Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families

J Hull, A Thomson, D Kwiatkowski

Abstract

***Background*—Respiratory syncytial virus (RSV) infects nearly all children by the end of their second winter. Why some develop bronchiolitis is poorly understood; it is not known whether there is a genetic component. The pathological features include neutrophil infiltration and high levels of interleukin 8 (IL-8), a potent neutrophil chemoattractant.**

***Methods*—Common genetic variants of the promoter region of the *IL-8* gene were identified by sequencing DNA from 36 healthy individuals. Genetic correlates of IL-8 production were assessed using whole blood from 50 healthy subjects. To investigate genetic correlates of disease severity 117 nuclear families were recruited in which a child had required hospital admission for RSV bronchiolitis.**

***Results*—A common single nucleotide polymorphism (allele frequency 0.44) was identified 251 bp upstream of the *IL-8* transcription start site. The IL8–251A allele tended to be associated with increased IL-8 production by lipopolysaccharide stimulated whole blood (p=0.07). Using the transmission disequilibrium test, the frequency of this allele was significantly increased in infants with bronchiolitis (transmission = 62% (95% confidence interval (CI) 53 to 71), p=0.014) and particularly in those without known risk factors (transmission = 78% (95% CI 62 to 93), p=0.004).**

***Conclusion*—Disease severity following RSV infection appears to be determined by a genetic factor close to the *IL-8* gene. Further analysis of this effect may elucidate causal processes in the pathogenesis of RSV bronchiolitis.**

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Keywords: respiratory syncytial virus bronchiolitis; interleukin 8 gene; genetics

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Acute bronchiolitis is one of the most common causes of hospital admission in infancy in the UK.[1] It is a viral respiratory infection that occurs in winter epidemics, causing breathlessness and occasionally fatal respiratory failure. In at least 70% of cases it is caused by respiratory syncytial virus (RSV) which is widespread in the community, infecting nearly all children by the end of their second winter.[2]

It is not understood why a few RSV infected children develop breathing difficulties that require hospital admission while most have a relatively minor illness.[3 4] Epidemiological studies have identified a number of risk factors—notably, prematurity, having older siblings, and pre-existing lung or heart disease—but these do not provide a full explanation. An episode of bronchiolitis predisposes to further wheezing episodes in the first three years of life[5] but the relationship of bronchiolitis to atopy remains open to debate.[6–8] However, it is clear from murine models and from the disastrous consequences of a vaccine strategy adopted in the 1960s that certain immune responses to the virus may be deleterious to the host.[9 10]

The present investigation was prompted by the observation that neutrophils are the dominant cell type in bronchoalveolar lavage fluid from infants with RSV bronchiolitis.[11] The most potent known neutrophil chemoattractant is interleukin 8 (IL-8) and it has been shown experimentally that RSV infected airway epithelial cells secrete high levels of IL-8 as well as other pro-inflammatory cytokines,[12 13] while clinical studies have identified high levels of IL-8 in plasma and nasal secretions of infants with RSV bronchiolitis.[14 15]

These observation raise the question of whether IL-8 is an important factor in the pathogenesis of RSV bronchiolitis, or merely an epiphenomenon of the disease process. One way of resolving this issue is to determine whether the clinical outcome of RSV infection is determined by genetic factors that modulate IL-8 production or function. Here we identify a common variant of the *IL-8* gene promoter region and investigate its association with IL-8 production and susceptibility to RSV bronchiolitis. To exclude population artefacts we have used the transmission disequilibrium test to analyse disease association within families where a child had been admitted to hospital with proven RSV bronchiolitis.

Methods

RECRUITMENT OF AFFECTED FAMILIES

We compiled a database of all infants admitted to the John Radcliffe Hospital, Oxford with a clinical diagnosis of bronchiolitis together with positive immunofluorescence or culture for RSV during the period 1992–9. All cases and their parents were invited by letter to take part in the study; 254 out of 651 families agreed and were sent mouth swab kits for DNA sampling. Out of 140 kits returned, 15 lacked an adequate sample from one family member and these families were excluded from the analysis.

