Interleukin 8 Gene Polymorphism Is Associated with Increased Risk of Nephritis in Cutaneous Vasculitis

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ABSTRACT. ***Objective.*** To assess the influence of interleukin-8 (IL-8), epithelial cell-derived neutrophil-activating peptide (ENA-78), and regulated upon activation normal T cell expressed and secreted (RANTES) gene polymorphisms in the susceptibility and clinical expression of patients fulfilling classification criteria for Henoch-Schönlein purpura (HSP).

Methods. Fifty patients (25 men) from Northwest Spain with primary cutaneous vasculitis classified as HSP according to proposed criteria were studied. All patients were required to have had at least 2 years' followup. Patients and ethnically matched controls were genotyped for IL-8, ENA-78, and RANTES gene polymorphisms.

Results. No allele or genotype differences between patients fulfilling HSP classification criteria and controls were observed for any of the chemokines. However, a significantly increased frequency of allele A of the IL-8 gene polymorphism was found in patients with HSP who developed renal manifestations compared with patients without renal involvement ($p = 0.02$; $p_{corr} = 0.036$). Moreover, the genotype distribution in HSP patients with and without renal involvement showed statistically significant differences ($p = 0.02$).

Conclusion. In unselected patients with cutaneous vasculitis, carriage of IL-8 allele A influences the susceptibility to renal involvement. (J Rheumatol 2002;29:2367–70)

Key Indexing Terms:
HENOCH-SCHÖNLEIN PURPURA CC CHEMOKINE RENAL INVOLVEMENT
CXC CHEMOKINE IL-8 RANTES ENA-78

Henoch-Schönlein purpura (HSP) is the most common small blood vessel leukocytoclastic angiitis in children and a rare condition in adults[1,2]. Palpable purpura, joint, and gastrointestinal (GI) manifestations constitute a well known triad of manifestations in this syndrome. However, renal involvement, manifested generally by the presence of hematuria with or without proteinuria and renal insufficiency, is the most serious complication and the major cause of longterm morbidity and mortality in HSP[2].

Susceptibility to HSP and associated clinical heterogeneity in HSP may be conferred by a number of genetic loci. Chemokines consist of 70-130 amino acids characterized by the presence of conserved cysteines linked by disulfide bonds[3]. They are chemotactic cytokines that activate and direct the migration of leukocytes. Chemokines act on responsive leukocyte subsets through G protein-coupled transmembrane receptors[4]. Thus, chemokines and chemokine receptors are involved in leukocyte trafficking across different compartments from the tissue of origin and blood to sites of homing, host defense, or disposal. Due to this, they are implicated in many pathological conditions including inflammation and autoimmunity[5].

Investigation of the regulatory factors implicated in the expression of chemokines may be important for our understanding in the study of inflammatory conditions. In this regard, we examined the influence of the polymorphism of interleukin-8 (IL-8) and epithelial cell-derived neutrophil-activating peptide (ENA-78) genes (both CXC chemokines), and regulated upon activation normal T cell expressed and secreted (RANTES) gene (CC chemokine) in the susceptibility and clinical expression of patients with cutaneous vasculitis fulfilling classification criteria for HSP.

MATERIALS AND METHODS

Study population. Patients classified with HSP were recruited from the Divisions of Pediatrics and Rheumatology of the Hospital Xeral-Calde (Lugo, Northwest Spain). Clinical characteristics of the patients (n = 50; 25 men and 25 women, 39 of them younger than 21 years) have previously been reported[6]. The mean age at the onset of the disease was 14 years (median: 7 years; range: 2-62 years). All of them presented with palpable purpura involving mainly the legs. Severe GI manifestations (GI bleeding and/or bowel angina) were found in 41 patients. All 32 patients with renal manifestations had hematuria (> 10 red blood cells/hpf) and 18 also had proteinuria (> 500 mg/24 h). After a median followup of 8 years (minimum

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of 2 years), 10 patients had persistent renal involvement (renal sequelae), most of them hematuria.

