Variable Resistance to Palivizumab in Cotton Rats by Respiratory Syncytial Virus Mutants

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Background. Palivizumab (PZ) is the only monoclonal antibody in use against a human infectious disease. PZ is given as prophylaxis against infection with respiratory syncytial virus (RSV). An RSV escape mutant, MP4, has been shown to resist PZ prophylaxis in cotton rats.

Methods. To further define the potential of RSV to resist prophylaxis, additional PZ-resistant viruses were selected in cell culture and were tested for susceptibility to PZ in cotton rats.

Results. Mutant MS412 had an A→C mutation at nucleotide position 827 in the F gene, resulting in an amino acid change from Lys to Gln at position 272. Mutant F212 had an A→T mutation at position 816, leading to an amino acid change from Asn to Ile at position 268. In vitro, F212 had impaired growth kinetics. In cell culture, F212 was partially and MS412 was completely resistant to PZ neutralization. A single prophylactic dose of 15 mg/kg PZ protected cotton rats from infection with F212 but not with MS412.

Conclusion. Both in vitro and in vivo, individual RSV PZ escape mutants varied in their susceptibility to PZ. Mutations associated with resistance to PZ did not always result in failure of PZ prophylaxis.

Respiratory syncytial virus (RSV) is the leading cause of respiratory infection in infants and young children worldwide. Children born prematurely and those with bronchopulmonary dysplasia, congenital heart disease, and certain immunodeficiencies are more likely to suffer severe RSV disease [1–5]. A humanized monoclonal antibody (MAb), palivizumab (PZ), neutralizes RSV through interaction with the RSV fusion (F) protein and is used prophylactically in select high-risk populations. In one study, passive immunization via monthly injections of PZ for 5 months spanning the annual RSV epidemic period reduced hospitalization rates in high-risk children [6]. RSV also causes severe respiratory illness in immunocompromised individuals, such as stem cell–transplant (SCT) recipients, and PZ has been administered to SCT recipients [7]. Increasing use of PZ in high-risk children, especially in patients with impaired cellular immune responses and prolonged viral replication, provides opportunities for PZ-resistant viruses to be encountered and transmitted among humans.

RNA viruses are highly mutable, as their RNA-dependent RNA polymerase lacks proofreading-repair capacity. Thus, like other RNA viruses, RSV is highly adaptive to selective pressure. Under selective pressure, viruses with a selective advantage may rapidly emerge from the viral quasispecies [8]. A previously described RSV escape mutant, MP4 [9], was selected by passing RSV A2 virus in cell culture in the presence of PZ. In the present study, we characterized additional RSV escape mutants that vary in their genotypes, growth phenotypes, and resistance to PZ prophylaxis in cotton rats.

MATERIALS AND METHODS

Selection of mutants. Palivizumab (Synagis; Medimmune) was purchased from a hospital pharmacy. Mutant viruses MS312, MS412, and MS512 were selected by a process similar to that described elsewhere for the selection of mutant MP4 [9, 10]. Parental A2 was neutralized and propagated in HEp-2 cells in separate wells in the presence of increasing concentrations (0.04–40 μg/mL) of
PZ for 5 passages. At passage 5, abundant cytopathic effect (CPE) was shown in 3 wells in the presence of 40 μg/mL PZ. Plaque purification was conducted under an agarose overlay that contained 4 μg/mL PZ. One mutant from each of the 3 wells showing CPE was selected from a single plaque pick and was regrown in HEp-2 cells without PZ [9].

The resistant virus F212 was derived as follows: parental A2 was neutralized with 4 μg/mL PZ in the presence of guinea pig complement (GPC), was used to infect Vero cells, and was incubated under an agar overlay that contained 0.4 μg/mL PZ [11]. Single plaques were picked and moved to 96-well plates (Corning) and were regrown in the presence of 40 μg/mL PZ in the medium. Virus was passed to fresh cells with 0.04 μg/mL PZ when a CPE was observed. Virus was passed a further 10 times in PZ, and, at passage 11, a viral CPE was evident but not extensive. The resistant virus was plaque purified twice, designated F212, and grown in the presence of 4 μg/mL PZ, to make viral stock.

**Microneutralization assay.** The microneutralization assay was performed as described elsewhere, with slight modifications [9, 12]. One thousand plaque-forming units of A2, MP4, or MS412 or 5000 pfu of F212 were incubated with serially diluted PZ in the presence of GPC for 1 h at 37°C. In response to reduced F212 growth in cell culture, as described below, the amount of F212 was increased, to ensure that the absorbance of F212 would be similar to that of the other viruses during the final step of the assay. The neutralized viral solutions were inoculated onto HEp-2 cells in triplicate wells in a 96-well plate for 1 h at 37°C. After incubation for 3 days, the cells were fixed with 80% acetone. The amount of viral antigen present, as measured by a G-protein–specific ELISA, was used as a measurement of viral replication; the mean absorbance at 450 nm was recorded for triplicate wells.

