Characterization of a Neuraminidase-Deficient Influenza A Virus as a Potential Gene Delivery Vector and a Live Vaccine

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We recently identified a packaging signal in the neuraminidase (NA) viral RNA (vRNA) segment of an influenza A virus, allowing us to produce a mutant virus [GFP(NA)-Flu] that lacks most of the NA open reading frame but contains instead the gene encoding green fluorescent protein (GFP). To exploit the expanding knowledge of vRNA packaging signals to establish influenza virus vectors for the expression of foreign genes, we studied the replicative properties of this virus in cell culture and mice. Compared to wild-type virus, GFP(NA)-Flu was highly attenuated in normal cultured cells but was able to grow to a titer of $>10^6$ PFU/ml in a mutant cell line expressing reduced levels of sialic acid on the cell surface. GFP expression from this virus was stable even after five passages in the latter cells. In intranasally infected mice, GFP was detected in the epithelial cells of nasal mucosa, bronchioles, and alveoli for up to 4 days postinfection. We attribute the attenuated growth of GFP(NA)-Flu to virion aggregation at the surface of bronchiolar epithelia. In studies to test the potential of this mutant as a live attenuated influenza vaccine, all mice vaccinated with $\geq 10^5$ PFU of GFP(NA)-Flu survived when challenged with lethal doses of the parent virus. These results suggest that influenza virus could be a useful vector for expressing foreign genes and that a sialidase-deficient virus may offer an alternative to the live influenza vaccines recently approved for human use.

A variety of viruses have been tested for their potential as gene therapy and/or vaccine vectors in the treatment or prevention of cancer, as well as neurological, immunologic, hereditary, and infectious diseases (2, 15, 34, 41). Influenza viruses have been evaluated for their use as vaccine vectors against infectious diseases (e.g., human immune deficiency, malaria, and arenavirus infection) and cancer (4, 8, 24, 31, 38, 43). Although human influenza viruses replicate primarily in respiratory organs, they are capable of inducing mucosal immune responses not only in their replication sites but also in distal mucosa including genital and intestinal tracts, suggesting their potential as vaccine vectors to combat agents that initiate infection at mucosal surfaces (8, 24). Moreover, recent technological innovations, including plasmid-based reverse genetics, have made it possible to manipulate influenza viruses to enhance their candidacy as vaccines and gene delivery vectors (28).

Influenza A viruses cause both pandemics and epidemics, contribute substantially to annual mortality rates in humans, and are responsible for enzootic outbreaks in other animals, leading to substantial economic loss (42). Treatment with neuraminidase (NA) inhibitors clearly improves the course of influenza in patients (14), but these antiviral compounds must be administered early in the infection to be effective. Moreover, the emergence of drug-resistant variants remains a concern (1, 35), although resistance to NA inhibitors is less com-

mon than resistance to amantadine or rimantadine (12, 25, 37). Thus, vaccination of large human populations affords the best protection against influenza outbreaks. The efficacy of current inactivated vaccines depends on the extent of their antigenic match with circulating viruses, with protective effects typically seen in 70 to 90% of cases. Such vaccines have only a limited capacity to induce cell-mediated and mucosal immune responses. Cold-adapted live vaccines have shown promising results in young children (33), but preliminary results indicate that they may not be appreciably more effective than the current inactivated vaccine in adults (7). Moreover, the limited number of attenuating amino acid changes in vaccine strains raises concern over the emergence of virulent revertants, although such vaccines have been phenotypically stable in clinical trials (5).

We recently generated an influenza virus [GFP(NA)-Flu] containing a mutant NA segment in which most of the coding region was replaced with the enhanced green fluorescent protein (GFP) gene (9). Because this virus lacks functional sialidase activity, it was predicted to be attenuated in animals. To characterize GFP(NA)-Flu as a potential gene delivery vector and to determine its efficacy as a live attenuated vaccine that would induce both humoral and cellular, as well as mucosal, immune responses, we tested several of its properties both in vitro and in vivo, including cell tropism after GFP expression, and then evaluated its protective efficacy in a mouse model.

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MATERIALS AND METHODS

Cells. 293T human embryonic kidney cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Madin-Darby

FIG. 1. Schematic diagram of the NA(183)GFP(157) vRNA in which most of the NA coding region has been replaced with the GFP gene. The regions shown in blue correspond to the noncoding regions of this segment, and regions in beige indicate the coding regions. This insertion yields a fusion protein consisting of 61 N-terminal residues of the NA protein and intact GFP. The lengths of the regions are not drawn to scale.

canine kidney (MDCK) cells and MaKS cells, a derivative of MDCK cells expressing a reduced level of sialic acid (17), were maintained in minimal essential medium containing 10% fetal bovine serum.