Table 1 Clinical details of infants included in the study

Total number of infants	117
Mean (range) age (months)	4.8 (0.25–18)
Number of boys	59 (50%)
Required oxygen	75 (64%)
Required oxygen >2 days	45 (38%)
Ventilated for this illness	10 (8%)
Mean (range) length of stay (days)	4.4 (1–16)

Table 2 Numbers of infants with identified pre-existing risk factors for bronchiolitis

Total number of infants	117
Born ≤36 weeks gestation	10 (8%)
Any siblings	86 (73%)
Pre-existing cardiac disease*	5 (4%)
Pre-existing lung disease†	2 (2%)

*Two large ventricular septal defects, one Fallot plus double outlet right ventricle, one Fallot with Blalock-Taussig shunt, one atrioventricular septal defect.
†Both chronic lung disease of prematurity.

The medical records of the remaining 125 infants were inspected. For this study, bronchiolitis was defined by the presence of tachypnoea, retractions and inspiratory crackles on auscultation; wheeze alone was not accepted. Using these criteria, a further eight infants were excluded. Duration of oxygen therapy (given if oxygen saturation, measured by pulse oximetry, fell below 92%) or requirement for mechanical ventilation were used as markers of severity. The number of siblings, gestation, and presence of pre-existing heart or lung disease were also recorded. The clinical details of the study group are shown in table 1 and the number of infants with identified risk factors is shown in table 2. To validate estimates of relative risk we also analysed cord blood samples from 180 healthy babies born at the John Radcliffe Hospital. The study was approved by the Central Oxford research ethics committee.

DNA COLLECTION

DNA was collected by mouth swab. Briefly, cotton wool buds were rubbed vigorously inside the cheek and placed in buffer (10 mM EDTA, 10 mM Tris and 0.5% sarkosyl) at 4°C for one week. After centrifugation the supernatant was incubated overnight at 37°C with proteinase K, guanidine hydrochloride, and ammonium acetate. DNA was extracted with chloroform, precipitated with ethanol, and quantified using a PicoGreen spectrofluoroscopic assay (Molecular Probes, Leiden, The Netherlands). Primer extension preamplification (PEP) of DNA was performed by polymerase chain reaction (PCR) with N15 oligonucleotides according to the manufacturer's instructions (GenPak, Brighton, UK).

IDENTIFICATION AND GENOTYPING OF *IL-8* PROMOTER VARIANTS

The 5' flanking region of *IL-8* extending to –1480 nt was analysed by dye primer cycle sequencing in 36 healthy white blood donors using an ABI 377 automated sequencer (PE Applied Biosystems, Warrington, UK). This identified a single nucleotide polymorphism at –251 nt relative to the transcription start site which was typed in families with bronchiolitis by the amplification refractory mutation system[16] using positive and negative controls that were verified by sequencing. Allele specific primers were CCACAATTTGGTGAATTATCAAT (–251A) or CCACAATTTGGTGAATTATCAAA (–251T). The consensus primer was TGCCCCTTCACTCTGTTAAC, giving a PCR product of 336 bp. In each reaction a second set of primers for exon 3 of the HLA-DRB1 gene (forward TGCCAAGTGGAGCACCCAA, reverse GCATCTTGCTCTGTGCAGAT, product size 796 bp) was used as a control for PCR efficiency. Reactions were carried out using Biotaq enzyme and buffer (Bioline UK Ltd) with 1.6 mM magnesium under the following conditions: 96°C for 60 s; four cycles of 96°C for 35 s, 68°C for 45 s, 72°C for 35 s; 20 cycles of 96°C for 25 s, 61°C for 50 s, 72°C for 40 s; five cycles of 96°C for 35 s, 58°C for 60 s, 72°C for 90 s.

WHOLE BLOOD ASSAY FOR IL-8 PRODUCTION

Blood samples were collected from 50 healthy donors attending the National Blood Transfusion Centre at the John Radcliffe Hospital. A portion of the sample was collected into sterile tubes with heparin 20 IU/ml, diluted with an equal volume of RPMI 1640, and incubated with or without 10 μg/ml lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Sigma, Poole, UK) in 5% carbon dioxide at 37°C.[17] Supernatants were harvested after four, eight, 12, and 24 hours and stored at –70°C. The concentration of IL-8 was measured by enzyme linked immunosorbent assay (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The concentration of IL-8 was corrected for the total white cell count, as measured in the original blood sample by the routine haematology service. A portion of the original sample was retained for DNA extraction (Nucleon extraction kit, Nucleon Biosciences, Scotland, UK) and *IL-8* genotyping as described above.

