Comparison of cytokine gene polymorphisms among Greek patients with invasive meningococcal disease or viral meningitis

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INTRODUCTION

Invasive meningococcal disease (IMD) is a rare life-threatening condition caused by Neisseria meningitidis. It is characterized by meningitis and/or septicaemia. Viral meningitis (VM) is more common than IMD in Greece (Table 1) but is a much less severe disease. Inflammatory responses play an important part in the pathology of these two diseases, but the responses in the cerebrospinal fluid differ significantly in the later stages of the infections. For VM, the predominant leukocytes are lymphocytes, whilst for meningococcal meningitis, neutrophils are predominant.

Some single-nucleotide polymorphisms (SNPs) affect levels of cytokine responses to infectious agents. Genetic predispositions for low pro-inflammatory and high anti-inflammatory responses have been observed among first-degree relatives of patients who died of IMD (Westendorp et al., 1997). In contrast, high levels of pro-inflammatory cytokine responses are associated with the severity of meningococcal disease (Balding et al., 2003; Read et al., 2000, 2003). Among ethnic groups in which IMD is more prevalent and/or more severe (e.g. Aboriginal Australians), there is a predominance of cytokine gene profiles predicted to result in high pro-inflammatory responses and low anti-inflammatory responses (Balding et al., 2003; Moscovis et al., 2004a, b, 2006).

In northern European populations, SNPs in genes encoding interleukin (IL)-1β, IL-6 and IL-10 were associated with altered susceptibility to meningococcal infection and/or severity of IMD (Balding et al., 2003; Read et al., 2000, 2003). Whilst there is some information about cytokine gene polymorphisms in ethnic Greek populations (Costeas et al., 2003; Theodoropoulos et al., 2006), there has been no systematic assessment of these genetic markers in relation to

High levels of pro-inflammatory cytokines are implicated in the severity of invasive meningococcal disease (IMD) and viral meningitis (VM). This study compared single-nucleotide polymorphisms (SNPs) in pro- and anti-inflammatory cytokine genes among patients with VM or IMD. Patient DNA samples were prepared by the National Meningitis Reference Laboratory in Athens: n=98 for IMD and n=53 for VM. The results for both patient groups were compared with data published for healthy Greek control data. Real-time PCR was used to assess the interleukin (IL) gene SNPs IL6 G→174C, IL1B C→511T, IL1RN T+2018C, IL10 G→1082A and IL8 A→251T and the tumour necrosis factor α (TNF-α) SNP TNFA G→308A. Differences were compared by Fisher's exact test. The genotype for high IL-6 responses was predominant among IMD (51%, P=0.0008) and VM (74.5%, P<0.0001) patients compared with the controls (31%). The genotype associated with high TNF-α responses was 5% among controls and lower for IMD (1.1%, P=0.0014) and VM (0%, P=0.052). There was no difference for IL-8 SNPs between controls and IMD (P=0.162), but the difference was significant for VM (P=0.0025). IL-6 (P=0.024) and IL-8 (P=0.00004) SNPs differed between IMD and VM. Reports on associations between IL-8 SNPs and cytokine responses differ. Because of its role in neutrophil attraction, differences in frequencies of the IL-8 SNP for IMD and VM require further investigation.

Abbreviations: CI, confidence interval; CSF, cerebrospinal fluid; HWE, Hardy-Weinberg equilibrium; IL, interleukin; IMD, invasive meningococcal disease; OR, odds ratio; SNP, single-nucleotide polymorphism; TNF-α, tumour necrosis factor α; VM, viral meningitis.
IMD or VM in Greece. In this study, we tested the hypothesis that there might be differences in SNP profiles for cytokines involved in the pathogenesis of these diseases, particularly IL-8, which is a neutrophil chemoattractant.

**METHODS**

This study adhered to the Declaration of Helsinki. All participants provided informed consent for this study, and ethics permission for this study was obtained from the relevant hospitals in Greece and the Hunter New England Research Ethics Committee. The patient population has been described previously (Tsolia et al., 2003; Tzanakaki et al., 2003). DNA from samples extracted from blood and/or cerebrospinal fluid (CSF) samples of 98 patients with laboratory-confirmed IMD were tested. Patient ages ranged from 2 months to 49 years. Information on the serogroup of the *N. meningitidis* isolate was available for 79 patient samples: 65 serogroup B, eight serogroup A, six serogroup C, six serogroup W-135, two serogroup Y and six were non-serogroupable.

