Influenza Virus Neuraminidase Contributes to Secondary Bacterial Pneumonia

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Secondary bacterial pneumonia is a common cause of death during influenza epidemics. We hypothesized that virus-specific factors could contribute to differences in annual excess mortality. Recombinant influenza viruses with neuraminidases from representative strains from the past 50 years were created and characterized. The specific level of their neuraminidase activity correlated with their ability to support secondary bacterial pneumonia. Recombinant viruses with neuraminidases from 1957 and 1997 influenza strains had the highest level of activity, whereas a virus with the neuraminidase from a 1968 strain had the lowest level of activity. The high level of activity of the neuraminidase from the 1957 strain, compared with that of other neuraminidases, more strongly supported the adherence of Streptococcus pneumoniae and the development of secondary bacterial pneumonia in a mouse model. These data lend support to our hypothesis that the influenza virus neuraminidase contributes to secondary bacterial pneumonia and subsequent excess mortality.

Influenza A virus causes epidemics annually and pandemics several times a century. Two subtypes of influenza A virus, H3N2 and H1N1, are presently circulating in the human population. Epidemics caused by H3N2 are associated with higher mortality in human populations than are epidemics caused by H1N1 or influenza B virus [1, 2]. Viruses that cause pandemics are defined as those that acquire a new hemagglutinin (HA), have a high attack rate, and spread globally. Infection with them is generally considered to result in high mortality. Understanding what factors are associated with increased mortality will help us prepare for the next influenza pandemic.

It has been suggested that antigenic novelty accounts for increased mortality during pandemics, but a comparison of mortality estimates between different pandemic years and between interpandemic years indicates that other factors are also involved. The capability of influenza viral strains to predispose to bacterial super-infection may be a factor that determines mortality during an epidemic. Secondary bacterial pneumonia is an important cause of influenza-associated death during both pandemic and interpandemic periods. Results of studies performed during the influenza pandemics of 1957 and 1968 revealed that there was a bacterial etiology in ∼70% of patients with fatal or life-threatening pneumonia [3, 4]. During interpandemic periods, 44%–57% of patients hospitalized with influenza have bacterial pneumonia [5–8], and—although the estimate varies from year to year, depending on the viral strain that is circulating—on average, 25% of all influenza-associated deaths are due to secondary bacterial pneumonia [9].

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia [10] and is a major cause of pneumonia and other bacterial complications of influenza. We developed a mouse model of synergism between influenza virus and S. pneumoniae [11, 12], and, using this model, we established that viral neuraminidase (NA) is an important factor in viral-bacterial synergism [13, 14]. NA activity is needed by an influenza virus to release newly synthesized virus by cleaving sialic acid both from host cell glycoconjugates and from oligosaccharides of viral HA and NA. This action of NA...
also promotes adherence and invasion of *S. pneumoniae*, because cleavage of sialic acid from the surface of host cells exposes cryptic receptors for *S. pneumoniae* [15–18]. Bacteria that can successfully invade the lower respiratory tract typically express NA for this purpose. That generally higher levels of NA activity are found in modern H3N2 than in H1N1 viruses [19] is consistent with the hypothesis that high levels of NA activity lead to higher mortality from secondary bacterial pneumonia. We sought to define the viral-specific NA activity in several influenza viruses within a subtype and correlate it with the biological characteristics of secondary bacterial pneumonia.

**MATERIALS AND METHODS**

**Generation of recombinant influenza viruses.** Recombinant influenza viruses were produced using an established 8-plasmid reverse-genetics system [20] in a coculture of 293T and MDCK cells and parental viruses from the influenza virus repository at St. Jude Children’s Research Hospital. Internal gene segments of A/Puerto Rico/8/34 (subtype H1N1; hereafter referred to as “PR8”) [21], the HA gene segment of the human influenza virus A/Hong Kong/1/68 (subtype H3N2; hereafter referred to as “HK68”), and the HA and NA gene segments of the human influenza virus A/Fujian/411/02 (subtype H3N2; hereafter referred to as “Fuj02”) had been cloned into the pHW2000 plasmid previously. NA gene segments of the human H2N2 influenza viruses A/Singapore/1/57 (Sing57) and A/England/12/62 (Eng62); the human H3N2 influenza viruses HK68, A/Memphis/102/72 (Mem72), A/Leningrad/516/86 (Len86), and A/Sydney/5/97 (Syd97); and the chicken H9N2 influenza virus A/Chicken/Hong Kong/WF2/99 (WF2) were amplified by polymerase chain reaction (PCR) and cloned into the pHW2000 plasmid. HA and NA genes were sequenced, and their identities were confirmed by comparison of their sequences with those of the parental strain. Viral stocks were grown in embryonated chicken eggs, centrifuged on a 25%/75% sucrose cushion, pelleted, and resuspended in PBS.

