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MAJOR ARTICLE

Prevalence and Significance of Substitutions in the Fusion Protein of Respiratory Syncytial Virus Resulting in Neutralization Escape From Antibody MEDI8897

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Background. Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection among infants and young children. To date, no vaccine is approved for the broad population of healthy infants. MEDI8897, a potent anti-RSV fusion antibody with extended serum half-life, is currently under clinical investigation as a potential passive RSV vaccine for all infants. As a ribonucleic acid virus, RSV is prone to mutation, and the possibility of viral escape from MEDI8897 neutralization is a potential concern.

Methods. We generated RSV monoclonal antibody (mAb)-resistant mutants (MARMs) in vitro and studied the effect of the amino acid substitutions identified on binding and viral neutralization susceptibility to MEDI8897. The impact of resistance-associated mutations on in vitro growth kinetics and the prevalence of these mutations in currently circulating strains of RSV in the United States was assessed.

Results. Critical residues identified in MARMs for MEDI8897 neutralization were located in the MEDI8897 binding site defined by crystallographic analysis. Substitutions in these residues affected the binding of mAb to virus, without significant impact on viral replication in vitro. The frequency of natural resistance-associated polymorphisms was low.

Conclusions. Results from this study provide insights into the mechanism of MEDI8897 escape and the complexity of monitoring for emergence of resistance.

Keywords. MEDI8897; monoclonal antibody; neutralization escape; respiratory syncytial virus.

Respiratory syncytial virus (RSV) is a single-stranded, negative-sense ribonucleic acid (RNA) orthopneumovirus belonging to the family *Pneumoviridae* [1]. It is the most common cause of lower respiratory tract infection among infants and young children, resulting in annual epidemics worldwide [2, 3]. Although prevention of RSV illness in all infants has been a major public healthy priority for decades, there are no licensed vaccines [4]. The only currently approved prophylaxis for RSV disease is the RSV fusion (F)-specific humanized murine monoclonal antibody (mAb) palivizumab, which is indicated for use in high-risk pediatric populations and has been used since 1998 [5]. The restrictions implemented by local or national recommending bodies have further reduced the population of high-risk infants receiving palivizumab such that only a small portion of the total birth cohort is provided protection against RSV [5]. Because there is no RSV prophylaxis for most infants, a single intramuscular administration of MEDI8897, an RSV pre-F-specific human mAb engineered to possess extended serum half-life, is being studied clinically as a passive immunization of all infants entering their first RSV season [6–8].

Palivizumab binds a linear epitope in antigenic site A (site II) in RSV pre- and post-F protein [9, 10], whereas MEDI8897 targets a conformational epitope in antigenic site Ø in pre-F protein [6, 11]. The widespread clinical use of a mAb to prevent infection with an RNA virus possessing an error-prone RNA polymerase such as RSV raises potential concerns about emergence of antibody-resistant viruses. Palivizumab resistant viruses have been selected in vitro and in an in vivo animal model [12–16]. Variants containing these resistance-associated mutations were found at a low frequency (<1%) in circulating strains [17] and in patients receiving palivizumab (~5%) [16, 18]. It remains unclear whether selection of antibody-resistant viruses for palivizumab occurs commonly in patients or not. Reduced palivizumab potency against mutant viruses has always been linked to lower binding affinity to RSV F protein containing resistance-associated mutations [19, 20]. Viruses encoding palivizumab resistance-associated mutations showed impaired fitness in vitro [15, 16]. More than 4 million patients have been dosed since its approval in 1998, and there is no report to date showing that palivizumab escape mutants acquire any growth advantage or disseminate into the community. To assess the potential risk for emergence of MEDI8897-resistant viruses, it is important

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to understand the mechanisms of neutralization escape, the potential impact on viral replication, as well as the prevalence of naturally occurring resistance-associated amino acid (a.a.) substitutions in currently circulating RSV strains.

In this study, we report the characterization of in vitro-selected MEDI8897-resistant mutants (MARMs). We identified the critical residues that are associated with reduced binding and susceptibility of virus to MEDI8897 neutralization, and we examined their potential impact on viral growth kinetics in vitro. We investigated the prevalence and significance of naturally occurring resistance-associated polymorphism, which may provide insight into resistance monitoring during the clinical development of RSV mAbs.

METHODS

Cells, Viruses, and Antibodies

HEp-2 cells, RSV MI-A2 and RSV B9320 strains, were originally obtained from American Type Culture Collection. HEp2 cells were passaged in minimal essential medium supplemented with 5% fetal bovine serum, glutamine, and antibiotics. Respiratory syncytial viruses were amplified in HEp2 cells before use. Monoclonal antibody proteins were expressed and purified as described previously [21].

