BACKGROUND: Multiple sclerosis (MS) is an autoimmune disorder, with a considerable genetic influence on susceptibility and disease course. Cytokines play an important role in MS pathophysiology, and genes encoding various cytokines are logical candidates to assess possible associations with MS susceptibility and disease course. We previously reported an association of a combination of polymorphisms in the interleukin (IL)-*1B* and IL-1 receptor antagonist (*IL-1RN*) genes (i.e. *IL-1RN* allele 2+/*IL-1B*[+3959]allele 2−) with disease severity in MS. Extending this observation, we investigated whether IL-1β and IL-1ra production differed depending on carriership of this gene combination.

Methods: Twenty MS patients and 20 controls were selected based upon carriership of the specific combination. In whole blood, *in vitro* IL-1β and IL-1ra production was determined by enzyme-linked immunosorbent-assay after 6 and 24 h of stimulation with lipopolysaccharide.

Results: Carriers of the specific combination produced more IL-1ra, especially in MS patients, although not significantly. IL-1ra production was significantly higher in individuals homozygous for *IL-1RN* allele 2. In patients, Il-1ra production was higher and IL-1β production lower compared with controls. In primary progressive patients, the IL-1β / IL-1ra ratio was significantly lower than in relapsing-remitting patients.

Conclusion: Our results suggest higher *in vitro* IL-1ra production in carriers of *IL-1RN* allele 2, with an indication of an allelic dose–effect relationship.

Key words: Genetics, Interleukin-1, Interleukin-1 receptor antagonist, Multiple sclerosis, Cytokines

Interleukin (IL)-1 gene polymorphisms: relevance of disease severity associated alleles with IL-1β and IL-1ra production in multiple sclerosis

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Introduction

Genetics of multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system with a presumed auto-immune etiology. There is a strong genetic influence, with a concordance rate in monozygotic twins of approximately 30%.[1] The genetics of MS are thought to comprise a variety of genes, influencing both disease susceptibility and disease characteristics. Among these, the contribution of genes encoding cytokines is currently under elaborate investigation, as cytokines are important mediators in immune and inflammatory conditions, such as multiple sclerosis. Due to the large and still increasing number of cytokines and the complexity of their interactions, the mechanisms by which they apparently influence the outcome of disease processes are poorly understood.

Interleukin-1 and interleukin-1 receptor antagonist

Interleukin (IL)-1α and IL-1β are major pro-inflammatory cytokines, their actions being counter-regulated by the naturally occurring IL-1 receptor antagonist.[2] Several reports attribute a role to cytokines of the IL-1 family in MS pathophysiology. IL-1 is present in and around MS lesions, and might be involved in the destruction of central nervous system myelin.[3] Both during relapses and after interferon treatment, elevated IL-1ra levels are present in sera of MS patients,[4] and there is some evidence that an increase in disease activity is followed by increased IL-1ra serum levels.[5] Differences in either absolute cytokine production or in the ratio of pro-inflammatory and anti-inflammatory cytokines may influence the disease course; for example, by maintaining inflammatory activity.

Gene polymorphisms

In the IL-1 genes, like in several other cytokine genes, polymorphisms have been demonstrated,[6,7] adding an additional level of variation. Various associations of gene polymorphisms and disease characteristics have been published. A number of studies associate *IL-1RN* allele 2 with disease severity in chronic inflammatory conditions, such as systemic lupus erythematosus[8] and ulcerative colitis.[9] We reported earlier an association of a specific $IL\text{-}1B^{+3959}$/*IL-1RN* gene combination with disease severity in MS.[10] In this study, carriers of a specific combination (*IL-1RN* allele $2+$/$IL\text{-}1B^{+3959}$ allele $2-$) had a significantly higher progression rate, measured as time from disease onset to an Expanded Disability Status Scale score equivalent with a walking distance of 100 m. The observed associations might be explained by functional consequences of these gene polymorphisms. Assuming that cytokine production is regulated by the polymorphism present in the encoding gene, specific alleles might be linked to high or low production levels. This has been demonstrated for some polymorphisms in genes of the IL-1 family.[6,11,12] Furthermore, it was shown very recently that innate production of IL-1β and IL-1ra is a risk factor for susceptibility and progression of relapse-onset MS.[13]

Study rationale

In an attempt to explain our earlier observation, we investigated in patients and controls whether *in vitro* IL-1β and IL-1ra production differed between carriers and non-carriers of an $IL\text{-}1RN$ /$IL\text{-}1B^{+3959}$ gene combination, previously shown to be associated with disease severity in MS.