Plasmids. pPolINA(183)GFP(157), used for the production of negative-sense RNA, contains the 3' noncoding ends of NA vRNA and a complementary sequence encoding a fusion protein composed of 61 N-terminal NA codons and GFP (Clontech), two consecutive stop codons (TAA-TAG), and 185 bases of the 5' end of NA vRNA (Fig. 1) (9). It was produced by replacing the region corresponding to nucleotides 203 to 1109 (positive sense) of the A/WSN/33 (WSN; H1N1) NA gene in pT7Blue-NA with a *Bgl*II site by inverse PCR. The GFP gene was then cloned into this *Bgl*II site and a *Sma*I site at position 1226 (in the wild-type NA gene) in frame with the NA protein. The NA(183)GFP(157) gene was then inserted into the *Bsm*BI site of a PolI plasmid, pHH21. All plasmid constructs were sequenced to ensure that unwanted mutations were not introduced by PCR.

Generation of GFP(NA)-Flu. A virus possessing the NA(183)GFP(157) vRNA instead of NA vRNA [designated GFP(NA)-Flu] was produced as described previously (9, 28). Briefly, 293T cells were transfected with seven plasmids for the production of all WSN influenza vRNA segments, except the NA vRNA, and with pPolINA(183)GFP(157), together with protein expression plasmids for PA, PB1, PB2, and NP. The supernatant from these plasmid-transfected cells was incubated with MaKS cells in minimal essential medium with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin (2 μ g/ml) for 48 h. The stock virus was produced in these cells. In some experiments, GFP(NA)-Flu virus was concentrated 200-fold by ultracentrifugation before use. To test the stability of GFP expression by GFP(NA)-flu, MaKS cells (50% confluent) were infected with this virus at a multiplicity of infection (MOI) of 0.1 in the presence of

TPCK-trypsin (2 μ g/ml). Two days later, virus in the supernatant was harvested, and the procedure was repeated four more times.

Experimental infection of mice. Four-week-old female BALB/c mice were intranasally inoculated with 1.2×10^7 PFU of virus. On day 0 through days 5 and 7 postinoculation (p.i.), nasal turbinates and lungs were harvested, frozen, and embedded in paraffin or resin for histologic examination. Virus in the respiratory organs was titrated by plaque assay. The dose required to kill 50% of mice (MLD₅₀) was determined as previously described (10).

Virus for challenge experiments. Wild-type WSN virus was propagated in MDCK cells and stored at -80°C until use. The MLD₅₀ was evaluated by a previously described method (10).

Immunization and virus challenge. Four-week-old female BALB/c mice were intranasally inoculated with 50 μ l (1.1×10^4 to 1.1×10^7 PFU) of GFP(NA)-Flu virus. One month after immunization, the mice were intranasally challenged with 10 or 100 MLD₅₀ of the wild-type WSN virus. To determine virus titers in lungs and nasal turbinates, we harvested and homogenized these organs 3 days after challenge, performing titrations on MDCK cells by plaque assay. The remaining animals were observed for 14 days for clinical signs and symptoms.

Detection of virus-specific antibody. Serum samples were examined for antibody by hemagglutinin inhibition (HI) and plaque reduction neutralization assays after treatment with receptor-destroying enzyme (HI tests) or heat inactivation at 56°C for 30 min (neutralization assay). A standard plaque reduction assay was performed to determine the WSN virus-specific neutralizing titer of the sera. Wild-type WSN virus (100 PFU) was mixed with an equal volume of serially diluted serum and incubated with MDCK cells at 37°C for 1 h. The neutralizing antibody titer is reported as the highest dilution of serum able to reduce the number of plaques by 50%.

RESULTS

In vitro properties of GFP(NA)-Flu. We first tested the growth properties of the NA-deficient virus in MDCK and MaKS cells. Because the latter cells express reduced levels of sialic acid on the cell surface, they allow mutant influenza viruses lacking sialidase activity to undergo multiple cycles of replication without exogenous sialidase (16, 17). Figure 2 shows the growth profiles of GFP(NA)-Flu and wild-type WSN virus in each cell line infected at an MOI of 10^{-4} . The wild-type virus replicated well in both cell lines, although a maximal titer was achieved more rapidly in MDCK cells than in MaKS

FIG. 2. Growth kinetics of GFP(NA)-Flu and wild-type WSN virus in cell culture. MaKS (KS; broken line) or MDCK (CK; solid line) cells were infected with either GFP(NA)-Flu or wild-type WSN virus at an MOI of 10^{-4} . At the indicated times after infection, virus titers in the supernatant were determined. The values are means of triplicate experiments. For GFP(NA)-Flu, the numbers of total plaques (open and closed black squares) and those of GFP-positive plaques (open and closed green triangles) were recorded separately.

