Sex-Biased Multiple Sclerosis Susceptibility and Gene Expression

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Background: Interferon (IFN) gamma (IFNG) allelic variants are associated with susceptibility to multiple sclerosis (MS) in men but not in women.

Objectives: To conduct a high-density linkage disequilibrium association study of IFNG and the surrounding region for sex-associated MS susceptibility bias and to evaluate whether IFNG allelic variants associated with MS susceptibility are associated with expression.

Design: Genotype case-control study, quantitative polymerase chain reaction (qPCR), and enzyme-linked immunosorbent assay expression analyses for IFN gamma.

Setting: Three independently ascertained populations from the Mayo Clinic, Rochester, Minnesota, Queen’s University of Belfast, Belfast, Ireland, and University of Leuven, Leuven, Belgium.

Patients: For linkage disequilibrium, 861 patients with MS (293 men and 568 women) and 843 controls (340 men and 503 women) derived from the US (population-based) and the Northern Ireland and Belgium (clinical-based) cohorts were studied. For expression analyses, 30 US patients were selected to enrich for homozygotes and to achieve a balance between men and women.

Interventions: Twenty markers were genotyped over the 120-kilobase region harboring IFNG and the interleukin 26 gene (IL26).

Main Outcome Measures: Expression of IFN gamma was evaluated by qPCR and enzyme-linked immunosorbent assay in stimulated peripheral blood mononuclear cells.

Results: Multiple markers were associated with MS susceptibility in men but not in women. The sex-specific susceptibility markers, of which rs2069727 was the strongest, were confined to IFNG. Carriers of rs2069727*G had higher expression than noncarriers. The effect of genotype in the qPCR experiments was also evident in men but not in women.

Conclusions: IFNG is associated with sex bias in MS susceptibility and with expression of IFN gamma in MS. These observations add to a growing body of literature that implicates an interaction between sex and IFN gamma expression in a variety of disease states.

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Multiple sclerosis is twice as common in women as in men. Men tend to have later disease onset and worse prognosis than women, suggesting sex-dependent factors in etiology and phenotypic variability. Whether genetic variations contribute to the sex-determined differences in susceptibility to or phenotypic variability of MS is unknown.

The balance between T11 vs T12 clonal expansion is pivotal to many autoimmune diseases, including MS and experimental autoimmune encephalomyelitis (EAE). Interferon (IFN) gamma is a key cytokine that stimulates T11 and inhibits T12 clonal expansion. Estrogens contribute to a shift from T11 to T12 cell response during pregnancy, and pregnancy ameliorates the course of EAE and reduces the effects of clinical exacerbations in MS. Interferon gamma is secreted primarily by naïve T and T11 cells as well as CD1d-restricted natural killer cells and microglia, and its expression is influenced by sex. Women with MS have higher IFN gamma expression and lower interleukin 5 (IL-5) expression in response to proteolipid protein stimulation than men with MS and women controls, suggesting a skew toward T11 reponse in women with MS.
cancer have more CD1d-restricted natural killer T cells expressing high levels of IFN gamma than men with cancer.14 A sex-specific TH1v sT H2 bias and IFN gamma expression could influence sex bias in susceptibility to MS. Expression of IFN gamma increases immediately before attacks of MS. Treatment with exogenous IFN gamma is deleterious to patients with MS.15-17 Low levels of IFN gamma expression by lymphocytes predict a favorable response to treatment with interferon beta, a drug used for prevention of attacks of MS.18 Expression of IFN gamma parallels disease severity in EAE.19,20 Transgenic mice that overexpress IFN gamma in the central nervous system under the influence of an oligodendrocyte-specific promoter develop extensive demyelination.21,22 Expression of IFN gamma may lead to demyelination either by direct deleterious effects on oligodendrocytes or by increasing major histocompatibility complex expression, thereby activating macrophages and microgli.a.23 In addition, IFN gamma has direct effects on neurons by inducing dendritic retraction and inhibiting

