Pathogenesis of Hong Kong H5N1 influenza virus NS gene reassortants in mice: the role of cytokines and B- and T-cell responses

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The severity of disease caused in humans by H5N1 influenza viruses remains unexplained. The NS gene of Hong Kong H5N1/97 viruses was shown to contribute to high pathogenicity of reassortants in a pig model. However, the molecular pathogenesis and host immune response underlying this phenomenon remain unclear. Here, in a mouse model, H1N1 A/Puerto Rico/8/34 (PR/8) reassortants that contained the H5N1/97 NS gene, the H5N1/01 NS gene, or an altered H5N1/97 NS gene encoding a Glu92→Asp substitution in NS1 was studied. The pathogenicity of reassortant viruses, the induction of cytokines and chemokine CXCL1 (KC) in the lungs and specific B- and T-cell responses was characterized. In mice infected with reassortant virus containing the H5N1/97 NS gene, the mouse lethal dose (50 %) and lung virus titres were similar to those of PR/8, which is highly pathogenic to mice. This reassortant virus required two more days than PR/8 to be cleared from the lungs of infected mice. Reassortants containing the altered H5N1/97 NS gene or the H5N1/01 NS gene demonstrated attenuated pathogenicity and lower lung titres in mice. Specific B- and T-cell responses were consistent with viral pathogenicity and did not explain the delayed clearance of the H5N1/97 NS reassortant. The reassortant induced elevated pulmonary concentrations of the inflammatory cytokines IL1α, IL1β, IL6, IFN-γ and chemokine KC, and decreased concentrations of the anti-inflammatory cytokine IL10. This cytokine imbalance is reminiscent of the clinical findings in two humans who died of H5N1/97 infection and may explain the unusual severity of the disease.

INTRODUCTION

In 1997 in Hong Kong, avian H5N1 influenza A virus (H5N1/97) was transmitted directly from poultry to humans with an overall case-fatality rate of 33 % (Claas et al., 1998; Subbarao et al., 1998). The severity and high mortality rate were not explained by underlying disorders (Yuen et al., 1998). Affected patients had a primary viral pneumonia complicated by acute respiratory distress, multiple organ dysfunction and haemophagocytosis (To et al., 2001; Yuen et al., 1998), all of which are associated with cytokine dysregulation (Fisman, 2000; Headley et al., 1997). Despite numerous studies in animal and cell models, the basis of the unusual pathogenicity of Hong Kong H5N1/97 influenza viruses remains unclear.

In the lungs and spleens of infected mice, all of the H5N1/97 viruses isolated from humans induce a high concentration of cytokines, such as interleukin (IL)-1β and interferon (IFN)-γ, and of the chemokine macrophage inflammatory protein (MIP)-1α, (Tumpey et al., 2000). In human primary monocyte-derived macrophages, the H5N1/97 viruses induced much higher transcription of pro-inflammatory cytokines, particularly tumour necrosis factor (TNF)-α and IFN-β, than did H3N2 or H1N1 viruses...
The laboratory human virus strain A/Puerto Rico/8/34 (H1N1) (PR/8), the H5N1 human isolate A/Hong Kong/156/97 (HK/156/97) and the avian H5N1 virus A/Chicken/Hong Kong/YU562/01 (Ck/HK/YU562/01) were obtained from the repository of St. Jude Children’s Research Hospital. Viruses were propagated in 10-day-old embryonated chicken eggs. All work with H5N1 viruses was conducted in a biosafety level 3 facility approved by the US Department of Agriculture.

Madin-Darby canine kidney (MDCK) cells and 293T human embryonic kidney cells were used for rescue of reassortant viruses from plasmids. MDCK cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). 293T cells were cultured in Opti-MEM I (Life Technologies) containing 5% FBS.

Isolation of viral RNA, reverse transcription, PCR amplification and sequencing. Extraction of viral RNA, synthesis of cDNA and PCR were performed as described previously (Hoffmann et al., 2000), with minor modifications. Sequencing was performed by the Hartwell Center for Biotechnology at St. Jude Children’s Research Hospital with the rhodamine dye-terminator cycle sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (Perkin-Elmer Applied Biosystems).