TABLE 1. Replication of GFP(NA)-Flu virus in mice^a

Tissue	Time p.i. (days)	Virus titer (mean $\pm$ SD log ₁₀ GFP-positive plaques/g)	Virus titer (mean $\pm$ SD log ₁₀ PFU/g)	% (GFP-positive plaques/PFU)
Nasal turbinate	1	3.2 $\pm$ 0.1	3.5 $\pm$ 0.3	53
	2	3.7 $\pm$ 0.4	4.1 $\pm$ 0.2	43
	3	— ^b	—	—
	4	—	—	—
	5	—	—	—
	6	—	—	—
	7	—	—	—
Lungs	1	4.4 $\pm$ 0.1	4.8 $\pm$ 0.1	44
	2	3.9 $\pm$ 0.1	4.4 $\pm$ 0.1	29
	3	2.7 $\pm$ 0.1	3.2 $\pm$ 0.1	30
	4	2.8 $\pm$ 0.1	3.2 $\pm$ 0.1	36
	5	—	—	—
	6	—	—	—
	7	—	—	—

^a BALB/c mice, anesthetized with isoflurane, were intranasally infected with 50 μ l of virus (1.2×10^7 PFU). Three mice from each group were euthanized on days 1, 2, 3, 4, 5, and 7 p.i. for virus titration.

^b —, no virus was isolated.

cells. Although GFP(NA)-Flu virus replicated more slowly than WSN virus in MaKS cells, it reached a titer only 1 log lower than that of WSN virus. Importantly, the growth of GFP(NA)-Flu was severely attenuated in MDCK cells, reaching a titer of only 10^3 PFU/ml. The percentage of GFP-expressing virus remained the same up to five passages in MaKS cells (>61%), suggesting stable maintenance of the vGFP(NA) segment in the virus population.

Growth properties of GFP(NA)-Flu virus in vivo. To determine the growth properties of GFP(NA)-Flu virus in mice, we intranasally inoculated mice with 1.2×10^7 PFU/50 μ l of virus. All mice intranasally infected with this virus survived but showed reduced activity for several days after infection. In contrast, the wild-type WSN virus was highly lethal to mice (with 50% lethal dose [LD₅₀] of 3.4 log₁₀ PFU). GFP(NA)-Flu was recovered until day 4 from lungs, but only up to day 2 from nasal turbinates (Table 1). However, unlike the wild-type WSN virus, which grew to high titers in lungs (>7.4 log₁₀ PFU/g; day 3 p.i.) and nasal turbinates (>4.6 log₁₀ PFU/g; day 3 p.i.) (Table 2, control), replication of GFP(NA)-Flu was limited (Table 1). To determine the pattern of GFP(NA)-Flu infection in respiratory organs and the cell types infected, we examined GFP expression in frozen sections. GFP fluorescence could not be detected immediately after intranasal inoculation of virus (Fig. 3A) but was readily observed in nasal epithelia (Fig. 3B) on day 1 p.i. GFP fluorescence was detected in the epithelial cells of the bronchioles on day 1 p.i. (Fig. 3C) and in alveolar cells on day 2 p.i. (Fig. 3D), with the number of fluorescence-positive cells in alveoli decreasing at 3 to 4 days p.i. (Fig. 3E and F). Electron microscopy revealed an aggregation of progeny viruses on the surface of degenerating bronchiolar epithelia at 12 h p.i. (Fig. 3G and H), a finding consistent with a lack of sialidase activity (19, 30).

