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Evaluation of the Role of the NSI Protein in Influenza A Virulence

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Evaluation of the Role of the NS1 Protein in Influenza A Virulence

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College Scholars Program
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Studies have implicated the NS1 protein of the A/Hong Kong/156/97 (H5N1) in conferring resistance to antiviral cytokines and perhaps playing a role in the enhanced virulence of human H5N1 viruses. Other studies have suggested that inserting the NS gene from A/Hong Kong/156/97 into a mouse-adapted strain (A/Puerto Rico/8/34) enhances the virulence of the virus in the mouse model. To further investigate the role of the NS gene in influenza A virulence, we generated recombinant viruses using the mouse-adapted H3N2 influenza A virus HKx31 as the parental strain. The NS segments from A/Hong Kong/156/97 and A/Vietnam/1203/04, both fatal human H5N1 isolates, were used in our experiments, as were the NS segments from two other contemporary human isolates, A/Fujian/411/02 (H3N2) and A/New Caledonia/20/99 (H1N1). Expression of the NS segment from A/Hong Kong/156/97 did not enhance the virulence of the parental strain in mice. Notably, the NS segment from A/Vietnam/1203/04 substantially attenuated the parental virus. This attenuation was associated with stronger early B and $CD8^+$ T cell responses, which correlated with a more rapid clearance of the virus from the lungs. All viruses reached similar peak titers in the lungs on days 2 and 3, suggesting that differences in the magnitude of adaptive immune responses were not simply due to differences in antigen load. Infection with the more virulent parental virus resulted in an unusually high proportion of B cells in the draining lymph node on day 5, raising the possibility of defective T cell activation and/or proliferation. Our findings indicate that the mouse model may not always be appropriate for identifying genetic determinants of influenza virulence in humans. However, we clearly identify the NS segment as an influenza virulence factor in mice that impacts the magnitude of the adaptive immune response.

INTRODUCTION

The first confirmed cases of avian-to-human influenza A transmission were reported in Hong Kong in 1997. Of the eighteen reported cases of human H5N1 influenza, six proved fatal. Patients exhibited symptoms ranging from mild respiratory illness to severe pneumonia and multiple organ failure. Unlike deaths associated with the influenza A strains annually circulating in the human population, underlying conditions such as alcoholism, pregnancy, cardiovascular, or pulmonary disease did not appear to be predisposing factors (Cheung et al., 2002; To et al., 2001; Yuen et al., 1998). Postmortem examinations were performed on only two of those cases (To et al., 2001). The most prominent feature was reactive hemophagocytic syndrome, characterized by excess fever, splenomegaly, and excessive activation of monocytes (Fisman, 2000). Amantadine and Ribavirin were administered but had no effect (To et al., 2001). Gastrointestinal symptoms were also observed; while this is not unusual in children with influenza A, its presence in adults is rare (Yuen et al., 1998). In 2004, a 39-year old woman presented with only gastrointestinal symptoms; after nearly a week of hospitalization, respiratory symptoms appeared. Nasopharnygeal aspirates revealed the presence of H5N1 influenza A (Apisarnthanarak et al., 2004).

Molecular determinants of pathogenicity of avian influenza A are well documented; these determinants in mammalian influenza A are not so clearly understood (Cheung et al., 2002). In each of the three major influenza A pandemics of the twentieth century, a new hemagglutinin (HA) subtype was introduced into the human population; with no protective immunity, the viruses spread rapidly and cumulatively caused millions of deaths. Human viruses carrying new HA molecules are derived from animal influenza A viruses (Subbarao et al., 1998). Most experts agree that a "mixing vessel," such as pigs, is necessary for the adaptation of avian influenza

viruses to human hosts. However, sequence comparisons of an H5N1 virus isolated from a human case (A/HK/156/97) with an avian H5N1 influenza virus (A/chicken/Hong Kong/220/97) revealed that the virus did not undergo adaptation in an intermediate host. Sequence differences were found in all eight gene segments, but the homology was still greater than 99% for all segments (Suarez et al., 1998). Sequence analysis of other human H5N1 isolates indicates avian origin of all viruses; the fact that the isolates are similar, but not identical, indicates that multiple separate avian-to-human transmissions occurred (Claas et al., 1998). The HA of all isolates contained basic amino acids near the cleavage site; in domestic fowl, this feature allows cleavage by proteases found in tissues other than the lung, resulting in disseminated infection (Suarez et al., 1998). However, no disseminated infection was found in human cases (Yuen et al., 1998). While certain influenza genes, such as hemagglutinin and neuraminidase, have known roles in virulence, they are not the sole determinants. Many studies have suggested that influenza virulence is affected by multiple genes and even by multiple alleles within those genes. The role of individual genes in influenza virulence must be studied in genetically identical backgrounds because the phenotype of a particular gene may be affected by multiple genes (Brown, 2000). These findings indicate that virulence cannot be predicted because of the many sequence differences between isolates (Brown et al., 2001).

Because human influenza strains can cause respiratory infection in mice, the mouse model is particularly useful for the study of human influenza. While most human isolates can grow in murine lungs without prior adaptation, between six and twelve passages are generally required for the virus to cause severe illness (Brown, 2000). This results in the selection of variants that have acquired mutations that confer enhanced virulence (Brown et al., 2001). Interestingly, several human H5N1 influenza A isolates have been shown to be virulent in mice

without adaptation (Lu et al., 1999). Genomic sequencing of a highly virulent mouse-adapted strain (non-H5N1) revealed eleven mutations, several of which were also seen in the H5N1 viruses from Hong Kong; finding the same mutations in multiple isolates indicates that these mutations are particularly important in virulence and not just the result of spontaneous mutations.

Gene segment 8 of the influenza A virus encodes two proteins, the NS1 and NEP (nuclear export protein). While the first nine amino-terminal amino acids are the same, NEP is produced by an alternative splicing mechanism (splicing out a 479-nucleotide sequence). The NS1 protein's "non-structural" designation is due to the fact that it is not packaged into virions (Krug et al., 2003). The NS1 has three known domains involved in its actions: an RNA-binding domain (residues 19-38), nuclear localization signals (residues 34-38 and 216-221), and an effector doman (residues 134-161) (Basler et al., 2001). Parsimony analysis indicates that the NS1 sequence from the 1918-1919 influenza A pandemic strain phylogenitically lies near the root of all subsequent human and swine viruses; all functional domains are highly conserved among different species-adapted strains (Basler et al., 2001).

While it most commonly associated with attenuating the type I interferon response, the NS1 protein also plays a role in inhibiting the interferon-independent antiviral response. The protein is believed to be involved in the inhibition of the processing of cellular pre-mRNA, preventing the production of mature antiviral proteins. NS1 binds to both the poly-A binding protein II (PABII) and to a subunit of the cleavage and polyadenylation specificity factor (CPSF), both of which are involved in the 3' processing of cellular pre-mRNA (Krug et al., 2003). Processing and translation of cellular proteins is thus inhibited, however, processing of viral pre-mRNA is unaffected because poly(A) tails are added by a viral polymerase.

Influenza A viruses are innately sensitive to interferon and have evolved ways to circumvent these host defenses (Marcus et al., 2005). Within hours of viral infection, cells begin synthesizing type I interferons. Synthesis of IFN α/β induces the transcription of many genes through the binding of the interferon-stimulated regulatory element (ISRE) to specific downstream DNA promoter sequences (reviewed by (Garcia-Sastre, 2002). Many of these genes code for other antiviral proteins. The influenza A NS1 protein has long been known to affect the induction of type-I interferons IFN α/β) by preventing the activation of the transcription factors NF-κB and IRF-3, both of which are essential for transcription of IFN-β (Basler et al., 2001). Activation of NF-κB requires the phosphorylation of its inhibitor, IκB. Phosphorylation of IκB can be performed by protein kinase R (PKR), another target for NS1 (reviewed in (Wang et al., 2000). PKR is activated by dsRNA and can inhibit protein synthesis by phosphorylating eIF2, a translation initiation factor. Both cellular and viral protein synthesis would be halted. The NS1 protein binds dsRNA, although with low affinity. Though exactly how NS1 binding of dsRNA inhibits PKR activation is not clear, it is possible that the binding sequesters the dsRNA and makes it inaccessible to PKR (reviewed by (Krug et al., 2003). It has also been suggested that NS1 may bind directly to PKR and somehow directly prevents its activation (Wang et al., 2000).

NS1 deletion mutants have been shown to grow well in interferon-deficient systems and are even lethal in Stat1 -/- mice but have little effect on wild-type mice (Brown, 2000). Studies of genetically similar influenza A viruses have shown that the strains causing the greatest production of interferon were the most attenuated in both chickens and mice (Marcus et al., 2005). Because of their limited ability to grow *in vivo*, NS1 deletion mutants have been suggested as possible candidates for modified-live influenza viruses (Talon et al., 2000).

While the exact reason H5N1 influenza is so virulent in humans is still largely unknown, work from Seo, et al, indicates that alternations in the NS1 protein may play a role in the increased pathogenicity. Growth of A/Hong Kong/156/97 (H5N1) appeared to be unaffected in St. Jude porcine lung (SJPL) cells pretreated with type I or type II interferons, leading to the conclusion that these viruses have developed a mechanism to counteract their antiviral effects (Seo et al., 2002). Seo, et al, also found that the resistance is independent of cytokine dose. Since the NS1 protein is known to antagonize the type I interferon-mediated host response, recombinant viruses were generated, using A/Puerto Rico/8/34 (H1N1) as the parental strain, expressing either the wildtype NS segment or the NS segment from A/Hong Kong/156/97 (H5N1). Growth of the virus expressing the HK/156/97 NS in IFN-pretreated cells was unaffected; growth of the wildtype virus was inhibited (Seo et al., 2002). Sequence analysis revealed the presence of glutamic acid, instead of aspartic acid, at position 92. Experiments using site-directed mutagenesis to replace the glutamic acid with aspartic acid suggested that the presence of glutamic acid was required for cytokine resistance, as viruses containing the revertant NS were again sensitive to IFN (Seo et al., 2002). Position 92 is located very close to the RNA binding domain, but exactly how the presence of glutamic acid conferred this resistance remains unknown (Seo et al., 2004).