Among the 11 adults classified as having HSP, 8 had hematuria, associated with proteinuria in 5 of them. A renal biopsy was performed in the 2 patients (both men) who developed renal insufficiency in the course of the disease. In both cases the biopsy showed a glomerulonephritis with mesangial IgA deposits by immunofluorescence. Although we do not have complete antineutrophil cytoplasmic antibodies (ANCA) data available as 5 of the 11 adults were diagnosed before ANCA tests were routinely available at our hospital, ANCA tests were negative in the 6 patients who were tested. Rheumatoid factor, cryoglobulins, and antinuclear antibodies were negative. Hepatitis C virus serology, performed at the time of diagnosis or later in the followup, was negative. In children, a focal and proliferative glomerulonephritis with IgA mesangial deposits was confirmed in the 3 cases on whom renal biopsy was performed. Ethnically matched controls (n = 88) were from the same region.

Inclusion criteria. Patients with primary cutaneous vasculitis were classified as having HSP according to the criteria proposed by Michel, *et al*[7]. As previously described[6], in adults a skin biopsy showing leukocytoclastic vasculitis was always required. In children with HSP (age under 21 years), a diagnosis of cutaneous vasculitis was considered in most cases without skin biopsy if they had typical non-thrombocytopenic symmetric palpable purpura involving the lower extremities, and other conditions such as connective tissue diseases and infections had been excluded.

Molecular analysis of IL-8, ENA-78, and RANTES gene polymorphisms. DNA was extracted from anticoagulated blood collected in EDTA using a commercially available DNA extraction kit (Bioline™, London, UK).

IL-8 gene polymorphism. Adenosine/uridine (A/U)-rich sequences in the 3'-untranslated region (UTR) of the IL-8 gene have been suggested to contribute to its post-transcriptional regulation[8]. A polymorphism at position 2767 (A/G) in the 3'-UTR region of IL-8 gene was assessed using the following primers for the amplification of the region: Forward 5'-CTTTAGTGTTTTTATGTGCTCTCCA-3', Reverse 5'-GCAAATATGCT-TAGGCTTTAACC-3'. The polymerase chain reaction (PCR) was carried out in a volume of 25 μl containing 100 ng of genomic DNA, 1 × NH_4 buffer (Bioline), 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Bioline), 5 pmol of each primer, and 1 unit of Taq DNA polymerase (Bioline) and 1 mM Betaine (Sigma, Poole, UK). The DNA was denatured at 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 55°C for 45 seconds and 72°C for 45 seconds, and a final extension at 72°C for 2 minutes. The PCR product yielded 510 bp. Analysis of the PCR product was performed by enzyme digestion using 4.0 units of ApaLI (New England Biolab, Hitchin, UK). The restriction enzyme, which cut product from allele A, yielded a product of 120 bp and 390 bp. The digestion was incubated overnight at 37°C and its products were then visualized on a 3% agarose gel stained with ethidium bromide.

ENA-78 gene polymorphism. Assays have been designed for detection of the promoter polymorphism (-156 C/G) using the ABI PRISM SNapshot ddNTP primer extension Kit[9]. The genomic DNA was amplified using the following primers: Forward 5'-ACT CCC TTC TAG CTG GAG CC-3', Reverse 5'-GTG CCT TCT GCA CTC CTT TT -3'. For the promoter polymorphism a total of 100 ng genomic DNA was amplified in a 10 μl final volume PCR reaction containing 1 × NH_4 buffer (Bioline), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Bioline), 5 pmol of each primer and 1 U Taq polymerase (Bioline) and 1 mM Betaine (Sigma). Thermal cycling for the promoter polymorphism were as follows: 95°C for 2 minutes followed by 40 cycles each of 95°C for 45 seconds, 59°C for 45 seconds and 72°C for 45 seconds and a final extension of 72°C for 2 minutes. A PCR product of 246 bp was visualized on a 3% agarose gel stained with ethidium bromide. The probe used for the single nucleotide extension in the primer extension kit was: 5'-CAG ACA ATG GGA ACT GGT-3'. After extension and purification, the product was electrophoresed on a 3100 ABI analyzer and the results were analyzed with Genescan software.

