MAJOR ARTICLE

Role of Neuraminidase in Lethal Synergism between Influenza Virus and *Streptococcus pneumoniae*

Jonathan A. McCullers and Kimberly C. Bartmess

Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee

A lethal synergism exists between influenza virus and *Streptococcus pneumoniae,* accounting for excess mortality during influenza epidemics. Using a model of viral-bacterial synergism, we assessed the role that the influenza virus neuraminidase (NA) has in priming mice for pneumococcal infection. Administration of the selective NA inhibitor oseltamivir improved survival, independent of viral replication and morbidity from influenza. Both pathologic examination of the lungs and live imaging of pneumonic lesions, using a bioluminescent pneumococcus, suggested that the effect of NA inhibition was to limit the extent of pneumococcal pneumonia during early infection. Adherence assays and immunohistochemical staining for sialic acids in lungs from infected mice demonstrated that the influenza virus NA potentiates development of pneumonia by stripping sialic acid from the lung, thus exposing receptors for pneumococcal adherence. Selective NA inhibitors may be useful clinically to interrupt this novel mechanism of synergism and to prevent excess mortality from secondary bacterial pneumonia.

A synergism exists between influenza virus and bacterial pathogens, accounting for excess mortality during influenza epidemics [1–3]. Combined, influenza and pneumonia rank as the sixth leading cause of death worldwide and as the leading infectious cause of death [4]. The impact of interventions against influenza and *Streptococcus pneumoniae,* the bacterium that most frequently is the cause of community-acquired pneumonia [5], can be seen in directed vaccination studies, where, in at-risk populations, hospitalizations were reduced by 18%–52% and mortality was reduced by 35%–61% [6, 7]. Although the mechanisms underlying this synergistic interaction are poorly understood, it has been presumed that influenza virus–mediated alterations of the host predispose to secondary bacterial infections. Destruction, dysfunction, alterations during regeneration of the ciliated respiratory epithelium [8–10], or defects in phagocytic function [11–14] that are mediated by influenza virus might directly benefit bacterial pathogens—or the cytokine response to influenza might indirectly support bacterial adherence and invasion by altering receptors present in the lung [15, 16].

A new class of antiviral agents, the selective neuraminidase (NA) inhibitors, has recently entered clinical use. These drugs inhibit sialidase activity and are effective against influenza A and B viruses in studies of prevention or early (i.e., within 48 h of infection in animals or onset of clinical symptoms in humans) treat-

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Animals used in this study were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council), under an approved protocol from the Animal Care and Use Committee of St. Jude Children's Research Hospital. All work with infected animals was performed in biosafety level 2 facilities.

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Reprints or correspondence: Dr. Jonathan A. McCullers, Dept. of Infectious Diseases, St. Jude Children's Research Hospital, 332 North Lauderdale St., Memphis, TN 38105-2794 (jon.mccullers@stjude.org).

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ment [17–20]. Prophylaxis and early treatment reduce the duration and severity of acute influenza, the amount of virus shed, and the levels of proinflammatory cytokines in the upper respiratory tract [18, 21]. Limited experience in clinical trials suggests that early treatment of acute influenza in humans can reduce the incidence of both antibiotic use and secondary bacterial complications [22, 23]. Prevention of the clinical sequelae of infection, as a result of reduction of viral load in the lung and prevention of the induction of proinflammatory cytokines, may be responsible for this effect. Alternatively, selective NA inhibitors may prevent exposure of pneumococcal receptors by inhibiting the sialidase activity of the influenza virus NA. Exogenously administered bacterial NA has been demonstrated to increase adherence of pneumococcus in vitro to tracheal [24], eustachian-tube [25], and middle-ear [26] epithelium in an organ-perfusion model using chinchilla tissues. A similar mechanism—one mediated by the influenza virus NA—may predispose to development of bacterial pneumonia.

We recently developed and characterized a mouse model of lethal synergism between influenza virus and *S. pneumoniae.* Administration of influenza virus and then pneumococcus, separated by 7 days, leads to rapid mortality, caused by either sepsis or a severe, extensive bacterial pneumonia [16, 27], depending on the doses of infectious agents used. We investigate here, in both in vitro and in vivo models, the role that viral NA plays in lethal synergism between influenza virus and pneumococcus.

MATERIALS AND METHODS

Infectious agents. The Mount Sinai strain of mouse-adapted influenza virus A/Puerto Rico/8/34 (H1N1), hereafter referred to as "PR8," was grown in Madin-Darby canine kidney (MDCK) cells, from stock cultures from the influenza-virus repository at St. Jude Children's Research Hospital. The minimum dose of PR8 lethal for 50% of infected mice (MLD_{50}) was the equivalent of 1500 $TCID_{50}$. *S. pneumoniae* D39, a type 2–encapsulated strain, was grown in Todd Hewitt broth (Difco Laboratories). The MLD_{50} for pneumococcus, in unmanipulated mice, was equivalent to 5×10^5 cfu, as quantitated on tryptic soy-agar plates (Difco Laboratories) supplemented with 3% (vol/vol) sheep erythrocytes.

Mice. Female BALB/cByJ mice (Jackson Laboratory) 6–8 weeks old were maintained in a biosafety level 2 facility in the Animal Resource Center at St. Jude Children's Research Hospital. All experimental procedures were performed while the mice were under general anesthesia with inhaled isoflurane 2.5% (Baxter Healthcare Corporation).

