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In Vivo Selection of Respiratory Syncytial Viruses Resistant to Palivizumab

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Palivizumab (PZ) is the only monoclonal antibody currently available for use in humans against an infectious disease. PZ is administered prophylactically for respiratory syncytial virus (RSV) infections. RSV selected in cell culture for growth in the presence of PZ develops F gene mutations and can be resistant to PZ prophylaxis in cotton rats. Here, we evaluated the potential for PZ-resistant RSV mutants to arise in vivo. Cotton rats were immunosuppressed with cyclophosphamide, administered PZ, and inoculated intranasally with RSV. Lungs were harvested 12 weeks after RSV infection, reverse transcription-PCR-amplified F gene fragments were cloned into plasmids, and the nucleotide sequences of the cloned cDNAs were determined. Three of the five animals had mixed populations of lung virus, and over 50% of the clones from the three animals revealed F gene mutations associated with resistance to PZ. A virus completely resistant to PZ neutralization was recovered from the lung homogenate of a rat that had received PZ. Thus, prolonged pulmonary replication of RSV in the presence of PZ was followed by the appearance of viruses resistant to PZ. The potential for the development of resistance is a consideration as the antibody is used prophylactically against RSV and as passively administered antibodies are under development for other infections, including emerging viruses and agents of biodefense.

Respiratory syncytial virus (RSV) is the principal cause of viral respiratory infections among infants and young children and causes disease in adults, with the elderly at particular risk for severe disease (8, 14, 26). In addition, immunocompromised patients may suffer serious morbidity and even mortality due to RSV infections (13, 16). Palivizumab (PZ), is the first commercially available antibody for use against an infectious disease. PZ, a humanized neutralizing monoclonal antibody reactive with an epitope on the F glycoprotein of RSV, is used prophylactically for high-risk children with preterm birth or underlying cardiorespiratory disorders (1).

RSV escape mutants from monoclonal and polyclonal antibodies against the F and G proteins have been derived in cell culture (5, 9, 15, 23, 31, 33). We selected RSV resistant to PZ by replication of virus in the presence of PZ in cell culture (36). Point mutations occurred at two sites in the F gene. At positions 828 (A-T, virus MP4) and 827 (A-C, virus MS412) changes resulted in two different amino acid changes at position 272 in the F1 subunit (Lys to Met or Gln, respectively). Both changes were associated with resistance to PZ neutralization in vitro. In addition, viruses with these point mutations were completely resistant to the prophylactic effects of PZ in cotton rats. A point mutation at another site, 816 (A-T), led to an amino acid substitution from Asn to Ile at position 268 in the F1 subunit. This virus, F212, was partially resistant to PZ neutralization but remained fully susceptible to PZ prophylaxis (15 mg/kg of body weight) in cotton rats. Interestingly, F212 grew to lower titers than the parent A2 virus both in HEp-2 cells and in cotton rat lungs (35). All of these mutations are within antigenic site II (or site A) in the F protein (3, 22).

Cotton rats treated with cyclophosphamide (CY) allow RSV replication in nasal tissue and the lungs for up to 7 weeks (20, 34). Immunosuppressed humans also reveal prolonged RSV replication (13). PZ is being evaluated for prophylactic and therapeutic use in immunosuppressed patients (6). Prolonged viral replication in vivo may provide a unique opportunity for RSV PZ escape mutants to arise. We used the immunosuppressed cotton rat model to examine the possibility that PZ-resistant viruses may develop during prolonged replication in the presence of PZ.

MATERIALS AND METHODS

Animal model. Cotton rats (*Sigmodon hispidus*) obtained commercially (Harlan, Indianapolis, Ind.) were treated with intraperitoneal injections of CY: 50 mg/kg three times per week for at least 3 weeks prior to viral challenge. CY treatment was continued until the end of study. As previously described, this led to marked leukopenia, with >80% reduction in total white blood cell counts compared to animals that did not receive CY (20, 27, 35).

Viruses and antibody. A2 RSV and previously described PZ escape mutants derived from A2 virus, MS412 and F212, were grown in HEp-2 cells (35). Viral titers were determined by plaque assay on Vero cells and expressed as PFU per milliliter. PZ (Synagis; Medimmune, Inc.) was purchased from a hospital pharmacy.