STATISTICAL ANALYSES

Since IL-8 concentrations were found to be normally distributed after log transformation, comparisons between groups used two tailed *t* tests on log transformed data. Test for trend across genotypes was carried out according to the method of Cuzick.[18] Association of the IL8–251A allele with disease within families was analysed by the transmission disequilibrium test (TDT).[19] This test analyses whether the transmission of an allele from parents who are heterozygous at the marker tested to the probands deviates significantly from the expected value of 50%.

Results

IDENTIFICATION OF A COMMON *IL-8* PROMOTER POLYMORPHISM

Genomic DNA from 36 individuals was sequenced in the 5' flanking region of *IL-8*. A single nucleotide polymorphism was identified at –251 nt relative to the transcription start site, with the nucleotide A in 31/72 and nucleotide T in 41/72 chromosomes; 58% of the individuals studied were AT heterozygotes. No

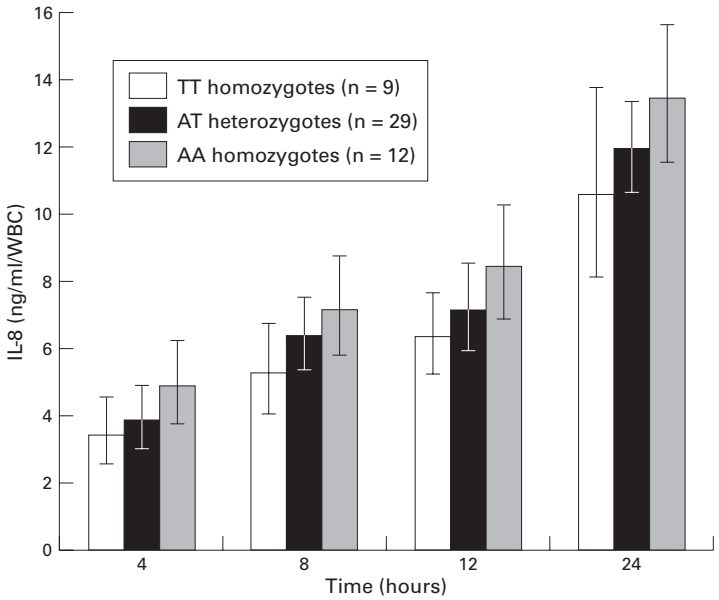

Figure 1 IL-8 production against time for each –251 genotype. The IL-8 concentrations, corrected for total white cell count, are geometric means; the error bars represent 95% confidence intervals.

other polymorphisms were identified within 1.4 kb 5' of the transcription start site.

RELATIONSHIP OF GENOTYPE TO IL-8 PRODUCTION IN VITRO

To explore functional associations with the IL8–251 polymorphism we measured the IL-8 response to LPS in whole blood from 50 healthy blood donors. To correct for variation in leucocyte numbers, since IL-8 can be produced by neutrophils, lymphocytes and monocytes, we divided the total IL-8 concentration by the total leucocyte count. Genotyping of the IL8–251 polymorphism revealed that 12 individuals were AA, nine were TT, and 29 were AT. As shown in fig 1, IL-8 production tended to be highest for the AA group and least for the TT group at each time point, with the AT group producing an intermediate amount, although there was considerable overlap. At 24 hours Cuzick's non-parametric test for trend gave p=0.07. Unstimulated samples produced low levels of IL-8 (geometric mean 0.41 ng/ml/WBC at 24 hours) which did not differ between the groups.

Table 3 Influence of disease severity on likelihood of transmission of the –251A allele

Marker of severity	*n*	*T*	*NT*	*%T (95% CI)*	*p value*
All infants	83	69	43	62 (53 to 71)	0.014
Infants needing oxygen	53	45	24	65 (54 to 76)	0.011
Infants needing >2 days oxygen	35	32	13	71 (58 to 84)	0.005
Infants needing mechanical ventilation	7	7	3	70 (42 to 98)	0.206

n = number of informative probands; T = transmitted; NT = non-transmitted; %T = percentage of –215A alleles transmitted. The p values, based on Pearson's test statistic, reflect the significance of the deviation of the % transmission from the expected 50%.