Immunization and protection tests. To evaluate GFP(NA)-Flu as a live vaccine, we intranasally infected mice with various amounts of the virus and then challenged them with 10 or 100 MLD₅₀ of the wild-type WSN virus 1 month after GFP(NA)-Flu infection. Mice immunized with $\geq 1.1 \times 10^6$ (PFU/50 μ l) virus were completely protected from lethal WSN virus chal-

TABLE 2. Protection against lethal challenge in mice immunized with GFP(NA)-Flu virus^a

Group	Virus titer (mean $\pm$ SD log ₁₀ PFU/g)		No. of deaths/ no. of mice tested ^b
	Lungs	Nasal turbinate	
Challenge 1 (100 MLD ₅₀)			
Control ^c	7.4 $\pm$ 0.1	5.7 $\pm$ 0.1	8/8
1.1 $\times$ 10 ⁴ PFU	7.0 $\pm$ 0.1	4.9 $\pm$ 0.1	4/8
1.1 $\times$ 10 ⁵ PFU	5.2 $\pm$ 0.2	1.6 $\pm$ 0.1	0/8
1.1 $\times$ 10 ⁶ PFU	— ^d	—	0/8
1.1 $\times$ 10 ⁷ PFU	—	—	0/8
Challenge 2 (10 MLD ₅₀)			
Control	7.7 $\pm$ 0.1	4.6 $\pm$ 0.4	8/8
1.1 $\times$ 10 ⁴ PFU	5.6 $\pm$ 0.3	3.9 $\pm$ 0.2	1/8
1.1 $\times$ 10 ⁵ PFU	5.1 $\pm$ 0.1	3.3 $\pm$ 0.5	0/8
1.1 $\times$ 10 ⁶ PFU	—	—	0/8
1.1 $\times$ 10 ⁷ PFU	—	—	0/8

^a One month after mice were intranasally immunized with different doses (1.1×10^4 to 1.1×10^7 PFU) of GFP(NA)-Flu virus, the animals were challenged with 10 or 100 MLD₅₀ (corresponding to $10^{4.4}$ and $10^{5.4}$ PFU, respectively) of wild-type WSN virus. Virus titers were determined in lung tissue or nasal turbinates at 3 days postchallenge.

^b Mice were monitored for 14 days after challenge.

^c Control mice were mock immunized with phosphate-buffered saline.

^d —, No virus was detected from any of the infected mice.

lenge, with no virus recovered (Table 2). Reduced levels of protection, as judged from virus titers in respiratory organs and/or animal death, were apparent in groups of mice immunized with $\leq 1.1 \times 10^5$ (PFU/50 μ l) of GFP(NA)-Flu. To determine the stability of GFP gene expression during replication in mice, we titrated mouse organs by plaque assay and determined the number of GFP-expressing plaques in total plaques. Even after replication in mice, a substantial percentage of the virus still maintained the intact GFP gene, although it was reduced compared to virus passaged in MaKS cells (29 to 53% [Table 1] versus 61 to 72% [data not shown]).

Levels of serum HI and virus-neutralizing antibody titers before and 3 days after virus challenge correlated with the extent of protection against WSN virus challenge (Table 3). In addition, on day 14 postchallenge, virus-neutralizing antibody titers did not differ among mice immunized with different doses of GFP(NA)-Flu.

DISCUSSION

Influenza viruses possessing genes or portions of genes of unrelated origins have been produced (4, 11, 21, 24, 26, 27, 29, 32, 38, 44). As a marker gene, we introduced the GFP gene into the NA segment of an influenza virus, where it was stably maintained during repeated passage of the virus in cell culture. GFP expression was also detected in intranasally infected mice up to 4 days postinfection, although the percentage of virus still expressing GFP declined during replication in this animal. Although the precise mechanism of such a loss was not further investigated in the present study, the level and duration of GFP expression suggest that foreign proteins expressed in this system likely induce sufficient levels of immunity. Hence, these findings indicate that this NA-deficient virus system could be

FIG. 3. GFP(NA)-Flu infection in respiratory organs of mice. GFP fluorescence was not detected immediately after virus inoculation (A) but was apparent in the nasal epithelium on day 1 postinfection (B). Intense fluorescence was apparent in bronchiolar epithelium on day 1 postinfection (C), shifting to the alveolus by day 2 (D) with diminishing intensity by day 3 (E) or 4 (F). (G and H) Aggregated virions were observed by electron microscopy on the surface of degenerating bronchiolar epithelium at 12 h postinfection. Bar, 500 nm.

exploited as a vaccine vector for the expression of foreign genes.

Intranasal infection of mice with an NA-deficient virus generated protective immunity against lethal influenza virus challenge (Table 2). Although the NA protein is a major surface antigen and can induce protection from influenza virus infection (6, 18, 22, 36), the hemagglutinin (HA) plays a more important role in eliciting protective immune responses (23). In fact, an avian influenza virus outbreak caused by an H7N1 virus in Italy in 1999 was finally controlled by an HA- but not an NA-matched inactivated H7N3 virus vaccine after attempts to control the outbreak by mass slaughtering of infected poultry failed (3). NA-deficient viruses are attenuated because they aggregate at the cell surface after their release from infected

cells (19, 20, 30). NA molecules can also stimulate the activation of transforming growth factor β , leading to the induction of apoptosis and to immunologic tolerance (13, 39). Thus, NA-deficient viruses may have the potential to serve as an alternative influenza vaccine for humans as well as animals.