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Cases (n=293)</th>
<th>Controls (n=340)</th>
<th>Combined (n=633)</th>
<th>Odds Ratio (95% Confidence Interval)</th>
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<td>rs3181034<em>G, rs2069727</em>G</td>
<td>0.388</td>
<td>0.476</td>
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<td>rs3181034<em>A, rs2069727</em>A</td>
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<td>1.49 (1.11 - 2.02)</td>
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<td>=</td>
<td>0.014</td>
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Figure 1. Haplotype constructs of the interferon gamma (IFNG) allelic variants and association with multiple sclerosis (MS) risk. A, Haplotype constructs begin with the outermost 2-marker haplotype and successively consider internal markers, thereby bracketing the region of association within IFNG (model 1) using the −log of max-stat statistic; results are shown for men and women separately. Significance level is marked by the horizontal gray line. The gray bar marks the chromosomal segment bound by the 2 markers constituting the significant haplotype. In addition, location of the markers in relation to the 120-kilobase (kb) chromosome 12q14 region is shown. Markers are aligned from centromere to telomere, from left to right. Orientation of IFNG and the interleukin 26 gene (IL26) are from telomere to centromere. B, To further define single-nucleotide polymorphisms (SNPs) essential for the haplotype association, adjacent 4-marker haplotypes were considered (model 2). In addition to the outermost 2 markers delimiting the chromosomal segment with significant association with MS susceptibility (gray bar), the 2 markers shared by 4 of the six 4-marker haplotypes, rs3181034 and rs2069727, are shown with arrows. C, The odds ratios from the most significant haplotypes from the 2-marker consecutive haplotype model (model 3) are shown for men. This analysis shows that the rs2069727 SNP is the marker responsible for the association of IFNG polymorphisms with susceptibility to MS in men. *Because of low frequency of this imputed odds ratios are unstable and not presented.
synapse formation. However, in some circumstances, the effects of IFN gamma in EAE and other animal models of central nervous system demyelination may be favorable. The IFN gamma receptor knockout increases susceptibility to EAE in mice and leads to a progressive form of EAE. On the other hand, absence of IFN regulatory factor 1 in T cells, a regulator of IFN-induced genes, protects patients from EAE. Neutralizing antibodies against IFN gamma increase Th1-related virus–induced demyelination and viral persistence. The opposing effects of IFN gamma may be due to the regulatory functions of IFN gamma on T-cell proliferation and apoptosis and depend on the prevailing balance within the immune system. However, although IFN gamma production by microglia and IFN gamma receptors on oligodendroglia are up-regulated in active MS lesions, other Th1 and Th2 cytokines and their receptors are similarly up-regulated, thereby defying oversimplification into a Th1 vs Th2 paradigm.

We and others have recently suggested that several allelic variants of IFNG and their corresponding haplotypes are associated with sex-determined differences in susceptibility to MS in Sardinia, North America, and Northern Ireland. A French study reported results that are consistent with an association of 1 of these allelic variants with MS, although the results of that study were not stratified by sex and were not significant after conservative correction for multiple genetic comparisons. We found that a single-nucleotide polymorphism (SNP) (rs2069727) adjacent to the 3’UTR region of the gene is associated with MS in men but not in women. In the present study, we report dense linkage disequilibrium (LD) mapping through the IFNG region on chromosome 12q14 and confirm that rs2069727 is the critical allelic variant underlying the association between IFNG and susceptibility to MS in men. In addition, we show that the expression of IFN gamma in MS is associated with rs2069727 and its haplotypes in a sex-dependent manner.

METHODS

STUDY POPULATION

We studied 861 patients (293 men and 568 women) and 843 controls (340 men and 503 women) from 3 independent populations in the United States, Northern Ireland, and Belgium. The US patients consisted of a population-based sample from Olmsted County, Minnesota, and age-, sex-, and ethnicity-matched controls; the other 2 populations were clinic based. The demographic details of these populations were previously reported. In the present study, we report an analysis of the combined population to conduct a dense LD map through the region. Informed consent for the study was obtained from all individuals, and the study was approved by the local ethics committees.