Cloning and generation of viruses by reverse genetics. The eight genes of PR/8 and the NS genes of HK/156/97 and Ck/HK/YU562/01 were cloned as previously described (Hoffmann et al., 2000; Hoffmann et al., 2001; Hoffmann et al., 2002). Plasmids were sequenced as described above, and the sequences were compared with those generated from the wild-type virus. Only clones that exactly matched the parental virus sequence were used for virus rescue by reverse genetics. Viruses were rescued by using the eight-plasmid system (Hoffmann et al., 2000), with minor modifications. Briefly, eight plasmids (1 μg of each) were incubated for 45 min with Trans-LTI (Panvera) in Opti-MEM I and were used to transfect a 1:1 mixture of 293T and MDCK cells as described previously (Hoffmann et al., 2000). Supernatant collected from transfected cells after 72 h was used to inoculate 10-day-old embryonated chicken eggs. PR/8 virus and the following NS-gene reassortant viruses were rescued from the plasmids: PR/8 × NS HK/156/97 (PR/8 × NS H5N1/97), PR/8 × NS Ck/HK/YU562/01 (PR/8 × NS H5N1/01) and PR/8 × NS192→Asp HK/156/97 (PR/8 × NS mut. H5N1/97). Allantoic fluid containing virus was harvested, and its infectivity was titrated in eggs; virus titres were expressed as log10 of the 50% egg infectious dose per 0.1 ml of fluid (log10 EID50 per 0.1 ml), according to the method of Reed and Muench (1938). Virus stocks were divided into aliquots and stored at −80°C.

Site-directed mutagenesis. We used PCR with overlapping internal primers (Seo et al., 2002) to substitute Asp for Glu at position 92 of the HK/156/97 virus NS1 protein. The reassortant virus that incorporated this gene in the background of the PR/8 virus was rescued as described above.

Mice and virus inoculation. Female, 6-week-old BALB/c mice (The Jackson Laboratory) were used for studies of viral pathogenicity and determination of the mouse 50% lethal dose (MLD50). Female, 10-week-old C57BL/6J mice (The Jackson Laboratory) were used for immunological experiments. To determine the MLD50 we anaesthetised mice in groups of four by isoflurane inhalation and infected them intranasally with 50 μl 10-fold serial dilutions of allantoic fluid in PBS. Animals were observed daily for 15 days after inoculation. MLD50 was calculated by the method of Reed and Muench (1938) and was defined as the number of EID50 resulting in 50% mortality. Viral pathogenicity (pulmonary viral titres, cytokine assays and histopathology) was studied in groups of 26 mice inoculated as described above with 50 μl PBS-diluted allantoic fluid containing 100 EID50 of virus per mouse. Three to six mice in each group were killed on days 3, 6, 7, 8, 9, 10 and 11 after inoculation, and lungs, brain and spleen were removed; an approximately 10% homogenate of each tissue was prepared as described previously (Lipatov et al., 2003) and stored at −80°C. Virus was titrated in lungs, brain and spleen, and cytokine concentrations were determined in lungs. Virus in tissue homogenates was titrated and the EID50 was determined in 10-day-old embryonated chicken eggs. The lower limit of virus detection was 0.1 log10 EID50 per 0.1 ml of tissue homogenate.

For the immunological studies, groups of 10 C57BL/6J mice were inoculated intranasally with 100 EID50 of virus as previously described (Sarawar & Doherty, 1994). Animals were killed on days 7
and 10 after virus inoculation; the cervical lymph nodes (CLN), mediastinal lymph nodes (MLN) and spleen were removed, and blood and broncho-alveolar lavage (BAL) specimens were obtained.

**Assay of cytokines and chemokine CXCL1 (KC).** Cytokine and chemokine proteins were measured in mouse lung homogenates collected on days 3, 6 and 9 after virus inoculation by using the Bio-Plex Mouse Cytokine 17-Plex panel (Bio-Rad Laboratories). Lung homogenate was clarified by centrifugation at 2000 g for 10 min. The assay was performed in 50 μl homogenate as specified by the manufacturer and was read on the Bio-Plex Protein Array system (Bio-Rad Laboratories).