Table 4 Influence of known risk factors on likelihood of transmission of the IL8–251A allele

Risk factor	*n*	*T*	*NT*	*%T (95% CI)*	*p value*
All infants	83	69	43	62 (53 to 71)	0.014
Infants with no siblings	26	26	10	72 (58 to 87)	0.008
Infants with no siblings and excluding those with prematurity	20	22	7	76 (60 to 91)	0.005
Infants with no siblings and excluding those with prematurity and those with cardiac disease	18	21	6	78 (62 to 93)	0.004

n = number of informative probands; T = transmitted; NT = non-transmitted; %T = percentage of –215A alleles transmitted.
The p values, based on Pearson's test statistic, reflect the significance of the deviation of the % transmission from the expected 50%.

TRANSMISSION OF THE IL8–251A ALLELE TO INFANTS WITH BRONCHIOLITIS

Out of 117 families with confirmed RSV bronchiolitis, 83 were informative for TDT analysis, having at least one parent who was heterozygous for the IL8–251 polymorphism. In 54 families one parent was heterozygous and in 29 families both parents were heterozygous. Table 3 compares the number of occasions on which the IL8–251A allele was transmitted or was not transmitted from a heterozygous parent to the affected offspring. p values are based on Pearson's test statistic and reflect the frequency of transmission of the IL8–251 allele compared with its expected frequency of 50% under neutral conditions. Considering all informative families, the proportion of occasions where the IL8–251A allele was transmitted to affected infants was 62% (95% CI 53 to 71), significantly more frequently than expected (p=0.014).

The relationship with disease severity was analysed in greater detail. Of the 83 infants in families that were informative for the IL8–251 polymorphism, 53 had required oxygen therapy, 35 had an oxygen requirement lasting more than two days, and seven had needed mechanical ventilation. As shown in table 3, the percentage transmission of the IL8–251A allele rose to 65% (95% CI 54 to 76) in families in which the affected infant required oxygen, to 71% (95% CI 58 to 84) in families in which the affected infant required oxygen for more than two days, and to 70% (95% CI 42 to 98) in families in which the affected infant required mechanical ventilation.

The effect of known risk factors for RSV bronchiolitis was also analysed. These included older siblings (thought to increase exposure to the virus), prematurity, and underlying heart or lung disease. As shown in table 4, when families with older siblings were excluded the proportion in which the IL8–251A allele was transmitted was greater (transmission = 72% (95% CI 58 to 87)). When families with all three risk factors were excluded (older siblings, prematurity, and congenital cardiac disease), the percentage transmission increased further to 78% (95% CI 62 to 93).

We sought to estimate the effect of the IL8–251A allele on risk of bronchiolitis within the general population. Family studies do not address this issue directly, but it has been argued that the frequency of the allele that is *not* transmitted to the affected child provides a good reflection of frequency distributions within the population as a whole. To test this prediction we genotyped 180 cord blood samples from babies born in the Oxford region. An identical IL8–251A allele frequency of 0.44 was observed in the cord blood samples (159/360 chromosomes) and in the non-transmitted alleles of bronchiolitis families (104/234 chromosomes). The proportion of bronchiolitis cases who carried the IL8–251A

allele—that is, AA homozygotes plus AT heterozygotes—was 83% (97/117) compared with 69% (124/180) for the cord blood samples. Therefore, with reference to the cord blood samples, the odds ratio associated with carriage of the IL8–251A allele was 2.2 (95% CI 1.2 to 4.1).

Discussion

These findings provide the first evidence of a genetic susceptibility determinant for RSV bronchiolitis. We found that over half of the UK population is heterozygous for a single nucleotide polymorphism located –251 nt relative to the *IL-8* transcriptional start site. Our data show a trend for increased IL-8 production in association with the IL8–251A allele when whole blood is stimulated in vitro with LPS. Analysis of cases of RSV bronchiolitis and their parents showed that the IL8–251A allele is significantly associated with disease severity. The effect is most marked for severe disease requiring oxygen therapy for more than two days, and for cases of bronchiolitis with no other known risk factors.