MARKER SELECTION AND GENOTYPING

Genomic DNA from the patients was typed for 20 markers. Five were reported in our previous analyses of the 120-kilobase region of chromosome 12q14 harboring IFNG and IL26: 4 microsatellites (D12S313, I1761CA, D12S2510, and D12S2511) and 1 SNP (rs2069727–3’[325]G→A). To better define LD through the region and to identify SNPs most responsible for the haplotype association with susceptibility to MS in men, we selected 15 additional SNPs from public databases, 4 of which were within IFNG (GenBank NT_029419, hs chromosome 12 contig reference assembly). The SNPs were selected based on a minor allele frequency of greater than 0.05 confirmed in 30 random individuals among our cases by sequencing and for their location in a region of association. In general, we strived to choose markers with R2 values less than 0.3 (for adjacent pairs of markers used in the full population; R2 range, 0.003-0.664). The locations of markers used in the final analyses are shown in Figure 1A. Genotyping of SNPs was performed using a single-base extension method, as previously described.

EXPRESSION STUDIES

We selected 50 patients from Olmsted County to achieve an adequate balance of men and women and to enrich for informative individuals homozygous for the individual alleles of the SNP most strongly associated with sex-specific susceptibility to MS in our study (rs2069727) and I1761CA, which had been associated in other studies with expression of IFNG.

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation with Ficoll Hypaque (Amersham Biosciences, Piscataway, New Jersey). Cells were divided and studied at the Mayo Clinic for expression by quantitative polymerase chain reaction (qPCR) and at Cleveland Clinic for protein expression; analyses were performed independently and blinded to genotype and sex. A total of 2 × 106 cells from each individual were stimulated with 6 µg/mL of phytohemagglutinin for 24 hours, and total messenger RNA (mRNA) was isolated (PURESCRIPT RNA Isolation kit; Gentra Systems, Minneapolis, Minnesota); complementary DNA (cDNA) was synthesized by reverse transcription (ThermoScript RT-PCR System; Invitrogen, Carlsbad, California).

Using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, California), cDNA encoding IFNG was measured by qPCR using a carboxyfluorescein fluorescent dye (FAM)–conjugated oligonucleotide probe that hybridized to the cDNA sequence spanning the IFNG exon 1–exon 2 junction. The reaction product was quantitated in real time with an ABI Prism 7700 apparatus (Applied Biosystems). An absolute standard curve was generated using a synthesized 40-nmol/L IFNG fragment recognized by the FAM probe and PCR amplifiers, including the sequence of exon 1–exon 2 splice site. Duplicate serial diluted samples ranging from 107 to 100 copies/µL yielded an absolute standard slope of −3.2. β-Actin was also quantitated as a constitutively expressed comparator gene. Threshold cycle (Ct) values in each assay were determined in duplicate, and variation between readings was required to be less than 0.6 Ct. Copy numbers of IFNG and β-actin genes were determined from the standard curve, and the relative number of IFNG cDNA to β-actin copies of mRNA was determined by the formula 2− ΔCt, method.

In general, little variation in copy numbers of β-actin cDNA was found among individuals compared with variation in IFNG cDNA. All results were indexed to the results of a single individual whose cDNA was included in each assay. Controls were included in each qPCR assay to correct for any variation among assays. Variation among assays for a given individual was minimal.

SCREENING FOR NOVEL GENETIC VARIATIONS

The cDNA isolated from PBMCs from each patient was sequenced; the screen sequence included the entire coding se-
sequence and untranslated region (GenBank NM_000619.1, IFNG mRNA). Sequence alignment was analyzed using Mutation Surveyor DNA Variant Analysis Software, version 2.61 (Softgenetics LLC, State College, Pennsylvania). More than 90% of the sequence was analyzed for sequence variation in both downstream and upstream orientations.