**Assay of virus-specific antibody-forming cells and antibodies.** Influenza-specific antibody-forming cells (AFC) were enumerated by ELISpot assay. Multiscreen HA 96-well filtration plates (Millipore) were coated with purified PR/8 influenza virus, and single-cell suspensions were plated and incubated as described previously (Sangster et al., 2000). Alkaline phosphatase-conjugated goat anti-mouse antibodies (Abs) specific for IgM, IgG1, IgG2b, IgG2c, IgG3 or IgA (Southern Biotechnology Associates) were diluted 1:500 in PBS plus 5% BSA and added to the plates. After overnight incubation at 4°C followed by extensive washing, the plates were developed at room temperature with 1 mg 5-bromo-4-chloro-3-indolyl phosphate (Sigma) mol−1 in diethanolamine buffer and washed and dried. Spots representing individual AFC were counted on an Olympus SZH Stereozoom microscope. Negative control plates were coated with purified Sendai virus. No spots were seen when lymph node cell suspensions from naive mice were tested on plates coated with either influenza virus or Sendai virus.

Influenza-specific serum Abs were measured by ELISA (Sangster et al., 2000), using plates coated with 0.5 μg per well of purified, detergent-disrupted PR/8 influenza virus. Briefly, threefold serial dilutions of sample were incubated in the plates. Bound Ab was detected with alkaline phosphatase-conjugated goat anti-mouse Abs specific for IgM, IgG1, IgG2b, IgG2c, IgG3 or IgA (Southern Biotechnology Associates), and colour was developed with p-nitrophenylphosphate substrate (Sigma). Absorbance was read at 405 nm on a SpectraMax 340 microplate reader with SoftMax Pro software ( Molecular Devices). The virus-specific Ab titre was defined as the reciprocal of the highest serum dilution giving an absorbance value greater than twice that of the samples from naive mice that were titrated in parallel.

**Flow-cytometric analysis.** The kinetics and magnitude of the virus-specific CD8+ T-cell responses were analysed by flow cytometry. CD8+ T cells in the disrupted splenic tissue were enriched (Hou et al., 1994) by incubation with mAbs to CD4 (GK1.5) and MHC class II glycoprotein (MS/114.15.2) and then with magnetic beads coated with anti-rat and anti-mouse IgG (Dynal). Lymphocytes were isolated from the mouse lungs by BAL, and macrophages were removed by incubation on plastic for 1 h at 37°C. The DNP586 and DP423 tetramers (Altman et al., 1996) were made by formation of a complex comprising H2Db plus the immunogenic NP586 (ASNENMETM) (Townsend et al., 1986) or PA423 (SSLNFRAYV) (Belz et al., 2000) peptides or H2Kb plus the polymerease 1 (PB1703, SSYRRVP) peptide (Belz et al., 2001). The lymphocytes were incubated for 60 min at room temperature with the PE-conjugated tetramers in PBS/BSA/azide (Flynn et al., 1998) and then stained with anti-CD8α-PerCP/Cy5.5 (PharMingen). Cells were sorted on a Becton Dickinson FACSscan instrument, and data were analysed by using CELLQUEST software (Becton Dickinson Immunocytometry Systems).

**Histopathologic analysis.** The lungs of mice infected with 100 EID50 of virus were harvested on day 6 after inoculation, washed in PBS, fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin and microscopically reviewed.

**RESULTS**

**Replication and MLD50 of reassortant viruses in mice**

The MLD50 is a quantitative estimation of pathogenicity that allows the comparison of different viruses. The laboratory strain PR/8, which was used as a background virus for the production of reassortants, caused high rates of morbidity and mortality in infected mice (MLD50 = 103.5). The reassortant virus PR/8 × NS H5N1/97 was also highly pathogenic in mice (MLD50 = 103.0), whereas the pathogenicity of reassortants PR/8 × NS mut. H5N1/97 and PR/8 × NS H5N1/01 was attenuated (MLD50 = 105.25 and 106.0, respectively). The original PR/8 virus and the NS reassortants had similar infectivity in embryonated chicken eggs (log10 EID50 = 7.75–8.0 per 0.1 ml).