Although there are many examples of genetic factors that appear to predispose to infectious disease, these are mostly derived from population based association studies that compare allele frequencies in cases and controls. The problem with this approach is that spurious associations may result from differences in the ethnic composition of case and control groups.[20] Such differences may be subtle and are difficult to exclude with confidence. This has led to a growing interest in statistical approaches such as the TDT that allow genetic associations with disease to be estimated in families, thereby ruling out the possibility of ethnic artefacts. The main prerequisite is to obtain DNA samples from affected individuals and their parents, and this approach is therefore particularly suited to the analysis of childhood diseases such as bronchiolitis. Our findings confirm that the TDT approach is capable of detecting genetic susceptibility factors for infectious disease, and indicate that in this case the genetic effect is strongest in individuals with no other risk factors for RSV bronchiolitis. This result is interesting since population based genetic studies are most prone to artefacts when there are strong environmental determinants of disease susceptibility, whereas family studies may allow gene-environment interactions to be analysed with greater confidence.

A limitation of family based analysis is that it does not provide a direct measure of relative risk for disease within the population as a whole. It has been proposed that this may be overcome by using the alleles that are *not* transmitted from heterozygous parents to affected offspring to estimate allele frequencies within the general population. Here we have tested the validity of this approach by studying healthy cord blood samples from the same population, and we found the frequency of the IL8–251A allele to be identical in cord blood samples and in the non-transmitted alleles of the family study. These data indicate that carriers of the IL8–251A allele have a twofold increased risk of developing severe bronchiolitis and, since they represent over two thirds of the UK population, this suggests that the IL8–251A allele may play a role in determining disease severity in the majority of infants with the disease.

In investigating disease associations with candidate gene regions, there is a strong argument for focusing on polymorphisms of functional significance.[21] Here we began by investigating genetic variation of the *IL-8* promoter region as this might alter transcriptional regulation of the *IL-8* gene. Our experimental data suggest that the IL8–251A allele is associated with increased IL-8 production, and thus our genetic findings might imply that high IL-8 production is a risk factor for developing bronchiolitis. To confirm this interpretation two issues need to be resolved by further investigation. Firstly, how does this polymorphism relate to IL-8 production within the RSV infected lung? This may be regulated differently from the LPS stimulated blood model that we have analysed here, but is less accessible to experimental investigation. Secondly, is the association with bronchiolitis due to the IL8–251A allele itself or is it simply a marker for a neighbouring functional polymorphism? To address this question we are currently defining other polymorphisms at the *IL-8* locus and recruiting a larger number of bronchiolitis families to allow multiple genetic markers to be analysed in detail. In summary, while the present data provide evidence that the *IL-8* gene region contains genetic determinants that influence susceptibility to bronchiolitis, the underlying mechanisms have yet to be determined.

There is growing expectation that molecular genetic epidemiology will enlarge our understanding of causal processes in complex diseases.[20] In the case of bronchiolitis, experimental studies in mice have revealed a variety of immunological mechanisms by which RSV can potentially damage the airways.[22] For example, an exuberant Th2 immune response to viral infection could lead to eosinophil mediated inflammation of the airways,[9] and this model is attractive because it provides a possible link with the persistent wheezing illness that is commonly seen after an episode of RSV bronchiolitis. An alternative model incriminates the neutrophil as a major cause of bronchiolar inflammation. This is supported by the high proportion of neutrophils found in the bronchial lavage fluid of infants with RSV infection[11] and the high IL-8 levels in plasma and respiratory secretions of infants with RSV infection.[14 15] However, these latter observations are correlative and do not address the question of whether neutrophil influx is causative or merely an epiphenomenon of the disease process. Our current findings indicate that further examination of the *IL-8* locus may help to elucidate causal mechanisms in the pathogenesis of RSV infection, particularly in relation to the neutrophil hypothesis, and there are strong grounds for establishing a larger collection of bronchiolitis families to examine this issue in

greater detail and to investigate other candidate gene loci.

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