**ENZYME-LINKED IMMUNOSORBENT ASSAYS**

Aliquots of PBMCs were frozen and shipped to the Cleveland Clinic. The PBMCs were gently thawed, washed twice, counted, suspended in complete media, and incubated for 48 hours at a concentration of 300,000 cells per well in either media alone or in the presence of anti-human CD3 monoclonal antibody (final concentration, 5 μg/mL; BD Pharmingen, Franklin Lakes, New Jersey) or phytohemagglutinin. Supernatants were transferred to 96-well polypropylene plates (AB0796; Abgene, Rochester, New York), sealed with Easy Peel Heat Sealing Foil (Marsh Biomedical, Rochester, New York), and stored at −80°C.

Culture supernatants were thawed at room temperature and centrifuged at 200g to remove cellular debris immediately before cytokine analysis. The IFN gamma production was detected using Ready-Set-Go! Human IFN gamma cytokine assay kits (E Bioscience, San Diego, California) analyzed on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, California). Samples with coefficient variations of greater than 10% between duplicates were retested. Samples with optical density readings greater than the highest standard (500 pg/mL) were diluted either 1:10 or 1:100 in assay diluent and retested. The concentration of IFN gamma in each culture supernatant was calculated from a standard curve (4-500 pg/mL) that was included on the assay plate.

**STATISTICAL ANALYSIS**

The LD in cases and controls was analyzed using the D' measure and R² for the initial LD screen in the smaller subpopulation of 30 individuals used to select markers. Haplotype frequencies were imputed for individuals with ambiguous haplotypes by an expectation-maximization algorithm and compared between cases and controls using a test for associations between traits and haplotypes in which phase is ambiguous using the haplo.score function within S-plus. Simulated P values were estimated to help eliminate spurious significant results that may result from multiple testing. Haplotypes with expected frequencies of less than 5% were removed from the analyses to increase model stability.

We first constructed 2-marker haplotypes, beginning with the outermost 2 markers and successively considering the next set of internal markers, thereby bracketing the region of association with IFNG (model 1) using the max-stat statistic and the corresponding permutation P values (Figure 1A). To further define SNPs essential for the haplotype association, 4-marker (model 2; Figure 1B) and consecutive 2-marker sliding haplotypes were considered (model 3; Figure 1C). Two-marker sliding haplotypes were also used to define the specific alleles of the SNPs responsible for the association (Figure 1C).

For individual odds ratios (ORs), the Cochran-Mantel-Haenszel statistic was used to calculate statistical significance adjusted for contribution of individual populations. Results are presented for carrier status and homozygote genotypes (Table). Note that the confidence intervals (CIs) of the ORs are conservative and do not correspond exactly to the P values listed. Expression levels and enzyme-linked immunosorbent assay (ELISA) results were summarized using medians and interquartile ranges (IQRs), and differences were assessed using the rank sum test. The SAS and S-plus software packages were used.

**LD ASSOCIATION STUDY**

Multiple genetic variants were independently associated with MS in men but not in women, precluding an initial conclusion as to the responsible marker (Table). The rs2430561 lies 3 bp upstream of the I1(761)CA₃ microsatellite and is in nearly complete LD with this microsatellite (D' = 0.86). Specifically, rs2430561*T is in LD with I1(761)CA₁₂ and rs2430561*A is with I1(761)CA₁₃.

Using model 1 (Figure 1A), the haplotypes defined by rs2069727 and rs2430561 were associated with MS in men (global P = .002) but not women (global P = .29). Specifically, the rs2069727*G, rs2430561*T haplotype was negatively associated (frequency, 0.442; P < .001) whereas the rs2069727*A, rs2430561*C haplotype was positively associated with MS in men (frequency, 0.336; P = .07). None of the other haplotypes was significantly associated, suggesting that either rs2069727 or rs2430561 was responsible for the association of IFNG allelic variants with MS in men.

Considering 4-marker sliding haplotypes in model 2, a total of 6 haplotypes limited by the outermost markers rs2216164 and rs2069705 were associated with MS in men according to global P scores (Figure 1B). Two markers were shared by at least 4 of six 4-marker sliding haplotypes: rs3181034 and rs2069727. Only 1 of these haplotypes limited by the outermost markers of rs1861494 and rs2069705 was weakly associated with MS in women.