Reactive hemophagocytic syndrome is commonly associated with dysregulation of cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, Interleukin (IL) 1, and Interleukin 6 (Fisman, 2000). Elevated levels of IL-6 and IFN-γ were observed in both cases on which postmortem exams were performed (To et al., 2001). The level of circulating interferon correlated closely with the decline of replication (Seo et al., 2002). When viewed in light of the

findings of Seo, et al, evaluation of the role of the NS gene in H5N1 influenza A virulence seems particularly appropriate.

Studies by Lipatov et al (2005) indicated that the NS segment from A/Hong Kong/156/97 (H5N1) actually enhanced virulence in a mouse model when inserted into the H1N1 influenza A virus Puerto Rico/8/34 (PR8). This gene segment was associated with high levels of pro-inflammatory cytokine production in the lungs of infected mice, and it was postulated that this contributed to the enhanced virulence. It was not associated with a deficiency in the B- or T-cell response. Since PR8 is highly virulent in mice, definitively determining that enhanced virulence is conferred by the NS segment is difficult. To further investigate the role of the NS gene as a virulence factor, we generated recombinants using the H3N2 influenza A virus HKx31 (x31) as the parental virus. The NS segments from A/Hong Kong/156/97 and A/Vietnam/1203/04, both fatal human H5N1 isolates were included in the study, as were the NS segments from the contemporary human isolates A/Fujian/411/02 (H3N2) and A/New Caledonia/20/99 (H1N1). We anticipated that any increase in virulence would be more readily apparent.

MATERIALS AND METHODS

Cell Culture and Propagation of Viruses A 1:1 mixture of Madin-Darby Canine Kidney (MDCK) cells and 293T human embryonic kidney cells were used for rescue of recombinant viruses from plasmids. MDCK cells were used for plaque assays and growth kinetics analyses. MDCK cells were cultured in MEM containing 10% fetal bovine serum. 293T cells were cultured in Opti-MEM I (Life Technologies, Gaithersburg, MD) containing 5% fetal bovine serum.

Generation of Viruses by Reverse Genetics Recombinant viruses were generated using a Pol-I/Pol-II eight-plasmid reverse genetics technique (Hoffmann et al., 2000). Briefy, 1 μg of

each plasmid was combined with 2 μl/μg TransIT; Opti-MEM was added to bring the volume of the mixture to 200 μl. This mixture was allowed to incubate at room temperature for 45 minutes. After incubation, the mixture was diluted with 800 μl of Opti-MEM to bring the final volume to 1 ml. This mixture was added to one well of a 6-well plate seeded the previous day with a 1:1 mixture of MDCK and 293T cells. The transfection mixture was allowed to sit for 6 hours at 37°C. After 6 hours, the mixture was removed and replaced with 1ml of Opti-MEM. The cells were incubated at 37°C for 24 hours. After this incubation, 1 ml of Opti-MEM containing 1 μg/ml of TPCK-trypsin was added. The cells were incubated at 37°C for 48 hours. After 48 hours, 1 ml of cell culture supernatant was collected. 100 μl was injected into 10-day-old embryonated hen's eggs. Eggs were incubated at 37°C for 48 hours and then chilled at 4°C. Allantoic fluid was collected and immediately stored at -80°C.

Segments encoding the internal proteins (M, NP, PA, PB1, and PB2) were derived from A/Puerto Rico/8/34 (H1N1). Segments encoding the envelope glycoproteins (HA and NA) were derived from A/Aichi/2/68 (H3N2). An x31 parental control virus was generated using the NS segment from A/Puerto Rico/8/34. Four other recombinant viruses were generated containing the NS segments from A/Hong Kong/156/97 (H5N1), A/Vietnam/1203/04 (H5N1), A/Fujian/411/02 (H3N2), and A/New Caledonia/20/99 (H1N1).

Sequencing RNA was extracted from allantoic fluid using the Qiagen RNeasy protocol. From this, viral cDNA was generated. Briefly, 1 μl of uni-12 primer (sequence available upon request) was added to 5 μl of RNA and incubated at 70°C for five minutes. Following this incubation, 10 μl of dNTP, 10 μl 5x RT-AMV buffer, 20 μl sterile water, 2 μl RNase inhibitor, and 2 μl of RT-AMV were added. This mixture was incubated at 42°C for 100 minutes and then inactivated by incubating at 70°C for ten minutes using a Hybaid Thermocycler. cDNA was

used for PCR amplification of all gene segments. Universal primers for all genes (Hoffmann et al., 2001), as well as H3 and N2-specific primers were used. PCR products were purified using the Qiagen QiaQuik PCR Purification protocol.

For sequencing, primers were diluted to 20 ng/µl. Rhodamine dye-terminator cycle sequencing was performed by the Hartwell Center for Bioinformatics and Biotechnology using the Ready Reaction Kit with AmpliTaq DNA polymerase FS (Perkin-Elmer Applied Biosystems, Boston, MA).

Determination of eID50 Ten-fold dilutions of allantoic fluid were made using sterile PBS as a diluent. One hundred µl of each dilution was injected into each of four 10-day-old embryonated hen's eggs. Eggs were placed in a 37°C incubator for 48 hours and then chilled at 4°C for 24 hours. Fifty µl of allantoic fluid was collected from each egg; 50 µl of 0.5% chicken red blood cells was added to each aliquot to test for hemagglutination. The eID_{50} was determined using the method of Reed and Meunch (Reed and Muench, 1938).

Plaque Assay Ten-fold dilutions of allantoic fluid were made using MDCK infection media (MEM supplemented with antibiotic/antimycotic and 4%BSA) as a diluent. One ml of each dilution was added to MDCK cells cultured in six-well plates. Plates were then placed in a 37°C incubator for 60 minutes. After incubation, virus dilutions were removed and the plates were washed once with sterile PBS. Three ml of MEM-1.8% Bacto-Agar containing 1 µg/ml TPCK-Trypsin was added to each well. The plates were incubated at 37°C for 72 hours. After incubation, plates were chilled, the agar was removed, and cells were stained with crystal violet. Plaques were counted to determine titer.

***In vitro* Growth Kinetics Analyses** MDCK cells were infected at a multiplicity of infection (MOI) of 0.01 and 5. Virus dilutions were prepared using MDCK infection media

containing 4% BSA. One ml of virus dilution was added to each well of a six-well plate; plates were incubated at 4°C for 30 minutes to synchronize infection. After 30 minutes, the plates were incubated at 37°C for one hour. Virus dilutions were then removed and the plates were washed once with sterile PBS. Following the wash with PBS, the plates were washed with an acidic (pH=2.2) 0.9% sodium chloride solution. Following this wash, the plates were then washed twice with sterile PBS. Three ml of infection media containing 4% BSA and 1 μg/ml TPCK-trypsin was added to each well. From the first well, a 200 μl aliquot was taken for T=0 hr. The plates were then placed in a 37°C incubator. For the plates infected at an MOI of 0.01, 200 μl aliquots were taken at T=12 hrs, 24 hrs, 36 hrs, and 48 hrs. For the plates infected at an MOI of 5, 200 μl aliquots were taken at T=4 hrs, 8hrs, 12 hrs, and 24 hrs. The aliquots were immediately stored at -80°C.

To determine titers at each time point, plaque assays were performed as previously described.

Infection and Sampling of Mice Eight to ten week old female B6 (B6) mice (The Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were infected intranasally with 10^6 eID_{50} (high dose) or 10^4 eID_{50} (low dose) in 30 μl sterile PBS. Inoculation and organ sampling were performed as previously detailed (Sangster et al., 2003). Serum samples were stored at -80°C until use in ELISAs. Right posterior mediastinal lymph nodes were collected on days 5, 7, and 9 for use in ELISpot assays to determine the prevalence of influenza-specific antibody secreting cells (ASCs). Cell populations were collected from the lung by bronchoalveolar lavage (BAL).

Lungs were sampled on days 1, 2, 3, 5, 7, and 9 and stored at -80°C. Lung homogenates were used to infect MDCK cells in 96-well plates; plates were incubated at 37°C and 5% CO_2 for

48 hours. Hemagglutination assays were performed on the tissue culture supernatant to determine titers.

ELISpot Assay for Antibody Forming Cells ELISpot assays were performed as previously described (Sangster et al., 2003). Briefly, 96-well multiscreen HA filtration plates (Millipore, Billerica, MA) were coated with disrupted influenza A x31 virus and incubated as described (Sangster et al., 2000). Single-cell suspensions generated from the harvested mediastinal lymph nodes were serially diluted and added to each well of the coated plate. Plates were incubated at 37°C for 3 hours and then washed thoroughly. Alkaline-phosphate conjugated goat-anti mouse antibodies specific for IgM, IgA, IgG1, IgG2c, IgG2b, and IgG3 (Southern Biotechnology, Birmingham, AL) were diluted in PBS-5% BSA and added to appropriate wells. After overnight incubation and washing, 5-bromo-4-chloro-3-indolyl phosphate dissolved (1mg/ml) in diethanolamine substrate buffer was used to develop the plates. Plates were allowed to develop all day then were washed and allowed to dry overnight. Spots were counted to determine the number of influenza-specific AFCs present in the lymph node.

ELISA for Circulating Influenza-Specific Antibodies ELISA assays were performed as previously described (Sangster et al., 2003).

Flow Cytometric Analysis All FACS analyses were performed using a FACSCalibur Flow Cytometer and CellQuestPro software. Fluorochome-conjugated anti-mouse antibodies specific for CD3 (FITC), CD19 (PE), CD4 (FITC), and CD8a (PE) were used (BD Biosciences Pharmingen, San Jose, CA). PE-conjugated tetramers specific for either influenza A PA or NP epitopes were obtained from St. Jude Childrens' Research Hospital, Memphis, TN. Staining was performed as previously described (Webby et al., 2003).

Statistical Analysis Statistical Comparisons were performed by one-way analysis of variance and by the Mann-Whitney *U* test.