Experimental design. Twelve cotton rats were divided into 4 groups: A, B, C, and D (Fig. 1). Group D cotton rats (2 rats) did not receive PZ or CY and served as a control for white blood cell counts. All other rats received CY. PZ was administered intramuscularly (i.m.) at 15 mg/kg. Group A (4 rats) received PZ 3 weeks post-CY treatment. Twenty-four hours later, cotton rats in groups A, B, and C were infected intranasally with 5×10^5 PFU (50 μl) of A2 virus. Infections were performed under ketamine-xylazine anesthesia. Groups A and B (2 rats) received PZ 6 weeks after the initial infections. Group C (4 rats) was treated with CY but did not receive PZ and served as a negative (no PZ) control for prolonged viral replication. Nasal wash samples were obtained under anesthesia weekly (except week 10 postinfection) from rats in groups A and B and inoculated onto HEp-2 cells for viral isolation. Infected HEp-2 cells provided RNA for reverse transcription (RT)-PCR and nucleotide sequence determination. Twelve weeks after the initial viral challenge, the experiment was terminated and the animals were sacrificed; lungs were harvested for RNA extraction and viral isolation.

Nasal wash and viral isolation. HEp-2 cells were split into six-well plates 1 day prior to sampling. Each animal's nose was washed with 25 μl of transportation medium (Eagle minimum essential medium supplemented with 2% fetal bovine serum and antibiotics) under light ketamine-xylazine anesthesia. Nasal wash

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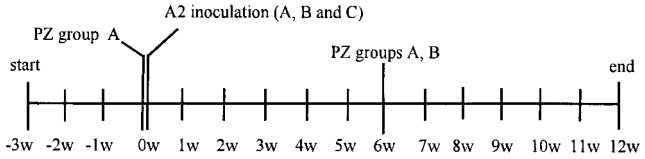

FIG. 1. Study design timeline. Study weeks are shown relative to the time of infection (in weeks [w]) under the horizontal line. Groups A, B, and C were treated with CY (50 mg/kg three times per week) for 3 weeks prior to viral challenge; treatment continued until the end of study. Rats in group A received 15 mg of PZ (i.m.)/kg 1 day prior to infection. Rats in groups A, B, and C were challenged intranasally with A2 virus at 0 weeks (3 weeks after beginning CY treatment). Groups A and B received 15 mg of PZ (i.m.)/kg 6 weeks after viral challenge. Group C never received PZ and served as a no-antibody control. Nasal wash samples were obtained weekly from groups A and B and inoculated onto HEp-2 cells to isolate virus. The experiment was terminated at 12 weeks post-first infection; lungs were harvested for RNA extraction and viral isolation.

samples in 1 ml of transportation medium were kept on ice. After vortexing for 30 s, 0.5 ml of medium was inoculated onto HEp-2 cells and absorbed for 2 h before being replaced with fresh 10% fetal bovine serum–Eagle minimum essential medium. Cultures were maintained in the absence of PZ until viral cytopathic effect (CPE) was evident or for at least 5 days. A second passage was conducted if no CPE was noted in passage one. Nasal wash samples with no CPE in the second passage were designated culture negative.

Cotton rat lung harvest and viral isolation. Cotton rats were sacrificed at 12 weeks post-viral infection, and their lungs were harvested for viral isolation and viral RNA extraction. Ground lung suspension (300 μl) was absorbed on HEp-2 cells for 2 h, washed twice, replaced with fresh medium, and maintained for 4 days in the absence of PZ. Little or no CPE was seen after the initial inoculation, and the medium was passed again onto fresh HEp-2 cells. Lung samples with no CPE shown in passage 2 were considered culture negative.

RNA extraction and F gene sequencing. Viral RNA was extracted from infected HEp-2 cells and ground lung tissue with Trizol reagent (Invitrogen), as described previously (36). The region from nucleotides 622 to 1225 in the RSV F gene was amplified by RT-PCR with primers F622For (5′-GTTACCTATTG TGAACAAGC-3′), corresponding to A2 F gene nucleotides 622 to 641, and F1225Rev (5′-GCTGCTTACATCTGTTTTTG-3′), which complements A2 F gene nucleotides 1206 to 1225, to yield a 603-bp fragment. PCR products were purified by agarose gel electrophoresis and DNA extraction (QIAQuick gel extraction kit; QIAGEN). Sequence data were determined directly from bulk RT-PCR products with the primer F622For on an automated sequencer (ABI model 3100) at the University of Alabama Center for AIDS Research DNA sequencing core facility. The region from nucleotides 660 to 1210 in the F gene was analyzed by using DNAsis, version 2.0. This region covered the point mutations observed (nucleotides 816, 827, and 828) previously during in vitro selection of PZ escape mutants and in an escape mutant of the original murine monoclonal parent monoclonal antibody (MAb) 1129 (5, 9).