**Pneumococci.** A type 3 strain of *S. pneumoniae* that had been transformed with the *lux* operon (provided by Kevin Francis and Jun Yu, Xenogen Corp.) was used in mouse studies. R6T, an unencapsulated laboratory strain of *S. pneumoniae*, was used in adherence assays.

**Characterization of recombinant influenza viruses in cell culture, embryonated eggs, and mice.** Allantoic fluid and concentrated viral stocks were titrated in MDCK cells and embryonated chicken eggs by standard methods to obtain the TCID<sub>50</sub> and EID<sub>50</sub>. Groups of 4 mice were infected with serial dilutions of the allantoic fluid stocks to obtain an MLD<sub>50</sub> by the method of Reed and Muench [22]. For lung viral titers, mouse lung homogenates were titrated in MDCK cells as described elsewhere [12].

**NA activity.** The total level of NA activity in concentrated influenza virus diluted in calcium saline buffer to a final substrate concentration of 10 μmol/L was determined by measuring the fluorescence of 4-methylumbelliferone cleaved from 2- (4-methylumbelleryl)-N-acetylneuraminic acid (Mu-NANA; Sigma) as described elsewhere [23]. The relative linkage specificity of NA was determined using N-acetylneuraminic acid (NANA) bound to lactose through either α(2–3) or α(2–6) linkage as a substrate. Fetuin was used as a substrate to measure the level of NA activity against a large molecule with both α(2–3) and α(2–6) sialic acid linkages. The amount of sialic acid released from NANA-lactose (substrate concentration, 0.1 mmol/L) or fetuin (substrate concentration, 6.1 mmol/L) was measured by the thiobarbituric acid assay, as described elsewhere [24]. All reactions were performed for 30 min at 37°C. To relate NA activity to the predominant viral proteins, virus concentrates were run on 10% Tris-HCl gels. After staining with Sypro orange (Amersham Pharmacia), HA and nucleoprotein (NP) bands were quantified using a laser-excited gel scanner.

**Immunoelectron microscopy (IEM).** IEM was performed, as described elsewhere [25], using a mixture of monoclonal antibodies against 7 different N2 NAs representing the range of NAs studied. Anti-NA monoclonal antibodies 152/6 (A/Japan/1/57), 25/4 (A/Tokyo/3/67), 5/2 (A/Aichi/2/68), 1/1 (A/Udorn/307/72), and E12-8 (A/Memphis/12/85) were provided by Dr. Robert Webster (St. Jude Children’s Research Hospital) [26, 27], and Mem4 and Mem5 (A/Memphis/31/98) were provided by Dr. Gillian Air (University of Oklahoma Health Sciences Center) [28]. Negative staining was performed with 2% phosphotungstic acid, and positive staining was performed with ethanolic uranyl acetate. Gold particles representing the amount of NA per virion were counted in positively stained samples.

**Adherence assay.** Adherence assays were performed using standard methods, as described elsewhere [13], using *S. pneumoniae* strain R6T after a 30-min incubation with influenza virus at 37°C. Controls were treated identically, except that influenza virus was not added. For inhibition of viral NA, 30 min before incubation with monolayers, the oseltamivir prodrug Ro 64-0796 (Roche Products) was added to the viral suspension at a concentration of 10 μmol/L (the concentration of the active metabolite oseltamivir carboxylate was not determined).

