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Influenza Viruses with Receptor-Binding N1 Neuraminidases Occur Sporadically in Several Lineages and Show No Attenuation in Cell Culture or Mice

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ABSTRACT

In nearly all characterized influenza viruses, hemagglutinin (HA) is the receptor-binding protein while neuraminidase (NA) is a receptor-cleaving protein that aids in viral release. However, in recent years, several groups have described point mutations that confer receptor-binding activity on NA, albeit in laboratory rather than natural settings. One of these mutations, D151G, appears to arise in the NA of recent human H3N2 viruses upon passage in tissue culture. We inadvertently isolated the second of these mutations, G147R, in the NA of the lab-adapted A/WSN/33 (H1N1) strain while we were passaging a heavily engineered virus in the lab. G147R also occurs at low frequencies in the reported sequences of viruses from three different lineages: human 2009 pandemic H1N1 (pdmH1N1), human seasonal H1N1, and chicken H5N1. Here we reconstructed a representative G147R NA from each of these lineages and found that all of the proteins have acquired the ability to bind an unknown cellular receptor while retaining substantial sialidase activity. We then reconstructed a virus with the HA and NA of a reported G147R pdmH1N1 variant and found no attenuation of viral replication in cell culture or change in pathogenesis in mice. Furthermore, the G147R virus had modestly enhanced resistance to neutralization by the Fab of an antibody against the receptor-binding pocket of HA, although it remained completely sensitive to the full-length IgG. Overall, our results suggest that circulating N1 viruses occasionally may acquire the G147R NA receptor-binding mutation without impairment of replicative capacity.

IMPORTANCE

Influenza viruses have two main proteins on their surface: one (hemagglutinin) binds incoming viruses to cells, while the other (neuraminidase) helps release newly formed viruses from these same cells. Here we characterize unusual mutant neuraminidases that have acquired the ability to bind to cells. We show that the mutation that allows neuraminidase to bind cells has no apparent adverse effect on viral replication but does make the virus modestly more resistant to a fragment of an antibody that blocks the normal hemagglutinin-mediated mode of viral attachment. Our results suggest that viruses with receptor-binding neuraminidases may occur at low levels in circulating influenza virus lineages.

The surface of an influenza virus contains about 400 trimers of hemagglutinin (HA) and about 100 tetramers of neuraminidase (NA) (1, 2). For decades, the understanding has been that the viral entry and release functions are partitioned neatly between these two proteins. Specifically, HA binds the virus to cell surface sialic acids and then, after the virus is endocytosed, mediates fusion of the viral and host membranes (3–5). NA is a sialidase that cleaves the same cell surface receptors that can be bound by HA, thereby facilitating the release of free virions from host cells and viral aggregates (6). While enzymatic activity of NA likely contributes to the ability of influenza virus to penetrate airway mucins *in vivo* to reach target cells (7), NA is completely (6) or largely (8) expendable for entry in cell culture.

However, the last few years have seen the characterization of several exceptions to this paradigm of neatly partitioned sialic acid binding and cleaving activities. The most striking of these exceptions is a recently characterized lineage of influenza virus in bats, where HA lacks detectable sialic acid binding activity and NA lacks detectable sialidase activity (9–12). But even in more canonical influenza virus lineages, a number of groups have described mutations that confer receptor-binding activity on NA. The first such mutation to be characterized was D151G in NA of a recent human H3N2 influenza virus (13). D151G alters a key residue in the NA active site, causing the protein to bind with increased affinity but then fail to cleave sialic acid moieties (14). A number of studies have presented evidence suggesting that D151G is not present in circulating viruses but arises in cell culture because it provides NA receptor-binding activity that helps compensate for the reduced HA affinity of recent human H3N2 viruses for many types of sialic acid (13, 15–18). According to this view, D151G is an interesting laboratory artifact that has little relevance for influenza virus in nature—and it certainly seems reasonable to suspect that D151G viruses that lack sialidase activity might be compromised in crucial aspects of their life cycle in natural settings.

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We recently described another mutation that confers receptor-binding activity on NA, this time without greatly compromising sialidase activity (19). Specifically, in a series of experiments with heavily engineered viruses, we found that the mutation G147R confers receptor-binding activity on the NA from the lab-adapted H1N1 strain A/WSN/33 (19). We showed that the G147R A/WSN/33 NA was able to rescue the growth of a virus in which the receptor-binding activity of HA had been eliminated by extensive mutagenesis and that virus-like particles expressing this NA but lacking HA could agglutinate red blood cells (19). Interestingly, G147R is located above rather than directly in the NA active site, and this mutation only slightly decreases sialidase activity in the context of A/WSN/33 (19). These facts suggest that G147R might enable NA to bind receptors without severely compromising its other functions. In tentative support of this notion, we noted that G147R is present in a small number of reported sequences of the human 2009 pandemic H1N1 (pdmH1N1), human seasonal H1N1, and chicken H5N1 lineages.

Here we characterize the effect of G147R on NAs from these three viral lineages. We show that in all three lineages, G147R enables NA to rescue the growth of a virus with binding-deficient HA—yet in all of the cases, the G147R NA retains substantial sialidase activity against the 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) substrate. We perform a detailed characterization of the mutation's effect in pdmH1N1 by reconstructing a virus that contains the HA and NA from a reported G147R isolate. We find that G147 and R147 variants of this virus are indistinguishable in their fitness in cell culture and pathogenesis in mice. However, G147R confers moderate resistance to neutralization by the Fab of a monoclonal antibody against the HA receptor-binding pocket. Overall, these results suggest that the G147R NA receptor-binding mutation occurs sporadically in circulating N1 lineages without greatly compromising replicative fitness.

MATERIALS AND METHODS

Analysis of N1 NA sequences and phylogenetic tree inference. N1 NA sequences in the Influenza Virus Resource (20) were analyzed for the occurrence of G147R. The input data, source code, detailed documentation, and a script enabling replication of the entire computational analysis are available at https://github.com/jbloom/NA_Mutation_Analysis for viewing and download.

