Respiratory syncytial virus escape mutant derived in vitro resists palivizumab prophylaxis in cotton rats

Xiaodong Zhao,a Fu-Ping Chen,a and Wayne M. Sullendera,b,*

a Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL 35233, USA
b Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35233, USA

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Abstract

Palivizumab (PZ) is the only monoclonal antibody in human use against an infectious disease. PZ is a humanized monoclonal antibody that recognizes the fusion protein of respiratory syncytial virus (RSV). PZ prophylaxis reduces the likelihood of hospitalization for young children at risk for severe RSV infections. The quasispecies nature of RNA viruses allows rapid emergence of viruses with a selective advantage. A PZ resistant virus was selected by passage of RSV in the presence of PZ in cell culture. The cell culture-derived virus was completely resistant to PZ prophylaxis in cotton rats. The increasing use of PZ, and in particular, the use of PZ in immunosuppressed patients, provide opportunities for resistant viruses to emerge. Whether such viruses will appear and be of clinical significance for humans is unknown. Preclinical studies in cotton rats predicted the efficacy of PZ in humans; these results suggest that if PZ resistant viruses arise in humans, PZ prophylaxis may be ineffective.

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Introduction

Infants born prematurely and those with chronic lung disease are at risk for severe disease due to respiratory syncytial virus (RSV) infection. A humanized monoclonal antibody [palivizumab (PZ)] neutralizes RSV through interaction with the RSV F glycoprotein. PZ monthly injections for the 5 months spanning the annual RSV epidemic period reduce the risk of hospitalization among high-risk children (IMPACT, 1998). RSV also causes severe respiratory illness in immunocompromised individuals such as stem cell transplant (SCT) recipients. PZ has been administered to SCT patients (Boeckh et al., 2001). Over 400,000 patients have received PZ and use continues to increase (Product Insert, 2002). For example, PZ has recently received Food and Drug Administration approval for prophylactic use in children with hemodynamically significant congenital heart disease.

The quasispecies nature of RNA viruses allows rapid emergence of viruses with a selective advantage (Domingo and Holland, 1994). Antibody selection of variant RSV in cell culture is well described, including mutant selection by the murine parent of PZ (Beeler and Van Wyke Coelingh, 1989). Increasing PZ use in high-risk children and in immunocompromised patients provides opportunities for PZ resistant viruses to arise and be transmitted among humans. The goal of this study was to determine whether a virus that resisted PZ in cell culture would also resist prophylaxis in vivo. If a mutant virus were shown to resist PZ prophylaxis in cotton rats, this would suggest that PZ prophylaxis would be ineffective against similar mutants in humans.

Results

Selection of escape mutant

RSV A2 strain virus was propagated in HEp-2 cells in the presence of increasing concentrations of PZ from 0.04 to 40 µg/ml for five passages. By pass 5 in 40 µg/ml PZ, there was abundant viral CPE; this was designated resistant to PZ
because the target PZ concentration to maintain in human subjects is 40 μg/ml (IMPACT, 1998). A single plaque pick from pass 5 was designated MP4. The resistance of MP4 to PZ was confirmed by microneutralization (Fig. 1). A2 virus revealed dramatic reduction in replication at PZ concentrations of 4 and 40 μg/ml, whereas the MP4 virus continued to replicate.

Nucleotide and amino acid changes

Nucleotide sequence analysis revealed a nucleotide change at F gene base 828 from A to T as compared to the parent A2 virus. This resulted in a deduced amino acid change in the F1 subunit at position 272 from Lys to Met. The murine parent MAb 1129 selected for a variant with an amino acid change at position 275 from Ser to Phe (Beeler and Van Wyke Coelingh, 1989; Crowe et al., 1998). The parent A2 virus F gene analysis revealed an ambiguity (G/T) at position 1591. F gene position 1591 is G in the published A2 and was T in the MP4 sequence (Collins et al., 1984). If the parent sequence gene nucleotide were T, there would be no coding change in MP4, if G, there would be an amino acid change at residue 526 from Met to Ile. The parent A2 virus was susceptible to PZ, suggesting that the nucleotide at position 1591 does not determine the resistance profile of the parent A2 or of the MP4 virus.

