorphisms associated with regulation of protein expression, may influence

TABLE 1. Allele frequencies for TNF-α, IL-1β, IL-6 and IL-10 promoter polymorphisms

Gene	Position	Allele	Nucleotide	Frequency	*n* (Males)	*n* (Females)	*n* (Total)
TNF-α	− 308	1	G	0.82	96	166	262
		2	A	0.18	22	34	56
TNF-α	− 238	1	G	0.955	88	94	182
		2	A	0.045	6	3	9
IL-1β	+3953	1	C	0.76	79	79	158
		2	T	0.24	31	19	50
IL-6	− 174	1	G	0.59	66	55	121
		2	C	0.41	42	43	85
IL-10	− 1082	1	A	0.525	98	112	210
		2	G	0.475	102	88	190
IL-10	− 819	1	C	0.775	75	80	155
		2	T	0.255	25	20	45
IL-10	− 592	1	C	0.775	75	80	155
		2	A	0.225	25	20	45

TABLE 2. Haplotype frequencies for IL-10 promoter polymorphisms

Haplotype*	Allele at position			Frequency	*n* (Males)	*n* (Females)	*n* (Total)
	− 1082	− 819	− 592				
1	G	C	C	0.475	51	44	95
2	A	C	C	0.3	24	36	60
3	A	T	A	0.225	25	20	45

* from ref 5.

clinical outcome: for example in transplant-related complications,[20–23] in immune or autoimmune diseases,[24–26] and in malignant diseases.[27–29]

Four cytokines have been examined in some detail in order to establish an association between specific gene polymorphisms and transcriptional activity. The rationale for these studies is that a proven association would constitute an a priori reason for their effect on clinical outcome.

Within the TNF-α gene, the presence of an A at position − 308 in the promoter region has been shown to strongly enhance transcriptional activation.[3,30] The polymorphism has been shown to lie in a transcription factor binding site, although there is some disagreement as to whether the different alleles affect transcription factor binding.[3,31] Studies suggest that the − 308 polymorphism lies within an AP-2 element.[32] A second TNF-α promoter polymorphism, a G to A substitution at position − 238,[9] has been implicated independently of the − 308 polymorphism, in chronic hepatitis infection[11,12] and in alcoholic steatohepatitis.[10] This suggests that the − 238 polymorphism may also affect TNF-α expression and activity. However, direct correlation between this polymorphism and subsequent gene expression has not yet been demonstrated.[9]

Three base substitutions have also been detected in the IL-10 promoter region: Turner and colleagues have shown that combinations of these represent three putative haplotypes, and that in an ELISA-based assay, peripheral blood lymphocytes which lack an A residue at position − 1082 showed a significantly higher IL-10 production than those which possessed an A residue.[5] This polymorphism was also shown to lie within an ETS-like recognition site.[33]

For the IL-1β C to T polymorphism at position +3953,[8] the allele dosage of a T at +3953 was shown to be directly related to IL-1β expression, i.e. TT>CT>CC. This polymorphism occurs in exon 5 of the gene, but represents a conservative substitution, so it is at present unclear why this polymorphism should affect gene transcription: one possible explanation is that it is in linkage with one or more further polymorphisms, which themselves are more directly concerned with transcriptional regulation.

The IL-6 G to C promoter polymorphism at position − 174[4] lies immediately upstream of a multiresponsive element (MRE) located at positions − 173 to − 151 relative to the transcription start site. This element has been shown to be important in IL-1 and TNF modulated expression of IL-6.[34] Cloning of IL-6 promoters containing the − 174 polymorphism and expression in a luciferase reporter assay indicate a twofold increase in transcription associated with the C allele (Olomolaiye *et al.*, in preparation).