RESULTS

Production of Recombinant Viruses NS recombinant viruses were generated and a stock of each virus was grown in eggs. After extracting viral RNA from harvested allantoic fluid and using reverse-transcriptase PCR to amplify the product, each segment was sequenced to confirm its origin. All viruses were identical in seven of eight segments; the only segment differing among the recombinants was segment 8, the segment encoding the NS1 protein. The HA and NA segments of each virus were confirmed to be from A/Aiche/2/68 (H3N2). The internal segments M, PA, PB1, PB2, and NP were confirmed to be from A/Puerto Rico/8/34 (PR8). The parental virus also contained the NS segment from PR8. The reassortant x31-Hong Kong (x31-HK) contained the NS segment from A/Hong Kong/156/97 (H5N1). The reassortant x31-Vietnam contained the NS segment from A/Vietnam/1203/04 (H5N1). The final two reassortants, x31-Fujian and x31-New Caledonia, contain NS segments from A/Fujian/411/02 (H3N2) and A/New Caledonia/20/99 (H1N1), respectively.

The titer of each virus stock was determined using three different methods (table 1). Titers given in plaque-forming units (pfu) were derived from MDCK plaque assays; titers given in EID_{50} units were derived from egg 50% infectious dose determinations; titers given in $TCID_{50}$ units were derived from MDCK tissue culture 50% infectious dose determinations. For each method of titration, the titers of the parental and recombinant viruses were similar, indicating that all viruses grew equivalently well in eggs.

***In Vitro* Growth Kinetics Analyses** To show that all viruses were replication-competent and grew at equivalent rates *in vitro*, growth kinetics analyses were performed. MDCK cells

were infected at an MOI of 0.01 to show that viruses will grow to equivalent titers after multiple rounds of replication (figure 1A). All viruses grew well in culture, with less than a ten-fold difference between the peak titers. The parental RGx31, x31-HK, x31-New Caledonia, and x31-Vietnam recombinants grew at similar rates and reached nearly identical peak titers. The x31-Fujian recombinant grew at a consistently slower rate and reached a slightly lower peak titer, though it too grew relatively well in tissue culture.

To show that all recombinants achieved similar titers after only one round of replication, MDCK cells were infected at an MOI of 5 (figure 1B). Titers for all recombinants were nearly identical after four hours, but then slight differences began to appear. However, after 24 hours, there is still less than a ten-fold overall difference between the highest (x31-HK) and lowest (x31-Fujian) titers.

Mortality in C57BL/6 mice To analyze virulence *in vivo*, groups of 9-10 week old female B6 mice were infected with 30 μl of either a 1/5, 1/10, 1/100, 1/1000, or 1/10000 dilution of the original virus stocks (table 2A). Marked differences were seen, though the recombinants could be divided into two primary groups: those that were virulent and those that were of low virulence. The highly virulent group, containing the parental RGx31, x31-HK, and x31-New Caledonia, were lethal to at least 75% of the infected mice at the 1/10 dilution; the RGx31 and x31-Fujian recombinants were lethal to 75% of the infected mice even at a 1/100 dilution (corresponding a dose of $3x10^5$ eID_{50}). In contrast, the "low virulence" group was lethal to 25% or less of the infected mice at the 1/5 dilution (corresponding to a dose of $1.90x10^7$ eID_{50}).

The 50% mouse lethal dose (MLD_{50}) was determined using the method of Reed and Muench (Reed and Muench, 1938) (table 2B). The parental RGx31 had an MLD_{50} dose of $6.3x10^4$ pfu, while both the x31-Vietnam and x31-Fujian reassortants were greatly attenuated

with MLD_{50} doses greater than $1.5x10^6$ pfu. The x31-New Caledonia reassortant also had a lower MLD_{50} dose ($8.7x10^4$), and the x31-HK recombinant had an intermediate MLD_{50} dose ($6.5x10^5$). In subsequent experiments to investigate the basis for the virulence differences, a high dose (10^6 eID_{50}) and a low dose (10^4 eID_{50}) virus challenges were used. The high dose challenge corresponded to approximately 10^5 pfu. Three viruses were selected for experiments *in vivo*: the parental RGx31, x31-HK (highly virulent), and x31-Vietnam (low virulence).

Virus Replication in Lungs To analyze viral replication *in vivo*, groups of B6 mice were infected intranasally with 10^6 eID_{50} in 30 μl PBS (high dose group) or 10^4 eID_{50} in 30 μl PBS (lose dose group) of either RGx31, x31-HK, or x31-Vietnam.

In the high dose group, after 24 hours, similar titers were seen in the RGx31 and x31-HK groups, while the x31-Vietnam group had nearly a ten-fold lower titer (figure 2A). However, on days 2 and 3, all recombinants had reached nearly equivalent titers. By day 5, the titers of the x31-HK and x31-Vietnam groups had dropped significantly (almost 1000-fold) from their peak at day 2. The most telling differences were seen on day 7. Both RGx31 and x31-HK seemed to level off, but x31-Vietnam seemed to be nearly cleared from the lungs. By day 9, both x31-HK and x31-Vietnam had essentially been cleared, while RGx31 still had a measurable titer.

With the low-dose group, samples were taken on days 3, 7, and 9 (figure 2B). The three viruses reached equivalent titers on day 3 and all were cleared by day 9. Sample numbers were not sufficient to perform meaningful statistical analyses.

B Cell Response

To analyze the antibody response to the three recombinants, ELISpot assays were performed using single-cell suspensions made from the mediastinal lymph nodes. Again, groups of B6 mice were infected with 10^6 eID_{50}s in 30 μl PBS and sampled at intervals after infection.

Though day 5 is very early in the antibody response, the x31-Vietnam recombinant induces a stronger early antibody response than the parental RGx31 and x31-HK recombinants (figure 3A). The x31-Vietnam group also showed more isotype switching. This correlated with a lower x31-Vietnam lung titer on day 5, indicating that the magnitude of the early antibody response may play an important role in viral attenuation and clearance.

Marked differences were noted in the magnitude of the antibody response on day 7 (figure 4B). The parental RGx31 virus elicited a reasonably strong IgM response, but little isotype switching was seen. The x31-HK recombinant elicited a slightly weaker IgM response, and again little isotype switching was seen, though there seemed to be more switching to the IgG2c isotype than was seen with the parental strain. The x31-Vietnam recombinant elicited the most robust IgM response and also induced more isotype switching to all subtypes than was seen with the other two viruses. The x31-Vietnam recombinant elicited a significantly stronger IgG2b ($P<0.001$), IgG1 and IgG2c ($P<0.01$), and IgG3 and IgA ($P<0.05$) response.

By day 9, the overall picture had changed substantially (figure 4C). The antibody response to RGx31 was strongest in all subtypes, but particularly in IgM and IgG2c. While the IgM response to all three viruses was waning, production of various isotypes in response to both the parental RGx31 and X31-HK recombinant had increased considerably. They exhibited significantly higher numbers of IgG2c and IgG3 antibody secreting cells than the x31-Vietnam recombinant. The numbers of IgG1, IgG2b, and IgA ASCs did not differ substantially between the three groups. The day 9 result probably reflected the more rapid clearance of the x31-Vietnam from the lungs of the mice.

Total cell numbers in the MLN were consistent with a stronger immune response against x31-Vietnam than against the other viruses (figure 4). The difference between the RGx31 and x31-HK samples was statistically significant ($P<0.05$) on day 9.

To demonstrate that numbers of ASCs present in the mediastinal lymph node correlates with the level of circulating antibodies, we performed ELISAs on serum samples taken on days 5, 7, and 9. Though the samples have not yet been comprehensively analyzed, the preliminary data supports the ELISpot data, indicating that the antibody response to the x31-Vietnam recombinant is induced earlier and is greater in magnitude than the response to the RGx31 and x31-HK viruses. (Data not shown.)

B-cell and T-cell Proportions in the MLN Day 5 MLN single cell suspensions were analyzed using flow cytometry to determine the proportion of B- and T- cells present in the node (figure 5). B-cells were distinguished based on the presence of CD19 on the cell surface, and T-cells were distinguished by the presence of CD3 on the cell surface. The cell population in the MLNs from mice infected with RGx31 are skewed heavily towards B cells, while the MLNs from mice infected with x31-Vietnam were more balanced, with nearly equivalent proportions of B- and T-cells. MLNs from mice infected with x31-HK showed a higher proportion of T-cells, though not as high as seen with the RGx31 MLNs. The percentage of B cells in the lymph nodes responding to x31-Vietnam is significantly higher than that seen in lymph nodes responding to RGx31 ($P<0.001$) and x31-HK ($P<0.05$). The percentage of T cells in the lymph nodes responding the x31-Vietnam was significantly lower than that seen in response to RGx31 ($P<0.001$) and x31-HK ($P<0.05$).

Virus-Specific $CD8^+$ T-Cell Response Because PA and NP epitopes predominate in primary influenza infection, influenza PA- and NP-specific tetramers were used to analyze the

influenza A specific CD8+ T-cell response in the BAL fluid on days 7 and 9 after infection (figure 6). The trend was for a stronger response following infection with x31-Vietnam, but additional experiments are required to confirm this.

B-cell Response to Intradermal Inoculation To investigate whether the growth of these viruses is detrimental to dendritic cells, mice were inoculated intradermally on the cheek with 10^6 eID_{50} in 30 μl PBS. Intradermal inoculation was chosen because of the large number of Langerhans (epidermal dendritic) cells present in the epidermis. On day 7, cervical lymph nodes (CLN) were harvested, disrupted, and used in an ELISpot analysis (figure 7). Since only two mice were infected with each virus, the number of samples prevented comprehensive statistical analysis. However, differences appear to be minimal.

Mortality in B-Cell Deficient Mice Since an early, robust antibody response seems to correlate with earlier virus clearance and decreased mortality, we analyzed the virulence of the viruses in a system lacking B cells. Though μMT mice lack B cells, they have an otherwise intact immune system and can mount a T-cell response to infection. Using B6 mice as controls, 5 μMT mice were infected with 10^6 eID_{50} in 30 μl PBS of either RGx31 or x31-Vietnam and monitored daily for 14 days (figures 8A-B). By day 12, all μMT mice infected with RGx31 had succumbed to infection, as had 80% of the control group. In contrast, all μMT and control mice infected with x31-Vietnam survived. This demonstrates that the ability of mice to survive infection with x31-Vietnam cannot be attributed solely to a more robust B-cell response.