Infection model. Infectious agents were diluted in sterile PBS and were administered intranasally, in a volume of 100 μl (50 μl/nostril), to anesthetized mice held in an upright position. Mice were weighed and were monitored, at least daily, for illness and mortality. Mice found to be moribund were euthanized and were considered to have died that day. For experiments using a selective NA inhibitor, oseltamivir (Ro 64-0796; Roche Products Ltd.) diluted in sterile water was administered, at a dosage of 10 mg/kg/day, in 2 daily doses, by oral gavage.

Lung titers. Anesthetized mice were euthanized by cervical dislocation. Lungs were removed under sterile conditions, were washed 3 times in sterile PBS, and were placed into 500 μL of sterile PBS. Lung homogenates were used directly for bacterial cultures or were spun at 10 *g* for 5 min, and the supernatants were used for determination of viral titers. Quantitation of pneumococcal colony counts was done by means of 10-fold dilutions on tryptic soy-agar plates supplemented with 3% (vol/vol) sheep erythrocytes. Viral titers were determined by means of 10-fold serial dilutions on MDCK cell monolayers, to obtain the $TCID_{50}$.

Adherence assay. A549 cells were grown to confluence in F-12K media (American Type Culture Collection) supplemented with 5% fetal bovine serum, in 24-well plates. After 3 washings with sterile PBS, monolayers were overlaid with 250 μL/well of a suspension containing 5×10^3 $TCID_{50}$ of PR8 in F-12K media and 4% bovine serum albumin. After incubation for 30 min at 37°C, monolayers were washed 3 times with sterile PBS, were overlaid with 200 μL/well of a suspension containing 1×10^5 cfu of D39 in F-12K media and 4% bovine serum albumin, and then were incubated for 2 h at 37°C. Monolayers were washed 3 times with sterile PBS and then were detached with 0.05% trypsin-EDTA (Life Technologies) and were lysed with 0.025% Triton X-100 (Sigma). Serial dilutions in sterile PBS were plated on tryptic soy agar supplemented with 3% (vol/vol) sheep erythrocytes, to quantitate the numbers of bacterial cells adhering to the monolayer. Controls were treated identically, without the addition of virus. For experiments using oseltamivir, the prodrug Ro 64-0796 (Roche Products Ltd.) was added to the virus suspension, at a concentration of 10 μM, 30 min before incubation with monolayers. This dose is ~1000 times the 50%-inhibitory concentration for the PR8 NA and is ~10 times the 50%-effective concentration, by cell-associated ELISA, for PR8 grown in MDCK cells (data not shown), although the short incubation time of 30 min would allow effects only on enzymatic activity in the media, not on replication of the virus.

Imaging of live mice. Mice were infected with a strain of pneumococcus D39 that had been transformed with the *lux* operon (Kevin Francis and Jun Yu, Xenogen Corp.) but that was otherwise isogenic to the strain used for other experiments reported here. The mice, while under general anesthesia with methoxyflurane (Pittman-Moore), were then imaged for 1 min by an IVIS CCD camera (Xenogen Corp.). Total photon emission from selected and defined areas within the images of each

mouse was quantitated by the LivingImage software package (Xenogen Corp.), as described elsewhere [28], and was expressed as relative light units.

Pathology. Lungs were removed immediately after euthanasia and were fixed in 10% neutral buffered formalin. After 24 h fixation, the lungs were embedded in paraffin, were sectioned at 5 microns, were stained with hematoxylin and eosin, and were examined microscopically for histopathologic alterations.

Immunohistochemistry. Paraffin-embedded lungs were sectioned at 5 microns, were deparaffinized, and were stained for sialic acid. Paraffin was removed from lung sections by immersion in Histo-Clear (National Diagnostics) 3 times, for 5 minutes each time; then successively in 100% ethanol 2 times, for 2 minutes each time; then in 95% ethanol, 80% ethanol, and 70% ethanol, respectively, 1 time; and finally in distilled water 2 times. Deparaffinized slides were kept in 0.05 *M* Tris-buffered saline (pH 7.4) until ready for staining. Detection of sialic acids was by the DIG Glycan Differentiation Kit (Roche Diagnostics), according to the manufacturer's instructions. In brief, lung sections were incubated with a blocking solution and then were exposed successively to a solution containing digoxigenin-labeled lectins, a solution containing an anti–digoxigenin-AP conjugate, and a solution containing NBT/X-phosphate (4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate) to provide staining. Both sambucus nigra agglutinin (SNA), which recognizes sialic acid linked to α(2–6) galactose, and maackia amurensis agglutinin (MAA), which recognizes sialic acid linked to α(2–3) galactose, were used. Sections were examined and photographed under a light microscope.

Statistical analysis. Comparison of survival rates in the groups of mice was done by Mantel-Cox χ^2 test on Kaplan-Meier survival data; comparison of viral and bacterial titers in lungs was done by Wilcoxon rank-sum test; and comparisons of weight loss and the numbers of bacteria adherent to monolayers were done by Student's *t* test. $P < .05$ was considered significant for these comparisons.