Replication of MP4 in HEp-2 cells

The growth kinetics of the MP4 and A2 viruses were compared. Both the A2 and MP4 viruses grew to nearly identical levels, with peak titers exceeding 107 PFU/ml during the third and fourth day after inoculation (Fig. 2). Thus, as measured by production of infectious progeny, the replication fitness of MP4 in HEp-2 cells was normal. This suggested that the fusion protein was able to support cell entry via fusion as an early step required for replication. The presence of syncytia in HEp-2 cells infected with MP4 also indicated that fusion function was present.

Immunoblot reactivity of MP4 F protein

Immunoblot reactivity was assessed for the F proteins of the A2 and MP4 viruses. The F proteins expressed by A2 and MP4 in infected cell lysates were indistinguishable with primary antibody detection by the F protein MAb A8 (Fig. 3A) (MAb generously provided by Ed Walsh, Rochester, NY) (Walsh et al., 1986). Detection with PZ showed reactivity with the A2 but not the MP4 F1 protein (Fig. 3B). PZ detection of the A2 sample also revealed reactivity with a protein that migrated faster than the F1 subunit; this may be an F1 degradation product (Lopez et al., 1990).

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**Fig. 1.** Neutralization of A2 and MP4 viruses. Microneutralization was performed as described (Anderson et al., 1985) to evaluate the resistance of the A2 and MP4 viruses to neutralization of PZ. PZ concentrations are shown in μg/ml (X-axis) and mean absorbance (optical density) (Y-axis).

**Fig. 2.** Growth kinetics of A2 and MP4 viruses. HEp-2 cells were split into a 6-well plate the day prior to inoculation and infected with 2.5 × 10⁵ PFU of either A2 or MP4 viruses. At daily intervals, the virus-containing media were harvested, stored at −70 °C, and titrated on Vero cells. Growth capacity was expressed as the log value of plaque forming units per milliliter.

**Fig. 3.** Immunoblot detection of F proteins. Proteins in infected and mock uninfected cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. Peroxidase-conjugated secondary antibodies were detected by chemiluminescence. (A) F protein MAb A8 primary antibody (1:20000), goat anti-mouse peroxidase-conjugated IgG secondary antibody (1:20000); lanes 1: A2, 2: MP4, 3: Mock. (B) Palivizumab primary antibody (1:10000), goat anti-human peroxidase-conjugated IgG secondary antibody (1:20000); lanes 1: A2, 2: MP4, 3: Mock.
Table 1
Prophylaxis with palivizumab in cotton rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Prophylaxis</th>
<th>Challenge virus</th>
<th>Lung viral titer (log, mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (9)</td>
<td>PZ</td>
<td>A2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>B (12)</td>
<td>PBS</td>
<td>A2</td>
<td>4.2 ± 0.5*</td>
</tr>
<tr>
<td>C (7)</td>
<td>PZ</td>
<td>MP4</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>D (7)</td>
<td>PBS</td>
<td>MP4</td>
<td>5.4 ± 0.1</td>
</tr>
</tbody>
</table>

Data combined from two studies. Eight of nine group A animals had no virus detected in the lungs. Group B (PBS control, A2 challenge) had three animals with no virus recovered from the lungs, this control group should uniformly yield virus and thus animals with no virus likely reflected failed inoculations. Samples with no virus recovered from the lungs were assigned a log value of 1.7 based on the lowest detectable measure of 1 plaque in both wells at a 1:10 dilution.

* Student’s t test P value < 0.01 as compared to Group A.

Thus, the mutant MP4 F1 subunit lost antigenic reactivity with PZ. In addition, the identical mobility of the F1 subunits of both A2 and MP4 indicated that cleavage into subunits and processing, such as glycosylation, was likely unchanged for the mutant protein.