Alignment of NS1 Sequences An amino acid sequence alignment of RGx31, x31-HK, and x31-Vietnam was performed. Several differences appeared between the three sequences; the NS gene from A/Hong Kong/156/97 (H5N1) revealed the presence of a glutamic acid residue at position 92 as has been reported previously (Seo et al., 2002). The sequence of the NS gene

from A/Vietnam/1203/04 (H5N1) revealed a 5-amino acid deletion from residue 80 to residue 84 but position 92 contained an aspartic acid residue; the NS gene sequence from PR8 also contained an aspartic acid at position 92. Other differences existed between the three sequences, though they were not localized within a particular known functional domain; the determination of the individual contribution of each substitution is beyond the scope of this project. (sequence alignments available but not included.)

DISCUSSION

Influenza A virulence is the result of the combined effects of the viral gene constellation and the immunocompetence of the host that it infects. While researchers know much about what makes an influenza A virus particularly virulent in birds, much less is known about specific genetic traits conferring enhanced virulence in humans. To date, the avian H5N1 influenza A epidemic in southeastern Asia has claimed the lives of 51 of the 71 confirmed cases in Vietnam since 2003 (Minh, 2005); this 71.8% mortality rate is particularly alarming, especially considering that most of the fatalities occurred in previously healthy individuals with no underlying predispositions. Because these avian influenzas are so virulent in humans, it is imperative that every effort be made to understand the mechanisms by which this virulence is enhanced.

Previous reports (Lipatov et al., 2005; Seo et al., 2002; Seo et al., 2004) have implicated alterations in the NS gene in the enhancement of virulence. When inserted into a PR8 parental virus, the NS gene of A/Hong Kong/156/97 (H5N1) (HK/156/97) was found to confer greater resistance to antiviral cytokines and result in a longer period of virus shedding. However, this observation was not repeated in this study. When inserted into an x31 parental virus, the NS gene from A/Hong Kong/156/97 (H5N1) was found to attenuate the pathogenicity of the parental

strain. When the NS segment from A/Vietnam/1203/04 (H5N1) was inserted into an x31 strain, it too was found to attenuate the parental virus, but to a greater extent than was seen with the NS from HK/156/97. Because all viruses grew at comparable rates and reached comparable titers in tissue culture, differences in virulence *in vivo* can be attributed to differing interactions with the mammalian host. *In vivo*, all viruses reached a similar peak titer on day 2 after high-dose infection, suggesting that differences in virulence are independent of growth kinetics in the lungs. However, differences in lung titer begin to appear on day 5, and by day 9, the parental RGx31 is the only virus with a measurable titer after high dose infection.

ELISpot assays indicate a strong relationship between antibody response and virus clearance. As early as day 5, the antibody response to x31-Vietnam was more robust and more isotype switching was observed; x31-Vietnam also had the lowest lung titer on day 5. The day 5 antibody responses to RGx31 and x31-HK were weaker, correlating with higher viral lung titers. By day 7, the amount of isotype switching had increased in response to all three viruses, but the difference between RGx31 and x31-Vietnam was statistically significant for all isotypes other than IgM. By day 9, both x31-HK and x31-Vietnam viruses had been cleared from the lung and the antibody response to x31-Vietnam was already beginning to wane. Total cell counts from MLNs collected on all sampling days also support the observation of a stronger B-cell response to the x31-Vietnam recombinant. On day 9, after the x31-Vietnam recombinant had been cleared from the lungs, the MLNs collected from mice infected with x31-Vietnam still had a significantly ($P<0.05$) higher number of cells in the lymph node when compared to nodes collected from mice infected with RGx31.

These findings suggest that the x31-Vietnam recombinant induces a stronger early antibody response, resulting in faster clearance of the virus as compared to RGx31 and x31-HK.

Antibodies aid in preventing the spread of progeny virions by neutralizing the surface antigens hemagglutinin (HA) and neuraminidase (NA), which are involved in the release and binding of progeny virions.

The influenza A NS1 protein is a known interferon α/β antagonist and has been shown to significantly reduce the amount of type I interferons produced by preventing the activation of NF-κB by dsRNA. More specifically, studies done by Wang, et al, suggest that the dsRNA binding domain of the NS1 protein is involved in preventing NF-κB activation by sequestering viral dsRNA and thus preventing the activation of protein kinase R (PKR), which is required for the activation of NF-κB (Wang et al., 2000). Alterations in this property of the NS1 protein could play a role in the attenuation of the adaptive immune response to x31-HK and x31-Vietnam observed in this study. The production of type I interferons has been found to enhance humoral immunity and also to promote antibody isotype switching. However, results indicate that type I interferons do not act directly on B-cells but instead act on dendritic cells (Le Bon et al., 2001). Studies done by Lipatov, et al found that inserting the NS gene from A/chicken/Hong Kong/YU562/01 (H5N1), which contained a 5-amino acid deletion similar to that seen in A/Vietnam/1203/04, into PR8 completely attenuated the virus in mice (Lipatov et al., 2005). It is possible that this deletion adversely affects the virus's ability to prevent induction of type I interferons, which could explain the accelerated antibody response and isotype switching seen in response to the x31-Vietnam recombinant.

Though day 5 is early in the adaptive immune response, we used flow cytometry to analyze the proportions of B- and T-cells present in the MLN on day 5. Consistent, substantial differences were seen between the groups. Though the antibody response to RGx31 was the weakest on day 5, the lymph node contained the greatest proportion of B-cells. In contrast, the

proportions of B- and T-cells found in the MLNs responding to x31-Vietnam were approximately equal. However, the antibody response to x31-Vietnam on day 5 was the strongest; this could be explained by the stimulation of B cells of $CD4^+$ T-cells. Since the number of T cells seen in the MLNs responding to RGx31 and x31-HK was smaller, it is possible that fewer numbers of $CD4^+$ T cells were present, which could help account for the lower antibody response.

To determine whether the growth of the recombinant viruses had an adverse effect on dendritic cells and thus the resulting antibody response, we infected mice intradermally on the cheek. The ELISpot data from the CLNs harvested on day 7 does not indicate a difference in B-cell activation by dendritic cells. However, the environment in the lung is very different from that in the epidermis, so it is still possible that, with assistance from other factors not present in the epidermis, the viruses could have an adverse effect on dendritic cells. This could also help account for the difference in the antibody response to each of the recombinants.

To determine the significance of the B-cell response to the parental RGx31 and x31-Vietnam recombinants, B-cell deficient mice (μMT) were infected with a high dose of virus. All μMT mice infected with RGx31 succumbed to the infection by day 14 as did 80% of the wild type B6 mice. In contrast, none of the mice infected with the x31-Vietnam recombinant died from the infection. This indicates that, while the B-cell response is important in controlling an influenza infection, other factors play significant roles. Using B6 mice, we analyzed the $CD8^+$ influenza-specific T-cell response. PA- and NP-specific tetramer staining indicated a significantly stronger influenza-specific response to x31-Vietnam as compared to RGx31; this is likely to be a factor contributing to the low virulence of x31-Vietnam in mice.

Contrary to previous reports, the NS gene segment from A/HK/156/97 does not appear to confer increased virulence when inserted into a mouse-adapted parental backbone. In fact, the NS segment from that virus slightly attenuated the virus when compared to the parental strain. Additionally, the NS gene segment from A/Vietnam/1203/04 substantially attenuates the parental strain. However, both the B- and T-cell responses to the x31-Vietnam recombinant were significantly stronger. Though both NS gene segments came from fatal human cases, previous studies have indicated that virulence in humans does not necessarily correlate with the virulence of the viruses when put into the mouse model (Lu et al., 1999). Many factors could explain this discrepancy, but additional experiments are necessary. In order to confirm that the circulating antibody levels correlate with the number of antibody secreting cells observed by ELISpot assays, more comprehensive ELISAs must be completed. Also, cytokine analyses of the lung tissue homogenates may shed light on the differences in cytokine induction by the different viruses. Other studies have indicated that high doses of infection with influenza results in an aborted $CD8^+$ T-cell response, which could help explain the difference in the $CD8^+$ response to the differenct recombinants (Legge and Bracaile, 2005). More comprehensive $CD8^+$ T-cell analyses would also be beneficial, looking at both the influenza-specific response as well as induction of apoptosis. However, all data from this project indicates that the NS gene segments from HK/156/97 and Vietnam/1203/04 attenuate the parental RGx31 virus. Nevertheless, it has established a valuable system to probe the mechanisms of virulence conferred by the NS sequence. While no enhancement of virulence was seen as a result of either H5N1 NS segment, differences in virulence based on sequence were observed.

