Variants of the Chemokine Receptor CCR5 Are Associated with Severe Bronchiolitis Caused by Respiratory Syncytial Virus

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Respiratory syncytial virus (RSV) bronchiolitis is characterized by intense inflammation of the airways, and high levels of proinflammatory cytokines and chemokines can be found in respiratory secretions of affected infants. Important among these chemokines are RANTES (regulated on activation, normal T cell–expressed and –secreted) and macrophage inflammatory-protein α, MIP-1α, both of which show correlation with severe RSV bronchiolitis. It is not clear whether high levels of these chemokines are important in disease pathogenesis, and this study addresses this question by studying genetic variants of their major receptor, CC chemokine receptor 5. Results from both a case-control and family-based genetic-association analysis show that the −2459G and −2554T variants are associated with severe RSV bronchiolitis ($P = .01$). It is proposed that these CCR5 variants influence the inflammatory response, and these data provide further evidence of the important role that host genetic variability plays in the determination of disease severity in RSV bronchiolitis.

Respiratory syncytial virus (RSV) bronchiolitis is characterized by an intense inflammatory response of the airways. Studies in vitro and in vivo have suggested that, after being infected with RSV, the airway epithelium is able to initiate this inflammatory response by producing large quantities of proinflammatory cytokines. Important among these are the CC chemokines—RANTES (regulated on activation, normal T cell expressed and secreted) and macrophage inflammatory protein (MIP)–1α. These chemokines are strongly chemotactic for T cells, monocytes, basophils, eosinophils, and, to a lesser degree, neutrophils. Studies of respiratory secretions from children show that RANTES is particularly highly expressed during infections with RSV [1]. RANTES and MIP-1α are present at higher levels in infants with more-severe disease [2]. Mice deficient in MIP-1α show reduced inflammatory-cell recruitment after infection with RSV, a finding emphasizing the important role that this chemokine plays in the inflammatory process [3]. RANTES and MIP-1α are the major ligands for CCR5. Several promoter-region variants of the CCR5 gene have been described. These variants have been associated with several diseases (e.g., myocardial infarction [4], rejection after renal transplantation [5], chagasic cardiomyopathy [6], rheumatoid arthritis [7]) and, most notably, reduced progression to AIDS after infection with human immunodeficiency virus [8–10] Although the levels of RANTES and MIP-1α appear to correlate with the severity of RSV bronchiolitis, it is not clear whether they play an important role in causing severe disease or are merely elevated as a consequence of the disease. Genetic-association studies can help to determine which of these possibilities is more likely. Genetic variants that are shown to predispose to a disease must themselves play a key role in the pathogenesis of that disease. In the present study, we have used a genetic-association approach, with case-control and family-based designs, to study the role that CCR5 gene variants play in the determination of disease severity in young infants after they have been infected with RSV.

Subjects, materials, and methods. Infants with RSV-positive (by immunofluorescence or culture) bronchiolitis were identified in 10 hospitals in southeastern England. To be included in the study, patients had to be <12 months of age and had to have RSV-positive bronchiolitis characterized by tachypnea, retractions, and bilateral crackles (wheeze alone was not accepted). Infants were included only if their disease was considered severe enough to require gavage feeding, intravenous fluids, or oxygen. DNA samples were collected from 580 index cases and from both parents of each index case. The clinical details of the index cases are shown in table 1. DNA samples were also collected from 580 infants born consecutively at the John Radcliffe Hospital in Oxford during 1999–2000. All subjects included in the present study were white Europeans. Informed consent was obtained from the parents of all participants, and the study was approved by the East Anglian Multi-Centre Ethics Committee (27 May 1999).

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Table 1. Clinical details of index cases with severe respiratory-syncytial-virus bronchiolitis.

Characteristic	Data
Age, mean, weeks	16.4
Male	309 (53)
Length of hospital stay, mean (range), d	6.04 (1–34)
Oxygen therapy	
Cases	465 (80)
Length of therapy, mean (range), d[a]	4.4 (1–30)
Cases requiring gavage feeding or intravenous fluids	404 (70)
Cases ventilated	78 (13)
Preterm (gestation, <37 weeks) cases	116 (20)
Cases with preexisting heart or lung disease	58 (10)

NOTE. Data are no. (%), unless otherwise indicated.

[a] Used as an objective measure of disease severity.

Mouth swabs were used to collect samples from the patients and their parents; for the control subjects, cord-blood samples were used. DNA from the samples was extracted, preamplified, and stored as described elsewhere [11].