Proportion of viruses in the mixed population in the lungs. The 603-bp RT-PCR products from cotton rat lung samples or HEp-2 cells infected with parental A2 virus were gel purified and cloned into pCR2.1-TOPO (Invitrogen) by following the manufacturer's instructions. Between 20 and 50 colonies were picked from each cloned RT-PCR, and the nucleotide sequences of the insert cDNAs were determined. The proportion of differing genotypes in each population was calculated based on the total number of clones sequenced (4).

Microneutralization assay. The microneutralization assay was performed as described previously with slight modifications (2). Briefly, 1,000 PFU of the standard viruses A2 and MS412, 1,000 PFU of the recovered viruses MCRL5 (rat A-4), A2R3 (rat C-3), and A2R4 (rat C-4), or 5,000 PFU of standard virus F212 was incubated with serially diluted PZ in the presence of guinea pig complement for 1 h at 37°C. The amount of F212 was increased in response to reduced F212 growth in cell culture (35) and to ensure that the absorbance of the F212 wells would be similar to that of the other viruses in the final step of the assay. These neutralized viral solutions were used to inoculate HEp2 cells in triplicate in 96-well plates for 1 h at 37°C. After 3 days, the cells were fixed with acetone. The amount of viral antigen present, measured by using a G-protein-specific enzyme-linked immunosorbent assay, was used as a measure of viral replication. Mean absorbance at 450 nm was recorded for triplicate wells.

RESULTS

Viral shedding and mutant screening from nasal wash samples. Nasal wash samples inoculated onto HEp-2 cells revealed the presence of virus from all of the infected animals for at least 6 weeks postinfection (Table 1). After the first 4 weeks, many samples required a second passage on HEp-2 cells before the CPE was extensive. After 6 weeks, the recovery of virus from nasal washes was sporadic. Viruses recovered in cells provided RNA for RT-PCR amplification and nucleotide sequence determination of the F gene nucleotides 660 to 1210, encompassing previously identified PZ resistance mutations and most of the point mutations described in antigenic site II of the F protein (8, 35, 36). At 6 weeks post-initial infection, one animal (A-4) revealed a mixed population at position 827 of A and C, with A representing the parent A2 virus and C identical to a mutant (MS412) completely resistant to PZ (35) (Fig. 2). At 8 weeks, the viral population from rat A-4 appeared to be homogeneous for the point mutation 827 A-C, resulting in a predicted amino acid change from Lys to Gln. However, as noted below, virus analyzed directly from the lungs revealed a different population than virus regrown from the lungs and then analyzed. Thus, the results from the nasal wash samples may have been influenced by the growth of virus in Hep-2 cells prior to analysis.

Characterization of escape mutants from viral populations in cotton rat lungs. At 12 weeks postinfection, the lungs of all rats were harvested. Virus populations were assessed by RT-PCR amplification of F gene RNA and by recovery of virus in cell culture. The RT-PCR products were subsequently analyzed by (i)

TABLE 1. Virus shedding and escape mutant screening from nasal wash samples[a]

Rat no.	PZ treatment at wk	Result at postinfection wk:						
		4	5	6	7	8	9	11
A-1	0, 6	+	+P2	+P2	−	−	−	−
A-2	0, 6	+	+P2	+P2	−	−	+P2	+P2
A-3	0, 6	+	+	+P2	−	−	−	−
A-4	0, 6	+	+	+ (mixed population, base 827 A/C)	−	+ (mutation, 827 A/C)	−	−
B-1	6	+	+	+P2	+P2	+P2	+P2	+P2
B-2	6	+	+P2	+P2	−	−	−	−

[a] Timing of PZ treatment is indicated by week. +, viral CPE; −, no CPE; +P2, CPE at second pass, not first pass. Viruses recovered in HEp-2 cells underwent nucleotide sequence analysis for F gene bases 660 to 1210; mutations are shown by nucleotide position and base. Mutation to C at position 827 was identical to a previously described escape mutant, MS412, completely resistant to PZ in vitro and in vivo (35). All rats shed virus from the nose from weeks 1 to 4 postinfection, and no mutations were identified in the F gene (data not shown).