RESULTS

Prophylaxis and delayed treatment with oseltamivir improve survival from secondary bacterial pneumonia. To determine whether viral NA activity was responsible for the increased mortality seen in the synergism model, we administered oseltamivir to mice, as either prophylaxis or delayed treatment. The doses administered in this experiment were chosen for 2 reasons. First, on the basis of preliminary studies (data not shown), this dose of pneumococcus (1000 cfu) would be expected to yield, in mice not infected with influenza virus, an intermediate mortality in this experimental design (repeated anesthesia lowers the MLD_{50}). Thus, either positive or negative effects of drug treatment could be seen in the control groups. Second, in combination with influenza, this dose would be expected to result in rapid mortality, providing a stringent test of potential efficacy of drug treatment. Prophylactic and delayed-treatment regimens were studied to test the hypothesis that potential drug efficacy would be related to a reduction in viral titers and to clinical debilitation of the mice.

As seen in figure 1*A*, mice that received prophylaxis had significantly less weight loss than those that received placebo (9% vs. 24%, at the time of pneumococcal challenge), a finding that reflects the decreased morbidity seen with oseltamivir prophylaxis. Mice in the delayed-treatment group were not protected, however, and they experienced weight loss similar to that in the corresponding placebo group (22% vs. 28%; figure 1*B*). The mortality differences between the prophylaxis group and the delayed-treatment group were a reflection of decreased total virus load in the lungs—mice in the prophylaxis group had significantly lower viral titers at days 3 and 7, compared with mice that received placebo (figure 1*C*). In contrast, mice that received delayed treatment had viral titers similar to those in mice in the corresponding placebo group (figure 1*D*).

Survival was significantly prolonged in the prophylaxis group, compared with the placebo group (figure 2). Mice that died in the prophylaxis group had a mean survival of 7 days, compared with a mean survival of 1 day in the placebo group. Despite no difference in morbidity or viral titers (figure 1*B* and 1*D*), survival was also significantly prolonged in the delayed-treatment group (figure 2). Mice that died in the delayed-treatment group had a mean survival of 5 days, compared with a mean survival of 1 day in the placebo group. There was no statistical difference, in survival, between either treatment group and the control group that received pneumococcus alone. Survival in mice that received pneumococcus alone was not enhanced by administration of oseltamivir (figure 2 shows only the control group that received pneumococcus and placebo treatment; data are not shown for mice that received pneumococcus and oseltamivir treatment). Thus, in our mouse model of synergism, oseltamivir improves survival from secondary bacterial pneumonia following influenza; the data on the delayed-treatment group indicate that the mechanism is independent of weight loss and of total virus in the lung.

Effect of inhibition of viral NA on bacterial pneumonia. In our synergism model, to analyze the effect of oseltamivir on bacterial pneumonia, we determined, by bioluminescent imaging in live mice, the extent and distribution of pneumonia. Three groups of 12 mice were infected and challenged, per the delayed-treatment model detailed above. The control group was mock infected with sterile PBS, was given sterile water as mock therapy, and was challenged with D39. The placebo group was infected with PR8, was given sterile water as mock therapy, and was challenged with D39. The oseltamivir group was infected