Effectiveness of prophylaxis with palivizumab in cotton rats

Intramuscular prophylaxis with PZ was followed by RSV challenge with either the A2 or MP4 viruses (Table 1). Cotton rats administered PZ and challenged by A2 virus had no virus detected in their lungs (group A), a greater than 99% reduction of lung RSV titers as compared with the PBS prophylaxis control (group B). This was expected based on earlier work (Johnson et al., 1997). However, PZ prophylaxis provided no protection against challenge by the MP4 virus (group C vs. D). Thus, the in vitro selected PZ resistant virus MP4 was completely resistant to the prophylactic effect of PZ in cotton rats.

Discussion

The quasispecies nature of RNA viruses allows rapid adaptation to changing selective pressures (Domingo and Holland, 1994). Monoclonal and polyclonal antibody escape mutants of RSV have been readily selected in cell culture (Beeler and Van Wyke Coelingh, 1989; Crowe et al., 1998; Garcia-Barreno et al., 1990; Lopez et al., 1990; Sullender and Edwards, 1999; Walsh et al., 1998). There have been no reports as to the capacity of such in vitro derived mutants to escape antibody neutralization in vivo. MAb 1129 (the murine parent to PZ) and PZ select for resistant viruses in cell culture (Beeler and Van Wyke Coelingh, 1989; Johnson et al., 1997). The MAb 1129 resistant virus also had reduced susceptibility to neutralization by polyclonal hyperimmune serum (Beeler and Van Wyke Coelingh, 1989). Assessment of PZ in cotton rats included testing for the presence of antibody resistant viruses in the lungs after prophylaxis; apparently one resistant virus was identified (Johnson et al., 1997).

The murine parent of PZ, MAb 1129, was unable to neutralize one of the clinical isolates against which it was tested, indicating that circulating wild-type RSV may be intrinsically resistant to PZ (Beeler and Van Wyke Coelingh, 1989). A panel of RSV clinical isolates were susceptible to neutralization by PZ at a concentration of 400 μg/ml. Interestingly, 400 μg/ml is 10-fold the target concentration of 40 μg/ml for prophylaxis and exceeds the trough concentration of 72 μg/ml reported after 4 months of prophylaxis (IMPACT, 1998; Johnson et al., 1997).

How likely are PZ resistant viruses to arise? For other short-lived infections such as influenza virus, antigenic variants are thought to arise during serial transmission through a partially immune population, providing a selective advantage for viruses that are able to evade host immunity (Kilbourne, 1994; Webster, 1998). PZ is administered only to restricted populations and most RSV infections do not occur among PZ recipients. Thus, it seems unlikely there will be a selective advantage during sequential passage through the general population. A resistant virus might have a selective advantage during transmission through several human hosts if resistance to PZ is associated with reduced susceptibility to serum antibodies as described for the MAb 1129 mutant (Beeler and Van Wyke Coelingh, 1989). Further limiting the likelihood of spread of resistant virus is that for children with intact immune systems, the virus should be eradicated by cellular immune responses even if the virus evades PZ.

The use of PZ in the profoundly immunocompromised, such SCT patients, may provide a greater opportunity for the emergence of resistant viruses. Cellular immunity is critical for clearance of established RSV infections, and patients who lack cellular immunity replicate RSV for extended periods (Hall et al., 1986). PZ administered to these patients may result in a situation of prolonged RSV replication in the presence of antibody. Prolonged replication provides a longer period of selective pressure to foster the emergence of PZ resistant viruses. RSV replication also can be prolonged in immunocompetent children, with virus recovery for as long as 3 weeks (Hall et al., 1976).