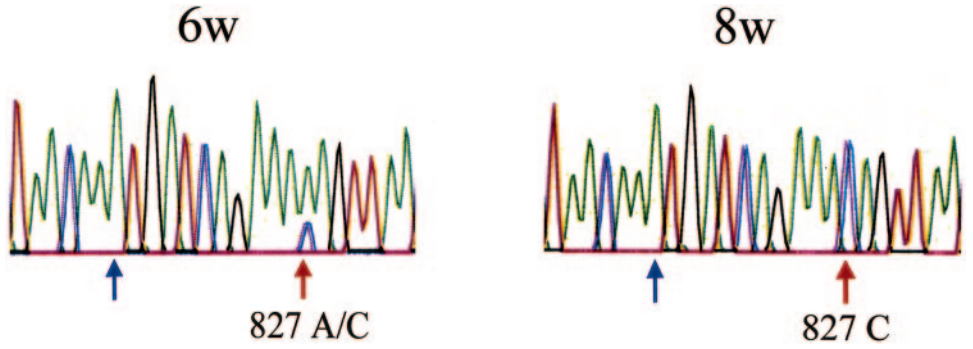

FIG. 2. RSV escape mutant in nasal wash samples from cotton rat A-4. HEp-2 cells infected with virus from the nasal wash samples at 6 and 8 weeks provided RNA for RT-PCR amplification and nucleotide sequence determination of the F gene (bases 660 to 1210); nucleotides 810 to 833 in the F gene are shown. Red arrows indicate nucleotide 827 of the F gene. At 6 weeks (6w), a mixed pattern with dominance of the parent virus base A (green) over mutant virus base C (blue) occurred, and at 8 weeks (8w), only the mutation to base C was seen. The mutation to 827 C was also seen in the in vitro-derived escape mutant MS412 (35). Blue arrows indicate position 816 in the F gene.

direct nucleotide sequence determination and (ii) cloning of RT-PCR products followed by nucleotide sequence determination.

Assessment of mixed populations in the lungs by nucleotide sequence determination of viral RNA from lungs. RT-PCR-amplified F gene products were obtained from 9 of the 10 infected animals' lungs and analyzed by nucleotide sequence determination (Fig. 3). One of the group A rats (rat A-3) was identical to the A2 virus F gene in this region with no evidence of mutations. Two of the group A rats (A-2 and A-4) revealed a mixed population at nucleotide 816, A was identical to the parent virus and T was a change seen in a previously described virus (F212) partially resistant to PZ in vitro (35). Two rats in group B revealed RT-PCR products: one (B-1) showed a mixed population at nucleotide 816, A/T, and the other was undistinguishable from the parent virus. The viruses recovered from rats that had not received PZ (group C) did not reveal mutations. Thus, the mixed populations observed among the rats that received PZ were not due simply to prolonged replication in an animal.

Assessment of mixed populations in the lungs by analysis of cloned RT-PCR products. Sequence data generated from RT-PCR products amplified directly from lung extracts revealed complexity of the viral populations (Fig. 3). To better define these mixtures, the RT-PCR products were cloned into plasmid vectors and the insert cDNA sequences were determined (Table 2). As controls, the input A2 virus and viruses from the lungs of infected cotton rats that did not receive PZ (group C) were analyzed. Of 24 clones from prototype A2 virus grown in HEp-2 cells, one (4%) showed a nucleotide change from A to G at position 816 compared to the A2 consensus sequence. This mutation would result in a coding change for amino acid 268 from Asn to Ser. Ninety-one clones were analyzed from 4 group C rats, and one clone showed a nucleotide change at position 828 from A to G. This point mutation has not been described among the in vitro-derived PZ escape mutants, and it resulted in a predicted amino acid change from Lys to Arg at position 272 in the F1 subunit. All other clones from group C rat lungs were identical to the parent A2 virus for the region analyzed.

The lungs of the five animals that received PZ revealed at least some mutations, but for two animals there were few mutants, similar to the results from the control samples (above). However, three of the five animals (A-2, A-4, and B-1) had greater than 50% of the clones with mutant sequences. Notably, mutants revealed nucleotide changes identical to those of previously described in vitro-derived mutants, including mutants (MP4 and MS412) associated with complete resistance to PZ prophylaxis in vivo (36). Additionally, a mutant with coding changes at positions 816 and 827 and another virus with a synonymous nucleotide change at 826 were observed. Thus, rats that received PZ had a much larger proportion of mutants in the mixed population than the control samples.