The Sequenom system was used to type single-nucleotide-polymorphism (SNP) variants. This system uses allele-specific primer extension followed by size detection by means of time-of-flight mass spectrometry. Successful Sequenom primers were developed for the following known CCR5 promoter SNPs: CCR5 −1835, CCR5 −2459, CCR5 −2554, and CCR5 −2733. The Δ32 variant was typed by size separation via polymerase chain reaction. These SNPs allow all of the common European CCR5 haplotypes identified by Gonzalez et al. [12] to be resolved. These SNPs were typed in the DNA samples from the 580 index cases and from the 580 control subjects. Markers showing a positive association in the case-control study were also typed in the parents of the index cases.

Haplotypes were constructed on the basis of the family data and data from unrelated individuals, by the computer program PHASE, version 2 [13]. Comparisons between cases and control subjects were made with simple 2×2 tables with calculated odds ratios (ORs). Subgroup analysis was performed by taking into account markers of severity (oxygen required for ⩾3 days) and known risk factors (prematurity and preexisting heart or lung disease). For the family analysis, the transmission/disequilibrium test (TDT) [14] was used, and only families for which there were no missing data were considered; the TDT considers the distortion, from the expected value of 50%, of transmission of a marker from parents to affected offspring.

Results. For all markers except −2773, for which 70% of the cases and control samples gave a reliable result, genotypes were successfully identified in >95% of samples. In the control samples, none of the allele distributions (including those for −2773) were different from those expected on the basis of Hardy-Weinberg calculations. Both the −2554T allele and the −2459G allele were found more frequently in cases than in control subjects (OR, 1.25 [$P = .01$ and 1.22 [$P = .02$], respectively) (table 2). The effect was slightly stronger when subjects homozygous for each of these alleles were compared, both in cases and in control subjects (both subgroups had an OR of 1.45 [$P = .04$ and $P = .01$, respectively]). None of the other markers showed significant frequency differences between the cases and the control subjects. In the subgroup analysis, the observed frequency differences between the −2554 variant and the −2459 variant were most marked in infants who had particularly severe disease (oxygen required for ⩾3 days) but who did not have the preexisting risk factors of prematurity or preexisting heart or lung disease ($n = 213$; both markers had an OR of 1.39 [$P = .004$ and $P = .005$, respectively]); the effect was also slightly stronger in homozygous individuals in this subgroup (OR for −2554, 1.63 [$P = .03$]; OR for −2459, 1.78 [$P = .002$]).

Six common haplotypes were identified. The −2554 variant and the −2459 variant are in complete linkage disequilibrium and occur together on the most common haplotype. This haplotype was found more commonly in cases than in control subjects (OR, 1.2 [$P = .03$]). The frequency of the other haplotypes did not differ between cases and control subjects. The −2459 SNP was typed in 518 complete (i.e., case-parents triad) families; the TDT showed significant transmission distortion ($\chi = 5.56$; $P = .018$; transmission frequency, 55%), with the −2459G allele being transmitted to affected offspring more frequently than expected.

Discussion. CCR5 is a key receptor for RANTES and MIP-1α. These chemokines have been found at elevated levels in respiratory secretions taken from infants with RSV bronchiolitis. It is unclear whether these elevated levels are a consequence or a cause of severe disease. The results of the present study show that a variant of the principle receptor of these chemokines, CCR5, is associated with severe RSV disease, suggesting an important pathophysiologic role for these chemokines.

We performed our initial study by using a case-control design. This design is more powerful than that of family-based

Table 2. Frequencies of CCR5 variants in 580 cases and in 580 control subjects.

CCR5 variant	Frequency		Odds ratio *(P)*
	Cases	Control subjects	
Δ32	0.12	0.11	1.02 (.87)
−2733	0.14	0.16	0.84 (.22)
−2554	0.38	0.33	1.25 (.01)
−2459	0.48	0.43	1.21 (.02)
−1835	0.08	0.09	0.88 (.41)

NOTE. For the −2733 variant, genotyping was successful in 70% of cases and control subjects; for all other variants, genotyping was successful in >95% of samples.

studies, allowing significant effects to be detected with a smaller sample size. For example, in the present study, in which all markers had frequencies >9%, our sample size was sufficient to detect ORs of 1.5, with a power of 85% at a significance of $P \geqslant .05$. The major disadvantage that case-control designs have with regard to genetic association is the possibility that ethnic variation in the samples will bias the results. We have confirmed the positive associations found in the case-control study, by using the TDT to analyze case-parents triads. Although this approach cannot be considered as offering an independent confirmation of the result, since the index cases were the same in both analyses, it does indicate that the observed result is not the effect of artifactual population admixture.