Selection of viral escape mutants has been described after active and passive immunization in animal and human populations, primarily under research conditions. Influenza virus and foot-and-mouth disease virus (FMDV) variants arise during passage of virus through immunized mice (influenza virus) and cattle (FMDV) (Hamre et al., 1958; Hyslop and Fagg, 1965). Vaccination of cattle against FMDV with synthetic peptides rapidly selected for FMDV antigenic variants (Taboga et al., 1997). Human immunodeficiency virus type 1 variants arose in a gp-120 vaccinated chimpanzee; the variants are resistant to neutralization by early serum samples (Nara et al., 1990). Hepatitis B virus (HBV) variants arise in humans after active immunization and in liver transplant patients.
Materials and Methods

Mutant selection

Palivizumab was purchased from a hospital pharmacy (Synagis, Medimmune, Inc.). A2 virus (1 × 10^5 PFU) was mixed with PZ (final concentration, 4 µg/ml) and guinea pig complement (final concentration 5%) at 37 °C for 1 h to allow neutralization of susceptible virus (Sullender and Edwards, 1999). Neutralized virus was inoculated onto HEp-2 cells and grown in the presence of PZ (Table 1). Viral CPE was evident within 3–4 days and a virus-containing cell suspension was harvested and clarified for the next pass. After the first two passes, the neutralization step was omitted. This process was repeated until pass 5 viral CPE was evident in 40 µg/ml PZ. A mutant (MP4) was obtained by plaque pick and used to produce viral stocks for subsequent studies.

Microneutralization assay

The assay was performed as described previously with slight modifications (Anderson et al., 1985). One thousand PFU of A2 or MP4 virus was incubated with serially diluted PZ in the presence of guinea pig complement for 1 h at 37 °C. These neutralized viral solutions were used to inoculate HEp-2 cells in triplicate in 96-well plate for 1 h at 37 °C. After 3 days of incubation, the cells were fixed with acetone. The amount of viral antigen present, measured using a G-protein-specific ELISA, was used as a measure of viral replication. Mean absorbance at 450 nm was recorded for triplicate wells.

F gene sequencing

RNA from A2 or MP4-infected HEp-2 cells was extracted, and the F gene was amplified by RT-PCR with primers F for (5'-eggggtacgGGGCAATTTAACG-AATGGAGTTGC-3'), corresponding to genome nucleotides 5601–5623 and nucleotides (lower case) that include restriction site KpnI, and Frev (5'-egggatccCA-GATGTAAGTTAACG-3'), which complements genome nucleotides 7515–7535 and nucleotides (lower case) that include restriction site BamHI. PCR products were purified by agarose gel electrophoresis and DNA extraction (QIA Quick Gel Extraction Kit, Qiagen). Sequence determinations were done directly from the bulk PCR products on an automated sequencing machine (ABI Model 377 or 3100) at the UAB CFAR DNA sequencing core facility.

Growth kinetics of A2 and MP4 in HEp-2 cells

HEp-2 cells in a 6-well plate were inoculated with same amount of A2 or MP4 virus. One milliliter of virus-containing media was harvested every 24 h. Viral titers were determined by plaque assay on Vero cells.

Immunoblotting

Immunoblots were performed as described (Sullender, 1995). The proteins in cell lysates were separated by electrophoresis on SDS–polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membrane was incubated sequentially in a blocking solution, diluted primary antibody, peroxidase-conjugated secondary antibody, and finally in a chemiluminescent reagent and exposed to radiographic film.

Prophylaxis in cotton rats

Cotton rats (Sigmodon hispidus) were obtained commercially (Harlan, Indianapolis). Prophylaxis and control groups were administered 15 mg/kg palivizumab (or a similar volume of PBS for the control groups) intramuscularly 1 day before virus inoculation (IMPACT, 1998). Cotton rats under ketamine/xylazine anesthesia were inoculated intranasally with 100 µl of RSV suspension containing 5 × 10^5 plaque-forming units (PFU) of A2 or MP4 viruses. After 4 days, the animals were sacrificed by carbon dioxide intoxication and lungs were obtained for virus titration. Virus titers were determined by plaque assay on Vero cells and expressed as plaque-forming unit per gram of lung tissue.
Acknowledgment

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References