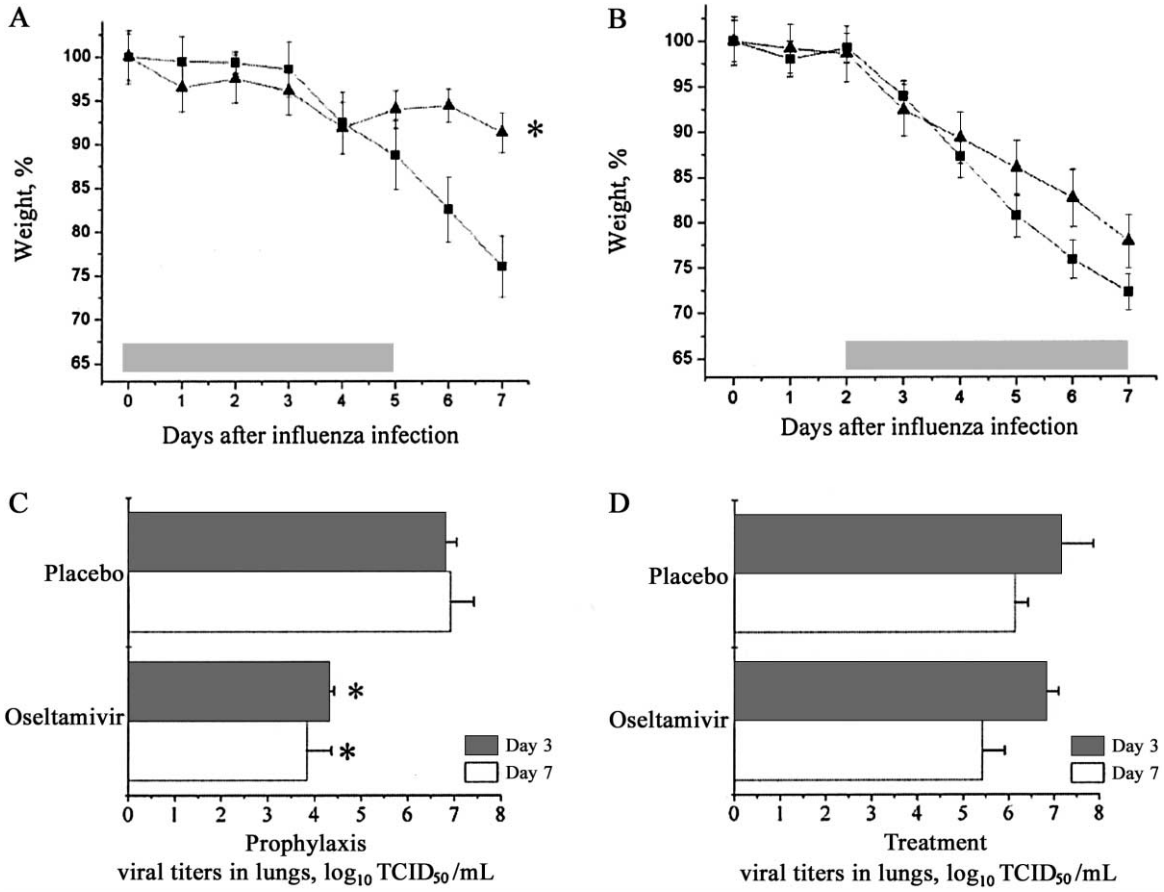

Figure 1. Comparative effects of oseltamivir and placebo. Oseltamivir prophylaxis improves morbidity and reduces total lung virus, whereas delayed treatment has no significant effect. Percentage weight loss, as a measure of morbidity, is plotted for groups of 10 mice that received either oseltamivir (▲) or water (■), as either *(A)* prophylaxis, for 5 days, beginning 4 h prior to influenza infection, or *(B)* delayed treatment, for 5 days, beginning 48 h after influenza infection. The gray bars in panels *A* and *B* represent the time periods, relative to influenza infection, during which the drug was administered. Whereas prophylaxis prevents weight loss, delayed treatment does not have a significant effect. Viral titers at days 3 and 7 after influenza infection are significantly decreased on the prophylaxis schedule *(C)* but not on the delayed-treatment schedule *(D)*. An asterisk (*) indicates a significant difference compared with controls ($P < .01$, by Student's *t* test, for weight data, and, by Wilcoxon rank-sum test, for titer data). Error bars indicate SD.

with PR8, was given oseltamivir as delayed treatment, and was challenged with D39. Mice intended for imaging were infected with D39 transformed with the *lux* operon. As can be seen in representative photographs (figure 3*A*), no focal infection could be visualized 24 h after pneumococcal challenge in any mice in the control group. All mice in the placebo group, however, had extensive pneumonia, with photon emission from bacteria visible throughout the thorax (figure 3*B*). Oseltamivir treatment affected the intensity and distribution of photon emission, resulting in lesions that were less intense and, typically, smaller in size, compared with those in the placebo group (figure 3*C*). Sequential imaging of pneumonic lesions in oseltamivir-treated mice showed, in some mice, progression toward more-extensive pneumonia (data not shown), but, typically, this progression occurred with a lag of several days, compared with mice in the placebo group, which uniformly had severe pneumonia at early times and rapidly succumbed to infection. In all cases, development of extensive pneumonia correlated with mortality.

Quantitation of photon emission from 4 mice/group, 24 h after pneumococcal challenge, demonstrated significant reductions in both the control group and in the oseltamivir group, compared with the placebo group (figure 3*D*). Bacterial lung titers from 4 mice/group also demonstrated significantly lower titers in both the control group and the oseltamivir group, compared with the placebo group. No bacteria could be recovered from mice in the control group (figure 3*E*). Repeating this experiment twice yielded similar results (data not shown). Although statistically similar photon counts were seen in the control group and in the oseltamivir group, the light distribution in the mice in the control group was diffuse, and, in contrast to what was observed in the oseltamivir-treated group, focal lesions could not be seen visually. This may be because

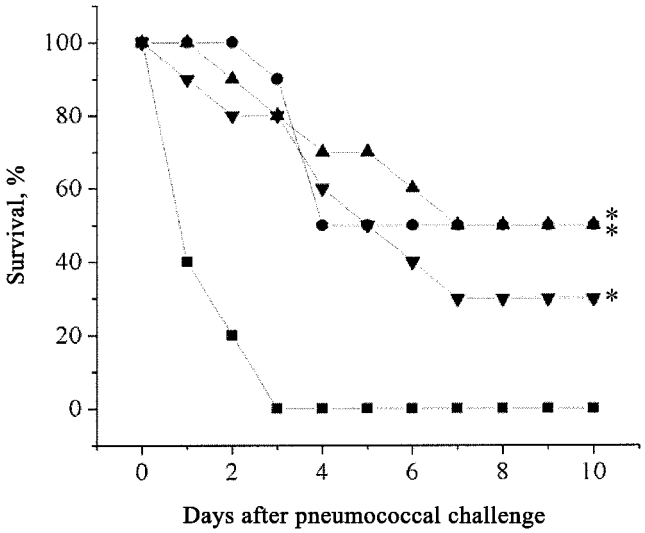

Figure 2. Effect of oseltamivir on survival from secondary bacterial pneumonia. The percentage survival is plotted for groups of 10 mice that received either oseltamivir, as either prophylaxis (▲) or delayed treatment (▼) after influenza infection or water after either mock infection (●) or influenza infection (■). Mice were challenged with pneumococcus D39 (1000 cfu) on day 0, 7 days after influenza infection. No significant difference was seen between mock-infected mice that received water and those that received oseltamivir (data not shown). An asterisk (*) indicates a significant difference compared with the control group ($P < .01$, by the Mantel-Cox χ^2 test on Kaplan-Meier survival data).

bacteria were present but nonviable—and thus not organized as a pneumonic process at that time.