Since these cytokine gene polymorphisms are potentially important as genetic predictors of disease

susceptibility or outcome, we wished to develop a simple and effective method for their detection which utilized the same technology and which provided clear advantages over the range of existing methods used: in particular, to avoid extensive post-PCR manipulations, or the use of probes and restriction endonucleases. In this report we demonstrate that the IHG method fulfils these requirements and provides unequivocal identification of key polymorphisms in the cytokine genes examined. We have achieved this by the use of artificially constructed IHG molecules which are identical in sequence to the target gene, except that they have been deliberately modified immediately adjacent to the polymorphic loci, either by the inclusion, deletion or substitution of bases. The data presented here involve the use of structurally similar IHGs with 4 or 5 adenine insertions either upstream (TNF-α −308, IL-1β +3953 and IL-6 −174) or downstream (TNFα −238, IL-10 −1082, −819 and −592) of the polymorphic nucleotide.

The gel patterns for the IL-6 and IL-1β polymorphisms showed remarkably similar mobility patterns, and both IHGs contained a 4-base adenine insert upstream of the polymorphic nucleotide. Similarly, the IHGs for the IL-10 −1082 and TNF-α −238 polymorphisms resulted in similar gel patterns, and both IHGs contained a 5-base adenine insert downstream of the polymorphic nucleotide. However, prediction of gel mobilities is not always possible, and the optimum design of individual IHGs is generally still achieved empirically.[13]

We consider that IHG-based genotyping has various advantages over other PCR-based methods. The method is highly flexible and can be applied to any single base substitution, deletion or insertion, or to a cluster of such mutations. It has been previously applied successfully to genotyping in a range of different inherited genetic disorders.[14] It is a rapid and inexpensive technique which, due to its simplicity, can be easily applied to population genotyping. In this report, we have addressed a previous criticism of IHG genotyping: namely that 10 cm × 8 cm minigel format electrophoresis[14] is unsuitable for high throughput analysis; and that using multiple 10 cm × 8 cm minigels gives rise to inter-gel variation in heteroduplex patterns. Here, we have used a triple-wide minigel system (see Materials and Methods) which permits simultaneous analysis of the entire complement of samples and controls from a 96-well PCR tray. Finally, the method provides unequivocal identification of both heterozygosity and homozygosity, a feature not always reliable in other PCR-based methods. Thus, we believe that IHG-based genotyping may prove useful in further studies correlating key cytokine polymorphisms with the aetiology of various clinical disorders.

MATERIALS AND METHODS

PCR primers

The following primer pairs were used for PCR amplification of genomic DNA samples, and of heteroduplex generators:

(1) TNF-α −308 polymorphism
Forward: 5′-TCCTGCATCCTGTCTGGAAG-3′
Reverse: 5′-GTCTTCTGGGCCACTGACTG-3′

(2) TNF-α −238 polymorphism
Forward:
5′-GTTCAGCCTCCAGGGTCCTACACA-3′
Reverse:
5′-GGGATTTGGAAAGTTGGGGACACA-3′

(3) IL-1β +3953 polymorphism
Forward: 5′-GAGGCCTGCCCTTCTGATT-3′
Reverse: 5′-CGGAGCGTGCAGTTCAGT-3′

(4) IL-6 −174 polymorphism
Forward: 5′-GCTTCTTAGCGCTAGCCTCAATG-3′
Reverse: 5′-TGGGGCTGATTGGAAACCTTATTA-3′

(5) IL-10 −1082 polymorphism
Forward:
5′-AATCCAAGACAACACTACTAAGGC-3′
Reverse: 5′-CTGGATAGGAGGTCCCTTAC-3′

(6) IL-10 −819 polymorphism
Forward: 5′-TACAGTAGGGTGAGGAAACC-3′
Reverse: 5′-GGTAGTGCTCACCATGACCC-3′

(7) IL-10 −592 polymorphism
Forward: 5′-GAAATCGGGGTAAAGGAGCC-3′
Reverse: 5′-AGTTCCCAAGCAGCCCTTCC-3′

Construction of induced heteroduplex generators (IHG)

IHGs were synthesized as single long oligonucleotides, using 0.2 μmole membrane columns on a PerSeptive Expedite 8900 Nucleic Acid Synthesis System. Following synthesis, deprotection, and precipitation, the oligonucleotides were amplified by PCR using the appropriate primers (above), and if necessary were purified by preparative polyacrylamide electrophoresis. Dilutions for use in PCR prior to IHG analysis were determined empirically. The nucleotide sequences of the IHGs, which incorporate poly-A residues adjacent to polymorphic residues in the target sequence,[13] are shown in Figure 2.

