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Toll-Like Receptor 4 Asp299Gly Polymorphism in Respiratory Syncytial Virus Epidemics

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Summary. The respiratory syncytial virus (RSV) antigen serves as ligand for Toll-like receptor (TLR) 4 that is a transmembrane signaling receptor in macrophages and dendritic cells. According to current evidence single nucleotide polymorphism involving amino acid 299 influences the susceptibility to severe RSV infections. The Asp299Gly allele has been shown to influence the TLR4-mediated signaling causing conformational change in the extracellular domain that recognizes pathogen-associated molecular patterns. The aim was to study the association between the *TLR4* Asp299Gly polymorphism and the susceptibility to severe RSV bronchiolitis in infants. Altogether 312 cases and 356 controls, selected on the basis place of residence, date of birth, gender, and gestation at birth, were studied. When adjusted for multiple dependent variables, no allele or genotype frequency difference was found between the cases and the controls. Post hoc analysis revealed that during the year 2000 epidemics, the Gly299Gly genotype associated with protection against severe RSV and during 2004 epidemics Gly299Gly genotype and 299Gly allele associated with severe RSV. To conclude, we could not confirm the association of the Gly299 allele with severe RSV. This is consistent with the evidence that the susceptibility to severe RSV infection is principally dependent on environmental and constitutional factors. We propose that the risk of severe RSV infection may additionally depend on the interaction between individual *TLR4* genotype and the particular RSV group causing bronchiolitis. **Pediatr Pulmonol. 2010; 45:687–692.** © 2010 Wiley-Liss, Inc.

Key words: respiratory syncytial virus; bronchiolitis in infants; innate immunity.

INTRODUCTION

Respiratory syncytial virus (RSV) is the main cause of severe bronchiolitis requiring intensive care in infants. Virtually all children are infected by RSV before the age of 3 years with clinical manifestations ranging from subclinical infection to death. Crowding, day care, and constitutional factors increase the risk of severe disease.[1] Prophylactic use of specific monoclonal antibodies decreases the severity of RSV infection among the high-risk patients.[2]

Genetic factors are likely to influence the severity of RSV infection. It has been shown that specific alleles of genes encoding surfactant protein A (SP-A) and SP-D, which are involved in innate immunity, associate with severe RSV bronchiolitis.[3,4] These results were recently confirmed.[5] SP-A and SP-D bind RSV and prevent severe pulmonary infection. According present evidence both SP-A and SP-D interact with Toll-like receptor (TLR) 4.[6]

The TLR-family of transmembrane receptors initiates signaling of innate host responses and may link innate and adaptive immunity.[7] Each TLR receptor recognizes some pathogen-associated molecular patterns.[8] A variety of components serve as ligands, including lipid–protein complexes from bacterial membranes, DNA, and proteins from fungi, viruses, and endogenous proteins. TLR4 is principally expressed in macrophages, dendritic cells, and in the other cell types. It serves as a transmembrane signaling receptor of lipopolysaccaride (LPS) from Gram-negative bacteria. TLR4 is also involved in an acute innate immune response to RSV. Previous studies have shown

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evidence that both TLR4 and CD14 are engaged in pattern recognition of RSV F glycoprotein[9] and that TLR4 expression is activated in RSV bronchiolitis.[10] TLR4-deficient mice suffer more prolonged, generalized infection due to RSV than the wild-type mice.[11] More recently, Tal et al.[12] reported evidence of an association of the minor 299Gly allele of *TLR4* with severe RSV infection in infants.

The *TLR4* gene is 19.0 kb long and located on chromosome 9q32–33.[13] Two polymorphic sites, Asp299Gly and Thr399Ile, are in very close linkage disequilibrium and they are involved in encoding of the extracellular protein domain.[14] The TLR4 encoded by the 299Gly allele has a blunted LPS response compared common Asp299 allele, influencing the conformational change in the extracellular domain that recognizes the ligand and promotes the TLR4-mediated LPS signaling.[14]