Generation of Monoclonal Antibody-Resistant Mutants

The MARMs were selected using a previously described procedure [16]. In brief, 10 × IC_{50} (half-maximal inhibitory concentration) of MEDI8897 was mixed with a stock of RSV MI-A2 or B9320 and incubated at 37°C for 1 hour. HEp-2 cells were then infected at a multiplicity of infection (MOI) of 0.3–1 plaque-forming units (pfus)/cell followed by 5–7 days incubation. The presence of cytopathic effects was assessed daily using light microscopy. The contents of each well were harvested and used to infect freshly seeded HEp-2 cells as described above. The selection process was repeated 2 more times. The MARMs were biologically cloned 1–2 times by sequential limiting dilutions in 96-well plates containing HEp-2 cells before neutralization testing against MEDI8897.

Reverse-Transcription Polymerase Chain Reaction Amplification, Sequencing, and Conservation Analysis

Ribonucleic acid was extracted, and viral complementary deoxyribonucleic acid (cDNA) corresponding to the entire F gene was amplified and sequenced by Sanger or Miseq Illumina as described previously (primer sequences and conditions are available upon request) [16, 22]. The sequence conservation of the MEDI8897 binding site was evaluated by examining 1158 F protein sequences derived from 573 RSV A and 585 RSV B clinical isolates that were collected from the OUTSMART surveillance program during the 2015–2016 and 2016–2017 RSV seasons from 25 regional laboratories in the United States [22, 23]. Netherlands RSVA/13-005275 (GenBank accession no. KX858757) and Netherlands RSVB/13-001273 (GenBank accession no. KX858756) were selected as references [6]

Generation of Recombinant Variants

The conditions used for recombinant (r)RSV cDNA construction containing the desired mutations and for rescuing rRSV were described previously [24]. The rRSV collected in the culture supernatant was biologically cloned 1–2 times in sequential limiting dilutions in 96-well plates containing HEp-2 cells. The viral clones were amplified 2–3 times in HEp-2 cells and the a.a. changes in the clone were sequence confirmed before characterization.

Microneutralization Assays

The RSV microneutralization assay was carried out as described previously [6]. In brief, RSV was incubated with an equal volume of serially diluted antibodies and incubated for 2 hours at 37°C. HEp-2 cells were added and cultured for 4–5 days. The replication of RSV was determined by quantification of expressed F protein by enzyme-linked immunosorbent assay measured at 450 nm (A450). The neutralizing titer (IC_{50}) is calculated as the antibody concentration that reduced the A450 value by 50%.

Virus Growth Kinetics

Growth kinetics were performed by inoculating HEp-2 cells with RSV or MARM variants at a MOI of 0.1 pfu/cell. The culture supernatant was collected at various time points postinfection, and viral titers were determined by a 50% tissue culture infective dose assay [25].

Kinetic Binding Measurement

The trimeric pre-fusion variants were expressed and purified as described previously [6]. Binding kinetics of F variants to mAb MEDI8897* (a version of MEDI8897 without mutations engineered in the Fc region for extended half-life [6]) were measured on an Octet QK384 (fortèBio). All assays were performed in manufacturer's kinetics buffer at 30°C with 1000 rpm agitation in black 96-well plates (Geiger One). Trimeric F variants (20 μg/mL) were loaded onto HIS1K biosensors for 300 seconds (sec), and Biosensors were then equilibrated in kinetics buffer for 60 sec, followed by mAb association (250–62.5 nM) for 60 sec and binding dissociation for 180 sec. Data analyses and 1:1 global curve fitting were performed using Octet data analysis software (version 6.4).

RESULTS

In Vitro Selection and Genotype Mapping of Respiratory Syncytial Virus Monoclonal Antibody-Resistant Mutants to MEDI8897

A total of 6 RSV MI-A2 and 9 RSV B9320 MARMs were isolated after 3 rounds of selection in HEp-2 cells in the presence of MEDI8897. Analysis of the F protein sequence from these RSV MARMs revealed a single a.a. substitution in all RSV A MARMs in F protein at position 208 from Asn (N) to Tyr (Y). In addition, a cell-culture-adaptive mutation at position 67 from N to Ile (I) was observed in both the parental RSV MI-A2 and the MARMs (Table 1). By contrast, sequence analysis showed that the 9 RSV B9320 MARMs encoded 4 different a.a. substitution patterns in F protein with single

Table 1. Genotypic Changes Selected in the MEDI8897 RSV MARMs and Their Susceptibility to MEDI8897 Neutralization