Briefly, we downloaded all of the full-length NA coding sequences for pdmH1N1, seasonal H1N1, and chicken H5N1. A total of 14,339 sequences were retrieved. We then aligned these sequences and counted the occurrences of each amino acid identity at site 147 in each lineage.

To build phylogenetic trees, it was necessary to reduce the number of sequences to make the data set computationally tractable. We therefore retained only seasonal H1N1 sequences isolated in 2007 or later (with one exception, all of the G147R isolates fall into this range), only chicken H5N1 sequences isolated in 2004 or later (all of the G147R isolates fall into this range), and all of the pdmH1N1 sequences. For all of the lineages, we retained all of the G147R isolates in the date ranges. For pdmH1N1, we also retained the six unique non-G147R sequences with the highest identity to each G147R isolate plus 10 randomly chosen sequences per year. For seasonal H1N1, we also retained the two unique non-G147R sequences with the highest identity to each G147R isolate plus six randomly chosen sequences per year. For chicken H5N1, we also retained the four unique non-G147R sequences with the highest identity to each G147R isolate plus 10 randomly chosen sequences per year.

After assembling these sequence sets, we inferred phylogenetic trees with CodonPhyML (21) by using the Goldman-Yang 1994 codon substitution model (22) with CF3x4 equilibrium frequencies (23), a single transition-transversion ratio estimated by maximum likelihood, and a single omega estimated by maximum likelihood. Branch supports were calculated by the SH-aLRT method (24). The trees were rooted by using Path-O-Gen (http://tree.bio.ed.ac.uk/software/pathogen/) and visualized by using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). For compact visual display, many of the non-G147R clades are collapsed in the figure shown here.

Reverse-genetic plasmids. We selected three G147R N1 NA sequences from the Influenza Virus Resource (20) for cloning into the pHW2000 reverse-genetic plasmid (25). The three sequences are those reported for A/Finland/614/2009 (pdmH1N1), A/Texas/74/2007 (seasonal H1N1), and A/chicken/Bangladesh/12VIR-7140-3/2012 (chicken H5N1). These sequences were created by either sequential site-directed mutagenesis of existing plasmids or gene synthesis. The NA sequences are shown in Table S1 in the supplemental material. We also created the G147 variant of each NA gene. Additionally, we used site-directed mutagenesis of an existing plasmid to clone the HA sequence reported in the Influenza Virus Resource (20) for A/Finland/614/2009 (pdmH1N1) into pHW2000; the sequence is shown in Table S1 in the supplemental material.

For some of our experiments, we used a binding-deficient HA and the binding-competent HA from which it was derived. Both of these HAs have been described previously (19). Briefly, the binding-deficient HA is that of H3N2 strain X-31 with the following modifications (in H3 sequence numbering): N-linked glycosylation sites added at sites 45, 63, 122, 126, 133, 144, and 246; mutations Y98F, H183F, and L194A at receptor-binding residues; deletion of the receptor-binding proximal loop spanning residues 221 to 228; and mutation K62E in the HA2 stalk (this is the variant referred to as PassMut in reference 19). The paired binding-competent HA used here has all of the added glycosylation sites but does not have the other point mutations or the loop deletion.

For some of our experiments, we generated viruses that packaged green fluorescent protein (GFP) in the PB1 segment and had all of the other internal genes from A/WSN/33. The plasmids for the internal genes for these viruses were pHH-PB1flank-eGFP (26), pHW181-PB2, pHW183-PA, pHW185-NP, pHW187-M, and pHW188-NS (25).

For some of our experiments, we generated viruses containing the internal genes from pdmH1N1 strain A/California/04/2009 (H1N1). The reverse-genetic plasmids for these genes have been described previously (27).

Viral rescue and titer determination. To test whether the G147 and R147 NAs were able to rescue the growth of a virus with a binding-deficient HA (or its binding-competent counterpart), we generated viruses carrying this HA, the indicated NA, PB1 packaging GFP, and all of the other genes from A/WSN/33. These viruses were rescued and propagated in the PB1-expressing 293T and MDCK-SIAT1 cells described in reference 26 by using the protocol described in reference 19. Virus titers were determined by flow cytometry as described in reference 19. Briefly, the transfection supernatants were collected at 72 h posttransfection and used to infect 1×10^5 MDCK-SIAT1-CMV-PB1 cells. The following day, cells were collected and analyzed by flow cytometry to determine the percentage of GFP-positive cells. The amount of virus in the original supernatant was then back-calculated by using the Poisson equation. Viruses containing the binding-competent HA with pdmH1N1 G147 and R147 NA, as well as virus with the binding-deficient HA and pdmH1N1 R147 NA packaging GFP, were also used for oseltamivir neutralization assays as described in reference 19.

To measure the growth kinetics and final titer of the pdmH1N1 virus with either G147 or R147 NA, viruses containing the A/Finland/614/2009 HA and either R147 or G147 pdmH1N1 NA along with the internal genes from A/California/04/2009 were rescued in cocultures of 293T and MDCK-SIAT1 cells as previously described. At 72 h posttransfection, viral supernatants were collected and titers were determined with the 50% tissue culture infective dose ($TCID_{50}$) assay. The Reed-Muench formula (https://github.com/jbloom/reedmuenchcalculator) was used to calculate the titer in $TCID_{50}$s/μl.

Measurement of NA surface expression, sialidase activity, and oseltamivir inhibition. The NA genes were cloned into an expression vector under the control of a cytomegalovirus (CMV) promoter with a C-terminal V5 epitope tag, followed by an internal ribosome entry site driving the expression of GFP to quantify transfection efficiency. All of the assays were performed with transfected 293T cells as described in reference 19.

Monoclonal antibody and Fab against HA. The IgG of human anti-HA monoclonal antibody 5J8 was produced as described in reference 28. The concentration was determined with Protein Determination Reagent (product no. GR133-500; Hoefer, Inc.). Full-length purified IgG was cleaved into Fab fragments with the Pierce Fab Preparation kit (product no. 44985; Thermo Scientific). Fab fragments were buffer exchanged into phosphate-buffered saline (PBS) with Slide-A-Lyzer MINI Dialysis Devices with a molecular weight cutoff of 20,000 (product no. 88405; Thermo Scientific) prior to neutralization assays.