According to present hypothesis the susceptibility to severe RSV infection is dependent on environmental and genetic factors of the host. On the basis of the strong interaction between the genetic and environmental factors the genetic component influencing the risk of the respiratory disease may be undermined or concealed. The genetic heterogeneity of population representing different ethic groups is a major problem in studies of hereditary risk factors of multifactorial diseases. The aim of the present study was to investigate the association between the polymorphism of *TLR4*, the signaling receptor containing the pattern recognition domain of the RSV antigen, and the susceptibility to severe RSV infection during two major epidemics during 1999–2000 and 2003–2004 in a genetically homogenous population of Northern and Central Ostrobothnia, Finland. We were unable to confirm the association between the *TLR4* 299Gly allele-associated susceptibility to severe RSV infection, previously described in Jewish population.[12] However, on the basis of post hoc analysis of patients in the two epidemics, *TLR4* associated with severe RSV infection. We propose that the allelic variation in *TLR4* may widen the repertoire of host responses in different RSV epidemics.

SUBJECTS AND METHODS

The study subjects were recruited and the study was conducted at Oulu University Hospital, Oulu, Finland, and Seinäjoki Central Hospital, Seinäjoki, Finland. The ethical committees at both hospitals approved the protocol. Written informed consent was obtained from the parents of all study subjects. The infants with RSV bronchiolitis fulfilled the following eligibility criteria: (1) positive RSV antigen test from nasopharyngeal aspirate; (2) admission to a pediatric ward a with diagnosis RSV bronchiolitis; (3) requirement of supplemental oxygen and monitoring of blood gases and oxygen saturation; (4) age <1 year; (5) no other underlying diseases; (6) birth after the 35th week of gestation; and (7) no prophylaxis with monoclonal RSV antibodies.

One to two controls per case were selected from the birth records of the hospitals; they were healthy infants matched for gender, the date of birth, gestational age, and place of residence. The controls had no underlying diseases, prematurity (gestation <35 weeks), or prophylaxis with monoclonal RSV antibody. They did not have any major airway infections in their medical history other than a maximum of two otitis media episodes.

Clinical data were collected from all RSV and control patients. The data were collected by means of a written questionnaire that was filled in by the parents until 1 year of age. The data were supplemented from the hospital medical records. The data collected included infections, vaccinations, hospitalizations, pregnancy history, neonatal diseases, surgical history, medications, allergies (patient and family members), nutrition, parental information, type of day care, and family structure. Altogether 20% of the controls were excluded due to excessive airway infections.

Sample Collection and DNA Extraction

The RSV cases were identified and the samples were collected prospectively during 12/1999 to 5/2000. In addition, the RSV cases were retrospectively identified from the hospital records during the 2003–2004 epidemics. With the exception of the prospectively identified infants with severe RSV, the informed consent from the parents was obtained retrospectively when the age of the child was at least 1 year. Altogether 345 RSV and 383 control samples were selected for genotyping. Buccal smear samples were collected using sterile cotton swabs (Copan, Brescia, Italy), and DNA extraction was done as previously described using Chelex-100 (Bio-Rad Laboratories, Hercules, CA).[3] Whole blood samples (20% of all) were collected into plastic EDTA tubes (Vacuette®, Greiner, Kremsmunster, Austria) and extracted using the Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN).

Genotyping of TLR4

TLR4 genotyping was carried out using a PCR-cRFLP-based technique. Two PCR reactions were used to amplify the polymorphic site. The A896G single-nucleotide polymorphism studied is located at amino acid 299. The primers used for the first PCR reaction were forward:

ABBREVIATIONS

RSV	Respiratory syncytial virus
SP	Surfactant protein
TLR	Toll-like receptor
LPS	Lipopolysaccaride

cctgtgcaatttgaccattg and reverse: tcatggtaataacaccattgaagc. The second set of PCR primes consisted of forward: agactactacctcgatgatat(c*)att and reverse: aagcattcccacctttgttg. The conditions for the first PCR were: denaturation 95°C, anneal 60°C, elongation 72°C, each for 1 min. The conditions for the second PCR were: denaturation 95°C, anneal 55°C, elongation 72°C, each for 30 sec. The PCR product was digested for 5 hr at 37°C with the *Nco*I restriction enzyme. The final products were separated on 2% agar gel electrophoresis and photographed after staining with ethidium bromide. The genotype was deduced from the photographs.