Subtype	Antibody Used for Selection	Virus	Amino Acid Substitution	Location of Substitutions in F Subunit	Average IC_{50} of MEDI8897 (ng/mL)	Fold Change Relative to Parental Virus in IC_{50}
RSV MI-A2[a]	None	Parental strain	N67I	F2	2.1	1
	MEDI8897	A17-B10	N67I/N208Y	F2, F1	998.3	475
RSV B9320	None	Parental strain	—	—	2.4	1
	MEDI8897	B14-B6	N208S	F1	35095	14623
	MEDI8897	B22-B4	K68N/N201S	F2, F1	13276	5532
	MEDI8897	B15-C2	N208D	F1	>600000	>250000
	MEDI8897	B12-B3	K68N/N208S	F2, F1	>600000	>250000

Abbreviations: F, fusion; IC_{50}, half-maximal inhibitory concentration; MARMs, monoclonal antibody resistant mutants; RSV, respiratory syncytial virus.

[a]Parental RSV MI-A2 laboratory strain contains a putative cell-culture adaptive mutation N67I.

a.a. changes at position 208 from N to Asp (D) or to Ser (S) (N208D or N208S); double substitutions at a.a. position 68 from Lys (K) to N in combination with another a.a. change either at position 201 from N to S (K68N/N201S) or at position 208 from N to S (K68N/N208S) (Table 1). As expected, all a.a. changes identified in these MARMs were located in the MEDI8897 binding site (a.a. 62–69 and 196–212) defined previously by structural determination and showed in Figure 1 [6].

The Phenotypic Susceptibility of Monoclonal Antibody-Resistant Mutants to MEDI8897

The representative MARM variants were clonally purified and evaluated in a microneutralization assay to determine their susceptibility to MEDI8897 neutralization. The MEDI8897-selected RSV MI-A2 clone A17-B10 containing mutations N67I/N208Y exhibited a 475-fold reduction in susceptibility to MEDI8897 compared with the parental RSV MI-A2 containing the cell culture mutation N67I alone (Table 1). The MEDI8897 RSV B9320 MARMs