**Sequencing of the F gene.** Viral RNA from infected HEp-2 cells was extracted by use of Trizol reagent (Invitrogen), and the entire F gene was amplified by reverse-transcription poly-

### Table 1. Nucleotide and amino acid changes of respiratory syncytial virus escape mutants resistant to palivizumab.

<table>
<thead>
<tr>
<th>Escape mutant</th>
<th>Nucleotide changes in the F gene (A2 mutation)</th>
<th>Amino acid changes in the F protein (A2 mutation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP4</td>
<td>828 (A→T)</td>
<td>272 (Lys→Met)</td>
</tr>
<tr>
<td>MS312</td>
<td>828 (A→T)</td>
<td>272 (Lys→Met)</td>
</tr>
<tr>
<td>MS512</td>
<td>828 (A→T)</td>
<td>272 (Lys→Met)</td>
</tr>
<tr>
<td>MS412</td>
<td>827 (A→C)</td>
<td>272 (Lys→Gln)</td>
</tr>
<tr>
<td>F212</td>
<td>816 (A→T)</td>
<td>268 (Asn→Ile)</td>
</tr>
</tbody>
</table>

**NOTE.** F, fusion.

**RESULTS**

**Mutations in the F gene.** Four new PZ-resistant RSV mutants were identified, 3 by use of methods described elsewhere for the selection of MP4 [9]. Two of these mutants, MS312 and MS512, possessed the same nucleotide change, from A to T, at
Figure 1. Neutralization of parental A2 and mutant viruses. Micro-neutralization was performed as described elsewhere [12], to evaluate the resistance of parental A2 and the mutants MP4, MS412, and F212 to neutralization with palivizumab (PZ). PZ concentrations are shown in micrograms per milliliter on the X-axis, and mean absorbance (optical density) is shown on the Y-axis.

Position 828 in the F gene, compared with the parental A2 nucleotide, leading to a deduced amino acid substitution from Lys to Met in the F1 subunit at aa 272, as seen in MP4. The third mutant, MS412, displayed a substitution from A to C at position 827 in the F gene, resulting in a coding change from Lys to Gln at aa 272. Thus, MS412 differs from MP4, with Gln, rather than Met, at aa 272.

A fourth PZ-resistant RSV mutant, F212, was derived by a different selection process that began with direct neutralization and a plaque pick under an agar overlay that contained PZ [11]. Different selection processes may yield different mutations [15]. F212 revealed a nucleotide substitution from A to T at position 816 in the F gene, leading to a deduced amino acid change from Asn to Ile at aa 268 in the F1 subunit (table 1). Thus, F212 was distinct in the location of its amino acid coding change, compared with that of the mutants described above (MS312, MS512, MS412, and MP4).

Position 1591 in the F gene is G in the published A2 sequence and was T in the MP4, MS312, MS412, and MS512 sequences [16]. As we have described elsewhere, the F gene of parental A2 analyzed in our laboratory revealed an ambiguity (G/T) at position 1591 [9]. If the parent sequence gene was T at position 1591, there would be no coding change at aa 526 in the F proteins of MP4, MS312, MS412, and MS512; if the parent sequence gene was G at position 1591, there would be an amino acid change at aa 526 from Met to Ile. Regardless of the ambiguity at position 1591, parental A2 was susceptible to PZ, suggesting that the nucleotide at position 1591 does not determine the resistance profile of either parental A2 or the escape mutants [9]. However, it is interesting to note that position 1591 in the F gene of the mutant F212 is G, raising the possibility that the mutation at position 1591 may play a role in resistance to PZ.

MP4 and F212 were assessed for the stability of their mutations, and, after 15 cycles of growth in HEp-2 cells in the absence of PZ, the mutations were shown by nucleotide-sequence analysis to have remained constant. Because the F proteins of MS312 and MS512 were identical to that of the previously described MP4 virus, these viruses were not characterized further. The new mutants MS412 and F212 were compared with parental A2 and MP4 in vitro and in vivo as described below.

Resistance to PZ neutralization in vitro. The resistance to PZ of A2, MP4, MS412, and F212 was assessed by microneutralization (figure 1). A2 revealed a dramatic reduction in replication at PZ concentrations of 4 and 40 µg/mL, with almost complete inhibition of replication at the highest PZ concentration. F212 showed partial resistance to PZ, and mean absorbance was reduced by 55% at a PZ concentration of 40 µg/mL. MS412 and MP4 were resistant to PZ, even at the highest PZ concentration tested.