Methods

Study design

All subjects were unrelated Dutch white Caucasian individuals. The study was carried out with approval of the Medical Ethical Committee and informed consent was obtained from all subjects. A biallelic polymorphism at position $+3953$ in the fifth exon of the *IL-1B* gene[6] and a penta-allelic polymorphism in intron 2 of the *IL-1RN* gene[7] had been determined for all subjects according to previously described methods.[9] We selected from our original study population of 148 MS patients in which we had established an association with disease severity[10] 10 patients that were carriers of the specific combination (i.e. *IL-1 RN* allele $2+$/$IL\text{-}1B^{+3959}$ allele $2-$) and 10 patients that were non-carriers (*IL-1RN* allele $2-$/$IL\text{-}1B^{+3959}$ allele $2-$). Carriers and non-carriers were matched for gender, and type of disease. Each patient group

consisted of four relapsing-remitting, four secondary progressive and two primary progressive patients. Likewise, 20 healthy controls that were carrier (nine controls) or non-carrier (11 controls) of the specific combination were selected. This design defined two subgroups of 20 subjects (patients and controls) differing only in carriership of *IL-1RN* allele 2. Subjects with exacerbations, steroid treatment, or infections within the past 3 months were excluded in order to avoid blood sampling in conditions that influence cytokine production. Due to deviations of the laboratory protocol the data of two controls were discarded, and the results are correspondingly based on the remaining 18 control subjects.

Whole blood stimulation

Blood was collected in two 4 ml endotoxin-free sodium heparin tubes (Chromogenix AB, Mölndal, Sweden) and whole blood samples were diluted 1:1 with endotoxin free RPMI 1640 (Flow Laboratories, Rockville, MD, USA) and incubated with the appropriate stimuli within 60 min after sampling. The production of cytokines was measured after stimulation with *Escherichia coli* serotype 0111;B4 lipopolysaccharide (LPS; Difco Laboratories, Detroit, MI, USA) at a final concentration of 10 ng/ml of LPS for 6 and 24 h in 96-well tissue culture plates at $37°C$ and 5% CO_2. The supernatants were centrifuged for 10 min and stored at $-20°C$ until assay with commercially available IL-1ra and IL-1β enzyme-linked immunosorbent assay kits (Biosource Europe S.A., Fleurus, Belgium). Incubation times were chosen on the basis of production curves generated from preliminary data of healthy volunteers. Procedures were performed according to the manufacturer's instructions. For every subject samples were run in duplicate with and without LPS stimulation, as control for contamination. Results are expressed in picograms per milliliter. Whole blood was used to maintain cell types in their *in vivo* ratios and to retain all blood components. As significant relations between cytokine production in peripheral blood mononuclear cells (PBMCs) and whole blood cultures have recently been reported,[14] comparisons with studies using peripheral blood mononuclear cells can be reliably made.

Statistical analysis

Production levels between groups and within groups were analyzed using one-way/two-way analysis of variance, Mann–Whitney and Kruskal–Wallis tests. Correlations were calculated using Spearman's rank correlation test. Significance levels were set at 5% (two sided, $p = 0.05$), and $p < 0.10$ was considered a trend.

Results

Relevant features of patients and controls are summarized in Table 1. Baseline levels (without stimulation) were all below 300 pg/ml. Average cytokine levels after LPS stimulation in patients and controls are shown in Figs. 1 and 2. Cytokine levels after 6 and 24 h correlated significantly ($p < 0.01$) for both IL-1ra ($r = 0.83$) and IL-1β ($r = 0.90$).

IL-1ra production

IL-1ra production was considerably higher in IL-1RN allele 2 carriers (MS and controls combined) than in non-carriers after 6 and 24 h (Fig. 1; 24% and 21%, respectively), although not significant ($p = 0.16$ and $p = 0.25$). IL-1ra production was similar in MS patients and controls after 6 h, but considerably higher (18%) after 24 h stimulation ($p = 0.303$). The higher production in MS patients was due to elevated levels (36%) in the *IL-1RN* allele 2+/*IL-1B*$^{+3959}$ allele 2− group. Although IL-1ra levels after 6 h were 16% higher in *IL-1RN* allele 2+/*IL-1B*$^{+3959}$ allele 2− controls, 24 h levels did not differ between *IL-1RN* allele 2 carriers and non-carriers (Fig. 2). The observed differences were not statistically significant. There was a trend ($p = 0.095$) towards higher production in controls and patients homozygous for *IL-1RN* allele 2 after 6 h, and a significant difference after 24 h (Fig. 3; $p = 0.043$). Gender, age and disease type did not influence cytokine production significantly.