Analysis of F gene of viruses recovered from rats A-4, C-3, and C-4. Viruses recovered at 12 weeks postinfection from the lungs of rats A-4, C-3, and C-4 after two passages on HEp-2 cells were designated as MCRL5, A2R3, and A2R4, respectively. The results of nucleotide sequence determination from the regrown viruses were compared to the results from RT-PCR products amplified directly from the lungs. Interestingly, results of the regrown virus from rat A-4, MCRL5, were profoundly different from the original results directly from lung tissue. Nucleotide 816 in the F gene of MCRL5 was identical to the parent A2 virus, with no evidence of a mixed population. At nucleotide 827, MCRL5 had a mutation from A to C, as previously seen in a virus completely resistant to PZ in vivo (MS412) (35). In contrast, the lung tissue analysis showed a mixed population at position 816 and a major genotype of A2 (A) at position 827. No difference was found between sequences of regrown viruses and that from lung tissue analysis in rats C-3 and C-4, which did not receive PZ.

Resistance of recovered viruses to PZ neutralization in vitro. The regrown viruses were assessed for resistance to PZ by microneutralization (Fig. 4). MCRL5 was completely resistant to PZ neutralization, even at 40 μg of PZ/ml. Controls, including the parent A2 virus and virus recovered after A2 growth in cotton rats (A2R3 and A2R4), and a virus with a phenotype of partial resistance in vitro (F212) revealed the appropriate patterns of susceptibility to PZ neutralization. The resistance of MCRL5 was expected because the same mutation was observed in the MS412 mutant that was resistant to PZ both in vitro and in vivo (35).

DISCUSSION

Passively administered polyclonal antibodies have a long history of use against infectious diseases (30). However, PZ is the

