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 IL-8–251A 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.

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

Acute bronchiolitis is one of the most common causes of hospital admission in infancy in the UK. 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.

It is not understood why a few RSV infected children develop breathing difficulties that require hospital admission while most have a relatively minor illness. 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 but the relationship of bronchiolitis to atopy remains open to debate.

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.

The present investigation was prompted by the observation that neutrophils are the dominant cell type in bronchoalveolar lavage fluid from infants with RSV bronchiolitis. 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, while clinical studies have identified high levels of IL-8 in plasma and nasal secretions of infants with RSV bronchiolitis.

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.
Hull, Thomson, Kwiatkowski

Pre-existing cardiac disease* 5 (4%)

atrioventricular septal defect.

outlet right ventricle, one Fallot with Blalock-Taussig shunt, one

*Two large ventricular septal defects, one Fallot plus double

Any siblings 86 (73%)

The 5' flanking region of

PROMOTER VARIANTS

IDENTIFICATION AND GENOTYPING OF

IL-8

ER (10 mM

DNA COLLECTION

er's instructions (GenPak, Brighton, UK).

oligonucleotides according to the manufactur-

polymerase chain reaction (PCR) with N15

Netherlands). Primer extension preamplifica-

tonic assay (Molecular Probes, Leiden, The

quantified using a PicoGreen spectrofluoro-

chloroform, precipitated with ethanol, and

proteinase K, guanidine hydrochloride, and am-

ant was incubated overnight at 37

for one week. After centrifugation the superna-

primer was TGCCCCTCCTCGTT-

taAC, giving a PCR product of 336 bp. In each

reaction a second set of primers for exon

3 of the HLA-DRB1 gene (forward

TGCCAAGTGAGGACCCCAA, 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 Transfu-

sion 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."

Supematants 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 extrac-

tion (Nucleon extraction kit, Nucleon Bio-

sciences, 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. Association of the

IL8–251A allele with disease within families

was analysed by the transmission disequilib-

rium test (TDT). This test analyses whether

the transmission of an allele from parents who

are heterozygous at the marker tested to the

proband deviates significantly from the ex-

pected 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 nucle-

otide T in 41/72 chromosomes; 58% of the

individuals studied were AT heterozygotes. No
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 Custick’s non-parametric test for trend gave p=0.07. Unstimulated samples produced low levels of IL-8 (mean geometric 0.41 ng/ml/WBC at 24 hours) which did not differ between the groups.

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 was estimated as 62% (95% CI 53 to 71), significantly more frequently than expected (p=0.014).
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. 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 a number of bronchiolitis 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. 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 nearby 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. In the case of bronchiolitis, experimental studies in mice have revealed a variety of immunological mechanisms by which RSV can potentially damage the airways. For example, an exuberant Th2 immune response to viral infection could lead to eosinophil mediated inflammation of the airways, 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 bronchial inflammation. This is supported by the high proportion of neutrophils found in the bronchial lavage fluid of infants with RSV infection and the high IL-8 levels in plasma and respiratory secretions of infants with RSV infection. 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.

The authors would like to thank Dr Mike Bunch for advice in setting up ARMS, Dr Nick Day for the cord blood samples, and Dr Mike Murphy and his staff at the National Blood Service. This study was funded by the Medical Research Council and Action Research.