IL-1β production

A trend towards higher IL-1β production in controls compared with patients after 6 h (32%) and 24 h (27%) stimulation was observed ($p = 0.80$ and $p = 0.094$). Also, a trend in primary progressive patients to produce less IL-1β after 24 h than relapsing-remitting or secondary progressive patients was seen (44%; $p = 0.059$). No significant differences were observed with regard to genotype, age or gender.

IL-1β /IL-1ra ratio

Assuming that a higher IL-1β/IL-1ra ratio reflects enhanced pro-inflammatory activity, this ratio was calculated from the cytokine production levels. No significant differences were observed with regard to genotype or gender. A significantly lower IL-1β/IL-1ra ratio was observed in primary progressive patients compared with relapsing remitting patients, with a ratio of 0.37 versus 1.14 after 6 h ($p = 0.038$) and of 0.17 versus 0.45 after 24 h ($p = 0.017$; Table 2). Both higher IL-1ra levels and lower IL-1β levels in the primary progressive patients underlie the observed difference.

Discussion

In 1999 we established an association of a specific combination of polymorphisms in the *IL-1B* and *IL-1RN* genes (i.e. *IL-1RN* allele 2+/*IL-1B*$^{+3959}$allele 2−) with disease severity in MS.[10] Although a large study in the UK did not show any evidence in favor of a role for the *IL-1RN* gene in susceptibility to MS or its progression,[15] other reports provided support for our original observation that polymorphisms in the IL-1 gene cluster are associated with disease severity.[16,17] Furthermore, the production of IL-1ra and IL-1β has very recently been proposed as a risk factor for susceptibility and progression of relapse-onset MS.[13] Hence, in an effort to explain our observed association, this study addresses whether a specific genetic make-up of the IL-1 genes is reflected in cytokine production. Although other studies have shown that inter-individual cytokine production is highly variable, intra-individual production is remarkably stable over time and appears to be characteristic of an individual.[11,14] In addition, we tried to limit influence of disease activity on cytokine production by blood sampling in periods without recent exacerbations. In favor of an effect on cytokine production is our observation that *IL-1RN* allele 2 carriers produced more IL-1ra than *IL-1RN* allele 2 non-carriers, as was anticipated from available data.[11,18] Furthermore, there was in individuals (patients and controls) homozygous for *IL-1RN* allele 2 a consistent trend

Table 1. Characteristics of MS patients and controls

	MS patients ($n = 20$)		Controls ($n = 18$)	
	IL-1RN 2+/*IL-1B*$^{+3959}$ 2−	*IL-1RN* 2−/*IL-1B*$^{+3959}$ 2−	*IL-1RN* 2+/*IL-1B*$^{+3959}$ 2−	*IL-1RN* 2−/*IL-1B*$^{+3959}$ 2−
Female	3	3	3	6
Male	7	7	4	5
Age ± SD (years)	43.4 ± 12.7	49.3 ± 15.3	30.9 ± 5.2	37.8 ± 9.5
RR	4 (3 M, 1 F)	4 (3 M, 1 F)		
SP	4 (3 M, 1 F)	4 (3 M, 1 F)	Not applicable	Not applicable
PP	2 (1 M, 1 F)	2 (1 M, 1 F)		