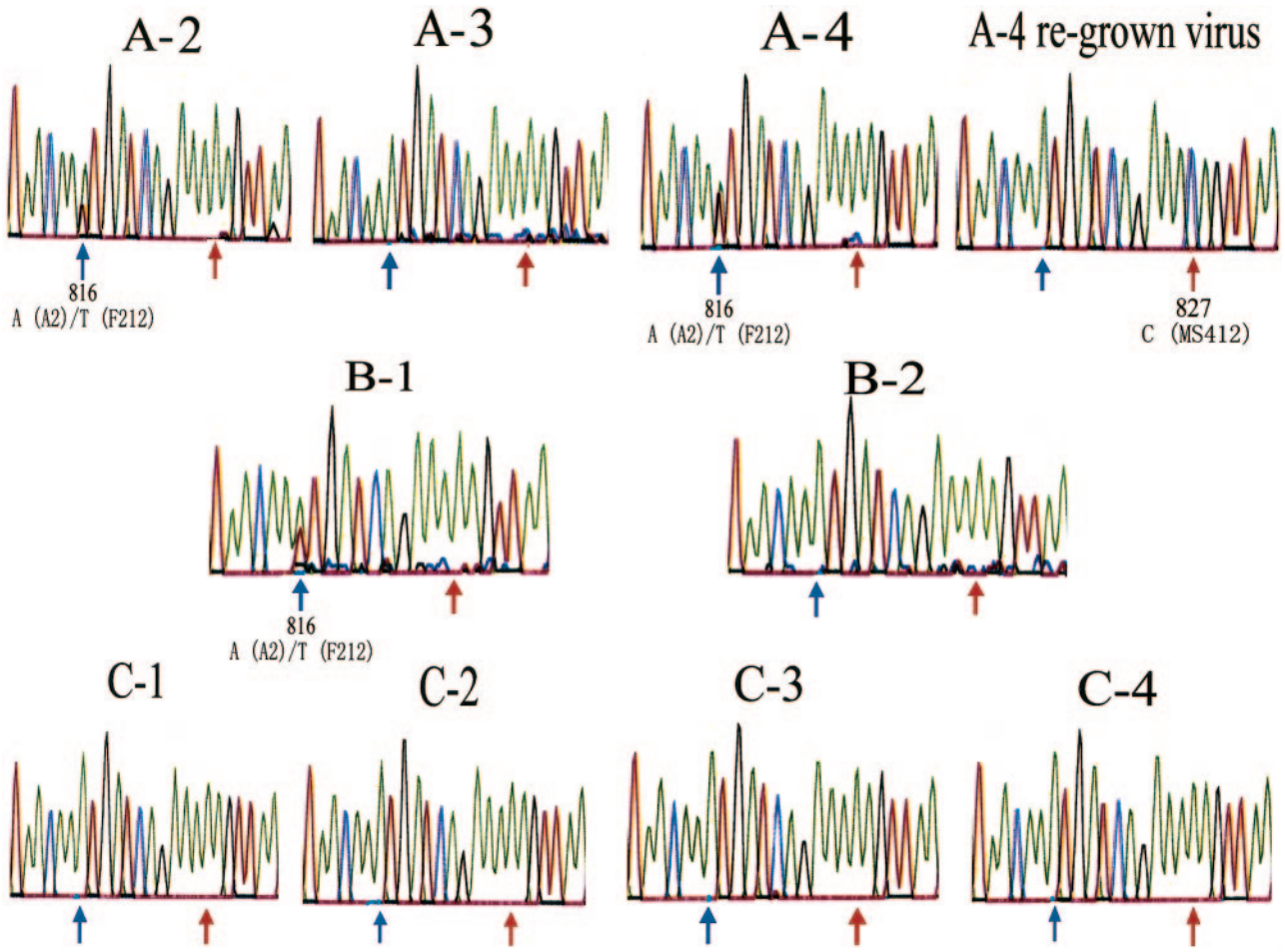

FIG. 3. Identification of mixed viral populations in cotton rat lungs. Lung samples were harvested (from rats A-2, A-3, A-4, B-1, B-2, C-1, C-2, C-3, and C-4) at 12 weeks postinfection, RNA was extracted, RT-PCR was performed, and nucleotide sequences were determined for the region from nucleotides 660 to 1210 in the F gene. For one sample, the A-4 regrown virus (MCRL5), virus was recovered in HEp-2 cells prior to characterization. The region shown is from nucleotides 810 to 833 in the F gene. Blue arrows indicate nucleotide 816 of the F gene. Base A (green) at position 816 is the parent A2 virus base. Lungs (A-2, A-4, and B-1) revealed mixtures at nucleotide 816, with both A and a mutation to T (red) present. The mutation to 816 T was also seen in the in vitro-derived escape mutant F212 (35). Red arrows indicate position 827 in the F gene. The A-4 regrown virus had the parental A2 virus base A instead of a mixed signal (A/T) at position 816; at position 827, the regrown virus revealed a mutation to C (blue) instead of the dominant base A (green) seen in the A-4 sample prior to recovery in cell culture.

first and, at present, the only monoclonal antibody commercially available for prophylaxis against an infectious disease. PZ is licensed by the Food and Drug Administration for protection against RSV infections in selected high-risk children, including certain preterm infants and young children with chronic lung disease or hemodynamically significant heart disease (1). There is no evidence of clinical benefit from PZ therapy of RSV infections in immunocompetent hosts (25). PZ therapy of RSV infections in profoundly immunocompromised stem cell patients is under evaluation (6).

RNA viruses exist as a quasispecies or complex distribution of mutant genomes. Selective pressure allows mutants with growth advantages to become dominant (11). Among immunocompetent individuals, normal immune responses will clear RSV infections. Patients with profound immunodeficiency, particularly deficiency of cellular immune responses, replicate RSV for extended periods (16). Thus, antibody preparations used against RSV in an immunocompromised host may result in exposure of virus to a selective agent through multiple rounds of replication. Antibody escape mutants of RSV have been selected in cell culture with monoclonal antibodies to the F and G proteins (including PZ) and with a polyclonal monospecific antibody to the G protein (5, 9, 15, 23, 31, 33). These results confirm the general principle of antibody selection but leave open the question as to whether such mutants would arise and replicate successfully in an in vivo setting.

We sought to determine whether PZ-resistant viruses would arise during prolonged replication of RSV in immunosuppressed animals. An established model of immunosuppressed cotton rats was combined with administration of PZ and infection with RSV (20, 27, 34). As previously described, virus was shed for extended periods, detectable by culture from the nose for at least 6 weeks and from the lungs of one animal at 12 weeks postinfection.

Virus recovered in cell culture from the nasal samples of one rat demonstrated a mixed population at 6 weeks and a dominant mutant population at 8 weeks postinfection. The rat had received a second dose of PZ at 6 weeks postinfection, sug-

TABLE 2. Analysis of molecular clones of escape mutants in mixed viral populations in cotton rat lungs[a]