To determine whether the NS genes of H5N1 viruses influence the organ tropism of reassortant viruses, we identified and titrated virus in the lungs, brains and spleens of infected mice. No differences were observed in organ tropism between the PR/8 and NS reassortant viruses: all studied viruses only replicated efficiently in the lungs. No viruses were detected in the brains or spleens of mice killed on days 2–11 after inoculation or in those of mice that died during MLD50 titration. Fig. 1 shows the kinetics of virus replication in the lungs of mice inoculated with 100 EID50 of PR/8 virus or NS reassortants. PR/8 virus replicated to high titres in mouse lungs on days 3–7 after inoculation and was completely cleared from the lungs on day 9. Reassortant PR/8 × NS H5N1/97 had titres only slightly

![Fig. 1. Virus titres in mouse lungs. Virus was titrated in 10% tissue homogenates after intranasal inoculation of mice with 100 EID50 of infective virus. Each value is the mean ± SD of the log10 EID50 determined in the lungs of three mice. The lower limit of virus detection was 0.1 log10 EID50 per 0.1 ml of tissue homogenate.](http://vir.sgmjournals.org)
The NS gene of Hong Kong H5N1/97 virus in mice

higher than PR/8, but a significant quantity of infectious PR/8 × NS H5N1/97 virus remained on day 9 after inoculation, and virus was not cleared from the lungs until day 11. Titres of PR/8 × NS mut. H5N1/97 virus approximated those of PR/8 only at day 3. On days 6–8 after inoculation, the kinetics of replication resembled that of PR/8 × NS H5N1/01 reassortant: both viruses were cleared from the lungs on day 9, and their titres were 1–2.5 log10 lower than those of PR/8 and PR/8 × NS H5N1/97 (Fig. 1).

The kinetics of virus replication was also determined in 10-week-old C57BL/6j mice, the host in which detailed immunological studies were performed. The results in C57BL/6j mice were very similar to those obtained in BALB/c mice (not shown), indicating similar kinetics of virus replication.

Cytokine and chemokine assays in the lungs of infected mice

We measured the concentration of cytokines and chemokine CXCL1 (KC) in the lungs of infected mice killed on days 3, 6 and 9 after inoculation. Mice inoculated with 50 μl sterile PBS served as negative controls. Eight cytokines and chemokines showed measurable concentrations greater than those of the controls. Fig. 2 shows the most significant differences among the viruses studied. Viruses that were highly pathogenic to mice, i.e. the original PR/8 and reassortant PR/8 × NS H5N1/97, induced much higher levels of inflammatory cytokines and chemokine CXCL1 in infected lungs than did the reassortant viruses with low mouse pathogenicity, PR/8 × NS mut. H5N1/97 and PR/8 × NS H5N1/01. Further, pulmonary concentrations of IL1α, IL1β, IL6, granulocyte macrophage colony stimulating factor (GM-CSF), and IL8-like chemokine CXCL1 (keratinocyte-derived chemokine, KC) were clearly increased in mice infected with the reassortant PR/8 × NS H5N1/97 as compared with mice infected with PR/8 (Fig. 2a, b, c, g, h). The reassortant PR/8 × NS mut. H5N1/97 induced lower levels of these cytokines and chemokine KC than did PR/8, and concentrations of IL1α, IL1β, IL6, GM-CSF and KC were close to negligible in the lungs of mice infected with PR/8 × NS H5N1/01 viruses (Fig. 2a, b, c, g, h). Levels of IL12 and IFN-γ were similar in the lungs of mice infected with the pathogenic viruses PR/8 and PR/8 × NS H5N1/97, were lower in the lungs of mice infected with PR/8 × NS mut. H5N1/97, and were very low in mice infected with PR/8 × NS H5N1/01 (Fig. 2e, f). The NS-reassortant viruses demonstrated interesting differences in their induction of the anti-inflammatory cytokine IL10: in the lungs of mice infected with PR/8 × NS H5N1/97, the concentration of this cytokine was only half that induced by PR/8, whereas the reassortant that contained the mutated NS gene, PR/8 × NS mut. H5N1/97, increased the concentration of IL10 to a level 2.5 times that induced by PR/8 × NS H5N1/97 (Fig. 2d). A trace amount of IL10 was detected in the lungs of mice infected with PR/8 × NS H5N1/01 virus (Fig. 2d).