GFP-based viral neutralization assays. All of the neutralization assays were performed with the viruses expressing GFP in the PB1 segment to quantify viral infection. Assays were read after 18 h before secondary infection caused spread of GFP fluorescence. The procedures are described in detail in reference 19.

Viral growth curves. To determine the growth kinetics of pdmH1N1 variants with the R147 and G147 NAs, MDCK-SIAT1 cells were plated in six-well dishes at a density of 2×10^5/well in influenza virus growth medium (19) with 4 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin. Cells were allowed to adhere for 4 h at 37°C and then inoculated at a multiplicity of infection (MOI) of 0.01. Every 24 h, an aliquot of the supernatant was collected and frozen at −70°C and the volume removed was replaced with an equal amount of fresh medium. All of the aliquots for a given time point then were thawed at the same time and titers were determined by $TCID_{50}$ assay.

For growth kinetics in A549 cells (ATCC CCL-185), 2×10^5 cells/well were plated in six-well dishes in D10. The plates were placed in a 37°C incubator for 6 h to allow cells to adhere. The medium was then changed to influenza virus growth medium with 4 μg/ml TPCK-trypsin following a wash with 1 ml PBS. The cells were then infected at an MOI of 0.1. Aliquot collection and titer determination were done as described for MDCK-SIAT1 cells.

Mouse experiments. Female BALB/cJ mice 8 weeks of age were purchased from The Jackson Laboratory. Mice were anesthetized by intraperitoneal injection of 2 mg of ketamine and 0.2 mg of xylazine in a final volume of 0.2 ml per mouse and then inoculated intranasally with a 20-μl volume containing 10^4 or 10^5 $TCID_{50}$s of pdmH1N1 carrying either the R147 or the G147 NA in the pdmH1N1 background. Control mice were inoculated in a similar fashion with PBS. Each treatment group consisted of three animals. The weight of each infected animal was monitored for 14 days after infection and reported as a percentage of the starting weight on the day of infection. Animal work was approved under Fred Hutchinson Cancer Research Center (FHCRC) IACUC protocol 1893.

RESULTS

NAs with G147R are found in multiple N1 viral lineages. We have described previously how G147R confers receptor-binding activity on the NA of lab-adapted H1N1 strain A/WSN/33 (19). In that previous work, we also noted that G147R is present in some reported human seasonal H1N1, pdmH1N1, and chicken H5N1 sequences.

To examine this issue more systematically, we analyzed all of the ~14,000 NA sequences in the Influenza Virus Resource (20) from seasonal H1N1, pdmH1N1, and chicken H5N1. Table 1 shows that the vast majority of sequences in all three lineages contain G147; however, we did find sequences with G147R and G147E in all of the lineages.

We next sought to assess how the G147R sequences are related to each other and to sequences lacking this mutation. In our previous work, we reported that phylogenetic trees inferred with NA protein sequences indicate that many of the G147R sequences are evolutionarily related (19)—however, protein-based (and nucleotide-based) phylogenetic trees are less accurate than those inferred by using codon sequences (29). To infer evolutionary relationships with maximal accuracy, we therefore used CodonPhyML (21) to construct phylogenetic trees by using a codon model of substitution (22). These trees are shown in Fig. 1. The G147R sequences are not monophyletic in any of the lineages, indicating that the occurrences of this mutation were not all due to a single evolutionary event. However, there are clusters of G147R sequences in both seasonal H1N1 and chicken H5N1, suggesting that related viruses with this mutation may have persisted in natural populations for a sufficient duration to be isolated multiple times.

TABLE 1 Counts of different amino acids at NA position 147 in seasonal H1N1, pdmH1N1, and chicken H5N1 sequences in the Influenza Virus Resource

Viral lineage	No. of sequences with:		
	G147	R147	E147
Seasonal H1N1	4,654	20	0
pdmH1N1	8,400	3	5
Chicken H5N1	1,242	8	2

Overall, these computational analyses support the notion that viruses with G147R NAs occur sporadically in multiple influenza virus lineages. However, computational analyses alone cannot determine whether this mutation confers receptor-binding activity on these NAs in the same way that it does for the lab-adapted A/WSN/33 virus, nor can they ascertain definitively whether G147R was really present in circulating viruses or simply arose as a lab or sequencing artifact. We therefore used our computational results to design the experiments described in the rest of this report.

G147R NAs from seasonal H1N1, pdmH1N1, and chicken H5N1 rescue the growth of a virus with a binding-deficient HA. Previously, we showed that G147R enabled the NA of A/WSN/33 to rescue the growth of a virus in which the receptor-binding activity of a HA had been obliterated by a combination of engineered point mutations and a deletion (19). To test if G147R NAs from pdmH1N1, seasonal H1N1, and chicken H5N1 possess similar activity, we selected one representative R147 NA from each lineage in Fig. 1. We selected A/Finland/614/2009 from pdmH1N1, A/Texas/74/2007 from seasonal H1N1, and A/chicken/Bangladesh/12VIR-7140-3/2012 from chicken H5N1. We cloned these R147 NAs into reverse-genetic plasmids (25) and also created an otherwise isogenic G147 version of each NA.

We then used reverse genetics in an attempt to rescue viruses with each NA paired with either our binding-deficient HA or the binding-competent HA from which it was derived (19). We efficiently rescued viruses with the binding-competent HA paired with either the R147 or the G147 NA for all three lineages that replicated to high titers (Fig. 2). However, we could efficiently rescue only viruses with the binding-deficient HA paired with the R147 NA—the binding-deficient HA did not support appreciable viral growth when paired with the G147 NA (Fig. 2). These results suggest that G147R confers receptor-binding activity on the NAs of pdmH1N1, seasonal H1N1, and chicken H5N1, much as it does for the A/WSN/33 NA.