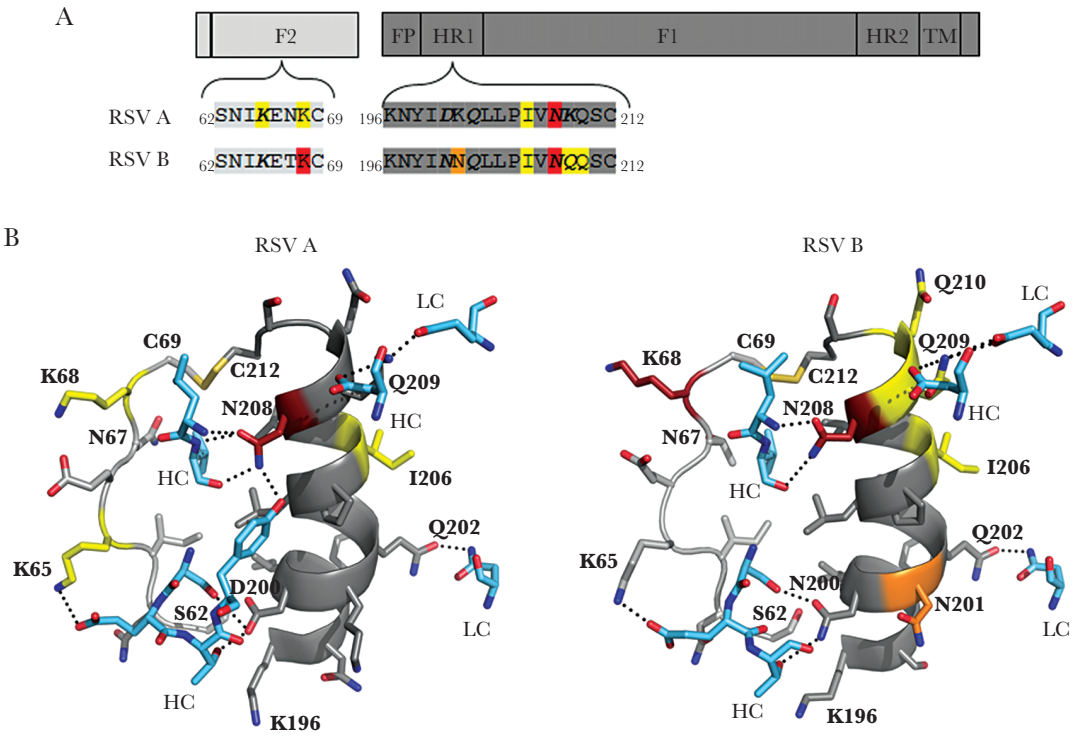

Figure 1. Mapping of residues identified in respiratory syncytial virus (RSV) A2 and B9320 monoclonal antibody-resistant mutants (MARMs) and natural isolates to MEDI8897 binding site. (A) Schematic of mature RSV F2 and F1 polypeptides (light gray and dark gray, respectively) with alignment of A and B strain consensus MEDI8897 binding site sequences. F protein amino acid residues with side chains making hydrogen bonds or salt bridges with MEDI8897 are bolded and in italics. F residues within the MEDI8897 binding site that were identified in MARMs, natural isolates, or in both a MARM and natural isolates are colored red, yellow, and orange, respectively. F2 and F1 polypeptides, fusion peptide (FP), heptad repeat 1 and 2 (HR1 and HR2) and transmembrane (TM) are indicated in the schematic. (B) Structural mapping of amino acid changes in MARMS and natural isolates to the RSV A2 and B9320 strain MEDI8897 binding sites (Protein Data Bank accession nos. 5UDC and 5UDD). F residues coloring is as described above. F protein residues are shown in cartoon with side chains in sticks, whereas MEDI8897 residues making polar contact to F are shown as cyan sticks. Dashed lines indicate polar contacts between the heavy chain (HC) or light chain (LC) MEDI8897 residues and side chains of RSV F residues. Nitrogen and oxygen atoms are colored blue and red, respectively.

Table 2. Susceptibility of Recombinant RSV Variants to MEDI8897 Neutralization

Parental rRSV	Amino Acid Substitution	Location in F Subunit	Average IC_{50} (ng/mL)	Fold Change to Parental Virus
rRSV A2	None	—	1.7	1.0
	N67I	F2	2.6	1.5
	N208Y	F1	1.8	1.1
	N67I, N208Y	F2, F1	174.3	102.5
rRSV B9320	None	—	2.2	1.0
	K68N	F2	8.3	3.8
	N201S	F1	142	64.5
	N208D	F1	>200 000	>90 000
	N208S	F1	54 161	24 618
	K68N, N201S	F2, F1	29 565	13 438
	K68N, N208S	F2, F1	>200 000	>90 000

Abbreviations: F, fusion; IC_{50}, half-maximal inhibitory concentration; rRSV, recombinant respiratory syncytial virus.

exhibited an even more significant reduction in MEDI8897 neutralization. Clone B14-B6 containing the N208S substitution and clone B22-B4 harboring the double mutation K68N/N201S displayed a 14 623-fold and 5532-fold reduction in susceptibility to MEDI8897, respectively, compared with the parental RSV B9320 strain. The RSV B clones B15-C2 and B12-B3 encoding N208D and K68N/N208S exhibited complete resistance to MEDI8897 (>250 000-fold), with no detectable neutralization at the highest MEDI8897 concentration tested (600 µg/mL) (Table 1). As expected, all MEDI8897 RSV A and B MARMs and their parental RSV strains were susceptible to palivizumab with similar IC_{50} values ranging from 70 to 350 ng/mL (data not shown).

Phenotypic Characterization of Recombinant Viruses Containing Amino Acid Substitutions Identified in Respiratory Syncytial Virus Monoclonal Antibody-Resistant Mutants

To assess the effect of the a.a. changes identified in the MARMs on the susceptibility to neutralization by MEDI8897, rRSV variants encoding the substitutions either alone or in combination were generated and evaluated using a microneutralization assay. The a.a. substitutions at position 208 (N208D or N208S) resulted in greatly reduced susceptibility of rRSV B9320 to MEDI8897 neutralization (>90 000- or >20 000-fold, respectively), whereas the reduced susceptibility (>100-fold) of rRSV A2 was only observed in the variant containing N67I/N208Y in combination but not in variants harboring N208Y or N67I alone (Table 2). The rRSV B9320 variants engineered to encode the single a.a. substitutions K68N or N201S exhibited approximately mild to moderate 4- and 65-fold reduction in susceptibility to MEDI8897 neutralization, respectively, compared with the parental wild-type rRSV B9320 virus. However, when the K68N substitution was introduced in combination with N201S or N208S, the resulting viruses exhibited a greater level of resistance, with calculated IC_{50} values that were 10 000-fold or 90 000-fold higher, respectively, than the parental strain (Table 2).

