Molecular Evolution of Respiratory Syncytial Virus Fusion Gene, Canada, 2006–2010

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To assess molecular evolution of the respiratory syncytial virus (RSV) fusion gene, we analyzed RSV-positive specimens from 123 children in Canada who did or did not receive RSV immunoprophylaxis (palivizumab) during 2006–2010. Resistance-conferring mutations within the palivizumab binding site occurred in 8.7% of palivizumab recipients and none of the nonrecipients.

Human respiratory syncytial virus (RSV) is the most common cause of acute respiratory tract infections (RTIs) and a major cause of hospital admission and death among children <5 years of age worldwide (1). Risk for severe RSV-associated illness is highest among children born prematurely or with chronic medical disorders (2). Palivizumab immunoprophylaxis is the only available measure to prevent severe RSV disease.

The RSV fusion (RSV-F) surface glycoprotein mediates virus fusion to host cells. It is a major antigenic determinant that elicits neutralizing antibodies and cytotoxic T-lymphocyte immunity (3). Palivizumab (MedImmune, Gaithersburg, MD, USA) is a humanized mouse monoclonal antibody that inhibits RSV-F by binding to a defined epitope (residues 262–276) (4,5). Palivizumab immunoprophylaxis is recommended for the prevention of serious lower RTIs caused by RSV in children at high risk (6). RSV strains with mutations in key amino acid residues within the palivizumab binding site are resistant to this antibody (7–9); however, little is known about the prevalence of such mutations in clinical samples. Furthermore, despite its role in RSV pathogenesis, immunity, and prevention strategies, few data on RSV-F molecular evolution are available (10,11) because previous phylogenetic studies have focused on the RSV-G glycoprotein (12,13). Therefore, we monitored evolutionary changes in RSV-F, particularly potential resistance mutations in the palivizumab binding site, among strains from children who did and did not receive palivizumab.

The Study

This cohort study was approved by the Centre Hospitalier Universitaire de Québec Research Ethics Board. Participants were <3 years of age and either received medical attention at an outpatient pediatric clinic or were hospitalized at a pediatric center for acute RTI during 4 winter seasons (2006–2010), in Québec City, Québec, Canada.

Clinical data were prospectively collected at study entry and after 1-month follow-up. For all patients, at the first visit a nasopharyngeal aspirate was collected. The aspirate was frozen at −80°C until subsequent testing by a multiplex PCR/DNA hybridization assay that detects RSV genotype-A (RSV-A), RSV-B, and 22 other respiratory viruses (Infiniti RVP assay; Autogenomics, Carlsbad, CA, USA) (14).

RSV infection was identified in aspirates from 467 (63.6%) of 734 hospitalized children (257 RSV-A, 210 RSV-B) and from 147 (48.2%) of 305 outpatient children (85 RSV-A, 62 RSV-B). During 2006–2010, a total of 724 children received palivizumab in the Québec City region (L. Cliche, pers. comm.). RSV-positive samples from all 12 study participants receiving palivizumab and from 100 not receiving palivizumab underwent RSV-F sequencing.

Additionally, F-gene analysis was performed on 11 RSV-positive clinical samples from palivizumab recipients retrospectively identified by using neonatal clinic registries at McMaster Children’s Hospital (Hamilton, Ontario, Canada) and Montréal Children’s Hospital (Montréal, Québec, Canada) during 2009–2010. Clinical data were collected by chart review.

RNA was extracted directly from nasopharyngeal samples by using a QIAamp Viral RNA Mini Kit (QIAGEN, Mississauga, Ontario, Canada). Random primers (Amersham, Piscataway, NJ, USA) and Superscript II RT Kit (Invitrogen, Carlsbad, CA, USA) were used for reverse transcription. PCR amplification was performed with QuantiFast Probe PCR+ROX Vial Kit (QIAGEN); primers and thermocycling conditions are available from G.B. upon request. RSV-F amplicons were sequenced by using an automated sequencer (Applied Biosystems, Foster City, CA, USA).
Microneutralization assays were performed as described elsewhere, with minor modifications (9). RSV was incubated (for 2 h at 37°C) with serially diluted palivizumab, then cultured in Vero cells (for 5 d). RSV replication and 50% inhibitory concentrations (IC_{50}) were subsequently determined by F protein quantification by using ELISA. The mean ± SD IC_{50} of C0910–1006A, a N276S strain, was 0.33 ± 0.04 μg/mL, similar to that of RSV-A2 wild type (IC_{50} 0.46 ± 0.04 μg/mL) and therefore was considered susceptible. Position-272 variants did not grow in culture and were not tested.