**Mice and infection model.** Experimental procedures were performed after the mice (8–10-week-old female BALB/c; Jackson Laboratory) were anesthetized with inhaled 2.5% isoflurane (Baxter Healthcare). Infectious agents were administered intranasally in a volume of 100 μL of PBS. For synergism between influenza virus and *S. pneumoniae*, mice were infected first with influenza virus and then 7 days later with *S. pneumoniae* and were monitored at least daily for illness and mortality. Groups of 9–10 mice were infected with 0.01 MLD<sub>50</sub> of influenza virus and 100 cfu of *S. pneumoniae* and were imaged daily. Instead of using a fixed number of infectious particles, we calculated...
doses of influenza virus in relation to the MLD_{50}, so that weight loss, viral lung titers, and damage to the respiratory tract before bacterial challenge were the same between groups. Mice found to be moribund were euthanized and were considered to have died that day. All animal experiments were approved by the St. Jude Children’s Research Hospital Animal Care and Use Committee and were performed under biosafety level 2 conditions.

Imaging of live mice. Anesthetized mice were imaged for 20 s by an IVIS CCD camera (Xenogen Corp.). The total emission of photons from selected and defined areas within the images of each mouse was quantified by LivingImage software (version 2.20; Xenogen Corp.), as described elsewhere [29], and was expressed as relative light units (rlu). Pneumonia was defined as detection of >20,000 rlu/min from the thorax.

Statistical analysis. Comparison of survival rates in the groups of mice was performed using the Mantel-Cox χ² test on Kaplan-Meier survival data, and comparisons of bacterial adherence were made using 1-way analysis of variance followed by Dunn’s test. P < .05 was considered to be statistically significant.

RESULTS

Recombinant influenza viruses differing only in NA. Influenza viruses with an N2 NA have been circulating in the human population since 1957. Representative NA genes were selected from 6 human pandemic and interpandemic H2N2 and H3N2 influenza viruses isolated between 1957 and 2004. Recombinant influenza viruses that differed only in their NAs were rescued on the common background of 7 gene segments (6 internal gene segments from PR8 and the HK68 HA gene). For comparison with these human influenza viruses, an influenza virus that had an NA gene from a 1999 chicken H9N2 influenza virus was also rescued. All the recombinant viruses grew to high titers in embryonated eggs (table 1). They caused morbidity and mortality in mice and thus were suitable for further study of viral-bacterial synergism in a mouse model. The MLD_{50} of the influenza viruses that had a human NA gene were similar, whereas the MLD_{50} of the influenza virus that had the chicken virus NA gene and of the mouse-adapted parental strain PR8 were 3–4 logs lower. An influenza virus that had the Fuj02 NA gene could not be rescued when the Fuj02 NA gene was paired with the HK68 HA gene, and it was therefore excluded from comparison with other isogenic influenza viruses. However, it was rescued when it had its own HA gene (Fuj02 HA), and a comparison influenza virus containing the Fuj02 HA gene and the Mem72 NA gene was also rescued. Because mutations may be generated during rescue of influenza viruses when the plasmid system is used and during passage of influenza viruses in eggs, NA gene sequences of the recombinant influenza viruses were compared with published NA gene sequences of the parental viruses. Site-directed mutagenesis was used to ensure that the NA gene of all influenza viruses matched the GenBank sequence of the NA gene of the target influenza viruses.

NA activity in recombinant influenza viruses. To characterize this set of viral tools, the level of NA activity in concentrated influenza viruses was measured using Mu-NANA as the substrate [23] and was expressed as a function of the amount of NA protein (figure 1). The level of NA activity was highest in the pandemic strain containing the Sing57 NA. The level of NA activity decreased and was lowest in the influenza virus containing the HK68 NA. From 1968 to 1997, a general increase in the level of NA activity was seen, such that the influenza virus containing the Syd97 NA had the second-highest level of NA activity. Although a direct comparison with the isogenic set of influenza

<table>
<thead>
<tr>
<th>Influenza virus</th>
<th>TCID_{50} log_{10} no./mL</th>
<th>EID_{50} log_{10} no./mL</th>
<th>MLD_{50} log_{10} no./EID_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sing57</td>
<td>7.75</td>
<td>8.75</td>
<td>7.0</td>
</tr>
<tr>
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<td>8.0</td>
<td>8.25</td>
<td>6.25</td>
</tr>
<tr>
<td>HK68</td>
<td>8.5</td>
<td>8.25</td>
<td>6.5</td>
</tr>
<tr>
<td>Mem72</td>
<td>8.0</td>
<td>8.5</td>
<td>6.75</td>
</tr>
<tr>
<td>Len86</td>
<td>7.5</td>
<td>8.75</td>
<td>6.5</td>
</tr>
<tr>
<td>Syd97</td>
<td>7.75</td>
<td>7.5</td>
<td>6.25</td>
</tr>
<tr>
<td>WF2</td>
<td>8.875</td>
<td>8.875</td>
<td>3.25</td>
</tr>
<tr>
<td>PR8</td>
<td>8.5</td>
<td>9.25</td>
<td>3.18</td>
</tr>
</tbody>
</table>