RANTES gene polymorphism. A biallelic polymorphism G/A at position –403 within the RANTES gene polymorphism was assessed as described[10]. The genomic DNA was amplified using the following primers: Forward 5'-GCC TCA ATT TAC AGT GTG-3', Reverse 5'-TGC TTA TTC ATT ACA GAT GTT -3'. PCR reactions were performed in a 25 μl final volume containing 1 × NH_4 buffer (Bioline), 1.5 mM Mg^{2+}, 0.2 mM dNTPs (Bioline), 6.3 pmol of each primer, 1 U Taq polymerase (Bioline) and 1 mM Betaine (Sigma). PCR cycles were the following: 95°C for 2 minutes followed by 35 cycles each of 95°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds. A final extension step was undertaken at 72°C for 5 min. PCR products were digested with Mae III enzyme in a 15 μl final volume using 4 units of Mae III enzyme and 5 μl of PCR product. The reactions were incubated at 55°C overnight. The wild allele G yielded two bands of 112 and 23 bp while the mutant allele A yielded a single uncut band of 135 bp.

Statistical analysis. The strength of association between HSP and alleles or genotypes of IL-8, ENA-78, and RANTES genes was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either chi-square or Fisher exact analysis. The same methods were used to examine the strength of association between HSP subgroups with or without renal manifestations and IL-8, ENA-78, and RANTES alleles. P values (p_{corr}) were corrected using the Bonferroni method. They were calculated by multiplying the p value by the number of alleles compared. For the analysis of HSP genotypes, when categorical variables had more than 2 mutually exclusive categories (i.e., HSP with or without hematuria or HSP with or without renal sequelae), a p value as an overall measure of statistical significance was provided. Statistical significance was defined as $p \leq 0.05$. Calculations were performed with the statistical package Stata V6.

RESULTS

Allele and genotype frequencies of IL-8 gene in patients with HSP and controls. In controls and patients fulfilling HSP classification criteria, no evidence of departure from Hardy-Weinberg equilibrium was observed (p = NS). When the allele and genotype frequencies of IL-8 polymorphism were compared to those of controls, no significant differences were found (Table 1). However, a significantly increased frequency of allele A was observed in patients with HSP who developed renal manifestations compared with patients without renal involvement [p = 0.02; p_{corr} = 0.036; OR: 2.9 (95% CI: 1.2–6.9)]. Moreover, the genotype distribution in HSP patients with and without renal involvement showed statistically significant differences (p = 0.02) (Table 1). In addition, allele A was also increased in HSP patients with renal manifestations compared with ethnically matched controls. This increase, however, was out of the range of significance when a Bonferroni correction was applied (Table 1). Likewise, although allele A was increased in HSP patients who suffered severe GI manifestations, such an increase did not achieve statistically significant differences when these patients were compared with those without GI bleeding and/or bowel angina. In this cohort no genotypic differences between younger and older patients was observed. Finally, IL-8 biallelic polymorphism was not associated with the development of renal sequelae.

Allele and genotype frequencies of ENA-78 and RANTES genes in patients with HSP and controls. The allele and genotype distribution in HSP patients and controls were

Table 1. Frequency of IL–8 polymorphism in Henoch-Schölein purpura (HSP) patients with and without renal or gastrointestinal (GI) manifestations and controls.

No. patients	Controls (n = 88)	HSP (Total) (n = 50)	HSP with renal Manifestations *,** Yes (n = 32)	HSP with renal Manifestations *,** No (n = 18)	HSP with GI Manifestations * Yes (n = 41)	HSP with GI Manifestations * No (n = 9)	HSP with Renal Sequelae† Yes (n = 10)	HSP with Renal Sequelae† No (n = 40)
Allele								
A	65[s]	70	78[‡,s]	56[s]	74	50	70	70
G	35	30	22	44	26	50	30	30
Genotype								
AA	41	48	56	33	54	22	40	50
AG	48	44	44	44	41	56	60	40
GG	11	8	0	22	5	22	0	10

* During the clinical course of the disease. ** Genotype distribution in HSP with and without renal involvement yielded statistically significant differences; p = 0.02. † Renal sequelae: Persistent renal involvement at the end of the study (at least 2 years' followup).
‡ Allele A was increased in HSP with renal involvement compared with those HSP without: p = 0.02; p_c = 0.036; OR: 2.9 (95% CI: 1.2–6.9).
[s]Allele A was increased in HSP patients with renal involvement compared with controls: p = 0.05; p_c = 0.098; OR: 1.9 (95% CI: 1.0–3.8).

similar (Table 2). This was also the case when HSP patients with and without renal or severe GI manifestations were compared (data not shown). Thus, ENA-78 and RANTES gene polymorphism does not seem to be associated with susceptibility or clinical expression of this vasculitis.