Results of histopathologic examination of lungs from 4 mice/group, 24 hours after pneumococcal challenge, supported the differences, in severity and distribution of the pneumonic process, seen in imaging of live mice at that early time. Lungs from control mice were essentially normal (not shown). Examination of lungs from mice in the placebo group revealed findings characteristic of the synergism model, as described elsewhere [16]. Multiple parenchymal foci with alveolar inflammation, alveolar epithelial-cell hypertrophy and hyperplasia, and alveolar necrosis and fibrin deposition were seen. Inflammatory cell infiltrates with lymphocytes, neutrophils, and macrophages were present, particularly around blood vessels. Extensive and severe consolidation of affected lobes and obliteration of alveolar architecture were striking findings (figure 4*A* and 4*B*). Affected areas of lungs from mice in the oseltamivir-treated group shared many of the features of those from mice in the placebo group, but the extent and distribution of pneumonic lesions were altered. Smaller, focal areas were abnormal, typically in a peribronchial distribution, in contrast to the extensive parenchymal disease seen in the placebo group. The majority of the lung parenchyma in the oseltamivir-treated group was histopathologically normal (figure 4*C* and 4*D*).

Adherence of pneumococci to A549 cells. Since the improved survival seen in the delayed-treatment group was not associated with lower virus load in the lungs, we hypothesized that the drug was blocking an effect of viral NA on the host. To test this thesis, we first modeled the effect in vitro, using A549 cells (a human lung epithelial-cell line). Six wells per group per day were assayed, and the experiment was repeated 4 times, on successive days, with the data being normalized to the internal control group for that day's experiment and with the results pooled for statistical comparison. After 30 min incubation with influenza virus, adherence of pneumococci to A549 cells was significantly increased, compared with that in controls, (figure 5). Since this incubation time is too short to produce viral-mediated effects on translation or transcription, the effect must be due to direct actions of either the virus or a component of the medium. The virus was grown in MDCK cells, in a medium with a composition similar to that of the medium used as the diluent for the treatment and control groups. Preincubation of virus with oseltamivir reversed the adherence, to levels indistinguishable from those in the controls, indicating that the increased adherence is specifically due to the action of viral NA (figure 5); in the absence of pretreatment with influenza virus, oseltamivir did not decrease adherence of pneumococci (data not shown).

Lectin staining in lungs. On the basis of the results of the adherence assays, we hypothesized that viral NA was cleaving sialic acid in the lungs of the mice, creating a milieu favorable for pneumococcal adherence. Thus, oseltamivir was inhibiting this side effect of viral residence in the lungs and was limiting the spread of pneumococcus during early infection. To determine whether cleavage of sialic acid in the lungs was being inhibited by oseltamivir, we stained lung sections from mice in the delayed–treatment group and from mice in the control group, for sialic acids in the $\alpha(2–3)$ and $\alpha(2–6)$ conformation, using SNA and MAA. The effect can best be seen in the airways, where little to no sialic acid was detected in mice that were infected with influenza and then pneumococcus and that received mock therapy with water (figure 6*A*). In contrast, mice in the delayed-treatment group had staining evident in the epithelial cells lining the bronchi (Fig. 6*B*), as well as in the lung parenchyma (not shown). Thus, oseltamivir prevents influenza NA–mediated stripping of sialic acids within the lung.

DISCUSSION

The synergistic interaction between influenza virus and *S. pneumoniae* is a multifactorial process. What these factors are and what the relative contribution of each is to excess morbidity and mortality is poorly understood. As studies of pneumococcal adherence and virulence have progressed over the last decade, there has been an increasing recognition that virus-mediated alterations in the receptor milieu in the lung can create cir-