NOTE. EID_{50}, doses of influenza virus infectious for 50% of embryos; MLD_{50}, log_{10} no. of EID_{50}’s; TCID_{50}, doses of influenza virus infectious for 50% of wells containing MDCK cells.

Figure 1. Neuraminidase (NA) activity in influenza viruses. The level of NA activity in influenza viruses differing from each other only in NA were measured using 2′-(4-methylumbelliferyl)-N-acetylanimeuramic acid as the substrate. Activity is expressed per amount of viral protein. The mean ± SD percentages from 3–5 independent measurements are shown as the percentage of the NA activity in the influenza virus containing the Sing57 NA. *P < .001, compared with all other influenza viruses (analysis of variance); **P < .05, compared with all other influenza viruses containing a human NA.
Figure 2. Amount of neuraminidase (NA) per virion. NA protein was detected by immunoelectron microscopy (IEM) using a mixture of monoclonal NA antibodies to bind gold particles to NA. A, Negatively stained IEM sample in which the viral structure is visualized more clearly than in positive staining, but the resolution of the gold particles (seen as black dots) is not as good. A clustered distribution of the NA can be seen. B, The relative amount of NA per virion, shown as the mean ± SD number of gold particles per virion. Gold particles from 31–49 virions of each influenza virus were counted. C, The amount of NA per virion, estimated by counting the gold particles from positively stained samples. Examples of 4 representative influenza viruses are shown. *P < .001, compared with influenza viruses containing the Sing57, Eng62, HK68, or Mem72 NAs (analysis of variance); **P < .05, compared with influenza viruses containing the Eng62 or Mem72 NAs.

viruses was not possible, both the wild-type Fuj02 influenza virus and the recombinant influenza virus that had the Fuj02 NA had NA activity levels that were similar to that of the influenza virus containing the Syd97 NA (data not shown). WF2 NA from a recent chicken strain of the H9N2 subtype had a very low level of NA activity—only 2% of that of the influenza virus containing the Sing57 NA. A similar pattern of NA activity was seen when values were expressed relative to the amount of the most abundant viral proteins, HA and NP, or when fetuin was used as a substrate: a decline in NA activity in the influenza viruses isolated from 1957 to 1968, then a gradual increase in those isolated from 1968 to 2002 (data not shown).

Changes in the amino acid sequences of the NAs of influenza viruses isolated between 1957 and 1962 have been associated with a decrease in the level of NA activity, which suggests that these structural changes are responsible for the observed differences [30]. To determine whether these differences might
reflect different preferences for α(2–3)- or α(2–6)-linked substrates, we performed additional experiments. IEM was used to quantify the amount of NA protein per virion (figure 2A and 2B). The differences in the amount of NA protein were minor (figure 2B) and did not correlate with the differences in the level of NA activity. Thus, it seems unlikely that changes in expression over time contributed significantly to changes in the level of NA activity. In addition to demonstrating the relatively stable amount of NA protein per virion, IEM studies confirmed the earlier finding of a clustered distribution of NA protein on virions [25].

Next, the substrate preference of the recombinant influenza viruses was examined. The ability of viral NA to liberate sialic acid from α(2–3)sialyllactose and α(2–6)sialyllactose as substrate was determined. All influenza viruses had the majority of NA activity against α(2–3) linkage of sialic acid. As expected, the influenza virus containing the Sing57 NA, as well as the chicken influenza virus WF2 NA, had activity almost solely (98%) against substrate with an α(2–3) linkage, which is the primary linkage of sialic acid in the gastrointestinal tract of aquatic birds [31]. As is consistent with previous findings [30, 32], the ratio of α(2–6) activity:α(2–3) activity increased in the influenza viruses isolated from 1957 to 1986. Even for NAs from influenza viruses circulating in 1986 or 1997, however, only ~11% of their activity was against the α(2–6) linkage. Thus, it seems unlikely that substrate preference is responsible for differences in the level of N2 NA activity. It is noteworthy that passaging influenza viruses in eggs causes selective pressure in favor of α(2–3) NA activity, because only α(2–3)-linked sialic acid is found in chicken egg allantoic cells [33].