PCR conditions

TNF-α −308 and −238 polymorphism.

PCR mixes (50 μl) contained 0.5 μM each of forward and reverse primers, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 1 × *Taq* polymerase buffer (75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% V/V Tween), 0.5 unit *Taq* polymerase (Advanced Biotechnologies) and either diluted IHG or 500 ng genomic DNA. PCR parameters (optimized for a Perkin Elmer 9600 Geneamp system) were: initial denaturation at 95°C for 5 min; 25 cycles of: 95°C for 1 min, 59°C for 1 min (58°C for 1 min for the −238 polymorphism), 72°C for 1 min; final extension at 72°C for 5 min.

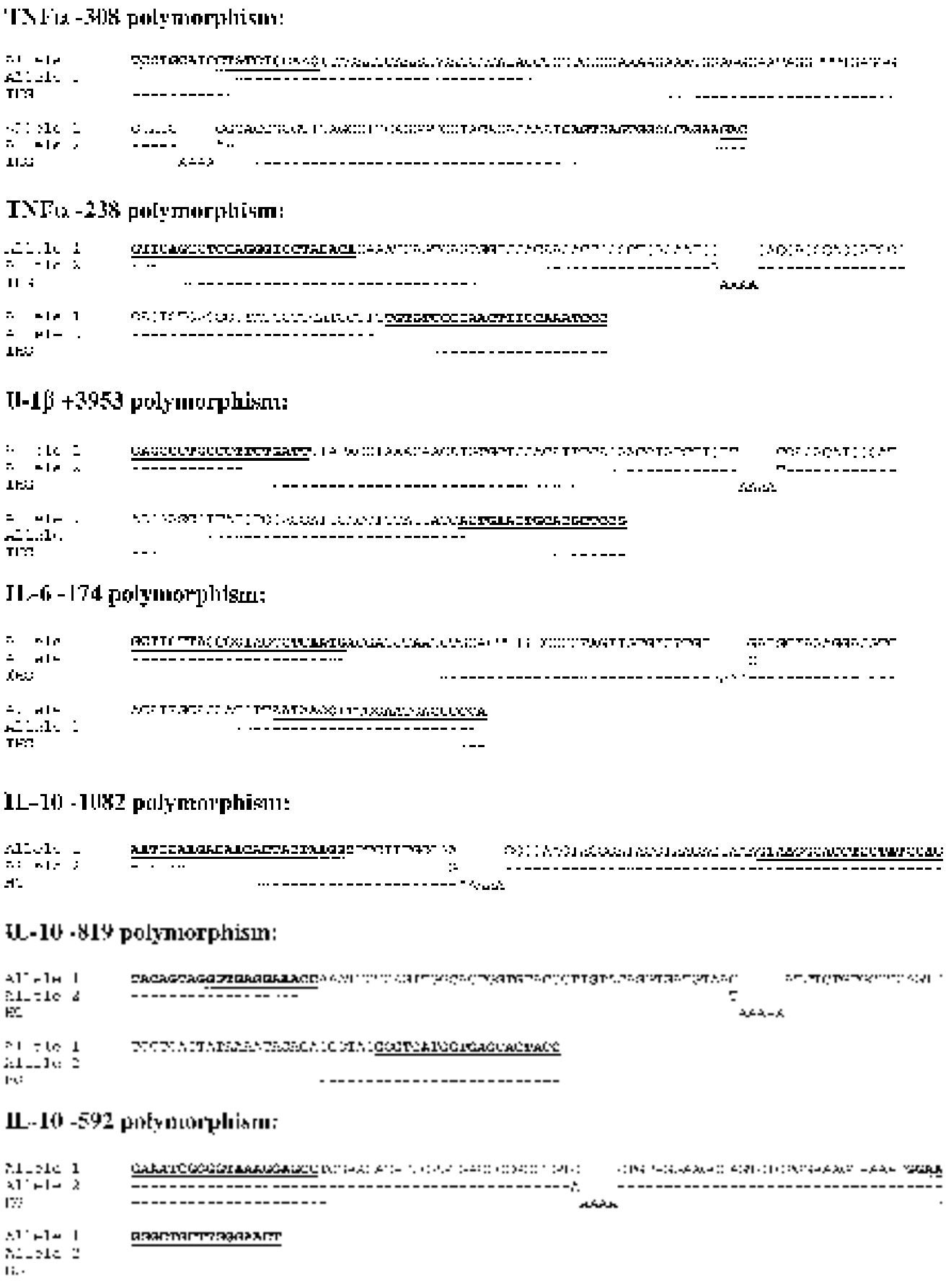

Figure 2. Nucleotide sequence alignments of cytokine gene regions containing polymorphic nucleotides (top and middle row) and IHG molecules (bottom row).

Forward and reverse primer annealing locations are underlined; nucleotide sequence homology is indicated by (–). Polypurine insertions in the IHGs are shown.

IL-1β +3953 polymorphism

PCR mixes (50 µl) contained 0.5 µM each of forward and reverse primers, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 × *Taq* polymerase buffer (67 mM Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% V/V Tween), 0.5 unit *Taq* polymerase (Advanced Biotechnologies) and either diluted IHG or 500 ng genomic DNA. PCR parameters (optimized for a Stratagene Robocycler Gradient 96 thermal cycler) were: initial denaturation at 95°C for 5 min; 30 cycles of: 95°C for 1 min, 60°C for 1 min, 72°C for 1 min; final extension at 72°C for 5 min.

IL-6 − 174 polymorphism

PCR mixes (50 µl) were as described above for the IL-1β polymorphism. PCR parameters (optimized for a Stratagene Robocycler Gradient 96 thermal cycler) were: initial denaturation at 95°C for 5 min; 30 cycles of: 95°C for 1 min, 60°C for 1 min, 72°C for 1 min; final extension at 72°C for 5 min.