RR, Relapsing-remitting; SP, secondary progressive; PP, primary progressive; SD, standard deviation; M, male; F, female.
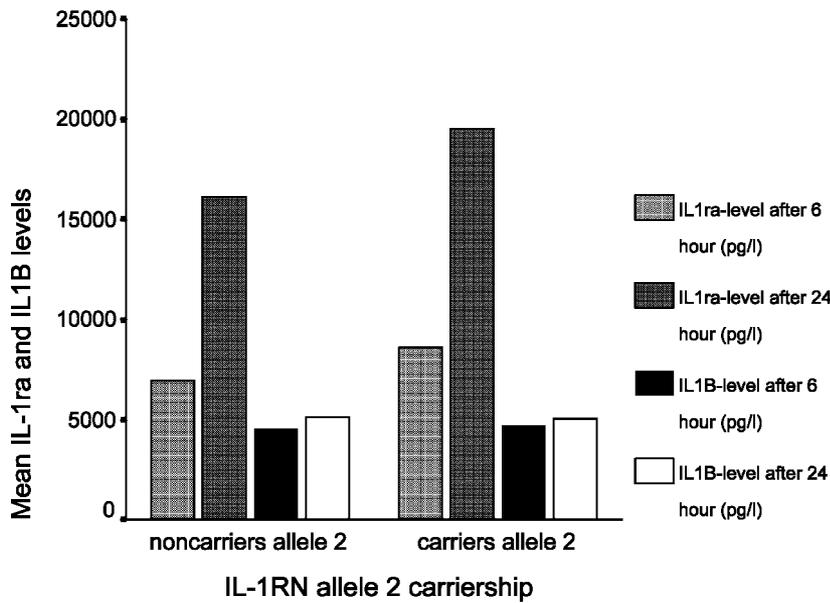
FIG. 1. IL-1ra and IL-1β levels after 6 and 24 h in carriers(*IL-1RN* allele 2+/*IL-1B*[+3959]allele 2−) and non-carriers (*IL-1RN* allele 2−/*IL-1B*[+3959]allele 2−) of IL-1RN allele 2.

towards higher IL-1ra production. This observation implies an allelic dose−effect relationship with regard to cytokine production, and is in line with an Italian study that showed that peripheral blood mononuclear cells from healthy controls homozygous for *IL-1RN* allele 2 produce significantly more IL-1ra.[19]

Unfortunately, due to the rarity of patients that are homozygous for *IL-1RN* allele 2 and non-carriers of *IL-1B*[+3959] allele 2, we were not able to further explore this observation in the present study. Despite the mentioned raised IL-1ra levels in *IL-1RN* allele 2 carriers, the observed differences between carriers

and non-carriers of *IL-1RN* allele 2 did not reach statistical significance. Obviously, this argues against a major influence of the studied gene combination on cytokine production. What, then, to conclude from this and earlier research? First, it should be noted that the size of this exploratory study may hamper detection of differences in production levels. Second, it has been shown that IL-1ra production is not only determined by the *IL-1RN* gene polymorphisms, but is co-regulated by the *IL-1B*[+3959] polymorphism.[12] Given the specific gene combination that we investigated, all individuals are *IL-1B*[+3959] allele 1 homozygous, and hence the observed differences in IL-1β
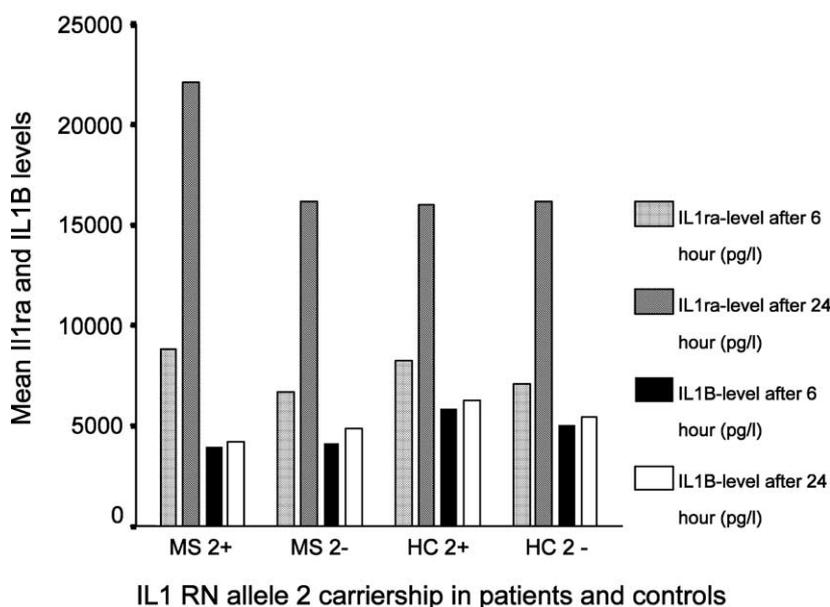
FIG. 2. Mean IL-1ra and IL-1β levels after 6 and 24 h in MS patients and controls (HC) after stratification for IL-1RN allele 2 carriership. 2+, *IL-1RN* allele 2+/*IL-1β* allele 2−; 2−, *IL-1RN* allele 2−/*IL-1β* allele 2−.
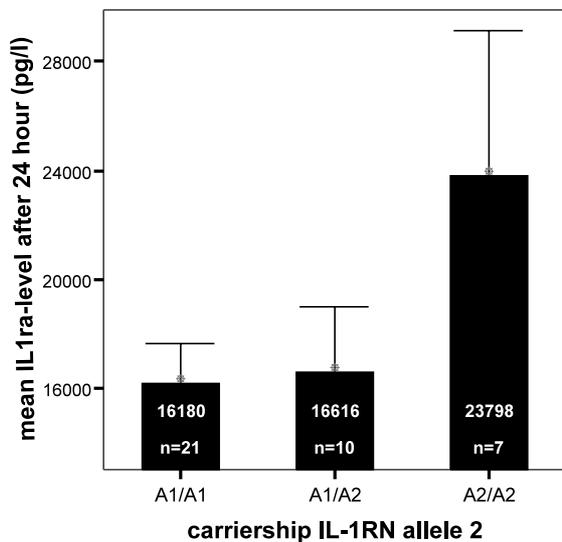
FIG. 3. Mean IL-1ra levels after 24 h and *IL-1RN* allele 2 number. Error bars represent standard error of mean; *n*, number of individuals; A1/A1, non-carriers of *IL-1RN* allele 2; A1/A2, carriers of one copy of *IL-1RN* allele 2; A2/A2, carriers of two copies of *IL-1RN* allele 2. *p*P = 0.043 A2/A2 versus other genotypes.