**Adherence of *S. pneumoniae* to human respiratory cells and NA activity.** We next tested the effect that the level of NA activity has on adherence of *S. pneumoniae* strain R6T. Before incubation on A549 cell cultures, the recombinant influenza viruses were diluted until they had the same amount of viral protein. At a viral protein concentration of 1 μg/mL, all influenza viruses clearly increased the adherence of *S. pneumoniae* strain R6T to cells, but incubation with the influenza virus containing the HK68 NA, which has a relatively low level of NA activity, increased the adherence of *S. pneumoniae* strain R6T by only 2.1-fold, compared with a 3.8-fold increase after incubation with the influenza virus containing the Sing57 NA and a 2.9-fold increase after incubation with the influenza virus containing the Syd97 NA. At a lower viral protein concentration of 0.2 μg/mL, the influenza virus with the most active NA, the one containing the Sing57 NA, still increased the adherence of *S. pneumoniae* strain R6T by 3.6-fold, whereas, at this concentration, the other influenza viruses caused only 1.3–1.7-fold increases in adherence (figure 3). The effect of all influenza viruses on the adherence of *S. pneumoniae* strain R6T was reversed when oseltamivir was added to the incubation mixture (only data related to the higher concentration of influenza virus are shown). Oseltamivir carbamoylate is a specific inhibitor of influenza virus NA and has no effect on bacterial NAs [13]. This reversal by oseltamivir in the effect of all influenza viruses on the adherence of *S. pneumoniae* strain R6T, together with a 30-min incubation time that is too short for the completion of mechanisms involved in viral replication, suggest that differences between influenza viruses in their effects on the adherence of *S. pneumoniae* are due to their intrinsic level of NA activity.

**Higher levels of NA activity and higher mortality from secondary bacterial pneumonia in mice.** Infection with influenza virus primes mice for subsequent lethal pneumonia caused by *S. pneumoniae* [11, 12]. We infected mice with influenza virus and, 7 days later, infected them with a strain of *S. pneumoniae* that had been transformed with the luciferase-expressing *lux* operon that permits bioluminescent imaging of pneumonia in live anesthetized mice [13]. We compared pairs of recombinant influenza viruses that differed ~2-fold in their level of NA activity (pair 1, the influenza virus containing the Sing57 NA and the influenza virus containing the Syd97 NA matched with the HK68 HA; pair 2, the influenza virus containing the Fuj02 NA and the influenza virus containing the Mem72 NA matched with the Lux02 HA; pair 3, the influenza virus containing the Mem72 NA and the influenza virus containing the Mem97 NA matched with the HK68 HA).
Figure 4. Lethal pneumococcal pneumonia in mice after infection with pairs of recombinant influenza viruses differing ~2-fold in the level of neuraminidase (NA) activity. Mice were challenged intranasally with 100 cfu of *Streptococcus pneumoniae* transformed with the *lux* operon 7 days after intranasal infection with influenza virus. A, Detection of pneumococcal pneumonia in mice infected with the influenza virus containing the Sing57 or Syd97 NA (9 mice/group). Pneumonia was defined as the detection of >20,000 relative light units (rlu) from the thorax by use of a live imaging system. B, Survival curves of mice infected with the influenza virus containing the Sing57 or Syd97 NA (19 mice/group). C, Detection of pneumococcal pneumonia in mice infected with the influenza virus containing the Mem72 or Fuj02 NA (9 mice/group). D, Survival curves of mice infected with the influenza virus containing the Mem72 or Fuj02 NA (9 mice/group). E, Daily live imaging of 2 representative mice. The mouse infected with the influenza virus containing the Sing57 NA died of pneumonia on day 6 after infection with *S. pneumoniae*. The mouse infected with the influenza virus containing the Syd97 NA survived and did not contract pneumonia. One mouse infected with the influenza virus containing the Syd97 NA and 1 mouse infected with the influenza virus containing the Fuj02 NA recovered from pneumonia; all other mice that developed pneumonia died. *P < .05, for comparisons of the development of pneumonia or survival between groups (Mantel-Cox χ² test on Kaplan-Meier survival data).
ampered with the Fuj02 HA). The mean weight loss at the time of infection with \textit{S. pneumoniae} was similar in paired groups of mice—7.9% in mice infected with the influenza virus containing the Sing57 NA versus 8.9% in mice infected with the influenza virus containing the Syd97 NA and 0.1% in mice infected with the influenza virus containing the Fuj02 NA versus 1.3% in mice infected with the influenza virus containing the Mem72 NA—which indicated that morbidity relating to infection with the 2 different influenza viruses was similar. However, a significant difference in survival after infection with \textit{S. pneumoniae} was observed: 8 (42%) of 19 mice infected with the influenza virus containing the Sing57 NA versus 15 (79%) of 19 mice infected with the influenza virus containing the Syd97 NA (\(P<.05\)) lived (figure 4A), and 3 (33%) of 9 mice infected with the influenza virus containing the Fuj02 NA versus 8 (89%) of 9 mice infected with the influenza virus containing the Mem72 NA (\(P<.05\)) lived (figure 4D). Daily imaging showed the development of pneumococcal pneumonia 2–3 days before death (figure 4A, 4C, and 4E; 2 representative mice are shown). Results of studies using several other recombinant influenza viruses indicated that there were similar differences when influenza viruses with high and low levels of NA activity were compared (data not shown). These data establish that even small differences in the level of NA activity are reflected in differences in the ability of influenza viruses to permit the development of secondary bacterial pneumonia in a mouse model. Data for the influenza virus containing the Syd97 NA (figure 4A and 4B) and the influenza virus containing the Fuj02 NA (figure 4C and 4D) are not directly comparable, despite the similarities in measured levels of NA activity, because the HA differs between the 2 pairs of viruses and could independently contribute to secondary bacterial pneumonia.