There were 53 samples of DNA extracted from patients diagnosed with VM; none had evidence of meningococcal disease by either culture or PCR analysis of blood and/or CSF. Both disease groups were compared with controls published previously for a healthy Greek control population (Theodoropoulos et al., 2008). DNA isolation. DNA was extracted from whole-blood samples using a Nucleospin Blood Quickpure kit (Macherey-Nagel) according to the protocol of Zambardi et al. (1995). In brief, 500 μl sample was centrifuged at 1700 g for 10 min. The supernatant was discarded to leave 150 μl, which was added to 650 μl sterile double-distilled H2O and 150 μl Chelex/Tween 80 buffer. The samples were heated at 100 °C for 30 min and centrifuged at 10 280 g for 8 min. A volume of 200 μl supernatant was used for the PCR assay.

**Assessment of cytokine gene polymorphisms.** The cytokine gene polymorphisms IL1B C–511T (dbSNP reference no. rs16944), IL1RN T+2018C (rs419958), IL6 G–174C (rs1800795) and IL10 G–1082A (rs1800896) were genotyped by PCR as described previously (Moscovis et al., 2004a, b, 2006). A commercial allelic discrimination assay was custom made to genotype IFNG T + 874A (rs2430561) and IL8 A–251T (rs4073) (PE Applied Biosystems). Primers and fluorescently labelled minor groove-binding probes were provided as a 40 × assay mix (Table 2). The tumour necrosis factor α (TNF-α) SNP TNFA G–308A (rs1800629) and IL10 C592A (rs1800872) were genotyped using commercial allelic discrimination assays (SNP ID: C___1747363_10 and C___7514879_10, respectively; PE Applied Biosystems). Primers and probes were provided as a 20 × assay mix (sequences and concentrations were not provided).

Each PCR (total volume 10 μl) contained DNA (50 ng), 1 × assay mix (50 nM each probe for IL4 assay) and 1 × TaqMan Universal PCR Master Mix (PE Applied Biosystems) diluted in sterilized MilliQ water (Millipore). DNA samples were genotyped in 96-well optical reaction plates (PE Applied Biosystems). All reactions were performed using an ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems) under the following thermal cycling conditions: 40 cycles of 50 °C for 2 min, 95 °C for 10 min, 92 °C for 15 s and 60 °C for 1 min. The PCR for IL8 A–251T required up to 60 cycles of 50 °C for 2 min, 95 °C for 10 min, 92 °C for 15 s and 56 °C for 1 min. Genotypes were auto-called at a quality value of 95.0 using SDS software version 2.1 (PE Applied Biosystems). Samples that failed the auto-calling were repeated. Samples that failed twice were not included in the dataset.

**Statistical analyses.** To assess differences between allele distributions and specific genotypes in the patient and control populations, Fisher’s exact test (2 × 2 table) was used generating an odds ratio (OR) with Instat software (Graphpad Software). Comparison of genotype distributions between patients and controls was done using Fisher’s exact test (2 × 2 table; http://faculty.vassar.edu/lowry/fisher2x3.html). Each SNP was assessed for variance from Hardy–Weinberg equilibrium (HWE).

**RESULTS**

The allele and genotype frequencies for each SNP were compared between patient groups and control subjects. Six

<table>
<thead>
<tr>
<th>Year</th>
<th>No. cases</th>
<th>Incidence*</th>
</tr>
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<tbody>
<tr>
<td>VM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>406</td>
<td>3.66</td>
</tr>
<tr>
<td>2001</td>
<td>984</td>
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<td>2002</td>
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<tr>
<td>2005</td>
<td>232</td>
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<tr>
<td>2006</td>
<td>233</td>
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<td>2007</td>
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</tr>
<tr>
<td>2008</td>
<td>350</td>
<td>3.15</td>
</tr>
<tr>
<td>IMD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>261</td>
<td>2.4</td>
</tr>
<tr>
<td>2001</td>
<td>234</td>
<td>2.1</td>
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<tr>
<td>2002</td>
<td>233</td>
<td>2.2</td>
</tr>
<tr>
<td>2003</td>
<td>131</td>
<td>1.2</td>
</tr>
<tr>
<td>2004</td>
<td>89</td>
<td>0.81</td>
</tr>
<tr>
<td>2005</td>
<td>98</td>
<td>0.93</td>
</tr>
<tr>
<td>2006</td>
<td>114</td>
<td>1.03</td>
</tr>
<tr>
<td>2007</td>
<td>106</td>
<td>0.95</td>
</tr>
<tr>
<td>2008</td>
<td>74</td>
<td>0.67</td>
</tr>
</tbody>
</table>

*Incidence per 100 000 of the population.
of the patients could not be genotyped, five were single
samples from CSF or blood culture, and for one patient
samples from both CSF and blood were tested.