Table 2. IL-1β/IL-1ra ratios after 6 and 24 h

	n	IL-1β/IL-1ra	
		6 h	24 h
Multiple sclerosis			
Relapsing-remitting	8	1.14	0.45
Secondary progressive	8	0.52	0.25
Primary progressive	4	0.37[a]	0.17[a]
All patients	20	0.72	0.31
Controls (HC)	18	0.77	0.40
IL-1RN allele 2 carriers (MS)	10	0.56	0.25
IL-1RN allele 2 carriers (HC)	7	0.78	0.46
IL-1RN allele 2 carriers (all)	17	0.65	0.34
IL-1RN allele 2 non-carriers (MS)	10	0.89	0.38
IL-1RN allele 2 non-carriers (HC)	11	0.77	0.36
IL-1RN allele 2 non-carriers (all)	21	0.82	0.37

IL-1RN allele 2 carriers, *IL-1RN* allele 2+/*IL-1B*[+3959]allele 2−; IL-1RN allele 2 non-carriers, *IL-1RN* allele 2−/*IL-1B*[+3959]allele 2−; HC = healthy controls.
[a] Primary progressive versus relapsing-remitting (*p* = 0.038 and *p* = 0.017, 6 and 24 h; Mann–Whitney test).

and IL-1ra production cannot be attributed to this gene polymorphism. In a recent report, despite significant differences in IL-1β and IL-1ra production levels between families of MS patients, no associations with *IL-1B* and *IL-RN* gene polymorphisms were established, leading the authors to suggest that possibly other polymorphisms are responsible for the differences they observed.[13]

As a separate observation, unrelated to the gene polymorphisms, we found in primary progressive patients a trend towards lower production of IL-1β and significantly lower IL-1β/IL-1ra ratios (after 6 and 24 h) than in relapsing-remitting patients. This is consistent with the current opinion that in primary progressive patients inflammation is a less prominent feature.[20] Since only four primary progressive patients were included, our findings must of course be interpreted with caution.

In short, the findings of this study cannot be viewed, nor are they presented, as conclusive. They do suggest, however, higher IL-1ra production in carriers of the *IL-1RN* allele 2, with a possible allelic dose–effect relationship. Furthermore, the *IL-1RN* gene polymorphism might not be the only determinant of IL-1ra production, but other factors that could well be other gene polymorphisms are probably also involved. Larger studies combining clinical, genetic, and functional data (both *in vitro* and *in vivo*) are needed to obtain definite answers concerning the role of cytokine genes like those of the IL-1 gene cluster in MS and other diseases.

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