The experiment using the influenza viruses containing the Sing57 and Syd97 NAs was repeated, and lung viral titers were measured 3 and 7 days after infection to make certain that the kinetics of viral infection in the 2 groups of mice were comparable. On day 3 after infection, groups of 4 mice infected with the influenza virus containing the Sing57 NA had mean ± SD viral titers of 6.5 ± 0.4 log$_{10}$ TCID$_{50}$/mL, compared with 6.3 ± 0.3 log$_{10}$ TCID$_{50}$/mL in mice infected with the influenza virus containing the Syd97 NA. On day 7, the mean ± SD viral titers were 2.4 ± 0.7 log$_{10}$ TCID$_{50}$/mL and 2.5 ± 0.4 log$_{10}$ TCID$_{50}$/mL, respectively, and there was no detectable influenza virus in the lungs of 1 mouse from each group of 4 mice.

### DISCUSSION

Results of a previous study using a panel of reassortant duck influenza viruses containing human N2 NAs suggested that the level of NA activity in influenza viruses decreased markedly between 1957 and 1968 [30]. We have confirmed and extended this observation by use of human influenza viruses that were generated by reverse genetics to contain N2 NAs representative of influenza viruses isolated between 1957 and 2004. The level of NA activity decreased in influenza viruses isolated from 1957 to 1968 and increased again in those isolated from 1968 to 1997. This trend in NA activity correlates with the observed historic mortality caused by H3N2 influenza viruses, which was highest in 1957, decreased during the next decade, but increased again during the 1990s (table 2). Low levels of NA activity in the influenza viruses circulating in 1968 is consistent with lower mortality from this pandemic, compared with that in the 1957 pandemic or during the epidemics caused by H3N2 influenza viruses during the 1990s. It has been suggested that conservation of the N2 NA in the influenza viruses circulating in 1968 resulted in relatively low mortality during this pandemic, but this does not explain why influenza viruses circulating later that had no antigenic shift in HA or NA caused higher mortality. The N2 NA in the influenza virus circulating in 1997 had the second highest level of NA activity, and this influenza virus caused the highest mortality during an epidemic since the 1957 pandemic [2, 34, 36].