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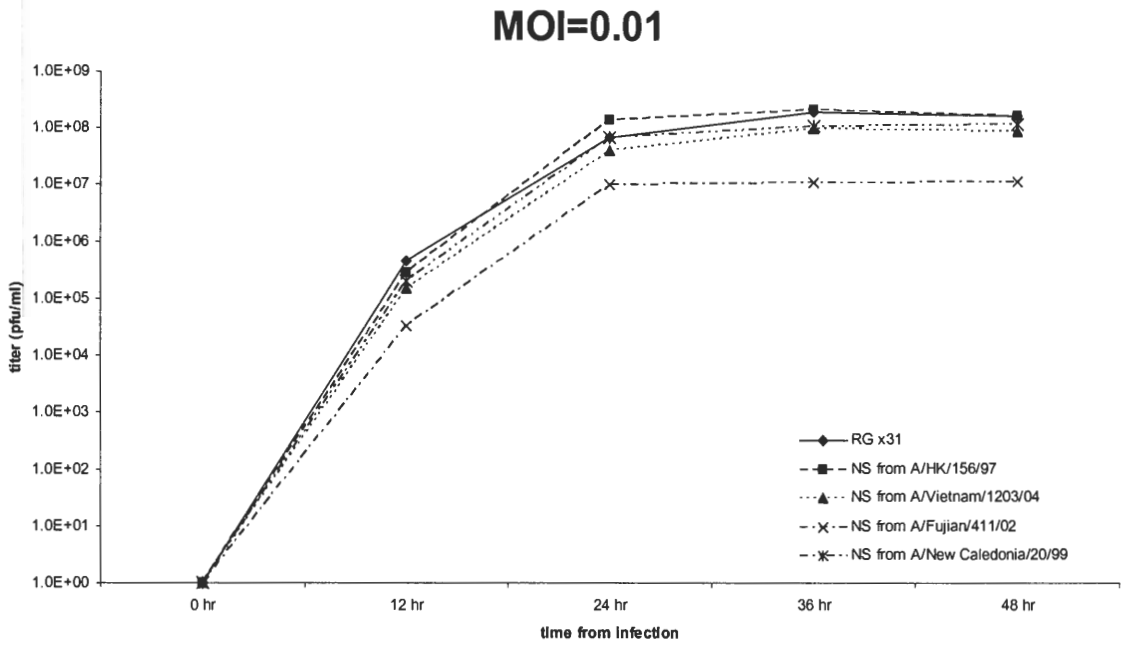
VIRUS	Plaque Assay Titer (pfu/0.1ml)	Egg 50% Infectious Dose (eID_{50}/0.1ml)	Tissue Culture 50% Infectious Dose ($TCID_{50}$/0.1ml)
RGx31	$4.50x10^7$	$1.00x10^9$	$4.88x10^8$
x31-NS(Hong Kong/156/97)	$8.00x10^7$	$3.16x10^8$	$2.50x10^8$
x31-NS(Vietnam/1203/04)	$3.00x10^7$	$3.16x10^8$	$2.50x10^8$
x31-NS(Fujian/411/02)	$2.70x10^7$	$3.16x10^8$	$1.58x10^8$
x31-NS(New Caledonia/20/99)	$7.30x10^7$	$3.16x10^8$	$1.25x10^9$

Table 1. Titers of each virus were determined in three different interferon-competent systems. MDCK plaque assays gave titers in plaque-forming units. Ten-day old embryonated hens' eggs gave titers in eID_{50} units. MDCK cells and hemagglutination assays were used to give titers in $TCID_{50}$ units.

A.



B.

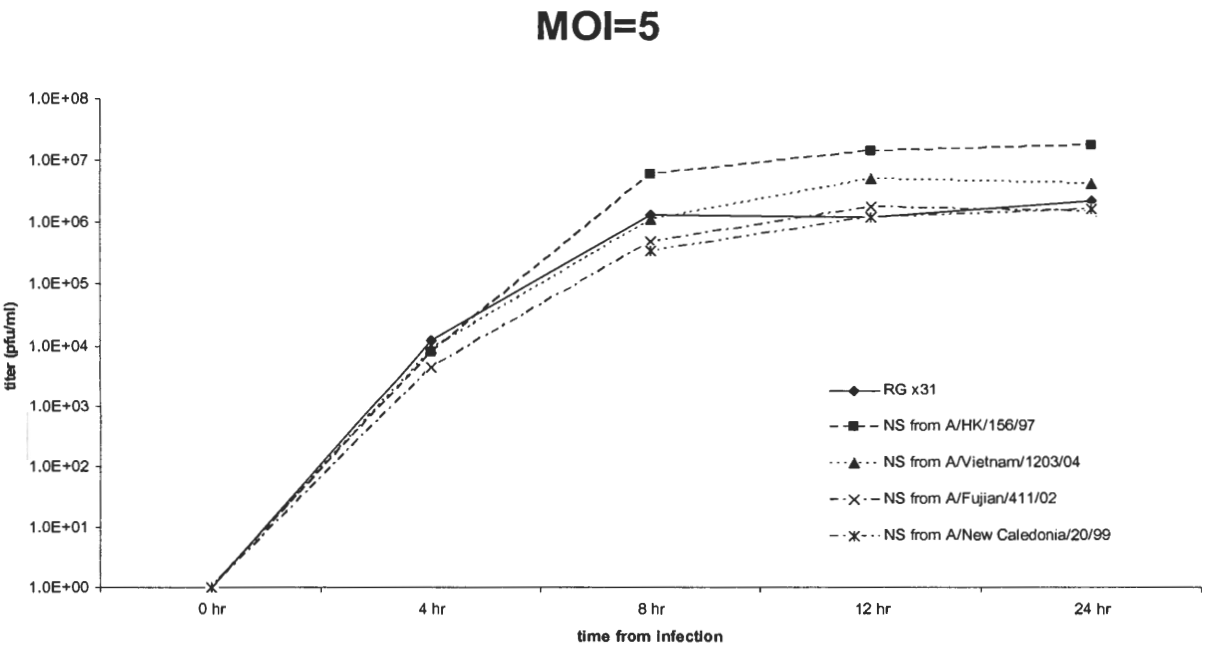


Figure 1. MDCK cells were infected with recombinant influenza A viruses. (A) Cells were infected at an MOI of 0.01 to evaluate replication after multiple rounds of replication. Aliquots were taken every 12 hours. (B) Cells were infected at an MOI of 5 to evaluate replication after very few rounds. Aliquots were taken every 4 hours.

A.

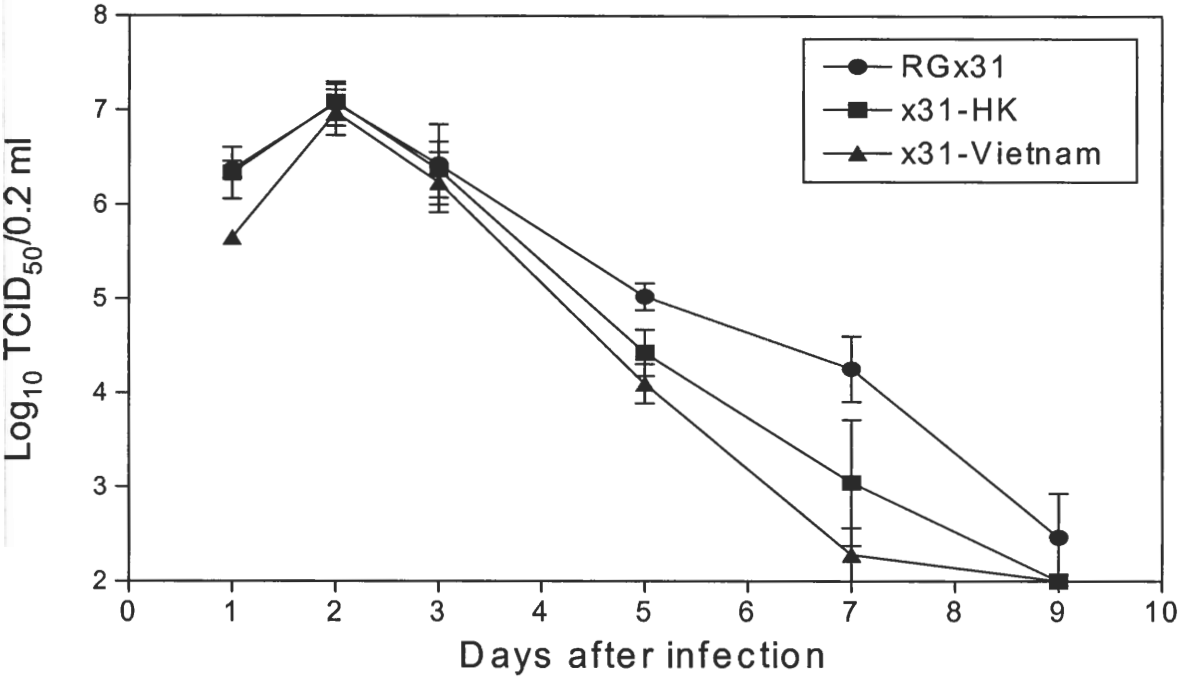
	# of mice dead per dose					
	dilution	1/5	1/10	$1/10^2$	$1/10^3$	$1/10^4$
Virus	**dose (elD_{50})**	$6x10^7$	$3x10^7$	$3x10^6$	$3x10^5$	$3x10^4$
RGx31		4/4	4/4	3/4	0/4	0/4
	dose (elD_{50})	$1.9x10^7$	$9.5x10^6$	$9.5x10^5$	$9.5x10^4$	$9.5x10^3$
x31-HK/156/97		4/4	4/4	0/3	0/4	0/4
x31-Vietnam/1203/04		0/4	0/4	0/4	0/4	0/4
x31-Fujian/411/02		1/4	0/4	0/4	0/4	0/4
x31-New Caledonia/20/99		3/3	3/4	3/4	0/3	0/4

B.

virus	mLD_{50} (pfu)
RGx31	$6.3x10^4$
x31-HK	$6.5x10^5$
x31-Vietnam	$>1.5x10^6$
x31-Fujian	$>1.5x10^6$
x31-New Caledonia	$8.7x10^4$

Table 2. (A) Mice were infected with various doses of each recombinant to determine virulence *in vivo*. From this data, a suitable dose for subsequent studies was determined. (B) Mouse 50% lethal dose determinations for each virus.

A.