Source of PCR products	No. of clones	PZ treatment	No. of clones with nucleotide change (% of clones identical) compared to A2 virus or in vitro-derived mutants					
			No change (A2)	816 A-T (F212)	827 A-C (MS412)	828 A-T (MP4)	816 A-G	828 A-G
Cell culture-grown virus A2	24	NA	23 (96)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)
Rat A-2	48	wk 0, 6	19 (40)	22 (46)	3 (6)	3 (6)	1 (2)	0 (0)
Rat A-3	45	wk 0, 6	43 (96)	0 (0)	0 (0)	2 (4)	0 (0)	0 (0)
Rat A-4	50	wk 0, 6	11 (22)	18 (36)	17 (34)	3 (6)	0 (0)	0 (0)
Rat B-1	22	wk 6	6 (27)	12 (55)	1 (5)	2 (9)	0 (0)	0 (0)
Rat B-2	23	wk 6	21 (91)	1 (4)	1 (4)	0 (0)	0 (0)	0 (0)
Rat C-1	23	None	23 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Rat C-2	24	None	23 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Rat C-3	22	None	21 (96)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5)
Rat C-4	22	None	22 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] RNA was extracted from cotton rat lungs, with the exception of infected HEp-2 cells used for cell culture-grown virus A2. RT-PCR products were cloned into a plasmid vector, and nucleotide sequences of the inserted DNA (nucleotides 660 to 1210 in the F gene) were determined. Differences compared to the A2 virus F gene sequence are shown; if the same mutation has been observed in previously described escape mutants, the name of the previous mutant is provided (36). The in vitro-derived RSV escape mutants MP4 and MS412 confer complete resistance to PZ in vitro and in cotton rats, and F212 confers partial resistance in cell culture and no resistance to PZ in cotton rats. NA, not applicable.

gesting that the additional selective pressure may have led to dominance of the mutant species. The mutant was identical to a previously described in vitro-derived virus, MS412, which is completely resistant to PZ neutralization in vitro and in vivo (35). Given the potential for viral changes during passage in cell culture and the differences we observed between results directly from the lungs compared to cell culture-recovered virus, the results from the virus recovered in cell culture must be interpreted cautiously. Future studies could incorporate more-sensitive RT-PCR assays to amplify directly from the nasal samples without initial recovery in cell culture.

Samples from the lungs at 12 weeks were analyzed by nucleotide sequence determination of F gene RT-PCR products, and the sequence chromatograms revealed mixed populations. Variability was observed at nucleotide 816, revealing the parent A2 virus nucleotide and a mutation identical to the in vitro-derived mutant F212 (35). These changes were not simply due to prolonged in vivo replication, since they were not observed in the control animals.

The mixed genetic populations in the lungs were further defined by analysis of lung RT-PCR products cloned into plasmid vectors (Table 2). Notably, the cloned samples revealed additional complexity of the populations that visual analysis of the chromatograms had not suggested (Fig. 3). Three of the five lungs analyzed revealed abundant mutant populations, including mutations known to be associated with in vitro and in vivo resistance to PZ (36).

Live virus (MCRL5) was recovered in cell culture from the lungs at 12 weeks postinfection from one PZ recipient animal. MCRL5 contained an F gene mutation at base 827, identical to that of mutant MS412, known to resist the prophylactic effects of PZ in cotton rats (35). Interestingly, this mutation was not evident in the chromatogram of the sequence from the lung-derived samples but was observed in one-third of the cloned samples from the lungs of rat A-4. It may be that passage in cell culture prior to analysis allowed dominance of the MS412 type mutation over the other members of the viral population. The PZ resistance of MCRL5 was confirmed in a microneutralization assay (Fig. 4).

The immunosuppression used in this study depletes white blood cells and allows prolonged replication of a virus that would normally be cleared in days (20, 27, 34). However, the animals were not rendered completely immunodeficient, as evidenced by the lack of unexplained animal deaths that may be due to opportunistic infections. Thus, the residual host immune response may also provide selective pressure on the infecting viruses. The infrequent development of F gene mutations and viruses with a PZ-resistant phenotype among the control animals indicated that the mutations that arose in the PZ recipient animals were due to antibody selective pressure.

Studies performed four decades ago revealed that viral variants arise with passage of influenza virus or foot and mouth disease virus through immunized animals (17, 18). Cattle immunized with foot and mouth disease synthetic peptides yield antigenic variants after viral infection (32). Recent studies in immunodeficient mice revealed the rapid evasion of neutralizing monoclonal antibodies by the parvovirus minute virus of mice (24). Thus, both RNA and DNA virus populations rapidly adapt to selective pressures in vivo. In humans, variant hepatitis B viruses appear after active immunization and among liver transplant patients receiving hepatitis B immunoglobulin (7, 28).