Statistics

The results were analyzed using non-paired tests. Comparisons of allele frequencies were carried out using χ^2 analysis. The allele and genotype distributions of the RSV cases and controls were compared using $2 \times k$ tables. Individual allele and genotype frequencies were calculated using 2×2 tables. To calculate the odds ratios and 95% confidence intervals, the Woolf (logit) method was used. When the expected values were <5, Fischer's exact test was used. The Hardy–Weinberg equilibrium was tested for the observed genotype frequencies using χ^2 analysis. Other than the genotype data are expressed as percentages or as means ± SD. The statistical significance of the difference in the distribution of alleles during the two epidemics was tested by conditional logistic regression model standardized for confounding factors. The software used for the analysis of the data was Arcus Quickstat (Longman Software Publishing, Cheshire, UK) in the χ^2 2×2 and Fisher's exact tests. SPSS for Windows version 14.0 (SPSS, Inc., Chicago, IL) was used for basic statistical analysis and logistic regression analysis. The clinical and genetic data were organized using the Microsoft Excel (Microsoft Corporation, Seattle, WA) program.

RESULTS

Altogether 312 RSV and 356 control samples were successfully genotyped out of the 345 RSV and 383 control DNA samples obtained. There were no significant demographic differences between the cases and the controls, with the exception of the tendency towards a higher number of children in the family of the severe RSV cases ($P = 0.05$) and the rate of infections affecting the respiratory tract. The latter is likely due to the selection criteria applied to the controls (Table 1).

There were no detectable differences in the allele frequencies between the RSV and control groups (Table 2). Gender or place of birth had no influence on the allele distribution (data not shown). In a further attempt to analyze the trends in genetic susceptibility, the patient population was divided between the two major epidemics that took place during 1999/2000 and during the winter months of 2004 (Table 3). This post hoc analysis revealed differences between the RSV and control groups. In the 1999/2000 epidemic, the allele A tended to be overrepresented (92.1 vs. 87.0, $P = 0.05$) and the allele G underrepresented (7.9 vs. 13.0, $P = 0.05$) in the severe RSV group. The genotypes AA and AG revealed no difference, whereas the GG genotype was underrepresented in the RSV group (0.0 vs. 4.2, $P = 0.02$). In contrast, during the 2004 epidemics, the allele A was underrepresented in population with severe RSV compared to the control population (86.1 vs. 92.0, $P = 0.014$) and the allele G was overrepresented in the RSV population (13.9 vs. 8.0, $P = 0.014$). In addition, the GG genotype was overrepresented in the RSV population (4.0 vs. 0.0, $P = 0.009$).

TABLE 1— Clinical Characteristics of the Infants With Severe RSV Infection and the Control Infants

Characteristic	RSV, n = 312	Controls, n = 356
Gestation at birth, days ± SD	274 ± 18	278 ± 13
Birth weight, grams ± SD	3460 ± 686	3542 ± 566
Percentage boys	60.3	56.7
Percentage girls	39.7	43.3
Mean n of children in family ± SD	3.7 ± 2.7	2.8 ± 2.2
Children in family, (%)		
1–3	65.0	78.9
≥4	35.0	21.1
Smoking in family, (%)	n = 300	n = 353
Father	21.3	19.0
Mother	5.7	3.1
Both	14.0	11.9
No smokers	59.0	66.0
Percentage of otitis media, (%)		
No	24.3	57.5
1–3	32.0	42.5
≥4	43.7	0.0
Percentage of daycare during infancy		
Home	90.0	96.9
Daycare center	10.0	3.1

Conditional logistic regression analysis standardized for confounding factors further confirmed the difference in the distribution of alleles during the two epidemics. The Hardy–Weinberg testing on the whole genetic material revealed a normal distribution, whereas the analysis performed separately for the two epidemics revealed a non-significant trend towards uneven distribution of the genotypes.