We previously reported the use of a replication-incompetent influenza virus as a potential vaccine (40). This virus infects cells only once and expresses proteins important for the induction of protective immune responses but does not produce progeny. Thus, it is safe (no emergence of virulent revertants is anticipated) and yet produces humoral, as well as cellular and mucosal, immune responses. However, to generate replication-incompetent influenza virus, we must transfect cells with plasmids, thus restricting its commercial value until a system is

TABLE 3. HI and neutralizing antibody titers in mice immunized with GFP(NA)-Flu virus^a

Group ^b	HI antibody titer (mean log ₂ ± SD) at:			Neutralizing antibody titer (mean log ₁₀ ± SD) at:		
	3 days before challenge	3 days after challenge	14 days after challenge ^c	3 days before challenge	3 days after challenge	14 days after challenge ^c
Immunized						
Control ^d	<3			1.1 ± 0.2		
1.1 × 10 ⁴ PFU	<3			1.8 ± 0.3		
1.1 × 10 ⁵ PFU	<3			2.1 ± 0.1		
1.1 × 10 ⁶ PFU	3.5 ± 0.7			3.5 ± 0.3		
1.1 × 10 ⁷ PFU	4.5 ± 0.7			3.1 ± 0.3		
Challenge 1 (100 MLD ₅₀)						
Control		<3	NA ^e		1.0 ± 0.2	NA
1.1 × 10 ⁴ PFU		<3	4.5 ± 0.7		1.6 ± 0.5	4.4 ± 0.7
1.1 × 10 ⁵ PFU		<3	5.5 ± 0.7		2.9 ± 0.4	5.2 ± 0.3
1.1 × 10 ⁶ PFU		4.0 ± 1.4	4.5 ± 0.7		3.4 ± 0.4	4.5 ± 0.4
1.1 × 10 ⁷ PFU		6.0 ± 1.4	4.5 ± 0.7		4.3 ± 0.2	4.5 ± 0.2
Challenge 2 (10 MLD ₅₀)						
Control		<3	NA		0.9 ± 0.3	NA
1.1 × 10 ⁴ PFU		<3	6.5 ± 0.7		2.5 ± 0.2	5.3 ± 0.3
1.1 × 10 ⁵ PFU		<3	7.5 ± 0.7		2.4 ± 0.2	4.3 ± 0.3
1.1 × 10 ⁶ PFU		4.0 ± 1.4	6.5 ± 0.7		3.6 ± 0.1	4.5 ± 0.2
1.1 × 10 ⁷ PFU		4.0 ± 1.4	6.5 ± 0.7		3.6 ± 0.1	5.0 ± 0.3

^a Virus-specific antibodies in samples from mice after immunization. HI and neutralizing antibody titers were detected by HI and plaque reduction tests as described in Materials and Methods. Results are reported as the mean dilution for complete HI (HI antibody test) or 50% plaque reduction (neutralizing antibody test).

^b Mice were intranasally immunized with the indicated amounts of virus.

^c Sera were collected from survivors.

^d Control mice were mock immunized with phosphate-buffered saline.

^e NA, not available, since mice died prior to day 14 p.i.

devised that will allow large-scale production of the virus. In contrast, the NA-deficient virus described here can undergo multiple cycles of replication in a cell line with reduced sialylation, although a system to produce a higher virus titer would be desirable for its use. Moreover, the vaccine efficacy of the NA-deficient virus is equivalent to that of the replication-incompetent virus (40).

We would point out that other genes in the NA-deficient virus do not contain attenuating mutations, which may raise concern over the safety of this virus as a vaccine. However, the deletion in the NA gene appears sufficiently extensive to prevent reversion of the vaccinating virus to the wild type. Also, insertion of the GFP gene was intended as a means to evaluate viral replication in vivo and therefore would not be required in production of the vaccine strain, avoiding any adverse effects from expression of this marker gene. In fact, instead of the GFP gene, a gene encoding a protein antigen from another infectious agent could be inserted into the NA segment, making such viruses multivalent live vaccines.