The levels of TNF-α induced by PR/8 × NS H5N1/97 were higher than those induced by the other viruses studied, but they barely exceeded the threshold of sensitivity and the control values (data not shown). These low concentrations may be explained by the small inoculum of infective virus: only 100 EID50 was used, in order to induce productive virus infection in the lungs of mice without causing mortality. The concentrations of TNF-α were too low to allow conclusions about the impact of this cytokine on pathogenesis.

Specific B- and T-cell responses

The enhanced virulence and delayed clearance of PR/8 × NS H5N1/97 virus that we observed can be consistent with diminished humoral and T-cell responses. We investigated whether adaptive immune responses were modified by the NS genes of H5N1 viruses. The mean frequency of influenza-specific AFC in the MLN on day 10 demonstrated that PR/8 and PR/8 × NS H5N1/97 elicited strong responses of similar magnitude (Fig. 3). Therefore, the delayed pulmonary clearance of PR/8 × NS H5N1/97 was not caused by a deficient humoral response. The AFC response in the MLN was negligible on day 7 after inoculation with PR/8 × NS H5N1/01 (data not shown) but had emerged fully on day 10 (Fig. 3), perhaps reflecting low early virus titres in the lungs. Interestingly, the response to PR/8 × NS mut. H5N1/97 on day 10 lacked the usually prominent IgA component; this finding may also be related to diminished replicative capability. The serum antibody concentrations determined by ELISA on days 7 and 10 after inoculation corresponded completely with the results of ELISpot assays (data not shown).

Fig. 2. Pulmonary cytokine and chemokine CXCL1 (KC) concentrations. At the indicated times after infection, lungs were removed and frozen at −80°C. The concentration of IL1α (a), IL1β (b), IL6 (c), IL10 (d), IL12 (p40) (e), IFN-γ (f), GM-CSF (g) and CXCL1 (h) was measured in 10% tissue homogenate. Shown are the mean concentrations ± SD. The x axis crosses the y axis at the detection threshold, which differed for each cytokine and chemokine.
H5N1/97 groups (P<0.05; Table 1). It is likely that virus-specific CD8\(^+\) T-cell immunity was not induced because of the low infective dose of virus used and the resulting attenuated virus replication in the respiratory tract. The PR8 × NS H5N1/01 virus generated an intermediate virus-specific CD8\(^+\) T-cell response, a result that again suggests that attenuation of virus contributed to the virus-specific CD8\(^+\) T-cell response.

**Histopathology**

Histological examination revealed that the lungs of mice inoculated with PR/8 and PR/8 × NS H5N1/97 were similarly affected, except for greater inflammatory cell infiltration in the latter cases (Fig. 4a, b). The bronchioles were minimally affected, with necrosis of individual epithelial cells and focal intraluminal aggregates of necrotic cellular debris. The alveoli, interstitial septa and perivascular spaces were extensively infiltrated by a mixture of inflammatory cells, predominantly mononuclear cells. Mice inoculated with PR/8 × NS mut. H5N1/97 had minimal lung involvement; only a few small areas of lung showed an inflammatory mononuclear cell infiltrate in the alveoli, interstitial septa and perivascular spaces (Fig. 4c). The lungs of mice inoculated with PR/8 × NS H5N1/01 were the least affected, with a minimal increase in the cellularity of the interstitial septa and no inflammatory infiltration of the alveoli and perivascular spaces (Fig. 4d).