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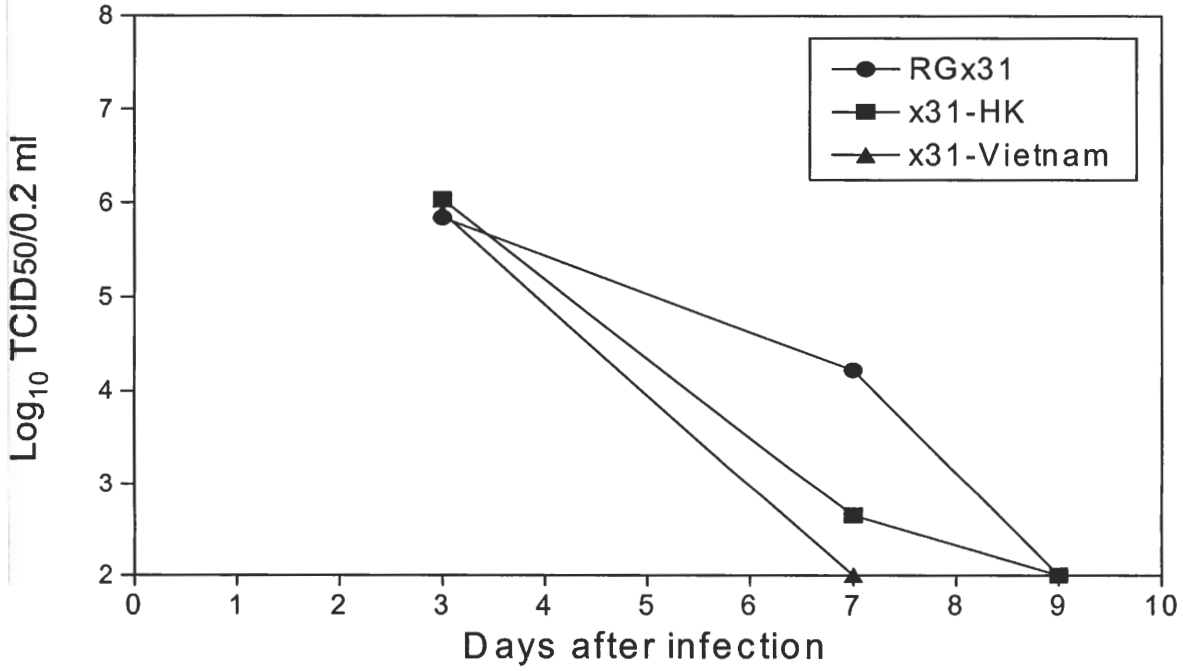
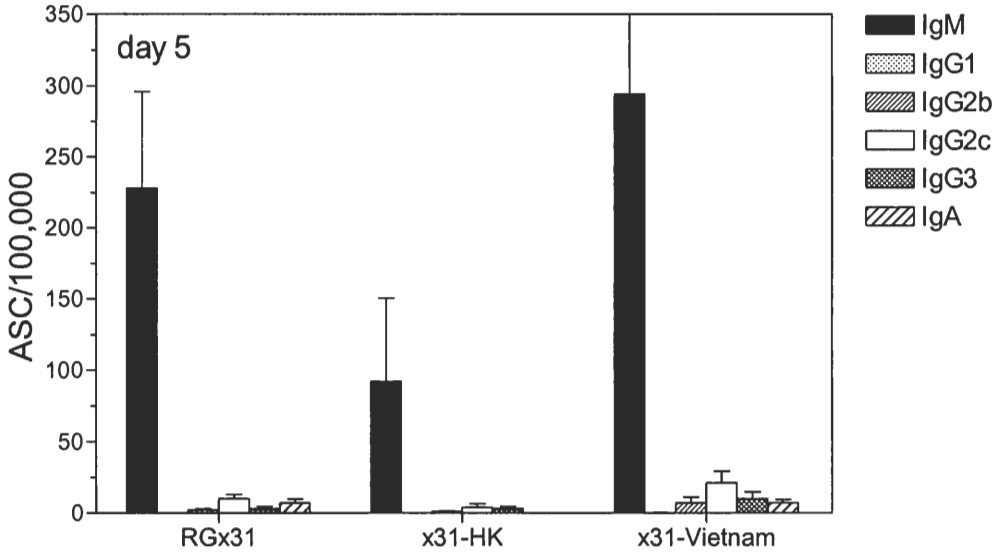
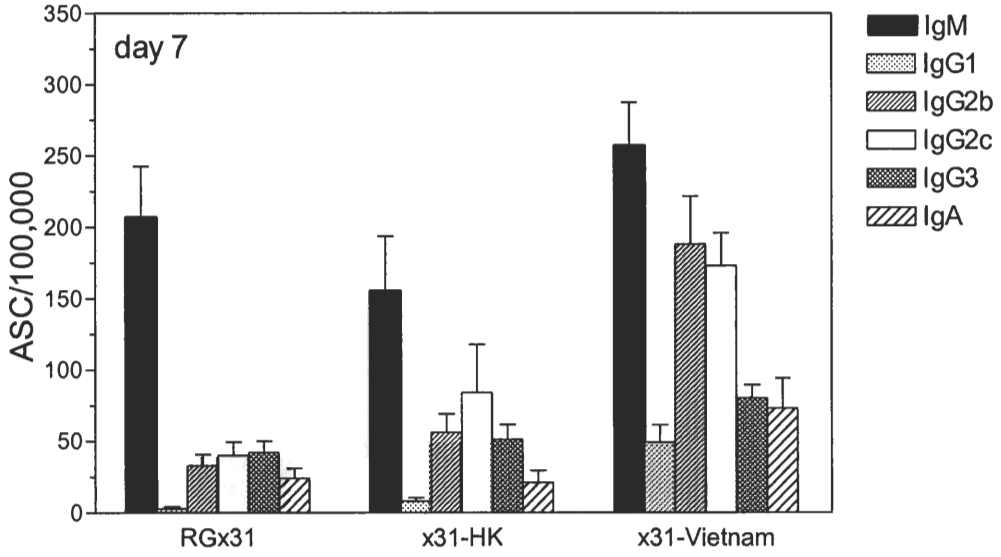


Figure 2. To analyze *in vivo* growth kinetics, lungs were sampled at intervals after infection. (A) Mice were infected intranasally with 10^6 eID_{50} in 30 μl PBS (high dose). On day 7, the difference in titer between RGx31 and x13-Vietnam was statistically significant. (B) Mice were infected with 10^4 eID_{50} in 30 μl PBS (low dose); on day 3 PI, titers were nearly identical.

A.


day 5
ASC/100,000
350
300
250
200
150
100
50
0
RGx31
x31-HK
x31-Vietnam
IgM
IgG1
IgG2b
IgG2c
IgG3
IgA

B.


day 7
ASC/100,000
350
300
250
200
150
100
50
0
RGx31
x31-HK
x31-Vietnam
IgM
IgG1
IgG2b
IgG2c
IgG3
IgA

C.

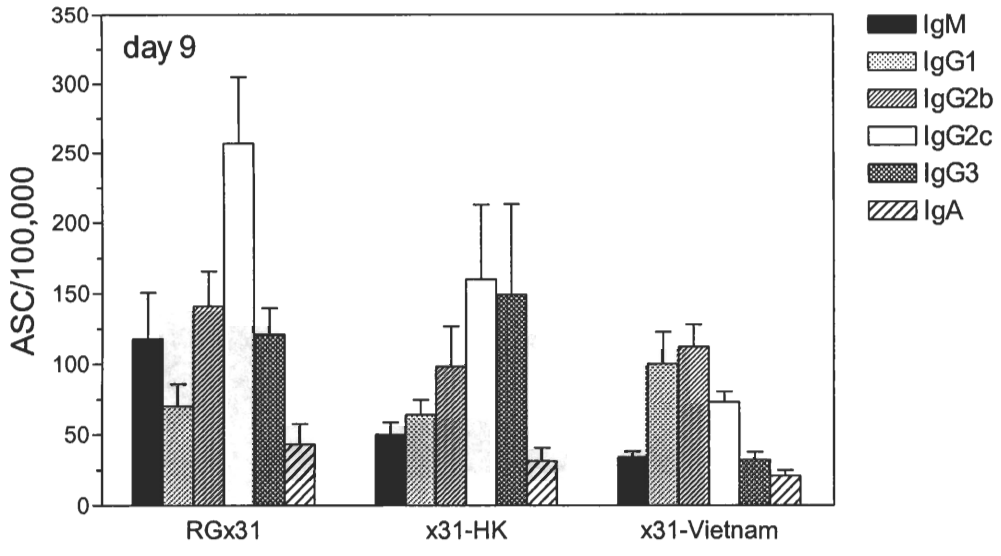

day 9
ASC/100,000
350
300
250
200
150
100
50
0
RGx31
x31-HK
x31-Vietnam
IgM
IgG1
IgG2b
IgG2c
IgG3
IgA

Figure 3. Mediastinal lymph nodes were harvested after 5, 7, and 9 days post-infection. ELISpot assays for IgM, IgG, IgG1, IgG2c, IgG2b, IgG3, and IgA were performed after each sampling. (A) Results from day 5. (B) Results from day 7. The magnitude of the switched-isotype antibody response to x31-Vietnam is significantly greater than the response to RGx31: IgG2b ($p<0.001$); IgG1 and IgG2c ($p<0.01$); and IgG3 and IgA ($p<0.05$). (C) Results from day 9. Results were reported as the number of influenza-specific antibody secreting cells (ASC) per 100,000 nucleated cells.

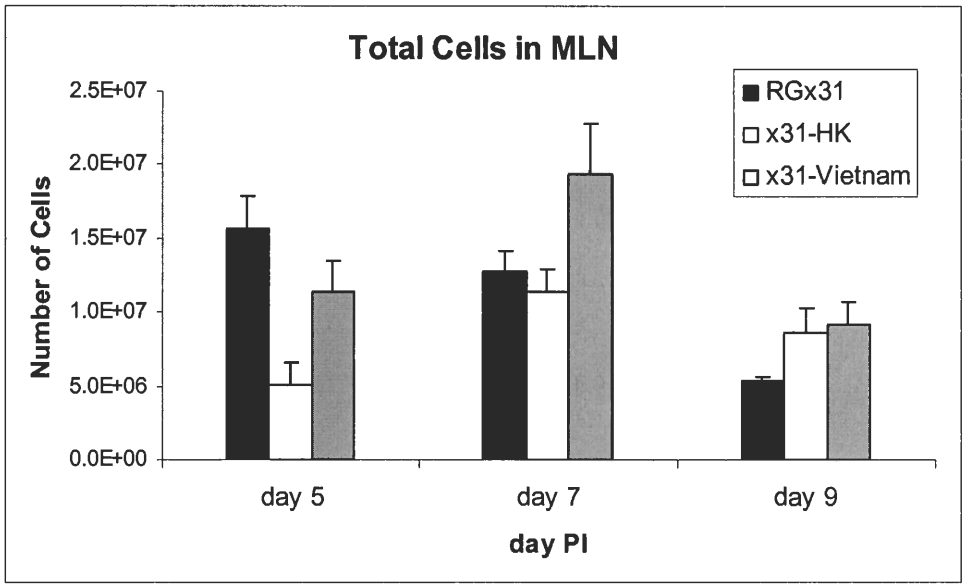


Figure 4. Cells from each MLN were counted for each sampling. The number of cells observed in MLNs responding to x31-Vietnam was significantly greater ($P<0.05$) than the number of cells observed in the MLNs responding to RGx31.