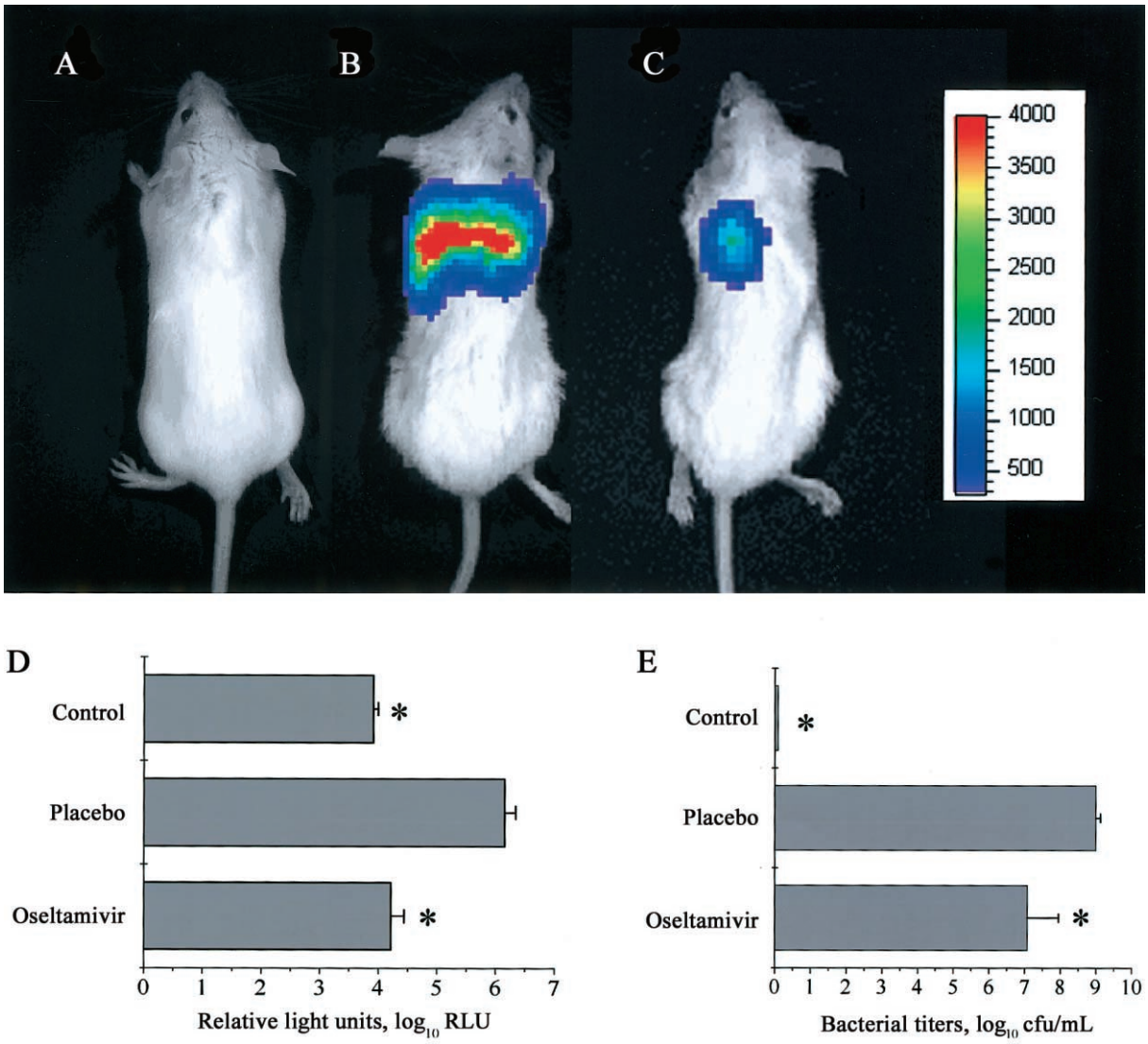

Figure 3. Imaging of secondary bacterial pneumonia in live mice. Oseltamivir reduces both the severity and the extent of secondary pneumococcal pneumonia. Groups of 12 mice received either PBS, followed 7 days later by pneumococcus (Control), or influenza, followed 7 days later by pneumococcus accompanied by delayed treatment with either water (Placebo) or oseltamivir (Oseltamivir). Imaging of bioluminescence from live mice 24 hours after challenge with pneumococcus D39 transformed with the *lux* cassette demonstrates the presence and extent of pneumonic lesions in the secondary-pneumonia model. Mice in the placebo group *(B)* have extensive pneumonia, compared with control mice which have cleared infection by 24 h *(A);* have higher photon emission from the thorax, compared with control mice *(D);* and have higher bacterial lung titers, compared with control mice *(E).* Mice that received oseltamivir have smaller pneumonic lesions *(C),* lower photon emission *(D),* and lower bacterial lung titers *(E),* compared with mice in the placebo group. An asterisk (*) indicates a significant difference compared with the placebo group ($P < .01$, by the Wilcoxon rank-sum test). Error bars indicate SD.

cumstances favorable for pneumococcal infection (reviewed in [29]). In the present study, we examined one potential contributor to synergy: the role that viral NA plays in priming the lung for pneumococcal infection. Our data indicate that viral NA activity contributes significantly to lethal synergism in a mouse model of secondary bacterial pneumonia.

A variety of evidence indicates that NA activity is important in the pathogenesis of pneumococcal infections involving epithelial surfaces. Colonization [30], exposure of receptors on eustachian-tube epithelium [26, 31] and mouse nasopharyngeal epithelium [32], and virulence in a pneumonia model (J.A.M., unpublished data) are decreased in a knockout mutant of pneumococcus deficient in *NanA*, the major pneumococcal NA gene. In an intraperitoneal-challenge model, in which adherence is not required, virulence is not affected [33] (J.A.M., unpublished data). The clear implication is that pneumococcus utilizes this cell surface–associated protein to clear terminal sialic acid from the carbohydrate residues to which it adheres