DISCUSSION

We recently described a genetic association with HLA-DRB1*01 in HSP patients from Northwest Spain[6]. This condition was also associated with HLA-DRB1*01 in Italians[11]. However, HLA-DRB1*01 patients with severe GI manifestations or with renal involvement did not have any specific HLA-DRB1 association other than the underlying association with HLA-DRB1*01. Additional studies have shown that in patients from Northwest Spain, ICAM-1 polymorphisms alone were not associated with development of HSP but patients not carrying the codon 469 K/E genotype were at decreased risk of developing severe GI complications[12]. In this study we assessed for first time the potential role of IL-8, ENA-78, and RANTES chemokine gene polymorphisms in the susceptibility to and clinical expression of a primary small-sized blood vessel vasculitis.

Table 2. Allele and genotype frequencies of ENA–78 and RANTES gene polymorphisms in a series of Henoch-Schönlein purpura (HSP) patients and controls *.

ENA-78	Controls n = 107	HSP n = 49
Allele		
G	86	90
C	14	10
Genotype		
GG	74	82
CG	23	16
CC	3	2
RANTES	n = 65	n = 50
Allele		
G	89	86
A	11	14
Genotype		
GG	77	74
GA	23	24
AA	0	2

* No statistically significant differences between the group of HSP patients and controls were observed.

In Northwest Spain, RANTES gene polymorphism was found to be associated with the susceptibility to isolated polymyalgia rheumatica (PMR)[10]. However, in the large blood vessel vasculitis, giant cell arteritis (GCA), a vasculitis frequently associated with PMR, no implication of this polymorphism seemed to occur. This study extends the analysis of the influence of RANTES gene polymorphism to small blood vessel vasculitides. As described in GCA, RANTES gene polymorphism was not implicated in either susceptibility or clinical expression of HSP. This was also the case for ENA-78 gene polymorphism. However, we have identified a possible role of IL-8 gene polymorphism in the susceptibility for nephritis in unselected patients with cutaneous vasculitis who fulfilled classification criteria for HSP.

The pathogenic mechanisms of HSP, a vasculitis due to IgA-mediated inflammation of small vessels are not completely understood. Vascular damage is due to the deposition of those immune complexes in vessel walls with activation of the complement. These immune complexes promote platelet aggregation and are capable of activating complement leading to the release of chemotactic factors, in particular C5a, which in turn recruit polymorphonuclear leukocytes at the site of deposition. The release of lysosomal

enzymes due to the ingestion of immune complexes by the polymorphonuclear leukocytes results in vessel damage[2]. However, the possible influence of IL-8 gene polymorphism in the development of nephritis in HSP needs to be elucidated.

IL-8 induces a rapid and active change in neutrophil shape resulting from the polymerization and de-polymerization of actin[5]. IL-8 would be implicated in the up-regulation and activation of integrins through which the leukocyte adhere to endothelial cell before emigration[13]. With respect to this, the chemokine receptors CXCR1 and CXCR2 are almost exclusively found in neutrophils. These receptors are principally implicated in antibacterial defense[5]. Of note, infectious agents, in particular, beta hemolytic streptococcus, seem to be implicated in the development of HSP[14]. The implication of these receptors in the acute response might explain why IL-8 polymorphism was associated with disease susceptibility to renal and possibly to GI manifestations as a trigger factor of inflammatory response against infectious agents but not linked to development of chronic disease. Based on that, investigations of chemokine antagonists of IL-8 or specifically their receptors might have a clinical implication. Other genes, however, may be implicated in the development of persistent and chronic renal damage. In this regard, in our patients the IL-1 receptor antagonist (IL-1ra) gene polymorphism was associated with severe renal involvement and renal sequelae in HSP[15]. IL-1ra gene polymorphism was directly implicated in severity and outcome, but not in susceptibility, of unselected patients with cutaneous vasculitis. No association of this polymorphism was observed in patients with vasculitis limited to skin, but the carriage of IL-1 receptor antagonist allele 2 in HSP patients was associated with a higher risk of severe renal manifestations and renal sequelae[15].

Additional studies in other populations are required to further delineate the role of IL-8 in the clinical spectrum of HSP.

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