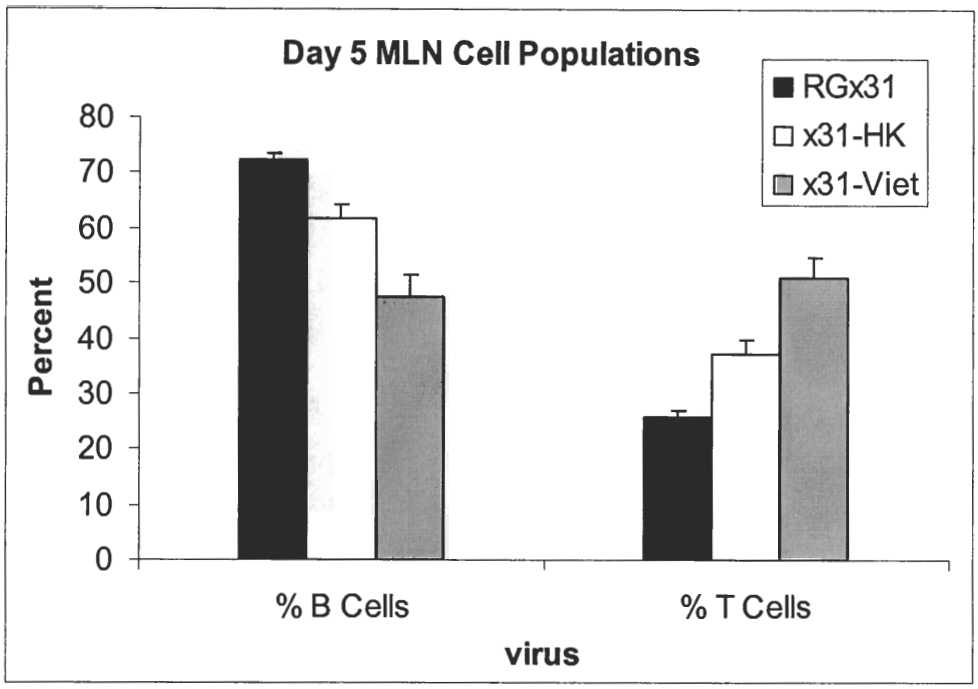


Figure 5. FACS analysis of the day 5 MLN lymphocyte population. B-cells were distinguished based on the presence of CD19 on the cell surface. T-cells were distinguished based on the presence of CD3 on the cell surface. The percentage of B cells in the lymph nodes responding to x31-Vietnam is significantly higher than that seen in lymph nodes responding to RGx31 ($P<0.001$) and x31-HK ($P<0.05$). The percentage of T cells in the lymph nodes responding the x31-Vietnam was significantly lower than that seen in response to RGx31 ($P<0.001$) and x31-HK ($P<0.05$).

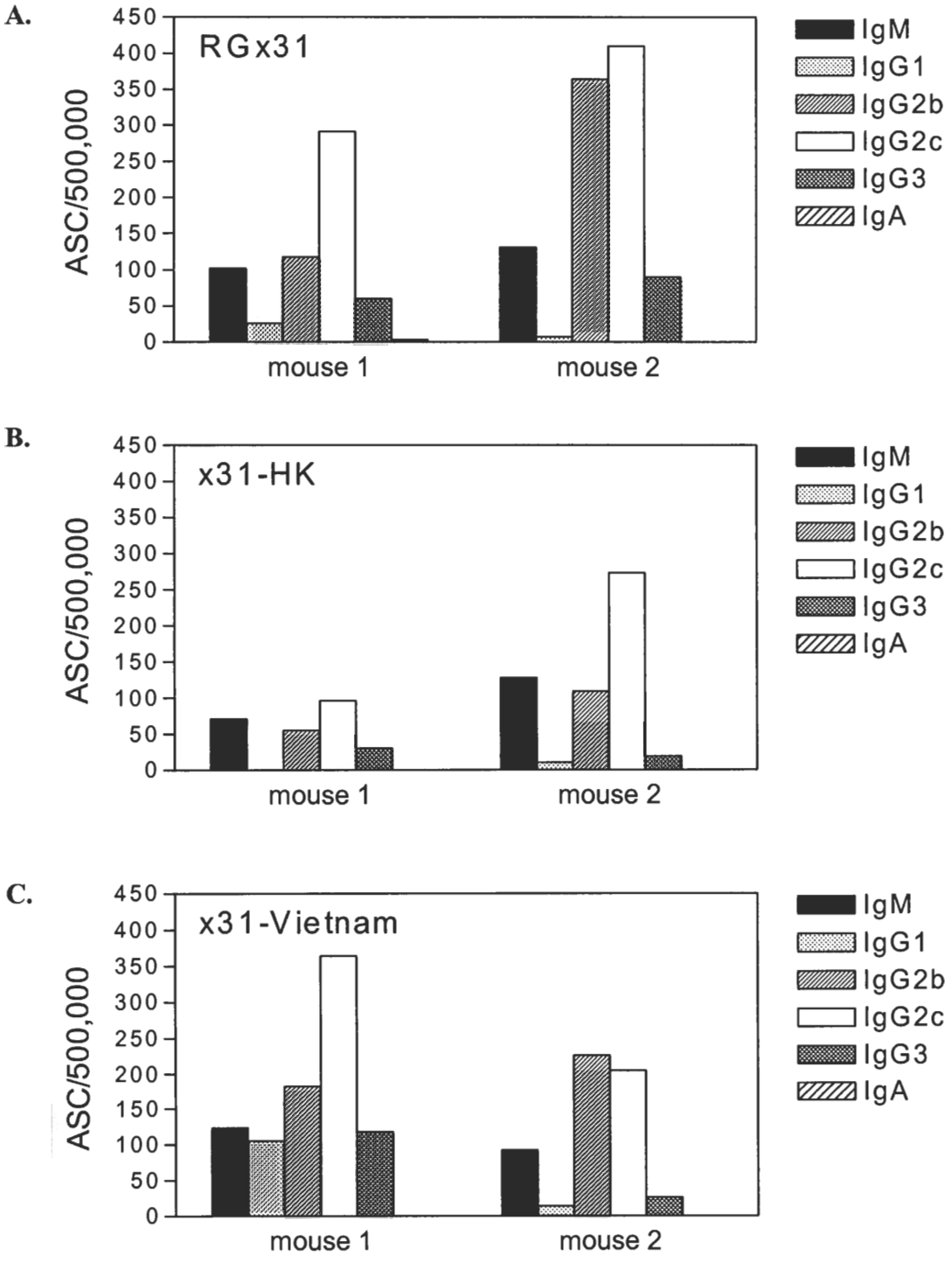
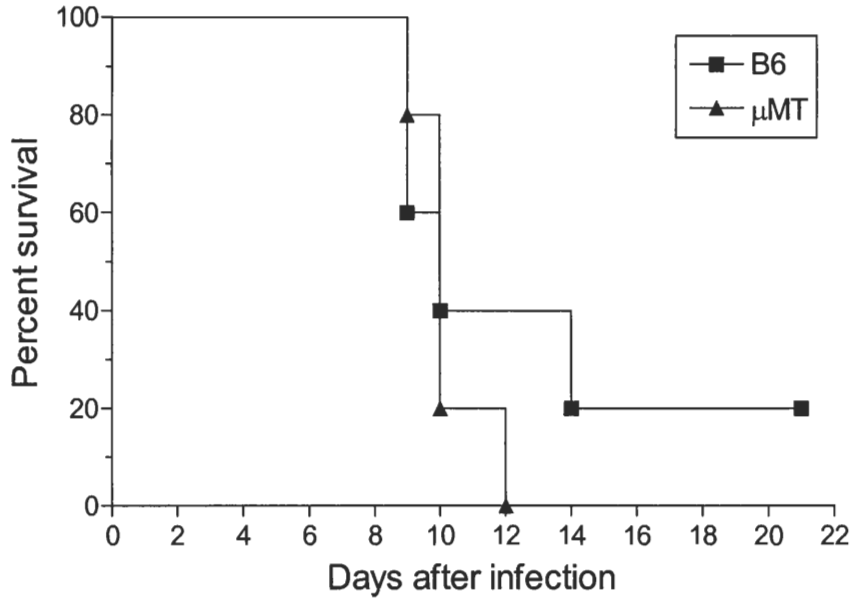


Figure 7. Mice were infected intradermally on the side of the cheek with 10^6 eID_{50} in 30 μl PBS. On day 7 PI, cervical lymph nodes (CLN) were harvested. ELISpot assays were performed to analyze the antibody response. (A) CLN antibody response to parental RGx31. (B) CLN antibody response to x31-HK. (C) CLN antibody response to x31-Vietnam. Results were reported as the number of influenza-specific ASCs per 50,000 nucleated cells.

A.



B.