Serogroup information was available for 87 of the 98 IMD
isolates. There were no differences in distribution of the
genotypes between individuals infected with serogroup B and
those infected with the other serogroups. There were no
significant differences in the genotype distribution between
controls and either patient group for IL1B C→151T, IL1RN
T→2018C or IL10 G→1082A. Significant differences between
controls and the patient groups were observed for IL-8, IL-6
and TNF-α.

**IL6 G→174C**

Samples from 88 patients with IMD and 51 patients with
VM were successfully genotyped. HWE testing showed no
development for IMD (P=0.75) or VM (P=0.07) patients.
The Greek controls were borderline for HWE (P=0.055)
(Table 3).

Genotype distributions were significantly different between
patients with IMD and the Greek controls (P=0.0008). GG
and CC genotype proportions were significantly different
between patients with IMD and controls (P=0.0002): indi-
cividuals with the GG genotype were at increased risk of
IMD compared with individuals with the CC genotype
[OR 4.395, 95 % confidence interval (CI) 1.900–10.162]. A
significant difference was observed for GG and C-allele
carriage between patients with IMD and the controls
(P=0.0024): individuals with the GG genotype were at
higher risk of IMD than carriers of a C allele (OR 2.224,
95 % CI 1.331–3.714). Allele distributions were signifi-
cantly different between IMD patients and controls
(P<0.0001); the G allele was associated with susceptibility
to IMD compared with the C allele (OR 2.130, 95 % CI

A significant difference was observed between the genotype
distribution of patients with VM and the control group
(P<0.0001). Allele distribution was significantly different
between the two groups (P<0.0001): the G allele was
associated with a greater risk of VM (OR 4.672, 95 % CI
2.645–8.251). A significant difference was observed for GG
and CC distributions (P<0.0001) and for GG and C-allele
carriage between the VM and control groups (P<0.0001).
The risk of VM was increased in individuals with the GG
genotype relative to those with the CC genotype (OR 9.896,
95 % CI 2.885–33.942) and the GG genotype compared
with carriage of the C allele (OR 6.212, 95 % CI 3.095–
12.465).

There was a marginally significant difference in genotype
distributions between patients with IMD and patients with
VM (P=0.0237). A significant difference was observed for
allele distribution between the two groups (P=0.0134).
There were no significant differences in GG and CC
distribution (P=0.3378); however, there was a significant
difference in GG and C-allele carriage (P=0.0074).

**IL8 A→251T**

There were 90 IMD patients and 53 VM patients
successfully genotyped for IL8 A→251T. The HWE test
showed no significant deviation for the IMD (P=0.62) or
VM (P=0.48) patients or the published Greek controls
(0.33) (Table 3).

Genotypes for patients with IMD were not significantly
different compared with the controls (P=0.1615). No
significant difference was found in allele distribution
between IMD patients and controls (P=0.0840). No
significant difference was observed for AA and TT
genotypes between patients with IMD and controls
(P=0.0669). No significant difference was observed for
comparison of the AA genotype with carriage of the T allele
in patients with IMD and the controls (P=0.0720).

Genotype distributions were significantly different between
patients with VM and the controls (P=0.0025). A
significant difference was observed for the AA and TT
genotypes between the patients with VM and the control
group (P=0.0009): AA was associated with increased risk
of VM (OR 4.381, 95 % CI 1.792–10.710). A significant
difference was found for the AA genotype compared with
T-allele carriage between VM patients and controls
(P=0.0024): the AA genotype was associated with increased
susceptibility to VM (OR 2.811, 95 % CI 1.480–
5.340). Allele distribution between patients with VM and
the controls was significantly different (P=0.0004): the A
allele was associated with increased risk of VM (OR 2.242,
95 % CI 1.438–3.496).

Genotype distributions between IMD and VM patients
were significantly different (P<0.0001). Allele distribution
between patients with VM and IMD patients was
significantly different (P<0.0001). A significant difference
was also observed between AA and TT genotype distribu-
tions between the IMD and VM groups (P<0.0001) and
for the AA genotype and T-allele carriage (P<0.0001).