DISCUSSION

We investigated the association between the *TLR4* gene polymorphism and severe RSV bronchiolitis in infants representing homogenous Finnish population. We hypothesized that certain alleles and/or genotypes

TABLE 2— Percentage Distribution of TLR4 Alleles and Genotypes in Severe RSV and Control Infants

TLR4	RSV (%), n = 312	Control (%), n = 356	OR	95% CI	*P*
Alleles					
A	89.3	89.7	0.95	0.7–1.3	0.773
G	10.7	10.3	1.05	0.7–1.5	0.773
Genotypes					
AA	80.4	81.5	0.94	0.6–1.4	0.740
AG	17.7	16.5	1.08	0.7–1.6	0.718
GG	1.9	2.0	0.98	0.3–2.9	0.968

influencing tightly linked amino acids 299 and 399 in extracellular pattern recognition domain of TLR4 would be associated with the risk of the disease. This was consistent with the role of TLR4 serving as an inflammatory signaling receptor for RSV F glycoprotein and the hyporesponsiveness of 299Gly–399Ile haplotype as a result of defect in ligand binding.[14] Contrary to the expectations the present results did not confirm the data supporting the association between the polymorphism and the susceptibility to RSV bronchiolitis. However, post hoc analysis of individual RSV epidemics yielded evidence on concealed genetic polymorphism that may influence the susceptibility.

There was no association between the TLR4 gene variants and severe RSV bronchiolitis in the patient population during two wintertime epidemics separated by 4 years. However, post hoc analysis of the individual epidemics separately revealed a significant trend as the genotype associating with the risk of severe disease was not constant. While during the last epidemic the GG genotype G allele encoding 299Gly were risk factors, consistent with the study of Tal et al.[12] in Jewish infants, the GG genotype protected against severe disease during the 1999/2000 epidemic (Table 3).

Previous studies demonstrate genetic variation in antigenic structure of RSV, producing the two RSV serotype groups (A and B).[15] Both groups show variation in the G glycoprotein. Group A viruses have shown more variability in G glycoprotein than the group B.[16] It has been suggested that the variants play a role in allowing RSV to elude immune responses. The variation in the antigenic structure of RSV could therefore be related to the lack of persistent immunity against RSV infection.[15] The dominating RSV subgroup (A/B) was shown to change in 2 years cycles in Finland during 1981–1990.[17] Data on RSV subgroups isolated between 1990 and 2004 have been reported from Sweden, showing diversity in the subgroup A genotypes with the dominating genotype of GA5.[18] In six outbreaks in Boston from 1981 to 1987 the distribution of RSV groups were evaluated: in three

TABLE 3— Percentage Distribution of TLR4 Alleles and Genotypes in Severe RSV and Control Infants During the Individual Epidemics

TLR4	RSV (%), n = 161	Control (%), n = 180	OR	95% CI	*P*
(A) 1999–2000 epidemic					
Alleles					
A*	92.1	87.2	1.76	1.0–3.1	0.050
G*	7.9	12.8	1.76	1.0–3.1	0.050
Genotypes					
AA	84.3	78.3	1.50	0.8–2.8	0.195
AG	15.7	17.8	0.9	0.5–1.6	0.675
GG*	0.0	3.9			0.020[1]
TLR4	RSV (%), n = 151	Controls (%), n = 176	OR	95% CI	*P*
(B) 2003–2004 epidemic					
Alleles					
A*	86.1	92.0	0.5	0.3–0.9	0.014
G*	13.9	8.0	0.5	0.3–0.9	0.014
Genotypes					
AA	76.2	84.1	0.6	0.3–1.04	0.072
AG	19.9	15.9	1.3	0.7–2.3	0.350
GG**	4.0	0.0			0.009[1]

*$P = < 0.05$.
**$P = < 0.01$.
[1]Fisher's exact test.

periods A viruses and in one period B viruses dominated whereas in two periods A and B viruses were isolated in equal proportion.[19] The genetic heterogeneity of the microbe can influence the innate immune responses, the generation of specific antibodies and the susceptibility to disease. As compared between group A and B, remarkable differences in antigens as well as the differences in nucleotides and amino acids are evident. At least 7 of the 10 proteins originating from the viral genome have revealed sequence differences among group A and group B viral genes and proteins. Most remarkable differences were evident in the transmembrane G protein. Concerning the F protein, of the coding nucleotides and the amino acids were variable 21% and 11%, respectively, indicating remarkable differences in nucleotide and protein sequences between group A and group B RSV.[15]