Effects of Resistance-Associated Mutations on Binding of MEDI8897* to Respiratory Syncytial Virus Pre-Fusion Proteins

To investigate the mechanism of RSV antibody resistance, we determined the binding kinetics of MEDI8897* (a version of MEDI8897 without the modification in the Fc region for in vivo half-life extension) to immobilized recombinant RSV A2 or B9320 DS-Cav1 pre-F protein containing the resistance-associated a.a. changes [6]. MEDI8897* exhibited moderately reduced binding affinity to RSV B F containing K68N and N201S, whereas RSV A F protein harboring N208Y and N208Y/N67I mutations had K_D values reduced approximately 5- and 55-fold, respectively, compared with the parental F proteins (Table 3). This reduced binding affinity was largely due to a faster dissociation rate constant, k_{off}. In contrast, no binding activity was detected for MEDI8897* to RSV B F proteins containing the N208D, N208S, or double mutations K68N/N201S and K68N/

Table 3. The Binding Kinetics of MEDI8897* to Immobilized RSV A2 or B9320 DS Cav1 Pre-F Protein Variants Containing Resistance-Associated Amino Acid Changes

RSV Subtype	Amino Acid Substitutions in RSV F DS- Cav1	K_D (nM)	k_a (M^{-1} s^{-1})	k_d (s^{-1})	Fold-Change Relative Parental F in K_D
RSV A2	None	0.12	$3.11 \times 10^5 \pm 8.32 \times 10^3$	$3.74 \times 10^{-5} \pm 4.09 \times 10^{-5}$	1.0
	N67I	0.098	$2.92 \times 10^5 \pm 8.62 \times 10^3$	$2.86 \times 10^{-5} \pm 4.45 \times 10^{-5}$	0.8
	N208Y	0.552	$4.48 \times 10^5 \pm 1.48 \times 10^4$	$2.47 \times 10^{-4} \pm 5.24 \times 10^{-5}$	4.6
	N67I, N208Y	6.67	$9.08 \times 10^5 \pm 8.97 \times 10^4$	$6.05 \times 10^{-3} \pm 2.16 \times 10^{-4}$	55.6
RSV B9320	None	1.22	$4.69 \times 10^5 \pm 1.52 \times 10^4$	$5.71 \times 10^{-4} \pm 5.27 \times 10^{-5}$	1.0
	K68N	14.4	$3.70 \times 10^5 \pm 1.44 \times 10^4$	$5.32 \times 10^{-3} \pm 8.64 \times 10^{-5}$	11.8
	N201S	35.4	$3.26 \times 10^5 \pm 1.74 \times 10^4$	$1.16 \times 10^{-2} \pm 1.79 \times 10^{-4}$	29.0
	N208D	<Limit of Detection			
	N208S	<Limit of Detection			
	K68N, N201S	<Limit of Detection			
	K68N, N208S	<Limit of Detection			

Abbreviations: F, fusion; RSV, respiratory syncytial virus.

N208S that are associated with a greater level of viral resistance (Table 3). As expected, a significant correlation was observed between antibody binding and virus neutralization (Figure 2).

Effects of Resistance-Associated Mutations on In Vitro Replication

To determine whether the MEDI8897 MARMs had altered growth properties, we compared the growth kinetics of parental RSV MI-A2 or B9320 and their derived MARMs in HEp-2 cells. As shown in Figure 3A, the A2 MARM N67I/N208Y did not show enhanced growth kinetics in cell culture compared with the parental MI-A2 N67I strain in repeated studies, nor did the RSVB N208S, N208D, K68N/N201S, or K68N/N208S variants compared with wild-type RSV B9320 (Figure 3B). Overall, the parental A2 or B9320 and their derived MEDI8897 MARMs grew with comparable kinetics to similar peak titers.

Prevalence and Significance of MEDI8897 Resistance-Associated Mutations in Naturally Circulating Strains

The MEDI8897 binding site was previously reported to be highly conserved with most sequence polymorphisms found at a frequency of <1% [6]. This was based on the analysis of available F protein sequence databases derived from naturally circulating RSV strains collected from 1956 to 2014. To further evaluate the sequence conservation of the MEDI8897 binding site, we examined an additional 1158 F protein sequences from 573 RSV A and 585 RSV B clinical isolates that were collected during 2015–2016 and 2016–2017 RSV seasons in the United States, concurrent with MEDI8897 clinical investigations, with particular attention focused on sequence changes associated with MEDI8897 resistance in the MARM analysis.

Amino acid changes identified in the MARMs associated with large shifts in MEDI8897 neutralization susceptibility (N67I/N208Y in RSVA; N208S, N208D, K68N/N208S, and K68N/N201S in RSV B) were not found in any naturally occurring RSV isolates. Although the a.a. change K68N identified in the MEDI8897 RSVB MARMs was not seen in the previous sequence database derived from clinical isolates collected from 1956 to 2014, or among RSV isolates collected in the United States during the 2015–2016 RSV season, it did appear in RSV A F during the 2016–2017 season at the frequency of 2.1% (Table 4) [6]. The resistance-associated F protein mutation