### Table 3. Distribution of cytokine gene SNPs among patient
and control groups

<table>
<thead>
<tr>
<th>SNP/group</th>
<th>n (%)</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL6 G→174C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n=200)</td>
<td>GG 64 (32)</td>
<td>GC 86 (43)</td>
</tr>
<tr>
<td>IMD (n=88)</td>
<td>GG 45 (51)</td>
<td>GC 35 (39)</td>
</tr>
<tr>
<td>VM (n=51)</td>
<td>GG 38 (74)</td>
<td>GC 10 (19.6)</td>
</tr>
<tr>
<td><strong>IL8 A→251T</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n=196)</td>
<td>AA 42 (21.4)</td>
<td>AT 90 (45.9)</td>
</tr>
<tr>
<td>IMD (n=90)</td>
<td>AA 11 (12.2)</td>
<td>AT 44 (48.9)</td>
</tr>
<tr>
<td>VM (n=53)</td>
<td>AA 23 (43.4)</td>
<td>AT 22 (41.5)</td>
</tr>
<tr>
<td><strong>TNF G→308A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n=200)</td>
<td>GG 146 (73.0)</td>
<td>GA 44 (22.0)</td>
</tr>
<tr>
<td>IMD (n=90)</td>
<td>GG 82 (91.1)</td>
<td>GA 7 (7.8)</td>
</tr>
<tr>
<td>VM (n=46)</td>
<td>GG 41 (89.1)</td>
<td>GA 5 (10.9)</td>
</tr>
</tbody>
</table>
**TNFA G–308A**

For TNFA G–308A, 90 patients with IMD and 46 with VM were successfully genotyped. HWE testing of allele frequencies found no significant deviation for the IMD (P=0.09) or VM (P=0.70) group; however, the published Greek controls deviated from HWE (P=0.01) (Table 3).

Genotype distributions were significantly different between patients with IMD and the controls (P=0.001). Allele distribution was also significantly different between patients with IMD and the controls (P=0.0001): patients with the G allele were at increased risk of IMD (OR 3.619, 95% CI 1.758–7.449). The GG and AA genotypes did not differ significantly between patients and controls (P=0.1027), but a significant difference was observed for the GG genotype and presence of the A allele between these two groups (P=0.0003). No significant differences were observed for overall genotype distribution between patients with IMD and those with VM (P=0.6970), for GG and AA genotype distributions for these two groups (P=1), and for GG genotype and A-allele carriage (P=0.7618). Allele distributions between the groups were not different (P=1).

**DISCUSSION**

The results of this study demonstrated an association between pro-inflammatory cytokine SNPs and susceptibility to IMD and VM. The differences between these genetic factors observed for the two patient groups provide a basis for further studies on the role of inflammatory responses in the respective disease processes. Although age and sex are associated with these infections, analyses of the material in relation to these factors were not carried out. The basic susceptibility factor for both IMD and VM is lack of humoral or cellular immunity to the invading pathogen. In both cases, host defences depend on the innate inflammatory responses to deal with the infectious agent. The cytokine polymorphisms examined do not change with age and are not sex linked.

**IL-6**

A significant difference was observed between genotypes for patients with IMD or VM and controls for the IL6 G–174C SNP. IL6 –174 G/G and the –174G allele were associated with increased disease susceptibility. An association of the IL6 –174 G/G genotype and the –174G allele with increased susceptibility to IMD has not been observed in other populations; however, in Irish patients with IMD, the IL6 –174 G/G genotype was more frequent in non-survivors and in patients with severe disease (Balding et al., 2003). Among the six non-survivors in this study, four (67%) had the GG genotype. A literature search found no reported associations between VM and this genotype.

The IL6 –174G allele has been associated with higher levels of plasma IL-6 (Fishman et al., 1998; Villuendas et al., 2002). Elevated IL-6 levels in response to either bacterial or aseptic meningitis have been reported (Azuma et al., 1997; Fida et al., 2006; Krebs et al., 2005; Matsuzono et al., 1995; Möller et al., 2005; Sato et al., 2003); however, IL-6 levels were up to tenfold greater in infants with bacterial meningitis compared with those with aseptic meningitis (Duikerian et al., 1995). If the high levels of IL-6 play a significant part in the pathogenesis and tissue damage of IMD, this could partly explain the increased susceptibility associated with the IL6 –174G allele. IL-6 levels were correlated with leucocyte counts in the CSF of patients with VM (Dalal et al., 2003).

**IL-8**

For patients with IMD, there was no association with the distribution of genotypes or alleles for the IL8 A–251T SNP. There was a significant difference between patients with VM and patients with IMD or the healthy controls. The –251A allele was associated with a greater risk of VM compared with results for the healthy controls. The –251A allele has been associated previously with increased risk of severity of bronchiolitis due to respiratory syncytial virus, independently and in a disease haplotype (Hacking et al., 2004; Hull et al., 2000).