It is conceivable that the susceptibility to severe RSV bronchiolitis depends on heredity of the host, possibly on the proteins that recognize pathogen-associated molecular patterns. Indeed, as studied in human monocytes and in transfected bronchial epithelial cells, the first line defense against RSV is blunted due to the failure of translocation of the TLR4 receptor to the plasma membrane when only one (299Gly or 399Ile) of the two TLR4 variants was expressed.[20]

The present results show evidence of a variable association between severe RSV infection and *TLR4* genotype. Considering the stable and genetically homogeneous population throughout the study period of two major epidemics 4 years apart, we propose that instability of the minor allele as a significant risk factor of severe RSV was not due to a chance. Instead we propose that the antigenic structure of RSV and the variability of the genome of RSV may influence the ability of the individual *TLR4* allelic variants to initiate a particular innate immune response, eventually leading to the formation of the syncytium of epithelial and viral membranes in severe RSV.[21] Severe RSV bronchiolitis is associated with wheezing during recurrent respiratory tract infections. It is well accepted that besides the environmental and constitutional factors, the genotype of the host may influence the susceptibility infections and inflammatory airway disease.[22]

Two more recent studies have published on the association of the Asp299Gly polymorphism and the risk of RSV infection. According to Awomoyi et al.[23] the risk of symptomatic RSV in the population of mostly preterm infants was confined to heterozygous carriers of the Asp299Gly polymorphism (89.5% vs. 10.5% in controls). The DNA from RSV cases was extracted from archived nasal specimens and compared to historical controls.[23] The extremely high heterozygous Asp299Gly carrier rate among RSV-susceptible preterm infants has not been repeated. In the study of Paulus et al.[24] of 236 children with mild to severe RSV infection and 219 controls revealed that *TLR4* Asp299Gly polymorphism had no influence in the susceptibility. Both RSV serotypes A and B were evaluated in this mixed population of children ranging from 1 week to 18 years of age. In monocytes from the carriers of either *TLR4* Asp/Gly or *TLR4* Asp/Asp genotypes, LPS produced a similar induction of IL-1β, TNF-α, and IL-6.[24] The cells from respiratory epithelium were not studied. The results of the present study addressing two epidemics in a homogenous population of term born infants and controls adjusted for gestation, time of birth, and residence do not contradict with these reports.

Recently the association of TLR2 and TLR6 with activation of RSV virus replication was examined. Interactions of TLR2 with RSV promoted neutrophil migration and dendritic cell activation within the lung. These studies performed on knockout mice in vivo suggested that TLR2 directly interacts with RSV providing the recognition and promoting innate immune response and Th1 activation.[25] A preliminary exploration on polymorphism of seven TLRs (1, 2, 3, 5, 6, 9, and 10) revealed no strong candidate genes.[26] Thus, far few genes expressed in epithelial cells and closely interacting with luminal plasma membrane of the peripheral lung—TLRs, SP-A, and SP-D—have been identified as candidates influencing the risk of severe RSV bronchiolitis. The cell membranes fuse with proteins produced by the genome of this Paramyxovirus. The proximity of mammalian and viral proteins is consistent with the possibility that the genetic risk factors of the host and the pathogen interactively influence the virulence.

To summarize, further investigation is required to address the hypothesis that the genotypes affecting the extracellular ligand-binding TLR4 domain influence the function of the signaling receptor and the susceptibility to infections. Current findings on the Asp299Gly-related conformational change in the extracellular domain of TLR4[14] and the genotype-related translocation of TLR4[20] need to be extended to include functional studies using both different *TLR4* genotypes and different RSV strains. We propose that analysis of genetic and environmental risk factors on populations exposed to different RSV epidemics may have to be complemented by studies of RSV types in viral isolates from patients. This complicates the possibility of defining the individual genetic risk. However, genetic studies involving both the host and the pathogen may further our understanding on virulence, chronic consequences, and on effective prevention of RSV disease.

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