Considering 2-marker sliding haplotypes in model 3, the strongest association with MS in men was observed with haplotypes defined by SNPs rs3181034 and rs2069727 (Figure 1C). Specifically, when compared with the most frequent (43.3%) imputed haplotype rs3181034*G, rs2069727*G, male carriers of rs3181034*A, rs2069727*A (28.7%; OR, 1.57; 95% CI, 1.18-2.10), and rs3181034*G, rs2069727*A haplotypes (26.4%; OR, 1.49; 95% CI, 1.11-2.02) were more likely than noncarriers to have MS. The haplotypes associated with MS susceptibility in men shared the allele of rs2069727 but not that of rs3181034, suggesting that the association is more likely explained by rs2069727 (3’[325]G→A).

**IFN GAMMA EXPRESSION STUDIES**

Data for qPCRs from 35 patients with analyzable data are shown in Figure 2. Analyses were stratified by genotype and sex. IFN gamma expression varied significantly by genotype. Carriers of rs2069727*G had higher levels of phytohemagglutinin-stimulated expression than noncarriers (median, 743 pg/mL; IQR, 191-2889 pg/mL; P = .04). Conversely, carriers of rs2069727*A had lower levels of expression than noncarriers (median, 290 pg/mL; IQR, 164-620 pg/mL; P = .07). Heterozygotes had intermediate and more variable levels of expression; however, heterozygotes were deliberately underrepresented in the analyses because they were expected to be less informative than individuals who were homozygous for each
allele (Figure 2). Similarly, carriers of I1(761)CA13, which is in significant LD with rs2069727* A, were associated with lower expression than noncarriers (median, 267 pg/mL; IQR, 145-697 pg/mL; P = .03). When stratified by sex alone, there was a trend for women (n=20) to have higher expression (median, 759 pg/mL; IQR, 218-1762 pg/mL) than men (n=15; median, 290 pg/mL; IQR, 164-612 pg/mL) independent of genotype (P = .10), as would be predicted from previous studies.11-13 When stratified by sex and genotype, despite the smaller number of men compared with women, the observed associations between these genotypes and IFN gamma expression were present in men but not in women (Figure 2). The small number of individuals in this subgroup precluded statistical analysis.

The ELISA results are illustrated in Figure 3. Sufficient viable PBMCs and optimal culture conditions allowed the measurement of mitogen-stimulated IFN gamma in the culture supernatant from 36 patients. Phytohemagglutinin and CD3 stimulation results were strongly correlated (Spearman correlation, R = 0.82; P < .001). The ELISA results were similar to findings from qPCR studies; individuals with the rs2069727*G allele had higher levels of expression (CD3 stimulation: median, 2062 pg/mL; IQR, 568-8210 pg/mL; phytohemagglutinin stimulation: median, 1808 pg/mL; IQR, 1064-15 730 pg/mL) than individuals with the A allele (CD3 stimulation: median, 1919 pg/mL; IQR, 560-3472 pg/mL; phytohemagglutinin stimulation: median, 1463 pg/mL; IQR, 456-8134 pg/mL). However, the results were not statistically significant (CD3 stimulation: G vs AA, P = .72; A vs GG, P = .30; phytohemagglutinin stimulation, G vs AA, P = .42; A vs GG, P = .54). Given the small number of men with sufficient viable PBMCs and optimal culture conditions allowing the measurement of mitogen-stimulated IFN gamma in the culture supernatant and with genotypes underrepresented in men with MS compared with women (1 individual each with genotype rs2069727* GG and rs2069727* AG), we could not stratify the ELISA data by sex.

SCREENING FOR CODING POLYMORPHISMS

Sequencing cDNA from these 35 individuals did not reveal any other mutation in the coding sequence of IFNG that could explain the positive association of IFNG rs2069727 and haplotypes defined by this SNP and MS in men (data not shown).
Using a dense LD map, we confirmed that the association between IFNG and MS in men is evident with multiple independently genotyped SNPs but is most robust with rs2069727 (3' [C325]G → A).\(^\text{33-37}\) This effect is stronger for homozygotes than for heterozygotes.\(^\text{36}\) We cannot completely rule out a collective (rather than independent) effect of several individual allelic variants or mutations of low frequency. As illustrated in Figure 1A, the haplotype association weakens even within a relatively short distance from the rs2069727.