IL-10 − 1082, − 819 and − 592 polymorphisms

PCR mixes (50 µl) were as described above for IL-1β polymorphism. PCR parameters (optimised for a Biometra thermal cycler) for all genomic DNA and heteroduplex generators, were: initial denaturation at 95°C for 5 min; 30 cycles of: 95°C for 1 min, 57°C for 1 min (62°C for 1 min for the − 592 polymorphism), 72°C for 1 min; final extension at 72°C for 5 min.

Heteroduplex analysis

Equal volumes of aliquots (7.5 µl–10 µl) of amplicons from genomic DNA and IHGs were mixed, denatured at 95°C for 5 min and allowed to cool slowly from 95°C to 37°C over a 30 min period. Heteroduplexes were resolved by electrophoresis for 90 min at 200 V in 15% non-denaturing polyacrylamide ''triple-wide'' minigels (30 cm × 8 cm: CBS Scientific Company, Del Mar, USA; gel constitution 37.5:1 (W/V) acrylamide: bisacrylamide; National Diagnostics, containing 1 × TBE electrophoresis buffer). Gels were stained for 5 min in 1 × TBE containing 0.5 µg/ml ethidium bromide and examined using a 302 nm UV trans-illuminator, or alternatively with a Glyko FACE® digitising CCD imager.

Population genotyping

Archived DNA samples from a population of healthy Caucasian volunteer blood donors were analysed in order to determine the allele and haplotype frequencies for the mutations described.

Acknowledgements

We are indebted to Dr D. M. Turner, Dr P. J. Sinnott and Prof. I. V. Hutchinson for providing genotyped reference materials, and for sharing results prior to publication. We thank Dr G. Gallagher for useful information on IL-10 mutations. HRM is supported by a programme grant from the Leukaemia Research Fund. OOO is supported by a grant from Howmedica. LJK is supported by a grant from the South West Regional NHS Research and Development Directorate.

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