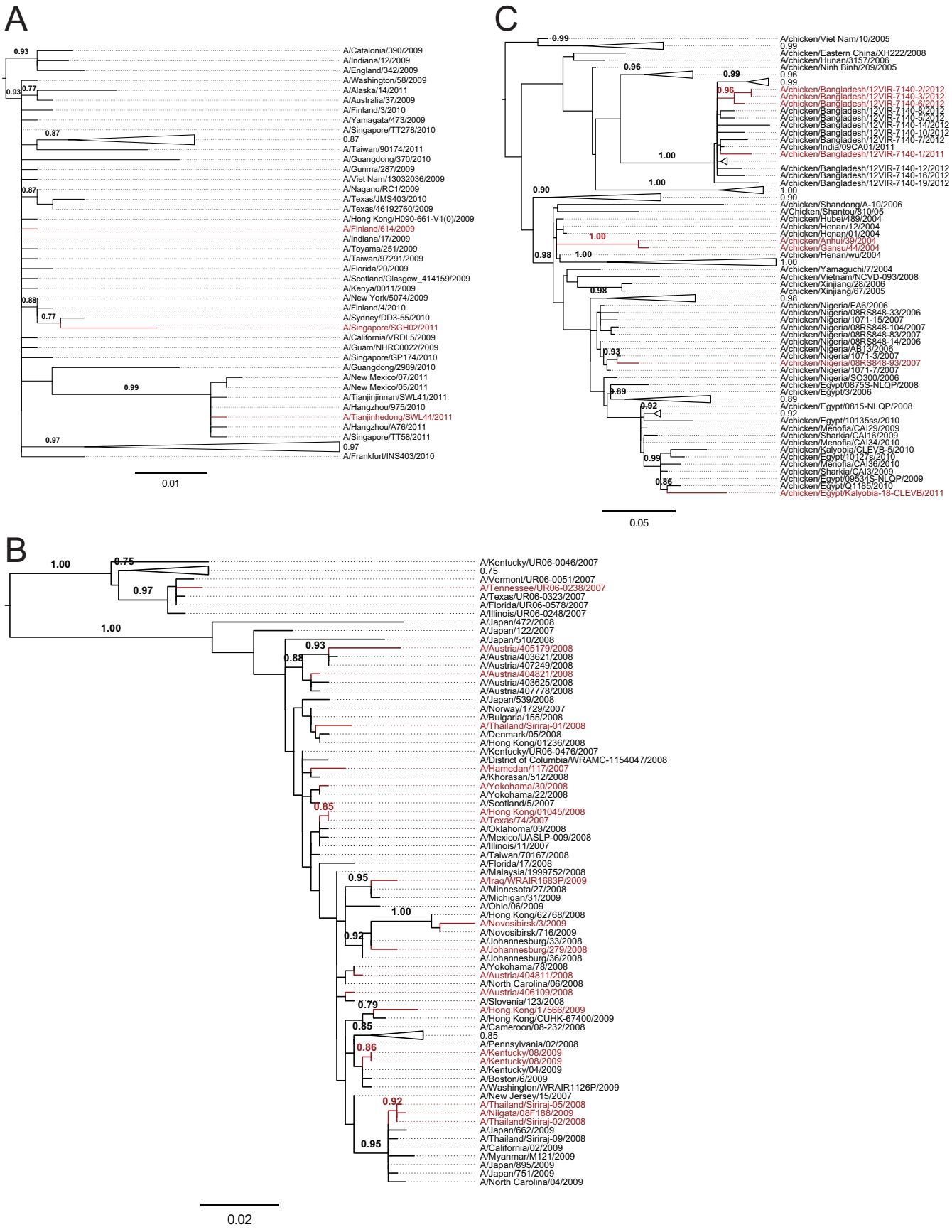


FIG 1 Phylogenetic trees showing sequences with G147R (red) in pdmH1N1 (A), seasonal H1N1 (B), and chicken H5N1 (C). Branch supports are shown for key nodes; scale bars are in numbers of codon substitutions per site. The code and data used to generate these trees are available at https://github.com/jbloom/NA_Mutation_Analysis.

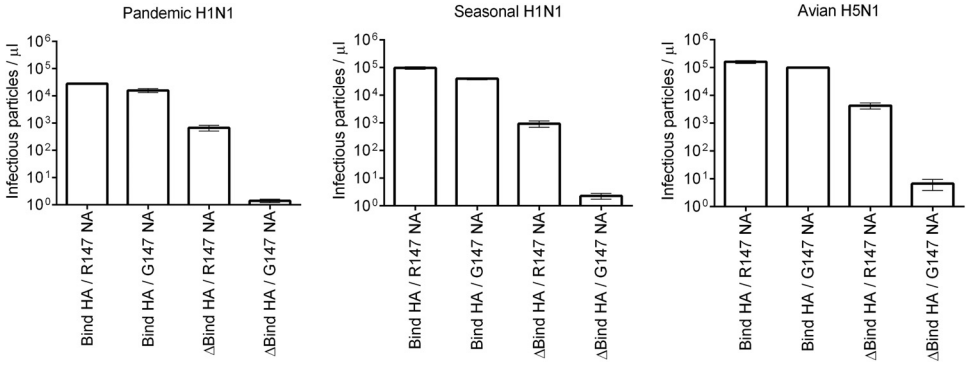


FIG 2 G147R NAs from all three viral lineages rescue the growth of virus with a binding-deficient HA. We used reverse genetics (25) to attempt to rescue viruses with either a binding-competent (Bind) or a binding-deficient (ΔBind) HA (19), the indicated G147 or R147 NA, and internal genes from A/WSN/33 with GFP packaged in the PB1 segment (26). Shown are the viral titers in the supernatant at 72 h posttransfection as determined by flow cytometry for GFP-positive cells. Virus with the binding-competent HA can be rescued efficiently with either the G147 or the R147 NA, but virus with the binding-deficient HA is rescued efficiently only with the R147 NA. Shown are the mean values and standard errors of three independent replicates.