In this study, IFNG mRNA expression varies according to IFNG genotype and haplotypes. Because I1(761)CA\(^\text{12}\) is in near-complete LD with rs2430561\(^*\)T and I1(761)CA\(^\text{13}\) is in near complete LD with rs2430561\(^*\)A, both in this and other studies,\(^\text{47}\) our results can be interpreted as the rs2430561\(^*\)T-I1(761)CA\(^\text{12}\)-rs2069727\(^*\)G haplotype being associated with high expression and the rs2430561\(^*\)A-I1(761)CA\(^\text{13}\)-rs2069727\(^*\)A haplotype being associated with low expression. This finding is in agreement with previous independent studies that suggested that I1(761)CA\(^\text{12}\) is associated with high expression\(^\text{41,42}\) and rs2430561\(^*\)A is associated with low expression.\(^\text{48}\) Our study is the first to document this effect of IFNG in patients with MS. It implicates rs2069727 as the SNP with the strongest association. Both the ELISA and mRNA results were in the same direction.

We observed 2 sex effects in this study: sex-IFNG genotype interaction and a sex-genotype–IFN gamma expression interaction. Either of these interactions is hard to
explain by the location of the allelic variants comprising the rs2430561-I1(761)CA n-rs2069727 haplotype. Sequencing IFNG cDNA did not reveal additional coding SNPs or mutations. However, we cannot completely rule out unknown intronic SNP effects.

A G-to-A nucleotide change at rs2069727 is predicted to modify a paired box gene 2 (PAX2) (a sex hormone–controlled transcription factor) binding site to a CCAAT/enhancer binding protein β (CEBPB) (an IL-6–activated transcription factor) binding site. A T-to-A nucleotide change in rs2430561 abolishes a nuclear factor–B (NF-B) (a glucocorticoid-controlled pivotal transcription factor) binding site. Whether these transcription factor binding site modifications influence gene expression in a sex-dependent fashion remains to be studied. The influence on gene expression could be a result of the combined effect of these allelic variants, which are in strong LD.

It is also unclear if the 2 sex influences observed in this study are interdependent. If there were a sex-dependent functional effect of an allelic variant, selective genetic pressure could lead to sex-dependent genotypes to accumulate and associate with certain disease states compared with healthy individuals. For a critical cytokine such as IFN gamma, one would also expect a sex-genotype–gene expression interaction in diseases other than MS, the pathogenesis of which involves T_{H}1/T_{H}2 cell skewing, such as autoimmune or infectious diseases. There is mounting evidence that IFNG rs2430561 and I1(761)CA n, associated with IFN gamma expression in this and other studies, may be associated with susceptibility to a variety of diseases unrelated to MS.

Evidence has also accumulated that in a variety of normal physiologic and disease states, IFN gamma levels vary by sex. After infection with the D variant of encephalomyocarditis virus, IFN gamma production is higher in spleenocytes from diabetes mellitus–resistant female than male mice. Expression of IFN gamma is higher in nonobese diabetic female than male mice, and estrogen increases IFN gamma expression, whereas testosterone decreases IFN gamma expression. Production of IFN gamma during mucosal anti–human immunodeficiency virus response is higher in female than in male mice. Periportal fibrosis in Schistosoma mansoni infection is less common in women with high than low levels of IFN gamma, but this relationship is not observed in men.

A common sex-dependent mechanism may influence a variety of complex disorders characterized by chronic inflammation, such as MS, in which IFN gamma plays a pivotal role. However, whether the sex–IFN gamma expression interactions observed in other disease states can be linked to the sex-genotype interaction that we observed in MS remains to be investigated.
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REFERENCES


**Announcement**

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