**Conclusions**

We report the prevalence of resistance-conferring mutations in RSV-F among children receiving or not receiving palivizumab. Although infrequent (8.7% of infections in palivizumab recipients), residue 272 mutations were significantly associated with palivizumab exposure and not observed at all in nonexposed patients.

We identified 2 new clinical specimens with position 272 mutations (K272Q and K272M). We cannot exclude the possibility that additional specimens contained mixed viral populations with minor proportions of position-272 mutants not detectable by conventional sequencing methods. Changes at this position (from lysine to asparagine, glutamine, glutamic acid, methionine, or threonine) have produced palivizumab resistance in vitro (9) and in a cotton rat model (7). As previously reported, 1 (10.0%) of 10 sequences from our 2004–2005 study of palivizumab patients carried a 272 mutation (K272E) (13). The K272E substitution is the only substitution also demonstrated to confer resistance to motavizumab, an enhanced-potency monoclonal antibody developed by affinity maturation of palivizumab (9). We could not perform neutralization assays on position-272 variants because they did not grow in culture. This finding suggests that such changes adversely affect viral replicative capacity (7,9).

Phylogenetic analysis demonstrated that mutations in the palivizumab binding site occur in diverse genetic backgrounds; all 3 strains with substitutions at residue 272 grouped to different clades (Figure). Furthermore, these mutant strains caused mild disease treatable in an outpatient clinic and severe illness requiring hospitalization.

From 3 Canadian communities we detected a lineage harboring an N276S mutation in 44.4% of RSV-A sequences.
Table 2. Characteristics of 23 children in whom clinically significant respiratory syncytial virus respiratory tract infection developed while receiving palivizumab immunoprophylaxis, Canada, 2006–2010*