All of the G147R NAs are active sialidases that are inhibited by oseltamivir. One way that a mutation might confer receptor-binding activity on NA is by inhibiting its sialidase activity, so that the NA protein binds sialic acid moieties in its active site but then fails to cleave them. This receptor-binding mechanism is used by D151G, which appears to arise in the NAs of recent human H3N2 NAs upon passage in cell culture (13, 15–18). D151G N2 NAs have a markedly increased affinity for sialic acid (an ~30-fold lower Michaelis-Menten K_m) and a dramatically decreased rate of substrate cleavage (a >100-fold lower Michaelis-Menten k_{cat}) (14). In contrast, G147R has only modest effects (less than 2-fold) on the Michaelis-Menten enzymatic parameters of the A/WSN/33 NA on the surrogate substrate MUNANA.

To test the effect of G147R on the NAs of the seasonal H1N1, pdmH1N1, and chicken H5N1 viruses, we cloned the NA coding sequences for the R147 and G147 variants into an expression plasmid with a C-terminal V5 epitope tag. We then quantified the effect of G147R on both the cell surface NA protein expression and NA-mediated cleavage of the MUNANA substrate in transfected 293T cells (Fig. 3A and B). In all three lineages, G147R had only a small effect (less than 1.5-fold) on NA surface expression. In pdmH1N1 and seasonal H1N1, G147R also had only a small effect (less than 2-fold) on K_m and k_{cat}. In chicken H5N1, G147R did cause a more substantial (about 4-fold) decrease in enzymatic activity—but this effect still remains small compared to that observed for D151G in N2 NAs (14). Therefore, G147R has only a modest effect on the sialidase activity of the NAs of all three viral lineages on the MUNANA substrate.

We also tested whether G147R affects the inhibition of NA activity by the small-molecule drug oseltamivir (Fig. 3C). All of the G147 and R147 NAs were potently inhibited by oseltamivir, demonstrating that G147R is not an oseltamivir resistance mutation.

Oseltamivir inhibits the infectivity of a virus that depends on pdmH1N1 G147R NA for attachment. We focused the remainder of our studies on G147R in the NA of pdmH1N1, since this viral lineage is currently circulating in humans. In our previous work, we showed that the G147R NA of the A/WSN/33 strain mediates receptor binding via a mechanism that is dependent on the NA active site. To confirm that the same mechanism is also used by the G147R NA of pdmH1N1, we grew viruses containing our binding-deficient HA (19) and the receptor-binding R147 NA or viruses containing the matched binding-competent HA and either the R147 or the G147 NA. We then tested whether oseltamivir inhibited the infectivity of these viruses.

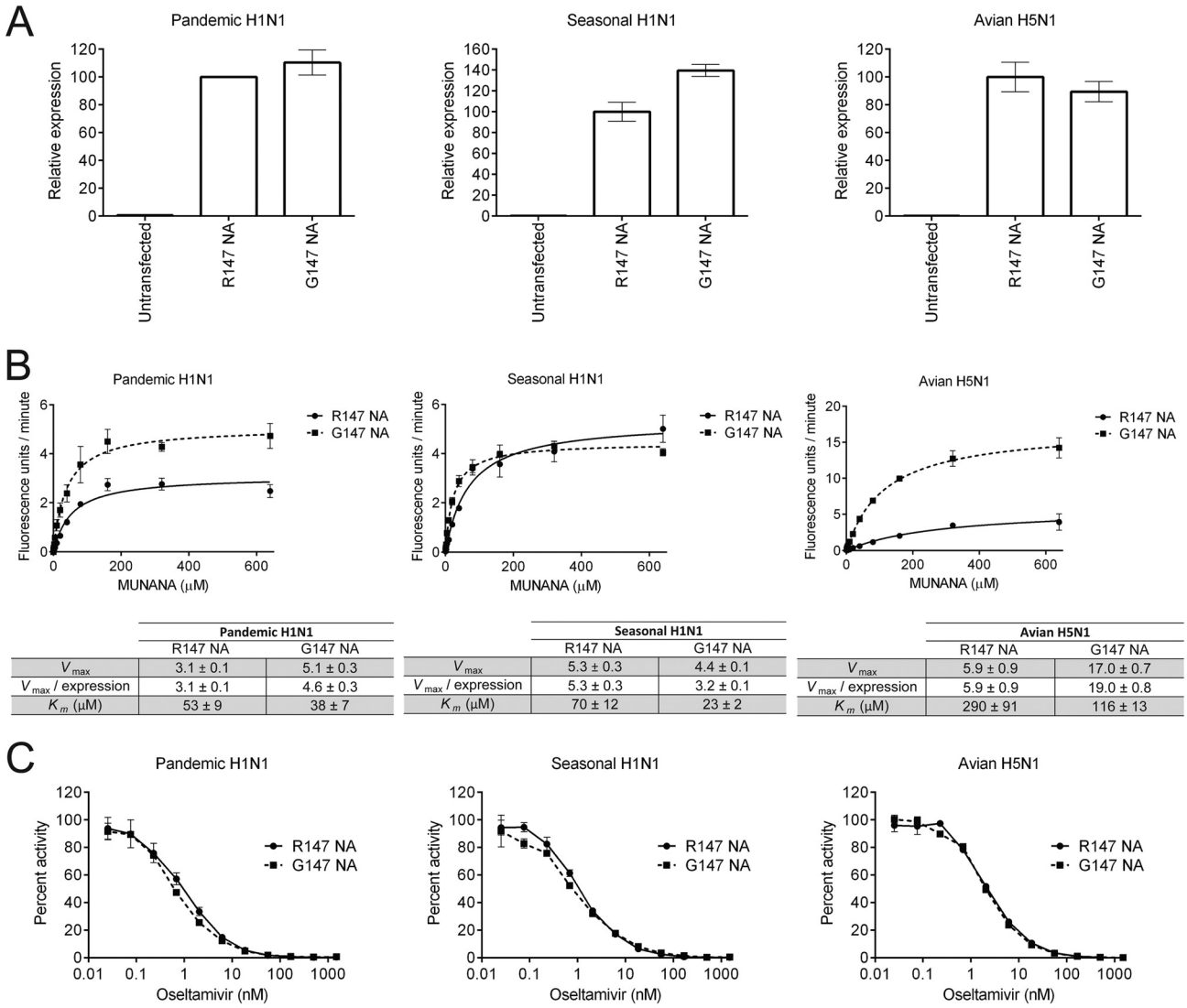
The infectivity of viruses with a binding-competent HA and the G147 NA was unaffected by oseltamivir (Fig. 4), a finding consistent with the accepted view that oseltamivir does not affect the cell culture infectivity of viruses that attach to cells via HA (6). However, oseltamivir ablated the infectivity of viruses with the binding-deficient HA and the R147 NA (Fig. 4), consistent with the idea that these viruses attach to cells via a mechanism that involves the NA active site. The infectivity of viruses with a binding-competent HA and the R147 NA was inhibited partially by oseltamivir (Fig. 4), consistent with the idea that these viruses can attach to cells via HA or NA.