Growth kinetics. The growth kinetics of A2, MP4, MS412, and F212 in HEp-2 cells were compared. MP4 and MS412 growth was similar to that of parental A2 (figure 2). However, F212 showed a lower capacity for growth, with a titer that was ~1 log lower than that of A2 on days 3 and 4 after inoculation. Thus, as measured by the production of infectious progeny, the replication fitness of F212 appears to be impaired. The observation of syncytia in HEp-2 cells infected with either A2, MP4,
Figure 3. Immunoblot detection of fusion (F) proteins. Proteins in infected and mock (uninfected) cell lysates were separated by SDS-PAGE and were transferred to polyvinylidene fluoride membranes. Peroxidase-conjugated secondary antibodies were detected by chemiluminescence. 

A, Primary antibody, F protein monoclonal antibody A8 (1:20,000); secondary antibody, goat anti–mouse peroxidase-conjugated IgG (1:20,000). Lanes: 1, parental A2; 2, MP4; 3, MS412; 4, F212; and 5, mock. 

B, Primary antibody, palivizumab (1:10,000); secondary antibody, goat anti–human peroxidase-conjugated IgG (1:20,000). Lanes: 1, parental A2; 2, MP4; 3, MS412; 4, F212; and 5, mock.

MS412, or F212 confirms that F protein capable of inducing cell-to-cell fusion was produced by virus.

**Immunoblot reactivity.** Immunoblot reactivity was assessed for the F proteins of A2, MP4, MS412, and F212. The F₁ subunits of each virus were revealed by primary antibody detection with the F protein MAb A8 (figure 3A). However, after substitution of PZ as the primary antibody, only the F₁ subunit of parental A2 was detected (figure 3B).

Thus, by immunoblot analysis, the F₁ subunits of the mutants MP4, MS412, and F212 did not react with PZ. In addition, the identical mobility of the F₁ subunits of A2, MP4, MS412, and F212 suggests that the cleavage of the F₂ subunit and subsequent processing (such as glycosylation) is likely unchanged for the mutant proteins. Immunoblots also revealed reactivity with a protein that migrated faster than the F₁ subunit; this may be an F₁ degradation product [17].

**Effectiveness of PZ prophylaxis in cotton rats.** Prophylaxis (im) of 6 groups (A–F) of cotton rats with either PZ (15 mg/kg) or PBS (control) was followed by RSV challenge with either parental A2, MS412, or F212 (table 2). Challenge with A2 revealed the expected >99% reduction in viral titers in the lung in the presence of PZ prophylaxis (group A vs. group B). Although F212 showed partial resistance to PZ in vitro, prophylaxis completely protected cotton rats from infection with F212 (group C vs. group D). In contrast, MS412 displayed complete resistance to PZ at the same prophylactic dose (group E vs. group F), a finding similar to the results seen previously with MP4 [9]. Of note, F212 (group D) grew to lower titers than did either A2 (group B) or MS412 (group F) in cotton-rat lungs.

**DISCUSSION**

RNA viruses exist as quasispecies, allowing rapid adaptation to changing selective pressures [8]. RSV MAb and polyclonal antibody escape mutants have been readily selected in cell culture [7, 10, 11, 18, 19]. We have previously reported a mutant virus,

<table>
<thead>
<tr>
<th>Group (no. of rats)</th>
<th>Prophylaxis</th>
<th>Challenge virus</th>
<th>Viral replication</th>
<th>No. of rats with no virus detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (6)</td>
<td>PZ</td>
<td>A2</td>
<td>Lung viral titer, mean ± SD, log pfu/g of lung tissue</td>
<td>6</td>
</tr>
<tr>
<td>B (6)</td>
<td>PBS</td>
<td>A2</td>
<td>1.7 ± 0.0</td>
<td>0</td>
</tr>
<tr>
<td>C (7)</td>
<td>PZ</td>
<td>F212</td>
<td>5.1 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>D (7)</td>
<td>PBS</td>
<td>F212</td>
<td>1.7 ± 0.0</td>
<td>7</td>
</tr>
<tr>
<td>E (7)</td>
<td>PZ</td>
<td>MS412</td>
<td>4.0 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>F (6)</td>
<td>PBS</td>
<td>MS412</td>
<td>4.8 ± 1.4</td>
<td>1</td>
</tr>
</tbody>
</table>

**NOTE.** Data combined from 2 experiments. Lung samples from which no virus was recovered were assigned a log value of 1.7, a value based on the lowest detected measure of log 1.699.

a. \( P < .01 \), compared with group A (Student’s t test).