The case-control analysis shows that both the −2554T allele and the −2459G allele were found more commonly in cases than in control subjects and that the effect seemed strongest in subjects who were homozygous for these alleles. The other variants, including the CCR5 Δ32 null variant, were not associated with severe disease. The subgroup analysis showed that infants with more-severe disease but without underlying risk factors had the highest frequency of these CCR5 alleles, in keeping with an independent genetic effect. The haplotype analysis identified 6 common CCR5 haplotypes at frequencies that have been reported elsewhere [12]. This haplotypic pattern would account for the observation that both the −2554 variant and the −2459 variant are associated with disease, since these 2 markers are in complete linkage disequilibrium. The only significant haplotype association that we identified was for the haplotype bearing both the −2554T allele and the −2459G allele. This finding was confirmed to be independent of ethnic artifacts by TDT analysis of the −2459 polymorphisms in >500 nuclear families.

The power of association studies lies in the identification of molecules important in the pathogenesis of the disease being studied. If functional genetic variants of a protein can be shown to confer either susceptibility or protection, then that protein must be involved in the process that leads to clinical disease. The size of the effect detected will be a combination of both the relative importance of the protein in the disease process and the degree to which the genetic variant alters the function of the protein. Most association studies choose relatively common genetic variants, typically present in the population at frequencies >10%, to allow sufficient statistical power. It is likely that many of these variants will have relatively subtle effects on gene function, such that, although they might increase susceptibility to one disease, they will confer protection to another. This type of balancing selection is necessary for the variants to be maintained within the population. Thus, although the apparent increases in risk of severe disease that were detected by this study were modest, with ORs of 1.25 for allelic comparisons and 1.45 for homozygotes, they nonetheless suggest that CCR5 and its ligands play an important role in the pathogenesis of severe RSV disease.

Genetic-association studies also address the question of why particular individuals are affected by a given condition. The degree to which the increased risks conferred by CCR5 gene variants contribute to the overall likelihood that an individual will be affected by severe RSV disease is difficult to assess. The disease-associated CCR5 variants are commonly found in the general population (at frequencies of 0.38 and 0.48 for the −2554 variant and the −2459 variant, respectively), which means that, at the population level, the small increases in risk that are associated with these markers can have a significant impact. Some studies (e.g., see [15]) have attempted to calculate population-attributable fractions for genetic risk, but their data must be interpreted with caution, since the sum of all susceptibility factors used in this way can be >100%. The size of the effect found in the present study is similar to those of effects found in other genetic-association studies of severe RSV disease. Elsewhere, we have reported an association with an interleukin (IL)–8 gene variant, with an OR of 2.1 [16], and others have reported an association with the IL-4 −590T allele, with ORs of 1.4–1.6 [17, 18]. It will be important to determine whether these genetic effects are independent of each other and whether a combination of genetic markers may indicate a disease risk increased to a level that would be clinically useful.

We speculate that the disease association that we have observed in the present study is due to the functional effects that the variants have on CCR5 transcriptional activity. Despite the interest in CCR5 gene variants, relatively little is known about the possible functional consequences of the SNPs in the promoter region. One study, using fluorescence-activated cell-sorter analysis, has reported increased expression of CCR5 on $CD3^+$ T cells from Chinese individuals homozygous for the −2459G allele [19]; another study, using unstimulated Jurkat T cells in vitro, has reported decreased transcriptional activity of a reporter-gene construct carrying the −2459G allele, compared with that of a reporter-gene construct carrying the −2459A allele [10]. There are no available data on the possible function of the −2554 variant.

The results of the present study suggest that the −2459G allele is associated with more-severe disease. Severe bronchiolitis is characterized by intense inflammation of the airway, and one might expect that a CCR5 variant that leads to increased numbers of surface receptors would be associated with more-severe disease. The effect of the variant may depend on the cell type, and this may partially explain the apparently contradictory functional studies discussed above. In addition, the haplotypic background may be important, and this is likely to be different in ethnically diverse individuals. The lack of an effect due to the CCR5 Δ32 null variant suggests that decreased expression does not influence disease severity and increases the likelihood

that the promoter variants result in increased CCR5 expression, at least in some cell types. Alternatively, the observed effects may be due to functional variants at more-distant genetic sites that are in strong linkage disequilibrium with −2459.

Although the present study has, to our knowledge, used the largest population available for analysis of susceptibility to RSV bronchiolitis, it is important to recognize that much larger studies will be needed to authenticate disease associations of the sort described here, where CCR5 variants appear to exert a significant but modest effect on susceptibility to disease. The possible explanations for this association can be resolved only by a combination of fine mapping (by use of more-extensive haplotypes) of the genetic association and further functional studies of the consequences of the existing CCR5 promoter variants.

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