G147 and R147 NA pdmH1N1 viruses grow similarly in cell culture. We next examined the effect of G147R in the authentic background of pdmH1N1 viruses. There are pdmH1N1 strains in the Influenza Virus Resource that lack G147R but have HA and NA protein sequences that are otherwise identical to those of A/Finland/614/2009. We used reverse genetics to generate viruses that had the A/Finland/614/2009 HA, either the R147 A/Finland/614/2009 NA or its G147 counterpart, and the internal genes from closely related pdmH1N1 strain A/California/04/2009.

To test the growth of these viruses in cell culture, we inoculated MDCK-SIAT1 cells at a low MOI and then monitored viral titers in the supernatant (Fig. 5A). The growth kinetics and peak titers of the G147 and R147 variants were nearly indistinguishable.

We next similarly determined growth curves in the human lung epithelial cell line A549 (Fig. 5B). Although the R147 NA variant appeared to grows slightly better than the G147 NA variant, this result was not statistically significant when a *t* test with *P* value cutoff of 0.05 was applied. Taken together, these results show that, at least in cell culture under the conditions tested, G147R does not have a strong effect on viral fitness.

G147 and R147 pdmH1N1 variants are similar in pathogenesis in mice. To test if G147R affects viral pathogenesis *in vivo*, we inoculated mice with 10^4 or 10^5 $TCID_{50}$s of either the G147 or the R147 pdmH1N1 virus and monitored them for weight loss over 14 days (Fig. 6). At both doses, both viral variants caused acute weight loss that peaked at 3 days postinfection, with all of the animals largely recovering within 14 days. There was no signifi-



	Pandemic H1N1	
	R147 NA	G147 NA
V_{max}	3.1 ± 0.1	5.1 ± 0.3
V_{max} / expression	3.1 ± 0.1	4.6 ± 0.3
K_m (μM)	53 ± 9	38 ± 7

	Seasonal H1N1	
	R147 NA	G147 NA
V_{max}	5.3 ± 0.3	4.4 ± 0.1
V_{max} / expression	5.3 ± 0.3	3.2 ± 0.1
K_m (μM)	70 ± 12	23 ± 2

	Avian H5N1	
	R147 NA	G147 NA
V_{max}	5.9 ± 0.9	17.0 ± 0.7
V_{max} / expression	5.9 ± 0.9	19.0 ± 0.8
K_m (μM)	290 ± 91	116 ± 13

FIG 3 G147R NAs are active sialidases that are inhibited by oseltamivir. Cell surface expression (A), sialidase activity (B), and sialidase inhibition (C) by oseltamivir for each R147 NA and its G147 counterpart. The NAs were cloned into an expression plasmid with a C-terminal V5 epitope tag and transfected into 293T cells, which were analyzed at 20 h posttransfection for cell surface NA expression and sialidase activity. G147R had little effect on cell surface expression or sialidase inhibition by oseltamivir. G147R did cause a modest decrease in the sialidase activity of pdmH1N1 and a fairly sizable decrease in that of chicken H5N1, but in all of the cases, NA retained substantial enzymatic activity. Shown are the mean values and standard errors of three independent replicates.

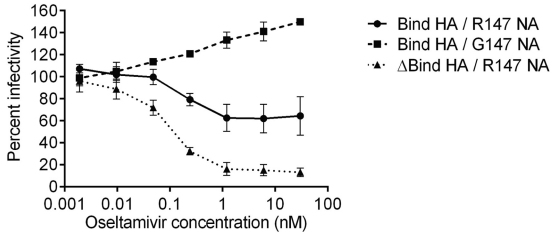


FIG 4 Oseltamivir inhibits the infectivity of a virus dependent on the pdmH1N1 G147R NA for receptor binding. We used reverse genetics (25) to create viruses with a binding-competent (Bind) HA and either the R147 or the G147 pdmH1N1 NA or with a binding-deficient (ΔBind) HA and the R147 pdmH1N1 NA (a virus with the binding-deficient HA and the G147 NA cannot be created [Fig. 2]). The internal genes were from A/WSN/33, with GFP packaged in the PB1 segment (26). We inoculated target cells with these viruses in the presence of increasing concentrations of oseltamivir and quantified the amount of infection by GFP fluorescence. Shown are the mean values and standard errors of three independent replicates.

cant difference in weight loss between animals infected with the two viral variants, indicating that G147R does not strongly affect pathogenesis in mice.

G147R-mediated NA receptor binding increases resistance to neutralization by an anti-HA Fab but not a full-length IgG. The dominant form of protective anti-influenza virus immunity is thought to be antibodies against the globular head of HA (30, 31). A major mechanism by which such antibodies neutralize influenza virus is steric blocking of the binding of HA to cellular receptors (32–34). In principle, receptor-binding G147R NAs could enable influenza virus to evade such antibodies by providing an alternative mechanism of cellular attachment.