**DISCUSSION**

The NS gene segment of influenza A viruses encodes two proteins, NS1 and nuclear export protein. The NS1 protein is known to contribute to viral pathogenesis, allowing the virus to disarm the host’s IFN defence system in multiple ways. NS1 binds double-stranded RNA via an N-terminal RNA-binding domain (reviewed by Krug et al., 2003), and

### Table 1. Virus-specific CD8\(^+\) T-cell response at day 10 after inoculation

Data represent the mean cell count (% cells stained/total cell number) ± SD in the spleen and BAL specimens of five C57Bl/6 mice.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Tetrameric complex</th>
<th>Mean no. of cells ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR/8</td>
<td>D(^n)P(_{366})</td>
<td>Spleen ((\times 10^5))</td>
</tr>
<tr>
<td>PR/8 × NS H5N1/97</td>
<td>D(^n)P(_{366})</td>
<td>3.7 ± 1.3</td>
</tr>
<tr>
<td>PR/8 × NS H5N1/97</td>
<td>D(^n)P(_{366})</td>
<td>3.9 ± 1.6</td>
</tr>
<tr>
<td>PR/8 × NS mut. H5N1/97</td>
<td>D(^n)P(_{366})</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>PR/8 × NS H5N1/01</td>
<td>D(^n)P(_{366})</td>
<td>1.2 ± 0.9</td>
</tr>
<tr>
<td>PR/8</td>
<td>D(^p)PA(_{224})</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>PR/8 × NS H5N1/97</td>
<td>D(^p)PA(_{224})</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>PR/8 × NS H5N1/97</td>
<td>D(^p)PA(_{224})</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>PR/8 × NS H5N1/01</td>
<td>D(^p)PA(_{224})</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td>PR/8</td>
<td>K(^a)PB1(_{703})</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>PR/8 × NS H5N1/97</td>
<td>K(^a)PB1(_{703})</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>PR/8 × NS mut. H5N1/97</td>
<td>K(^a)PB1(_{703})</td>
<td>0.3 ± 0.08</td>
</tr>
<tr>
<td>PR/8 × NS H5N1/01</td>
<td>K(^a)PB1(_{703})</td>
<td>0.2 ± 0.2</td>
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\(^*P<0.05\)
prevents activation of NF-κB, IFN-regulatory factor (IRF), and IRF7, thus inhibiting the IFN-inducible double-stranded RNA-activated kinase PKR and blocking INF-β production (reviewed by Garcia-Sastre, 2001; 2002). The NS1 protein is required for virulence in mice: when NS1 is absent or altered, the virulence of influenza A viruses is highly attenuated (Garcia-Sastre et al., 1998; Talon et al., 2000). Data showing that the NS1 protein functions as a type I IFN antagonist raise the question whether this protein can also increase viral pathogenicity in vivo. In mice, A/WSN/33 (H1N1) mouse-adapted virus reassortants with either the whole NS gene or only the NS1 gene of the 1918 pandemic influenza virus were less pathogenic than the original A/WSN/33 virus (Basler et al., 2001). The human origin of the NS gene may have significantly reduced the pathogenicity of the reassortant in mice. This explanation is supported by work showing that a virus containing the NS1 gene of the 1918 pandemic strain blocks the expression of IFN-regulated genes in human lung cells more efficiently than its parental A/WSN/33 virus (Geiss et al., 2002).

Seo and co-authors (Seo et al., 2002) first showed that the NS gene of a human isolate of Hong Kong H5N1/97 viruses dramatically increased the pathogenicity of PR/8 virus in pigs, and that this effect required the presence of Glu at residue 92 of the NS1 protein. The observed effect was attributed to resistance to the antiviral effects of IFNs and TNF-α conferred by the NS gene of these viruses (Seo & Webster, 2002; Seo et al., 2002). In contrast, recent studies using primary human monocyte-derived macrophages as an in vitro model found that the NS genes of H5N1/97 viruses induce the transcription of TNF-α and IFN-β, a process that can contribute to pathogenesis (Cheung et al., 2002).