| Location and patient ID | Age, mo/sex† | GA at birth, wk + d | Underlying comorbidities | No. doses PZB‡ | Delay, d§ | Clinical diagnoses H | Multiplex PCR/DNA results | Mutation |
|-------------------------|--------------|---------------------|--------------------------|----------------|-----------|----------------------|--------------------------|----------|
| Québec City, Québec (2006–2010) |              |                     |                          |                |           |                      |                          |          |
| C0607-1023              | 9/F          | 32 + 4              | Prematurity, LBW         | 3              | 21        | Bronchiolitis         | No                       | RSV-A; enterovirus type A | K272Q    |
| H0607-064               | 24/M         | 38 + 3              | Congenital myopathy      | 3              | 15        | Pneumonia; bronchospasm | Yes                      | RSV-B    | NF       |
| H0607-132               | 12/M         | 38 + 5              | Pulmonary artery stenosis| 5              | 7         | Bronchiolitis         | Yes                      | RSV-A    | NF       |
| H0708-199               | 4/M          | 30 + 4              | Prematurity, VLBW        | 4              | 14        | Bronchiolitis         | Yes                      | RSV-B    | NF       |
| H0809-037               | 11/F         | 27 + 5              | Prematurity, ELBW        | 3              | 14        | Bronchiolitis         | Yes                      | RSV-A    | NF       |
| C0809-1055              | 6/F          | 29 + 0              | Prematurity, ELBW, triplet| 4              | 27        | Bronchiolitis         | No                       | RSV-A    | N276S    |
| C0809-1056              | 6/M          | 29 + 0              | Prematurity, ELBW, triplet| 4              | 27        | Bronchiolitis         | No                       | RSV-A    | N276S    |
| C0899-1057              | 6/M          | 29 + 0              | Prematurity, VLBW        | 4              | 27        | Bronchiolitis         | No                       | RSV-A    | N276S    |
| H0910-004               | 4/F          | 39 + 5              | Choanal hypoplasia       | 1              | 16        | Apnea; upper RTI      | Yes                      | RSV-A    | N276S    |
| H0910-140               | 6/M          | 25 + 5              | Prematurity, ELBW        | 4              | 29        | Bronchiolitis         | Yes                      | RSV-B    | NF       |
| H0910-144               | 13/F         | 26 + 5              | Prematurity, VLBW        | 4              | 7         | Pneumonia             | Yes                      | RSV-A    | K272M, N276S |
| H0910-150               | 9/M          | 28 + 3              | Prematurity, VLBW        | 4              | 12        | Upper RTI; acute otitis media | No                      | RSV-A; adenovirus type C | N276S    |
| Montréal, Québec (2009–2010) |              |                     |                          |                |           |                      |                          |          |
| MCH0910-001             | 15/M         | 40 + 4              | Total anomalous pulmonary venous return | 3         | 26        | Pneumonia             | Yes                      | RSV¶     | N276S    |
| MCH0910-002             | 6/F          | 39 + 0              | Pulmonary valve stenosis, right aortic arch | 2         | 7         | Bronchiolitis         | Yes                      | RSV¶     | N276S    |
| MCH0910-003             | 5/M          | 39 + 6              | Cystic fibrosis          | 3              | 24        | Bronchiolitis         | No                       | RSV¶     | N276S    |
| MCH0910-004             | 7/M          | 36 + 2              | Prematurity, BPD hypotonia | 4         | 6         | Bronchiolitis         | Yes                      | RSV¶     | N276S    |
| MCH0910-005             | 15/M         | 40 + 4              | Neuromuscular disorder, recurrent aspirations | 4         | 13        | Upper RTI; acute otitis media | No                      | RSV¶     | N276S    |
| MCH0910-006             | 2/M          | 34 + 6              | Prematurity, LBW         | 1              | 14        | Bronchiolitis         | Yes                      | RSV¶     | N276S    |
| MCH0910-007             | 19/F         | 25 + 0              | Prematurity, ELBW, BPD   | 3              | 19        | Bronchiolitis         | Yes                      | RSV¶     | N276S    |
| MCH0910-008             | 2/F          | 38 + 1              | Neuromuscular disorder, ventricular septal defect | 2         | 12        | Bronchiolitis         | Yes                      | RSV¶     | N276S    |
| Hamilton, Ontario (2009–2010) |              |                     |                          |                |           |                      |                          |          |
| MAC0910-001             | 1/F          | 34 + 5              | Prematurity, LBW         | 2              | 3         | Bronchiolitis         | Yes                      | RSV¶     | N276S    |
| MAC0910-002             | 6/F          | 34 + 3              | Prematurity, LBW, twin   | 1              | 25        | Bronchiolitis         | Yes                      | RSV¶     | N276S    |
| MAC0910-003             | 6/F          | 34 + 3              | Prematurity, VLBW, IUGR, twin | 1         | 27        | Bronchiolitis         | Yes                      | RSV¶     | N276S    |

*Patient identification (ID) nomenclature: hospitalized (H) or clinic (C) prospective study participant, Montréal Children’s Hospital (MCH) or McMaster Children’s Hospital (MAC) patient. GA, gestational age; PZB, palivizumab; multiplex PCR/DNA, hybridization assay; mutation, mutation in respiratory syncytial virus fusion protein PZB binding site (residues 262–276); RSV, respiratory syncytial virus; LBW, low birthweight (1,500–2,500 g); NF, no mutation found in PZB binding site; VLBW, very low birthweight (1,000–1,499 g); ELBW, extremely low birthweight (<1,000 g); RTI, respiratory tract infection; BPD, bronchopulmonary dysplasia; IUGR, intrauterine growth restriction. †Median patient age 6.0 mo (range 3–24 mo). ‡Mean ± SD no. palivizumab doses received that winter: 3.0 ± 1.2 doses. §Median interval between last palivizumab dose and symptom onset: 15.0 d (range 3–27 d). ¶Retrospectively identified participants from Montréal Children’s Hospital or McMaster Children’s Hospital were RSV-positive by direct immunofluorescence assay (Chemicon International, Temecula, CA, USA) and were not tested by the multiplex PCR/DNA hybridization assay (14).
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from 2008–2009 and 100% from 2009–2010, unrelated to palivizumab exposure. Adams et al. have proposed that N276S led to palivizumab resistance in a clinical specimen (8). However, that sample also comprised a K272E subpopulation. Our microneutralization assay results and unpublished neutralization data using recombinant viruses and clinical isolates (Q. Zhu, pers. comm.) suggest that N276S does not confer resistance.

Although serious RSV RTIs during palivizumab prophylaxis remain uncommon, we observed an 8.7% prevalence of known resistance mutations among 23 medically attended patients receiving palivizumab. These findings underscore the need for continued monitoring of RSV-F evolution.

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