Elevated IL-8 levels in response to bacterial and aseptic meningitis have been reported (Hacking et al., 2004; Hull et al., 2001; Ishiguro et al., 1997; Ostergaard et al., 1996; Sato et al., 2003): higher levels of IL-8 were associated with bacterial meningitis compared with VM (Ostergaard et al., 1996). IL-8 is elicited by meningococci from meningeal cells as well as from leucocytes (Christodoulides et al., 2002). There is evidence from several studies that the A allele is associated with higher levels of IL-8 responses. The IL8 251A/A and A/T genotypes have been associated with significantly higher IL-8 levels relative to the T/T genotype. The A/A genotype was associated with higher neutrophil infiltration score in the gastric mucosa of patients with gastric cancer (Taguchi et al., 2005). The –251 A/A genotype has also been associated with significantly higher faecal IL-8 levels than those with the A/T or T/T genotype in patients with entericaggregative *Escherichia coli* diarrhea (Jiang et al., 2003).

The findings for the IL8 SNP were the opposite of predictions based on the neutrophil attractant activity of
IL-8. As neutrophils are the major leukocyte observed in the CSF of patients with meningococcal meningitis, genotypes associated with higher IL-8 responses were predicted for patients with IMD. The IL8 –251T allele has been associated with increased IL-8 levels (Lee et al., 2005; Selvaraj et al., 2006). The absence of an association in the IMD group might reflect moderation by other functional variants in the IL8 locus.

Increased susceptibility to VM for Greek patients with the IL8 –251 A/A genotype might be associated with higher IL-8 levels, which spike initially in the disease (Straussberg et al., 2003), or could reflect lower IL-8 levels causing reduced chemotactic activity for macrophages in the CSF. Interactions between IL-8 and TNF-α interact (data not shown). Interactions between IL-8 and TNF-α might reflect moderation by other functional variants in the IL6 locus.

There were 23 patients with VM who had the IL8 –251 A/A genotype; of these 20/23 (87%) also had the IL6 –174 G/G genotype (data not shown). Interactions between IL-8 and IL-6 in these diseases need to be assessed in greater detail.

**TNF-α**

For patients with IMD, the TNFA G–308A SNP was associated with increased susceptibility. This was not reported among Irish patients with IMD (Balding et al., 2003). For patients with VM, the GG genotype was associated with increased risk of VM relative to the A allele. Deviation from HWE was observed for TNF –308 in the Greek controls obtained from the literature; however, the allele distribution observed in this population is consistent with that observed in studies of Caucasian populations (Allen, 1999; Theodoropoulo et al., 2006).

The –308G allele is associated with lower levels of TNF-α than the A allele (Wilson et al., 1997) and appears to be protective against IMD and VM. The majority of individuals with the A allele in both patient and control groups were heterozygotes; however, carriage of a single A allele was still associated with higher TNF levels relative to the GG genotype (Louis et al., 1998). TNF-α levels were found to be significantly raised in a group of children with bacterial meningitis compared with those with aseptic meningitis (Tang et al., 2001; Mukai et al., 2006). The role of TNF-α in the initial responses to meningococcal and viral infections needs further investigation.

This study had several limitations. Not all samples were successfully genotyped, particularly if DNA was extracted from CSF. CSF is not a reliable source of DNA, as the concentration of cells varies. The normal range of leukocytes in CSF of a healthy adult is 0–5 cells μl⁻¹. For individuals with bacterial meningitis, this can be >500 cells μl⁻¹ (Venkatesh et al., 2000). Failure to obtain sufficient DNA from CSF might also reflect the presentation of septicaemia without meningitis.

Data on the severity of disease were limited to mortality. Whilst this is a very precise indicator of severity, this limited detailed patient analysis. Due to the low mortality of meningococcal disease in Greece, only seven samples were supplied from patients who had died; of these, only six could be genotyped.

Among patients in the comparison group with suspected viral infections, there might have been some selection bias; however, the genotype frequencies of IL10 G–1082A, TNFA G–308A and IL6 –174C were similar to those reported in the Greek Cypriot population (Costeas et al., 2003). There was no selection bias based on geographical area, as samples were sent to the reference laboratory from all areas of Greece.

In conclusion, our findings indicated that, compared with the general population, there are significant differences between the genotypes for IL-6, IL-8 and TNF-α among Greek patients with VM and for IL-6 and TNF-α genotypes among Greek patients with IMD. The differences noted for the IL-8 SNP genotype for patients with VM compared with those with IMD require further investigation into the role of inflammatory responses in the pathology and severity of these diseases.

Although the major risks identified for meningococcal disease and respiratory infections to date have been environmental factors such as smoking, crowding and poor living conditions, the SNPs associated with the increased risk of these infections are predominant in a number of populations in which meningococcal disease is more prevalent, such as IL6 –174 G/G among Indigenous Australians (Blackwell et al., 2005; Massey & Durrheim, 2008).

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**REFERENCES**


Cytokine gene polymorphisms and meningitis