The study presented here describes in detail the pathogenesis of infection caused by reassortant viruses containing the NS gene of H5N1/97 and H5N1/01 viruses in a mouse model, including the production of cytokines and chemokine CXCL1 in mouse lung and the characterization of adaptive immune responses. The NS1 protein encoded by the NS gene of the H5N1/01 avian viruses has a deletion of 5 aa (residues 78–82) and confers resistance to the antiviral effects of TNF-α, IFN-α and IFN-γ in cell culture (Seo et al., 2002), but the pathogenicity of reassortant virus carrying this gene had not been studied in vivo. Because the H5N1 viruses isolated from two human cases in Hong Kong in 2003 have a similar NS gene (Guan et al., 2004), we included the gene in this study.

Our results demonstrated that the NS gene of H5N1/97 viruses confers high pathogenicity in mice when inserted into the PR/8 background. PR/8 × NS H5N1/97 replicated efficiently in mouse lung, and clearance of this reassortant required 2 days longer than clearance of PR/8. In mice, as previously observed in swine, the pathogenic characteristics conferred by the NS gene originating from the H5N1/97 viruses required the presence of Glu at residue 92 of the NS1 protein. Reassortant virus with a Glu92→Asp mutation in NS1 caused a lower rate of mortality and lower mean pulmonary virus titres. These results demonstrate that the mouse model is suitable for studies of the pathologic effects caused by the NS gene of H5N1/97.

Surprisingly, the NS gene of H5N1/01 origin completely attenuated the pathogenicity of PR/8 virus in mice. Because

![Fig. 4. Histological examination of lungs from mice infected with PR/8 (a), PR/8 × NS H5N1/97 (b), PR/8 × NS mut. H5N1/97 (c) and PR/8 × NS H5N1/01 (d) viruses. Mice were inoculated intranasally with 100 EID₅₀ of infective virus. Lungs were harvested on day 6 after inoculation, washed in cold PBS, fixed in 10% neutral buffered formalin and stained with haematoxylin and eosin.](http://vir.sgmjournals.org)
this reassortant was resistant to the antiviral effects of IFNs and TNF-α in pig lung epithelial cells (Seo et al., 2002), we examined its pathogenicity in Yucatan miniature pigs, using PR/8 × NS H5N1/97 reassortant as a positive control virus. In this preliminary experiment, PR/8 × NS H5N1/01 virus replicated in the upper respiratory tract to lower titres than PR/8 × NS H5N1/97 and caused no signs of disease, whereas infection with PR/8 × NS H5N1/97 virus resulted in serious illness (data not shown). Therefore, despite the similar properties conferred upon PR/8 by the NS genes of H5N1/97 and H5N1/01 viruses in cell culture experiments, PR/8 reassortants with these genes exert opposite pathological effects in vivo, in both mouse and pig models. This finding suggests that the resistance to the antiviral effects of IFNs and TNF-α conferred by the NS genes of H5N1/97 and H5N1/01 viruses is not the main mechanism of the high virulence associated with the NS gene of H5N1/97 origin. The attenuated pathogenicity of PR/8 × NS H5N1/01 reassortant virus also raises questions as to whether the NS gene, which encodes the NS1 protein with a 5 aa deletion, similar to that observed in human H5N1/03 lethal virus isolates, can contribute to high virulence in humans? Mouse and pig models do not reflect exactly the pathogenicity of H5N1 viruses in humans. However, it is possible to propose that in the case of H5N1/03 human isolates high pathogenicity can be determined by other viral genes or gene constellations. To find an answer, additional studies of the influence of H5N1/01 and H5N1/03 NS genes in virulence are required including studies on human primary cells and in primate models.

To explore other possible explanations for the pathogenicity of the PR/8 × NS H5N1/97 reassortant virus, we assayed cytokines and IL8-like chemokine CXCL1 (KC) in the lungs of infected mice. Primary influenza A virus pneumonia in mice is known to induce the production of cytokines and chemokines in lung tissues (Hennet et al., 1992; Monteiro et al., 1998; Sarawar & Doherty, 1994). The cytokines have been proposed to contribute to the recruitment and activation of virus-specific T cells and the induction and development of immune response (Doherty et al., 1992; Hennet et al., 1992; Sarawar & Doherty, 1994). On the other hand, elevated levels of inflammatory cytokines are also associated with acute respiratory distress syndrome, multiple organ dysfunction (Headley et al., 1997) and haemophagocytic syndrome (Fisman, 2000) in humans. Increased levels of inflammatory cytokines, especially IL6, were associated with pathological signs in human volunteers experimentally infected with influenza viruses (Hayden et al., 1998; Kaiser et al., 2001; Skoner et al., 1999). Moreover, the full post-mortem reports available for two cases of H5N1/97 virus-induced pneumonia describe reactive haemophagocytic syndrome with increased concentrations of IL6, TNF-α and IFN-γ (To et al., 2001).