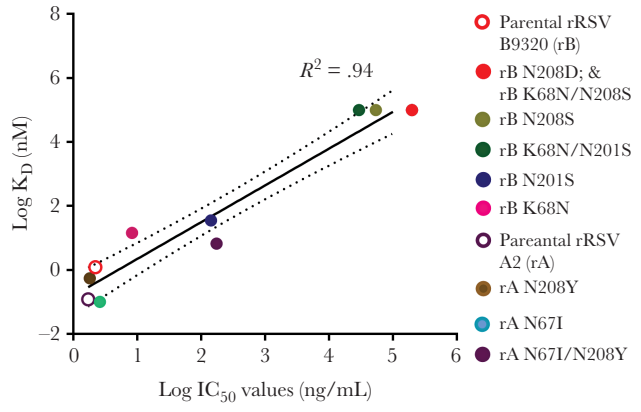

Figure 2. The relationship between MEDI8897 neutralization susceptibility of respiratory syncytial virus (RSV) variants and binding kinetics of MEDI8897* pre-fusion (F) protein variants containing the same amino acid substitutions identified in MEDI8897 monoclonal antibody-resistant mutants. The linear regression analysis was based on the results in Tables 2 and 3. Abbreviations: IC_{50}, half-maximal inhibitory concentration; rRSV, recombinant RSV.

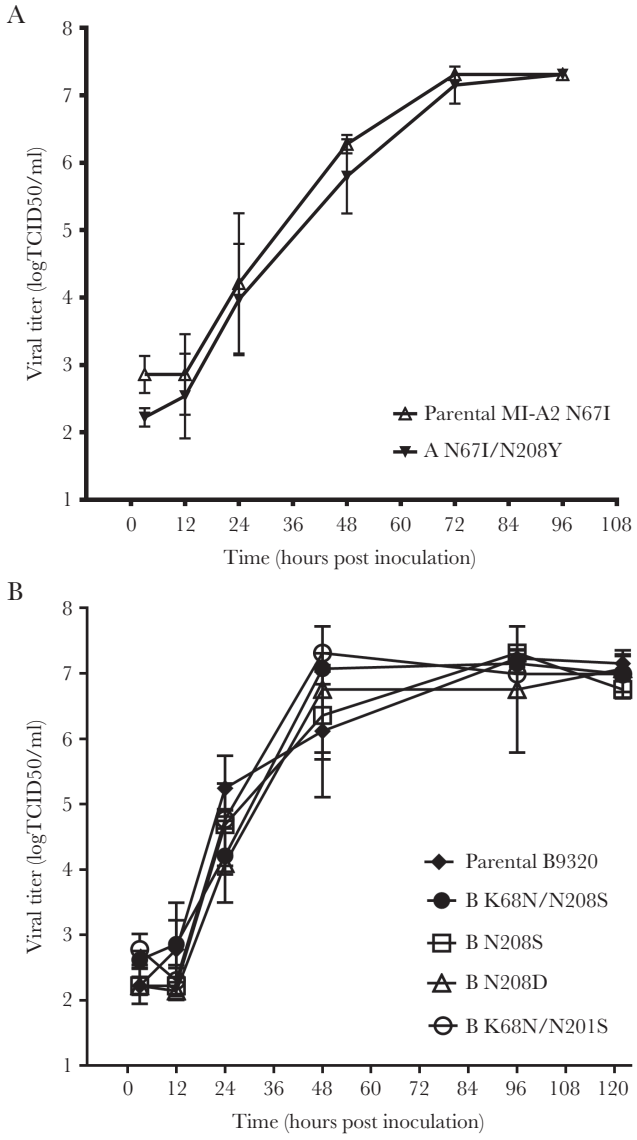

Figure 3. Growth kinetics of parental respiratory syncytial virus (RSV) and mutant viruses. HEp-2 cells were inoculated at a multiplicity of infection of 0.01 plaque-forming units/cell for RSV A and 0.1 pfu/cell for RSV B parental strain or mutant strains. The culture supernatant was sampled daily for 5 days and titered for the presence of virus on HEp-2 cells using a 50% tissue culture infective dose ($TCID_{50}$) assay. (A) Growth kinetics of parental RSV MI-A2 N67I and mutant variant A2 N67I/N208Y. (B) Growth kinetics of parental RSV B9320 and mutant viruses B9320 N208D, B9320 N208S, B9320 K68N/N201S, and B9320 K68N/N208S. Growth capacity is represented as a log value of $TCID_{50}$ units per milliliter on the y-axis.

N201S was found in combination with another sequence polymorphism Q209R in RSV B isolates collected during the 2015–2016 RSV season at a frequency (<1%) similar to that reported previously [6]. We also found a few other natural polymorphisms (K65R, E66D, I206T, Q209K, and Q210H) in the MEDI8897 binding site of RSV at a frequency of ≤1%. However, a novel I206M/Q209R double mutation was found in approximately 19% of RSV B viruses collected in the United States from 17 states during 2016–2017 season.