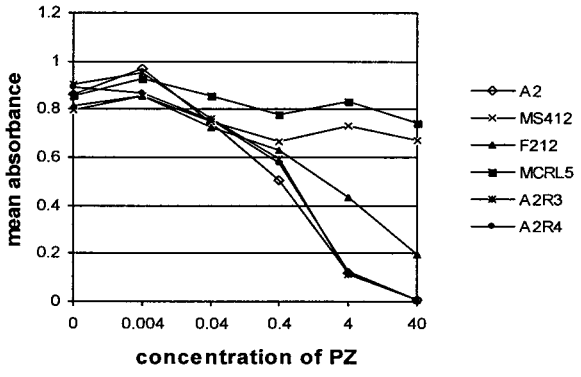

FIG. 4. Resistance of in vivo-derived viruses to PZ neutralization in vitro. The resistance to PZ of the parent A2 and in vitro-derived (MS412 and F212) and in vivo-derived (MCRL5, A2R3, and A2R4) viruses was assessed by microneutralization (36). PZ concentrations are show in micrograms per milliliter on the *x* axis, and mean absorbance (optical density) is shown on the *y* axis.

Circulating wild-type viruses may be resistant to PZ, as shown by the failure of the murine parent to PZ, MAb 1129, to neutralize one clinical isolate (5). Interestingly, a MAb 1129-resistant virus selected in cell culture also had reduced susceptibility to neutralization by polyclonal hyperimmune serum (5). Clinical isolates of RSV were susceptible to neutralization by PZ at a concentration of 400 μg/ml. Analysis of the lungs of cotton rats after PZ prophylaxis revealed the presence of one possibly resistant virus (21). We have shown that PZ-resistant viruses selected in cell culture can also evade PZ prophylaxis in vivo (36).

In preclinical testing, a PZ dose of 2.5 mg/kg resulted in concentrations in serum of 25 to 30 μg/ml and a 99% reduction of viral titers in the lung after RSV challenge in cotton rats (21). The target concentration in humans was chosen as 40 μg/ml based on the results in cotton rats. Children that received 15 mg of PZ/kg had levels in serum of 37 μg/ml after the first dose and >40 μg/ml after repeated monthly doses (19). In cotton rats, 10 mg/kg produced levels in serum of 118 μg/ml and no virus was detected after challenge (21). PZ at 15 mg/kg was equally effective in immunosuppressed and control cotton rats (27). The 15-mg/kg dose given to cotton rats in this study should exceed the levels produced by the same dose in humans and profoundly inhibit viral replication. However, as shown here, even at this dose not all virus was eliminated.

Surveillance of additional clinical isolates for resistance to PZ has been recently reported (10). Three hundred seventy-one viruses were tested, including 25 from PZ recipients. Viruses were initially isolated in cell culture and passed on HEp-2 cells before shipment to a central laboratory for testing. For testing, the viruses were grown in HEp-2 cells, PZ binding was assessed by using an immunofluorescence assay, and all viruses bound PZ. Some isolates were also tested in a microneutralization assay and found to be susceptible to PZ. Whether these viruses required additional passage in cell culture prior to assessment and what concentration of PZ was used to define susceptibility was not stated. In addition, the ability of these assays to detect the presence of PZ-resistant viruses present in a mixture with susceptible viruses is unknown. An immunocompromised child with an RSV infection who received PZ was assessed for the development of PZ-resistant viruses (12). Sequential isolates from the child were passed twice in cell culture prior to PZ binding assessment, and all five isolates tested bound PZ. In addition, F gene RT-PCR products from the cell culture samples were analyzed as a bulk population, and no amino acid changes were found among six sequential isolates.

In the present study, there were notable differences among viruses analyzed directly from the animals compared to those obtained from cell culture. Additionally, nucleotide sequence analysis of bulk RT-PCR products failed to reveal the presence of resistant viruses in a mixed population with susceptible viruses. RSV may accumulate mutations during cell culture passage (15). Influenza virus is known to undergo significant antigenic changes with adaptation to cell culture (29). These data suggest caution in interpreting results from clinical samples of RSV that have undergone cell culture passage prior to analysis.

This report demonstrates that viruses resistant to PZ arise during prolonged replication in the presence of PZ in immunodeficient hosts. Most of the identified mutations were identical to those previously described for viruses selected in cell culture for resistance to PZ. It is unknown whether such mutations will arise in humans receiving PZ and whether such mutant viruses will be clinically important. If the variant viruses that arise are less fit than the parent viruses, then they should be less likely to disseminate. Future studies of viruses resistant to PZ will include assessments of viral fitness. Surveillance for the development of resistant viruses among populations receiving PZ could contribute to our understanding of the use of PZ and of the potential for resistance as antibody products are deployed against other viral infections.

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