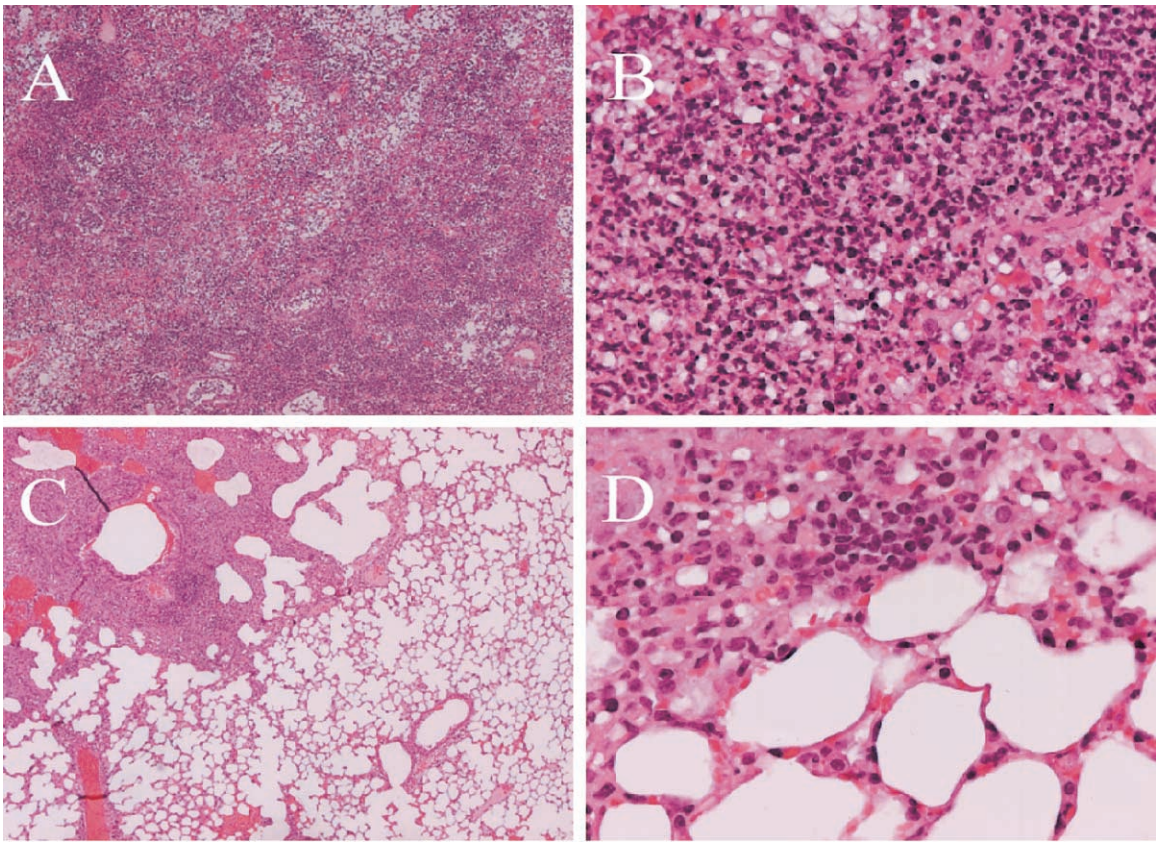

Figure 4. Effect of oseltamivir on the extent of secondary pneumococcal pneumonia. Hematoxylin-and-eosin staining of lung sections of mice that received delayed treatment with either water (*A* and *B*) or oseltamivir (*C* and *D*), at 10× (*A* and *C*) and 40× (*B* and *D*) magnification. Lungs were removed 24 h after pneumococcal challenge in mice infected with influenza virus 7 days earlier. Wheras extensive, severe pneumonia is seen in the lungs of mice that received mock treatment with water, pneumonic lesions are limited in mice that received delayed treatment with oseltamivir, and most of the lung parenchyma is histologically normal.

[34]. It has been demonstrated that either exogenous bacterial NA [25] or preinfection with influenza A virus [32, 35] can either enhance or substitute for this activity in the chinchilla middle ear and in the mouse nasopharynx. Therefore, it seems likely that viral NA could also contribute to the synergism displayed in our model of secondary bacterial pneumonia following influenza.

Perhaps the most important observation in this study is that inhibition of viral NA improves survival, independent of either total virus in the lung or weight loss in the animal. We reason that, if effects of viral replication—such as epithelial-cell destruction, cytokine-mediated receptor up-regulation, ciliary dysfunction, or immune suppression—were the predominant mechanisms responsible for synergism, then improved survival would be seen only after prophylaxis, not after delayed treatment. However, a survival advantage was gained in a delayed-treatment setting after viral titers had peaked (figures 1*D* and 2). Despite having no activity against bacterial NAs and pneumococcal NA [36 and present study (see Results; data not shown)], the effects of the NA inhibitor could be seen in the severity of both the pneumococcal pneumonia, as measured by photon emission (figure 3*D*), and of the bacterial lung titer (figure 3*E*). Thus, an alteration of either the host or the host response must be occurring. This does not exclude a contribution from other mechanisms of synergy, but it does implicate viral NA as an important factor in the pathogenesis of this interaction.

Taken together, the findings from the live imaging and from the necropsy examinations suggest that the effect of oseltamivir in this model is to alter the extent and development of pneumonic lesions, not to change their essential character. The strikingly severe and extensive pneumonia seen in the synergism model [16] is restricted at early times after treatment with a selective NA inhibitor. Extensive pneumonia (and death) develops only in a limited number of subjects and is delayed in both onset and progression. In the delayed-treatment setting, the viral titer reaches its peak and the virus spreads throughout the lungs before the start of therapy. Although much of this virus may remain inside the cells, protected from the drug, it is expected that any NA activity of extracellular virus would

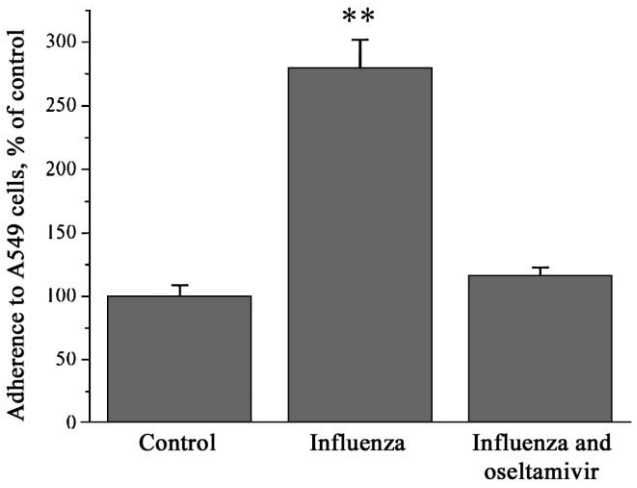

Figure 5. Comparative effects that various treatments have on pneumococcal adherence. Adherence of pneumococcus D39 to human lung epithelial-cell line A549 is significantly enhanced by pretreatment, for 30 min, with influenza virus PR8. This increased adherence is reversed by incubation of influenza virus with oseltamivir 30 min before exposure to the monolayer. The double asterisk (**) indicates a significant difference compared with controls and with the oseltamivir wells ($P < .01$, by the Student's *t* test). Error bars indicate SD.