When tested in the microneutralization assay using clinical and/or recombinant virus, variants containing the natural polymorphisms in the MEDI8897 binding site were all susceptible to MEDI8897 neutralization with average IC_{50} values similar to those obtained with the reference isolates or parental recombinant viruses (Table 4). It is interesting to note that the RSV B clinical isolate harboring the I206M/Q209R mutations exhibited moderately increased susceptibility (>5-fold) to neutralization by MEDI8897 (Table 4), which was confirmed using a recombinant RSV B9320 variant encoding the same I206M/Q209R polymorphisms (Supplementary Table 1).

DISCUSSION

Development of resistance to antiviral agents is considered to be a natural consequence of rapid replication of virus in the presence of selective pressure. Virologic assessment including understanding the potential mechanisms for viral escape are integral to the development of an antiviral drug products. MEDI8897, a highly potent extended half-life antibody, is being developed as a potential passive immunization approach against RSV for all infants. Widespread use of an antiviral mAb with extended half-life presents the potential risk of selecting resistant viruses. Formal proof that changes in virus gene sequences could impact clinical efficacy takes time to establish. However, laboratory data demonstrating that specific sequence changes in viral proteins are associated with loss of drug sensitivity and could impact clinical use have been used as guidance for surveillance and monitoring for the emergence of antiviral resistance in clinical studies.

In the present study, we isolated RSV variants in vitro under selective pressure with MEDI8897 and identified N208D, N208S, K68N/N208S, or K68N/N201S substitutions in the F proteins in RSV B MARMs and N208Y/N67I in RSV A MARMs. All of these a.a. changes are located in the MEDI8897 binding site defined by crystal structure analysis [6]. However, the contribution of each substitution to mAb binding and virus susceptibility to MEDI8897 varied when determined in microneutralization assays. In the crystal structure analysis, F protein residue 208 in both RSV A and B was found to make polar contact with MEDI8897 through a side-chain interaction, and indeed a.a. sequence changes at this position had a profound impact on the ability of MEDI8897 to neutralize RSV B (>10 000-fold change in IC_{50}). However, a change at this position in the F protein of

Table 4. The Frequency of Polymorphisms in the MEDI8897 Binding Site of 2015–2017 RSV Strains and Susceptibility of Variants Encoding These Polymorphisms to MEDI8897[a]

RSV Subtype	RSV Season	No. of Total Samples	Polymorphic Changes in Binding Site	No. of Sequences	Freq. of Polymorphism (%)	Average IC_{50} (ng/mL)	Fold Change to Reference Virus[b]	Reference
RSV A	2015–2016	186	K65R	1	0.5	1.2	0.6	This study
	2016–2017	387	K65R	1	0.3			
			K68N	8	2.1	2.2	1.2	This study
			I206T[c]	4	1	3.2	1	Unpublished data
			WT RSV A isolates	—	—	3.2[d]	1	[6]
			(range)			(0.5–15)		
			rRSV A2	—	—	1.9	1	This study
RSV B	2015–2016	128	Q209K	1	0.8	1.6	0.6	This study
			N201S/Q209K[c]	1	0.8	2.8	1	[6]
	2016–2017	457	E66D[c]	1	0.2	1.2	0.4	[6]
			I206M/Q209R[c]	86	18.8	0.4	0.1	This study
			Q209L	1	0.2	1.8	0.6	This study
			Q210H	1	0.2	2.6	0.9	This study
			WT RSV B isolates	—	—	2.9[e]	1	This study
			(range)			(0.3–59.7)		
			rRSV B9320	—	—	2.8	1	This study

Abbreviations: IC_{50}, half-maximal inhibitory concentration; rRSV, recombinant respiratory syncytial virus; RSV, respiratory syncytial virus; WT, wild type.

[a]IC_{50} values were from 1 or 2 independent experiments, quadruplicate per experiment.

[b]Fold change was calculated by dividing the IC_{50} values of recombinant or clinical variants with the IC_{50} of parental recombinant virus or with median IC_{50} values of a panel of RSV clinical isolates.

[c]IC_{50} determined using clinical isolate containing indicated polymorphism.

[d]Median IC_{50} values from 70 RSV A isolates.

[e]Median IC_{50} values from 49 RSV B isolates.