Although the correlation between historical excess mortality in humans and the NA activity and secondary bacterial pneu-

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**Table 2. Comparison of neuraminidase (NA) activity with excess mortality from influenza.**

<table>
<thead>
<tr>
<th>Season</th>
<th>Circulating influenza virus</th>
<th>NA activity, %</th>
<th>Deaths, Excess mortality/100,000 persons</th>
<th>Reference</th>
</tr>
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<td>1957–1958</td>
<td>Sing57</td>
<td>100</td>
<td>66,000</td>
<td>39</td>
</tr>
<tr>
<td>1962–1963</td>
<td>Eng62</td>
<td>24</td>
<td>46,000</td>
<td>25</td>
</tr>
<tr>
<td>1968–1969</td>
<td>HK68</td>
<td>12</td>
<td>28,100</td>
<td>14</td>
</tr>
<tr>
<td>1972–1973</td>
<td>Mem72</td>
<td>37</td>
<td>20,700</td>
<td>10</td>
</tr>
</tbody>
</table>

\(^a\) NA activity was measured in recombinant influenza viruses with a common background of hemagglutinin and internal genes.

\(^b\) Excess deaths by all causes in the United States above the seasonal baseline attributable to influenza. Excess mortality was calculated by Thompson et al. [2] by a method slightly different from that used by Liu et al. [35] and Housworth et al. [34]. These methods provide comparable estimates of mortality caused by influenza [2].
monia found in our model is intriguing, other factors must be involved. A large proportion of excess deaths related to influenza are coded as cardiovascular disease, cerebrovascular disease, or diabetes [37], and most of these conditions are presumably not related to bacterial superinfection. Virus-specific factors contribute to excess morbidity and mortality, and differences in these virulence factors account for differences in morbidity and mortality from season to season. Our data suggest that NA activity is such a factor and that its impact can be seen in deaths from secondary bacterial pneumonia. Other virulence factors—such as the antigenic novelty of the HA, the modulation of interferon [38] or cytokine expression by non-structural protein–I [39], or interactions between several genes—may be involved, and NA may have effects on the host unrelated to cleavage of sialic acid, as is suggested by its ability to activate tumor growth factor–β [40].

Results of our previous studies indicated that pharmacological inhibition of viral NA improves survival in secondary pneumococcal pneumonia after influenza [13, 14]. However, these studies were performed using a single influenza virus and could not establish whether a dose effect based on the activities of different influenza viruses might exist. In the present study, we created a set of recombinant influenza viruses that differed from each other only in their level of NA activity. A hierarchy of support for bacterial adherence and secondary bacterial pneumonia could be seen when recombinant influenza viruses were compared in cell culture and animal models of viral-bacterial interactions. The NA that had the highest level of activity (from Sing57, the strain that caused the 1957 pandemic) was capable of inducing more adherence by S. pneumoniae to cultured respiratory epithelial cells and higher mortality from secondary bacterial pneumonia in mice than an NA with 2-fold less activity (from Syd97). Similar weight loss and lung viral titers during the influenza phase of the infections indicated that differences in viral kinetics were not interfering in the comparisons. An influenza virus containing the NA of the Fuj02 strain, which has a level of NA activity comparable to that of an influenza virus containing the Syd97 NA, more effectively supported secondary bacterial pneumonia than did an influenza virus that had another 2-fold decrease in relative activity (Mem72) when tested on the same background (paired with the Fuj02 HA). An H3N2 virus (Fuj02) caused high mortality during the 2003–2004 influenza season and contributed to a number of well-publicized deaths from methicillin-resistant Staphylococcus aureus [41]. The activity of the NA from the influenza virus that caused the 1918 pandemic has not been reported, and activities of N1 NAs from influenza viruses circulating before 1957 have not been studied in a comprehensive fashion. Our data provide direct evidence that NA activity in influenza viruses is a predictor of mortality from secondary bacterial pneumonia.

These results, together with our previous data showing that oseltamivir treatment prevents secondary bacterial pneumonia in mice even when it is administered late during the course of the viral infection [13], warrant clinical studies of the prevention and treatment of secondary bacterial infections after influenza with NA inhibitors. Effectiveness studies of these drugs in the treatment of influenza have already shown that they decrease the number of complications, although the populations studied have most often not included those individuals who are most vulnerable, because of underlying illnesses or extremities of age, to secondary bacterial infections [42–44]. Preparations for the next influenza pandemic must take into account the possibility that many deaths will be caused by secondary bacterial pneumonia, and an increased focus on viral NA—including stockpiling of NA inhibitors—is therefore essential.

Acknowledgments

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References