b. \( P < .01 \), compared with group C (Student’s t test).
Interestingly, in HEp-2 cells, F212 replicated to a titer of ~30 μg/mL. Thus, a PZ concentration of 25–30 μg/mL was considered to be the target serum concentration for protection from RSV infection in humans. A dose of 10 mg/kg in cotton rats results in PZ serum concentrations of ~120 μg/mL [20]. In humans, serum concentrations of ~72 μg/mL have been observed after 4 months of PZ prophylaxis (15 mg/kg) [6]. Subsequent studies in cotton rats have administered PZ at a dose of 15 mg/kg [21]. The 15-mg/kg dose of PZ was used in the experiments reported here because it provided a rigorous test of the ability of the mutant viruses to escape PZ prophylaxis in vivo. Even at this high dose, MP4 and MS412 were completely resistant to PZ. However, F212 remained susceptible—it was not found in the lungs of cotton rats that received PZ prophylaxis. Thus, mutations that arise after passage of RSV in the presence of PZ in vitro are not necessarily associated with resistance in vivo. However, because the dose of PZ used here in cotton rats results in serum levels in excess of that produced in humans receiving PZ, it remains possible that mutants such as F212 would be less than completely susceptible to prophylaxis in humans.

Mutations that occur in the RSV genome due to the error-prone nature of RNA-dependent RNA polymerase and its lack of capacity to proofread often lead to a loss of viral fitness [8]. Interestingly, in HEp-2 cells, F212 replicated to a titer ~1 log lower than that of either parental A2 or MS412. This finding was mirrored in the lungs of cotton rats; F212 reached a titer ~1 log lower than that of A2. Whether this difference between the in vitro and in vivo replication abilities of F212 resulted from the mutation in the F gene or from an unidentified change elsewhere in the RSV genome is not known. Compared with F212, the other mutants, such as MP4 and MS412, are potentially of more concern; in the present in vitro and in vivo experiments, they revealed no obvious impairment to replication, compared with that of parental A2. If they are also fit for replication in humans, they may be just as capable of human-to-human spread as is the parent virus.

Of the 5 mutants selected in HEp-2 cells, 3 (MP4, MS312, and MS512) had the same nucleotide change (position 828, A→T) in the F gene and the same deduced amino acid substitution (aa 272, Lys→Met). MS412 had a nucleotide change at a different base (position 827, A→C), resulting in a deduced amino acid change of Lys to Gln at the same amino acid position in the protein as for the other mutants. Interestingly, MS412 and MP4 shared similar phenotypic characteristics, including growth capacity in HEp-2 cells and complete resistance to PZ neutralization in vitro and in cotton rats. It appears likely that F protein aa 272 Lys is critical for interactions with PZ. All of the PZ mutations that we have identified are in antigenic site II of the F protein [22].

The potential significance of PZ-resistant mutants and whether they will be encountered by humans receiving PZ prophylaxis remain to be determined. Initial testing has indicated that all reported clinical isolates are susceptible to neutralization by PZ at a serum concentration of 400 μg/mL [20]. We are not aware of any published reports on screening for PZ resistance among RSV isolates from infected PZ recipients. Interestingly, the murine parent of PZ, MAb 1129, failed to neutralize 1 of a panel of clinical RSV isolates, and a mutant virus resistant to MAb 1129 had reduced susceptibility to neutralization by polyclonal hyperimmune serum [11]. Antibody-resistant viruses might also arise during exposure to PZ in vivo, as shown by the identification of an apparently PZ-resistant virus from the lung of a cotton rat that had received PZ before RSV challenge [20]. The risk of developing viruses that are resistant to PZ may be heightened in patients who lack adequate cellular immunity and are unable to control the replication of RSV [23].

Escape mutants have been described after active and passive immunization of animals and humans against other viral pathogens [24]. Active hepatitis B immunization and administration of hepatitis B immunoglobulin to humans have been followed by the development of hepatitis B virus variants [25, 26]. Thus, there is precedence for the development of resistance to immunization in humans.

As shown in the present report, mutants that are resistant to PZ in vitro may vary in their degree of resistance. Also, PZ-resistant mutants may vary in their capacity for replication in vitro and in vivo. Mutants with impaired replication capacity in animals could be less likely to transmit effectively, particularly outside the selective pressure of the presence of PZ. Similarly, mutants with incomplete resistance to PZ should pose less of a threat to the efficacy of PZ prophylaxis in humans.

At present, PZ is the only commercially available MAb for use against a human infectious disease. Thus, study of the potential for the development of resistance and surveillance for the appearance of PZ-resistant RSV mutants in the human population may provide useful lessons. Such lessons may inform the use of PZ, the potential use of MAbs against other infectious diseases, and our understanding of the dynamics of transmission of RSV. 
References