be inhibited. Therefore, the effect of viral NA on host-cell sialic acid would be expected to be diminished. Indeed, in mice given oseltamivir, staining for sialic acids within the lung demonstrates clear differences. The sialic acid moieties preserved by this effect of viral NA inhibition may impair adherence of bacteria such as pneumococcus, a hypothesis supported by our data on adherence (figure 5). On the basis of these data, we argue that, during treatment with oseltamivir, pneumococcus no longer enjoys the advantage conferred by the presence of influenza virus and can cause pneumonia only in areas where its own NA is active. Because pneumococcal NA is a cell surface–associated enzyme and is not secreted, the spread of bacteria is limited to areas contiguous to those where bacteria are already adherent, providing an opportunity for the adaptive immune response to clear the pathogen before there is progression to respiratory failure and death.

An interesting area for speculation based on the importance of viral NA for viral-bacterial synergism is the subtype-dependent differences in excess mortality seen with influenza viruses. Although it is generally accepted that typical influenzal illness due to H3N2-subtype viruses is more severe than that due to H1N1 or B viruses [37, 38], the incidence of excess hospitalizations [2] and mortality [1, 3] is disproportionately higher during H3N2 epidemics. This may be explained by differences in the respective NAs of these viruses. The N2 from human H3N2 influenza viruses has a higher NA activity [39], which is required to balance the higher affinity of the H3HA receptor [39–41]. Either this higher activity or, perhaps, a higher affinity of the N2 NA for particular sialic acids could more efficiently prime the lungs for pneumococcal adherence, potentiating development of secondary bacterial pneumonia. The ability to replicate these subtype-specific differences in an animal model [42] suggests that this hypothesis could be tested experimentally.

If the findings presented in the present study can be extended to humans, the improved survival seen with treatment with oseltamivir has important implications for the use of selective NA inhibitors. Although not powered to detect differences in secondary bacterial infections, clinical studies of NA inhibitors suggest that there is an effect on both the incidence of complications and the need for antibiotic use [22, 23]. Our data support these limited observations and argue for further study

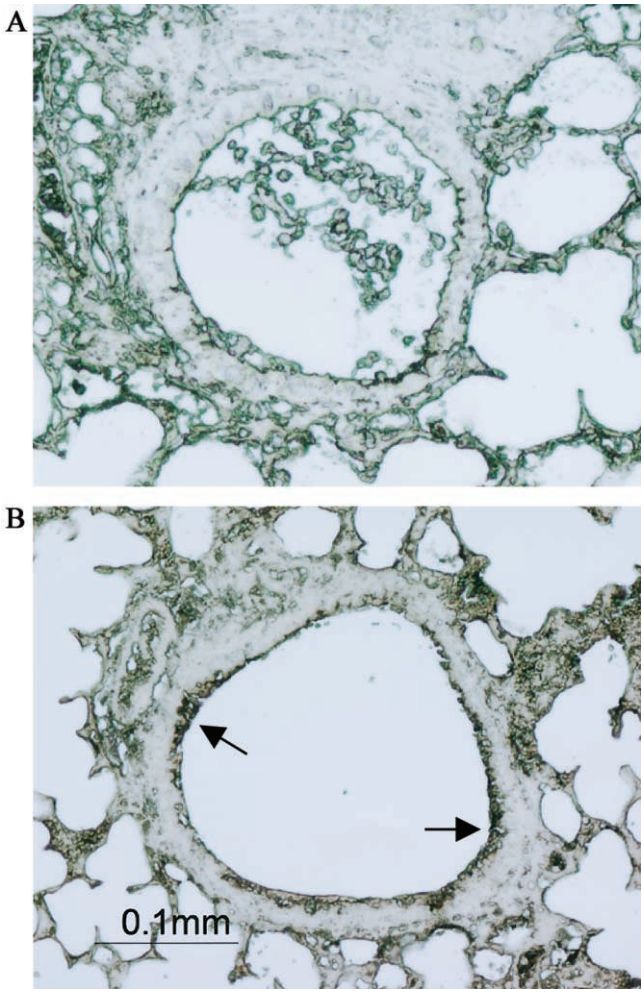

Figure 6. Effect that oseltamivir has on sialic acid. Oseltamivir prevents influenza-mediated stripping of sialic acid. Cross-sections of bronchioles are shown at 40× magnification, after being stained with SNA and MAA, lectins that recognize sialic acid. A representative lung section from a mouse that received influenza virus and mock treatment with water *(A)* has no appreciable staining. A similar section from a mouse that received delayed treatment with oseltamivir *(B)* has dark staining in the epithelial cells lining the bronchiole *(arrows)*.

of this class of drugs. Our findings suggest that, although currently approved only for prophylaxis and early treatment of influenza, selective NA inhibitors may prevent secondary bacterial complications, even when given in a delayed-treatment setting, where no substantive benefit to the course of the influenzal illness itself is expected. This potential preventive effect may change the way in which this class of drugs is prescribed [43], particularly in populations such as the elderly, who are at high risk for secondary complications and death.

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