To test this idea, we performed neutralization assays with the anti-HA human monoclonal antibody 5J8, which inserts its complementarity-determining region 3 loop into the receptor-binding pocket of the pdmH1N1 HA (28). The full-length 5J8 IgG

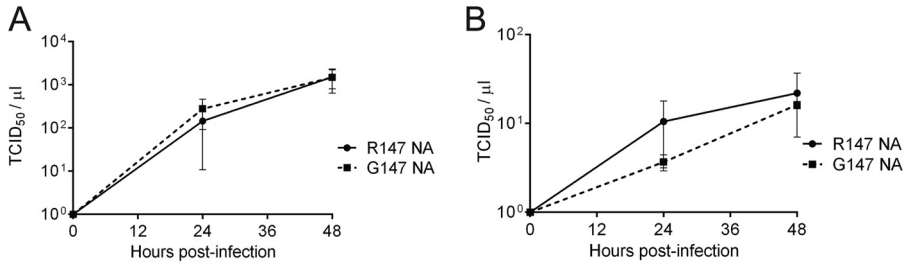


FIG 5 Variants of pdmH1N1 with G147 and R147 NAs exhibit indistinguishable growth kinetics in cell culture. Viruses with the HA from A/Finland/614/2009 and either the R147 NA of this strain or its G147 counterpart were rescued by reverse genetics with the internal genes from A/California/04/2009. MDCK-SIAT1 (A) or A549 (B) cells were then inoculated at an MOI of 0.01 or 0.1, respectively, and viral titers in the supernatant were determined at 24 and 48 h postinoculation by $TCID_{50}$ assay. Shown are the mean values and standard errors of three independent replicates. None of the differences between the G147 and R147 data points are significant according to a *t* test with a *P* value cutoff of 0.05.

neutralized both the G147 and R147 NA variants of pdmH1N1 with equal potency (Fig. 7A). We speculated that the inability of the R147 NA to provide resistance to neutralization by 5J8 might be because binding of this antibody to HA sterically inhibited receptor binding by NA. We therefore cleaved the full-length IgG to produce antigen-binding Fab domains that have a smaller steric footprint. Indeed, the R147 variant exhibited modestly (about 5-fold) greater Fab neutralization resistance than its G147 counterpart (Fig. 7B), indicating that the receptor-binding NA provided a partially redundant mechanism of attachment after Fab binding to HA.

To confirm that this effect was real and reproducible, we repeated the neutralization assays with a new preparation of antibody and Fab. The R147 variant again was about 5-fold more resistant to the Fab (Fig. 7C and D), confirming that the increased neutralization resistance is a genuine, if modest, effect.

If the increased resistance of the R147 variant to Fab neutralization is due to NA-mediated attachment, this effect should be abrogated by the addition of oseltamivir to block NA receptor binding. To test this idea, we performed neutralization assays in the presence of 100 nM oseltamivir, a concentration well above that which we showed in Fig. 4 is sufficient to completely inhibit the infectivity of R147 NA viruses with a binding-deficient HA. As expected, oseltamivir increased the sensitivity of the R147 pdmH1N1 to the anti-HA Fab to a level comparable to that of its G147 counterpart (Fig. 7D). Since oseltamivir alone did not neutralize pdmH1N1 variants with either G147 or R147 (Fig. 7E), this result indicates that R147 increases resistance to the anti-HA Fab by enabling NA to provide a partially redundant mechanism of viral attachment.

DISCUSSION

Prior work has clearly demonstrated that single amino acid mutations are sufficient to transform NA into a receptor-binding protein (13–15, 19). The question that we have attempted to address here is whether viruses with such receptor-binding NAs have the potential to be anything more than interesting artifacts that can arise occasionally when influenza viruses are passaged in the lab.

We focused our studies on the NA receptor-binding mutation G147R, which is found at low frequency in the reported sequences of viruses from several lineages with N1 NAs. The fact that a few of the occurrences of this mutation involve small groups of sequences that cluster on phylogenetic trees weakly suggests that this mutation might sometimes persist long enough in natural settings to be transmitted among several hosts. However, it also remains possible that the G147R sequences in public databases represent sequencing errors or variants selected during lab passage prior to sequence analysis. Because we do not have access to unpassaged samples of any of these reported G147R isolates, we instead investigated this mutation by experimentally characterizing its effect.

We found that G147R confers receptor-binding activity on NAs from three lineages: pdmH1N1, seasonal H1N1, and chicken H5N1. In all of the cases, the G147R NAs were active sialidases, recapitulating our earlier findings with the lab-adapted A/WSN/33 strain that G147R receptor binding does not come at the cost of enzymatic function (19). The exact mechanism of G147R-mediated receptor binding therefore remains unclear. G147R receptor binding clearly involves the NA active site since it is inhibited by oseltamivir, but it is not due to the simple elimination of catalytic activity that underlies the receptor-binding mechanism

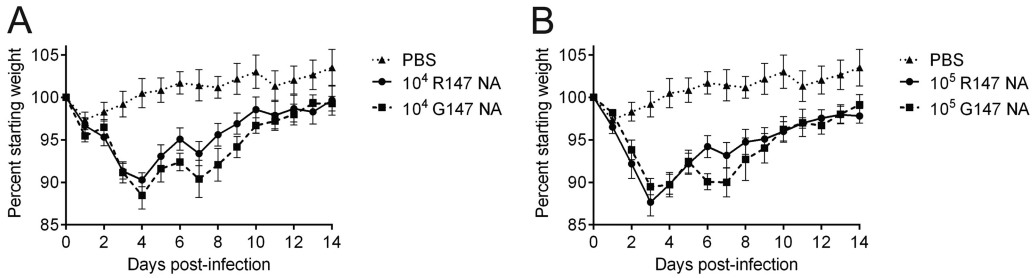


FIG 6 Variants of pdmH1N1 with G147 and R147 NAs are similar in pathogenesis in mice. Mice were inoculated intranasally with 10^4 (A) or 10^5 (B) $TCID_{50}$s of virus or with PBS. Animal weights were recorded for 2 weeks following infection. Each treatment group consisted of three animals, and the plots show the mean value and standard error of each group. None of the differences between the G147 and R147 groups are significant according to a *t* test with a *P* value cutoff of 0.05.