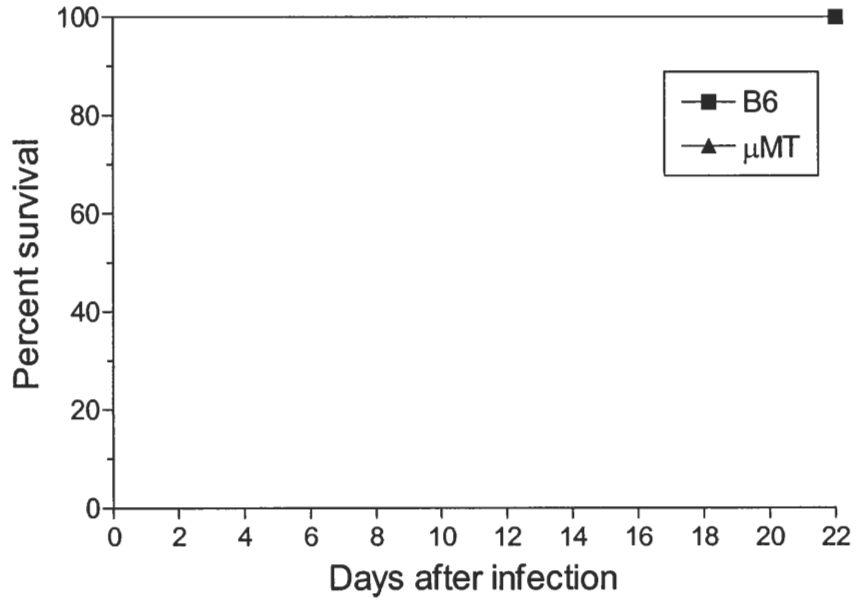
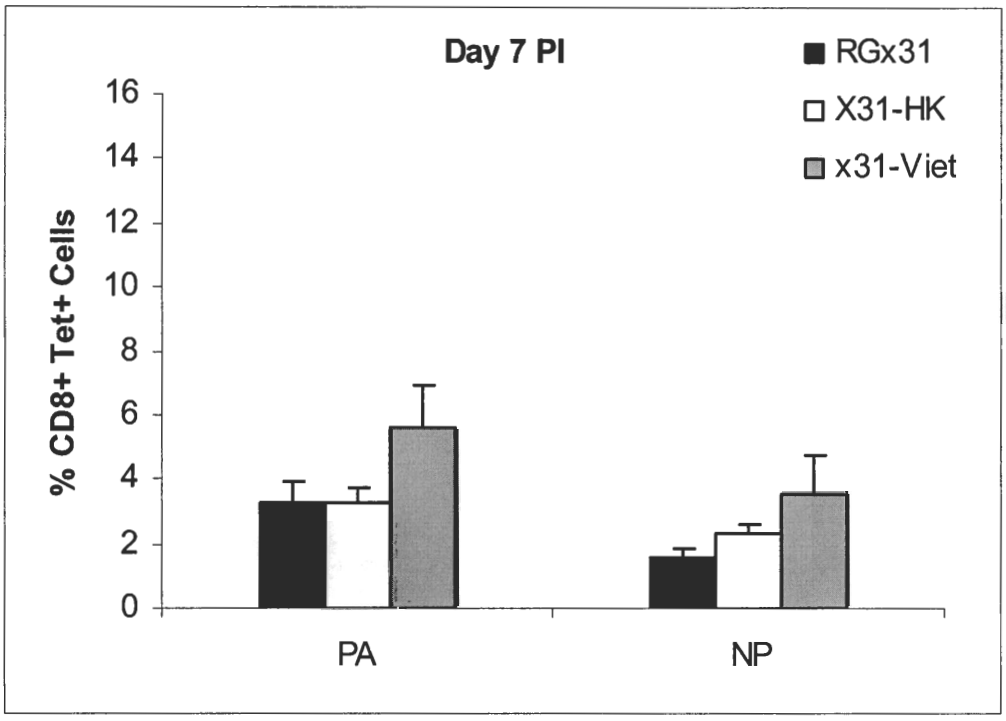
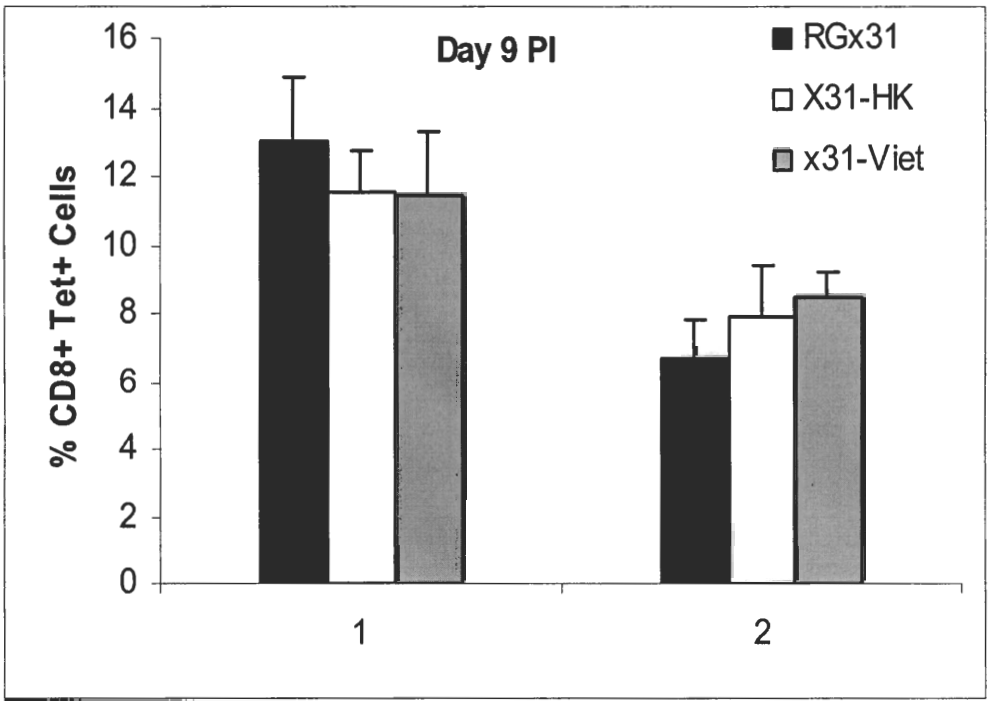


Figure 8. Results from mortality studies using wild-type B6 and B-cell deficient (μMT) mice. All mice were infected with 10^6 eID_{50} in 30 μl PBS and observed for 14 days. (A) Results from infection with parental RGx31. All B-cell deficient mice died; only one wild-type mouse survived. (B) Results from infection with x31-HK. All mice of both strains recovered.

A.


Day 7 PI
RGx31
X31-HK
x31-Viet
% CD8+ Tet+ Cells
16
14
12
10
8
6
4
2
0
PA
NP

B.


Day 9 PI
RGx31
X31-HK
x31-Viet
% CD8+ Tet+ Cells
16
14
12
10
8
6
4
2
0
1
2

C.

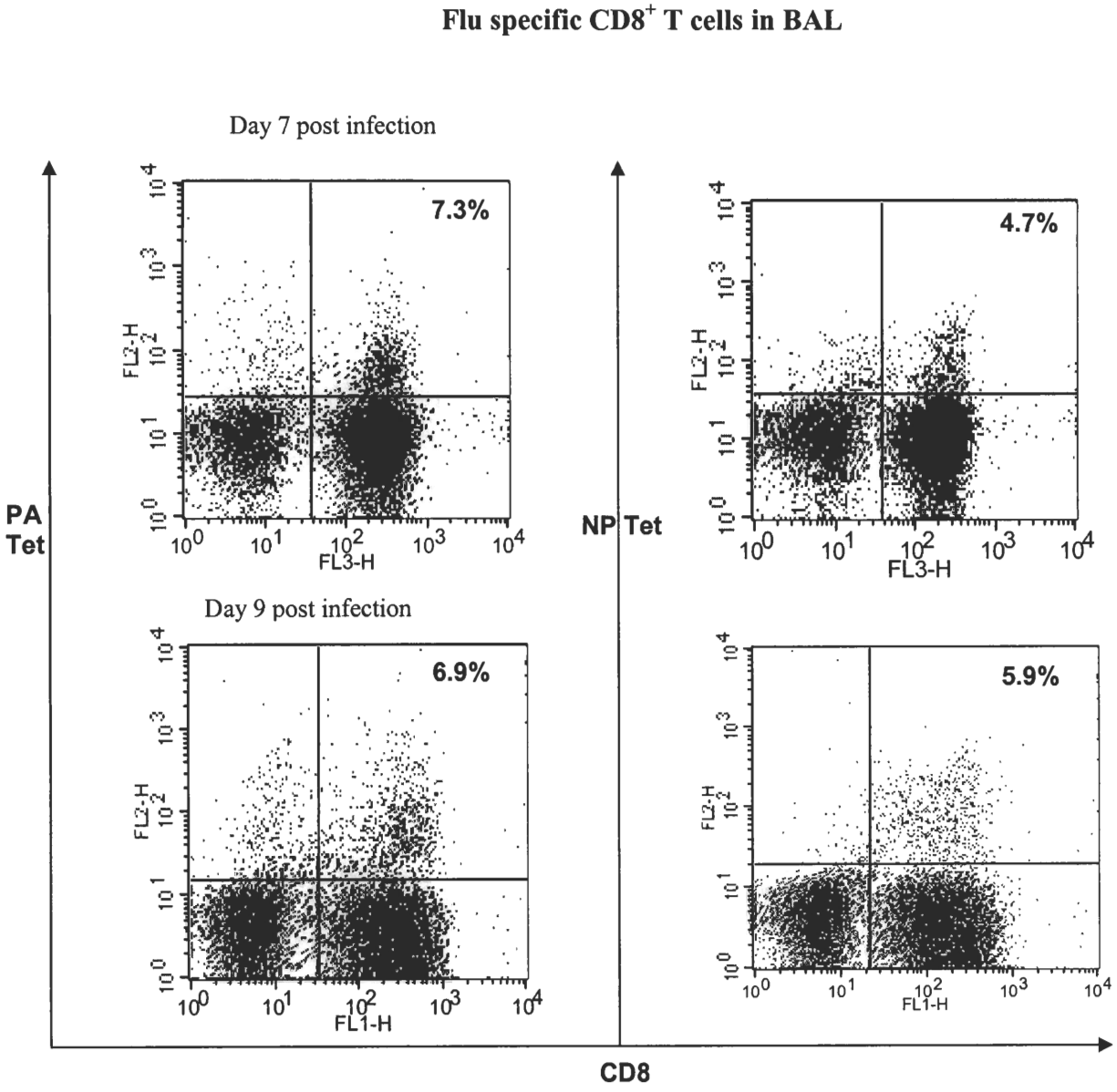


Figure 6. The influenza-specific $CD8^+$ T-cell response was analyzed using PA- and NP-specific tetramers. (A) The influenza-specific $CD8^+$ response on day 7 PI. (B) The influenza-specific $CD8^+$ response on day 9 PI. (C) FACS analysis of $CD8^+$ NP- or PA-specific response using PA- or NP- specific tetramers. Dot plots are from days 7 and 9 post-infection with x31-Vietnam and are shown as representative staining. The percentage of PA-specific $CD8^+$ T-cells in response to x31-Vietnam was significantly greater than the response to RGx31 ($P<0.05$) and to x31-HK ($P<0.01$). The PA- or NP-specific $CD8^+$ response is given as the percent of total $CD8^+$ T-cells in the sample.