RSV A (N208Y) affected MEDI8897 neutralization only when combined with another change at position 67 (N67I). It is not obvious from the crystal structure why neutralization of RSV B would be affected to a much greater extent by changes at F position 208 than RSV A. Moreover, it is not apparent from the crystal structure why the N67I/N208Y double variant of RSV A is highly resistant to MEDI8897 neutralization when the single mutants are not. Similarly, the double mutations K68N/N208S and K68N/N201S in RSV B F protein rendered virus more highly resistant to MEDI8897 neutralization than the individual changes alone. However, for both RSV A and B, we did find that the ability of MEDI8897 to neutralize virus encoding different F protein sequence variations was predicted by the ability (or lack thereof) of the mAb to bind pre-F protein containing these sequence changes. The correlation observed between antibody binding and virus neutralization suggests that the primary mechanism of viral resistance is to acquire a.a. changes that prevent or weaken antibody binding, allowing viral escape.

Among viruses currently in circulation, subpopulations may exist with natural resistance to antiviral agents. The outgrowth of these subpopulations can lead to the emergence of resistance and the clinical failure of an antiviral. It was reported previously that palivizumab resistance-associated polymorphisms existed in the natural circulating strains of RSV at a low frequency [17]. However, clinical failure of prophylaxis due to the emergence of palivizumab-resistant virus has not been reported in approximately 20 years of postmarketing analysis. Palivizumab binds the highly stable antigenic site A (or site II) on both the pre-fusion and postfusion forms of the RSV F protein. In contrast, MEDI8897 binds antigenic site Ø in RSV pre-F, which was reported to be the target of most neutralizing antibodies found in human serum in response to natural RSV infection [26, 27]. Although this region might be expected to contain more polymorphic variations than antigenic site A due to natural immune pressure, we previously reported that sequence conservation in the MEDI8897 binding site was high among naturally occurring RSV isolates collected from 1965 to 2014 [6]. Because additional antigenic site Ø variants could emerge over time, we examined contemporary clinical isolates of RSV collected in the United States between 2015 and 2017. We observed some of the same sequence polymorphisms within the MEDI8897 binding site that were previously identified (I206T in RSV A; Q209K, N201S/Q209K, and E66D in RSV B) ([6] and unpublished data), as well as some novel sequence variations (K65R and K68N in RSV A; I206M/Q209R, Q209L, and Q210H in RSV B). All variants appeared at a low frequency except for RSV B encoding the I206M/Q209R double mutation, which was found in approximately 19% of RSV B collected in the United States from 17 states during the 2016–2017 RSV season. It is interesting to note that none of the sequence changes found in the MEDI8897 binding site of contemporary clinical isolates had any impact on MEDI8897 neutralization as determined in microneutralization assays conducted with recombinant viruses encoding the RSV F sequence polymorphisms. It should be noted that the recombinant viruses used in these studies were derived from parental RSV A2 and B9320 viruses isolated almost a half century ago, and therefore the polymorphisms were presented in a F protein sequence context quite different from that found in contemporary RSV strains, especially in RSV A. To investigate the impact of the overall F protein sequence on susceptibility to MEDI8897, we compared MEDI8897 neutralization of recombinant viruses and contemporary clinical isolates containing various antigenic site Ø sequence polymorphisms including the I206M/Q209R mutations in RSV B that were reported previously and in this study [6]. No significant differences in MEDI8897 susceptibility were detected between a subset of natural isolates and recombinant viruses as calculated by the fold-change in IC_{50} values relative to parental virus (Supplementary Table 1). This result suggests that a.a. changes outside the MEDI8897 binding site likely do not contribute to the susceptibility of virus to neutralization by the mAb. Overall, results from this study are consistent with our previous studies that the frequency of viruses with natural resistance to MEDI8897 is low despite the selective pressure exerted by natural human immunity [6].

A retrospective analysis comparing the MEDI8897 sensitivity data and our initial structural predictions of residues most likely to alter MEDI8897 recognition upon mutation did not entirely align. It is known that both polar and nonpolar contacts are important for protein-protein binding and that defining the exact residues critical for the specificity and strength of protein-protein interactions can be very difficult by structure examination alone [28]. The data reported in this manuscript for MEDI8897 binding to RSV F protein indicate that although structural analysis provides insights, functional characterization of neutralizing and/or binding activity is required to fully understand the implications of changes to specific F protein residues.

CONCLUSIONS

In summary, the results from this study provide insights into mechanisms of resistance to MEDI8897 and provide guidance on surveillance and monitoring for emergence of MEDI8897 resistance. Similar to those observed with palivizumab, the frequency of viruses containing natural MEDI8897 resistance-associated mutations was low. In addition, the escape variants and their parental virus replicated at about the same growth rates in vitro, suggesting that the resistance-associated substitutions may not provide growth advantage over the natural circulating strains. Whether the broad use of MEDI8897, which is currently being evaluated in a multinational pivotal Phase 2b study (ClinicalTrials.gov Identifier: NCT02878330), will have impact on the emergence of clinical resistance to MEDI8897 remains to be seen.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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