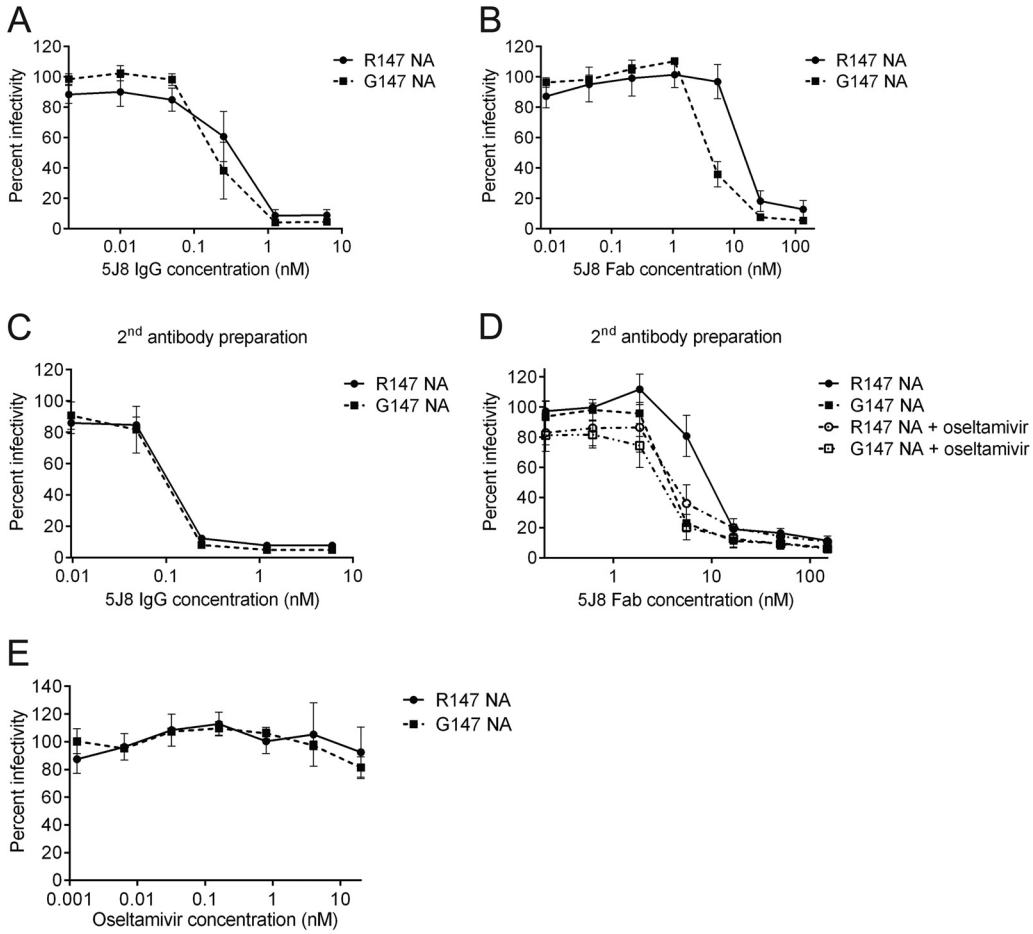


FIG 7 A pdmH1N1 virus with the receptor-binding G147R NA has modestly increased resistance to neutralization by the Fab of an antibody that targets the HA receptor-binding pocket but no change in sensitivity to the full-length IgG. (A) The full-length IgG neutralizes R147 and G147 variants of pdmH1N1 with equal potency. (B) However, the variant with the receptor-binding R147 NA has increased resistance to the antibody Fab. (C, D) These results were repeatable with a second independent antibody preparation. Oseltamivir abrogates the increased neutralization resistance of the R147 NA variant to the Fab (D), but oseltamivir alone has no neutralizing activity (E).

of the D151G mutation to N2 NAs (14). As discussed in our previous work (19), we hypothesize that G147R causes NA to bind to some specific glycan structure that it then cleaves inefficiently, thereby enabling the protein to promote viral attachment while at the same time cleaving its normal sialic acid substrates. However, rigorous testing of this hypothesis requires new experimental approaches that can provide more than the circumstantial evidence presented in our present and previous (19) work.

To assess whether G147R affects viral fitness, we reconstructed a pdmH1N1 virus with the HA and NA of an isolate reported to carry this mutation. We did not detect an effect of G147R on viral replication in cell culture or on pathogenesis in mice—indeed, the only phenotype of G147R that we were able to detect was a modest increase in resistance to neutralization by an anti-HA Fab. We did not test the effect of G147R on virus transmission, so it is possible that G147R still exerts a deleterious effect there. Nonetheless, the fact that G147R is compatible with efficient viral replication supports the idea that this mutation may authentically occur in some naturally occurring influenza virus strains. However, G147R does not appear to play an essential role in the cellular attachment of such viruses, since the HA of the G147R isolate that we characterized is fully sufficient to support viral growth without this NA receptor-binding mutation.

If NA receptor-binding mutations do indeed occur in circulating viruses, perhaps the most biomedically relevant question is whether they alter viral sensitivity to anti-HA antibodies. Our results suggest that any increased resistance to anti-HA antibodies in viruses with G147R is modest. The G147R pdmH1N1 variant is about 5-fold more resistant to an anti-HA Fab, but it has no additional resistance to the full-length IgG. The most likely explanation is that large antibodies bound to HA sterically inhibit NA-mediated cellular attachment; such antibodies might also impair viral fusion or promote intracellular neutralization. In any case, our results suggest that NA receptor-binding mutations are unlikely to cause wholesale changes in the effectiveness of anti-HA humoral immunity, although they might lead to mild changes in sensitivity to certain antibodies.

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