Our assays of a panel of cytokines in the lungs of infected mice showed that infection with reassortant virus bearing the NS gene of H5N1/97 induces high concentrations of the inflammatory cytokines IL1α, IL1β, IL6 and IL8-like chemokine (KC); concentrations were higher even than those induced by parental, pathogenic PR/8 virus. This characteristic was related to the presence of Glu at residue 92 of the encoded NS1 protein. Reassortant virus with a mutated NS1 gene encoding Asp at position 92 induced significantly lower levels of these inflammatory cytokines and chemokines. On the other hand, PR/8 × NS mut. H5N1/97 virus induced a higher level of the anti-inflammatory cytokine IL10 in infected mouse lungs than did the other tested viruses. This cytokine was also elevated in the lungs of mice infected with PR/8 virus, whereas it was decreased in the lungs of animals infected with PR/8 × NS H5N1/97 virus. IL10 is known to suppress the transcription of lipopolysaccharide-induced inflammatory cytokines (Hamilton et al., 2002; Moore et al., 2001). Thus, the lungs of mice infected with PR/8 × NS H5N1/97 demonstrated elevated levels of pro-inflammatory cytokines and chemokine, particularly IL6 and KC, and a low level of the anti-inflammatory cytokine IL10. The high level of inflammatory cytokines is also consistent with the histological findings: the pulmonary lesions in mice infected with PR/8 and PR/8 × NS H5N1/97 were similar in character, but the inflammation and cellular infiltration were more intense in the latter mice.

Primary infection of mice with the reassortant viruses, as well as with PR/8 virus, induced normal, non-deficient humoral and virus-specific T-cell responses. The enhanced virulence and delayed clearance of PR/8 × NS H5N1/97 virus is not consistent with diminished humoral and T-cell responses. In fact, a greater magnitude of CD8+ T-cell response was induced by the more pathogenic strains. Some observations, particularly the very low number of CD8+ cells in the spleen and BAL specimens of mice infected with PR/8 × NS mut. H5N1/97, could be both a cause and the effect of the attenuated pathogenicity and replication of this virus. Infection with PR/8 × NS H5N1/97 induced a large CD8+ T-cell response, especially in infected lungs. It is possible that this characteristic of the H5N1/97 NS gene requires the presence of Glu at position 92 of the encoded NS1 protein and is lost in virus with a mutated NS gene.

Our findings support two main conclusions. First, the NS gene of Hong Kong H5N1/97 viruses can generate and support high pathogenicity when inserted into a background virus that is pathogenic (like PR/8 in mice) or non-pathogenic (like PR/8 in pigs). The pathogenicity conferred by the H5N1/97 NS gene may be caused by a cytokine imbalance; that is, by elevated production of inflammatory cytokines and chemokine CXCL1 and the simultaneous decreased production of anti-inflammatory cytokine IL10. These functional properties of the H5N1/97 NS gene require the presence of Glu at residue 92 of the encoded NS1 protein. This residue has been identified only in the NS1 protein of Hong Kong H5N1/97 human isolates and phylogenetically closely related avian viruses. The cytokine imbalance caused by the virus bearing the H5N1/97
NS gene is consistent with the detailed post-mortem report on two humans who died in Hong Kong in 1997 of H5N1 pneumonia and explains, at least partially, the unusual severity of H5N1/97 influenza virus infection.

Second, the detailed pathological and immunological studies reported here show that the mouse model is useful for further dissection of the molecular mechanisms and pathways underlying the high pathogenicity conferred by the unique NS gene of H5N1/97 influenza virus origin.

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REFERENCES


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