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REFERENCES

- Boivin, G., N. Goyette, and H. Bernatchez. 2002. Prolonged excretion of amantadine-resistant influenza A virus quasispecies after cessation of antiviral therapy in an immunocompromised patient. *Clin. Infect. Dis.* **34**:e23–e25.
- Bramson, J. L., and Y. H. Wan. 2002. The efficacy of genetic vaccination is dependent upon the nature of the vector system and antigen. *Expert Opin. Biol. Ther.* **2**:75–85.
- Capua, I., C. Terregino, G. Cattoli, F. Mutinelli, and J. F. Rodriguez. 2003. Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. *Avian Pathol.* **32**:47–55.
- Castrucci, M. R., S. Hou, P. C. Doherty, and Y. Kawaoka. 1994. Protection against lethal lymphocytic choriomeningitis virus (LCMV) infection by immunization of mice with an influenza virus containing an LCMV epitope recognized by cytotoxic T lymphocytes. *J. Virol.* **68**:3486–3490.
- Cha, T. A., K. Kao, J. Zhao, P. E. Fast, P. M. Mendelman, and A. Arvin. 2000. Genotypic stability of cold-adapted influenza virus vaccine in an efficacy clinical trial. *J. Clin. Microbiol.* **38**:839–845.
- Chen, Z., T. Yoshikawa, S. Kadowaki, Y. Hagiwara, K. Matsuo, H. Asanuma, C. Aizawa, T. Kurata, and S. Tamura. 1999. Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein. *J. Gen. Virol.* **80**:2559–2564.
- Edwards, K. M., W. D. Dupont, M. K. Westrich, W. D. Plummer, Jr., P. S. Palmer, and P. F. Wright. 1994. A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. *J. Infect. Dis.* **169**:68–76.
- Ferko, B., D. Katfinger, A. Grassauer, A. Egorov, J. Romanova, B. Niebler, H. Katfinger, and T. Muster. 1998. Chimeric influenza virus replicating predominantly in the murine upper respiratory tract induces local immune responses against human immunodeficiency virus type 1 in genital tract. *J. Infect. Dis.* **178**:1359–1368.
- Fujii, Y., H. Goto, T. Watanabe, T. Yoshida, and Y. Kawaoka. 2003. Selective incorporation of influenza virus RNA segments into virions. *Proc. Natl. Acad. Sci. USA* **100**:2002–2007.
- Gao, P., S. Watanabe, T. Ito, H. Goto, K. Wells, M. McGregor, A. J. Cooley, and Y. Kawaoka. 1999. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. *J. Virol.* **73**:3184–3189.
- García-Sastre, A., T. Muster, W. S. Barclay, N. Percy, and P. Palese. 1994. Use of a mammalian internal ribosomal entry site element for expression of a foreign protein by a transfectant influenza virus. *J. Virol.* **68**:6254–6261.
- Gubareva, L. V., R. G. Webster, and F. G. Hayden. 2002. Detection of influenza virus resistance to neuraminidase inhibitors by an enzyme inhibition assay. *Antivir. Res.* **53**:47–61.

13. Haneda, K., K. Sano, G. Tamura, H. Shirota, Y. Ohkawara, T. Sato, S. Habu, and K. Shirato. 1999. Transforming growth factor- β secreted from CD4⁺ T cells ameliorates antigen-induced eosinophilic inflammation. A novel high-dose tolerance in the trachea. *Am. J. Respir. Cell Mol. Biol.* **21**:268–274.
14. Hayden, F. G. 2001. Perspectives on antiviral use during pandemic influenza. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356**:1877–1884.
15. Hermiston, T. W., and I. Kuhn. 2002. Armed therapeutic viruses: strategies and challenges to arming oncolytic viruses with therapeutic genes. *Cancer Gene Ther.* **9**:1022–1035.
16. Hughes, M. T., M. Matrosovich, M. E. Rodgers, M. McGregor, and Y. Kawaoka. 2000. Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice. *J. Virol.* **74**:5206–5212.
17. Hughes, M. T., M. McGregor, T. Suzuki, Y. Suzuki, and Y. Kawaoka. 2001. Adaptation of influenza A viruses to cells expressing low levels of sialic acid leads to loss of neuraminidase activity. *J. Virol.* **75**:3766–3770.
18. Johansson, B. E., and E. D. Kilbourne. 1994. Immunization with purified N1 and N2 influenza virus neuraminidases demonstrates cross-reactivity without antigenic competition. *Proc. Natl. Acad. Sci. USA* **91**:2358–2361.
19. Liu, C., and G. M. Air. 1993. Selection and characterization of a neuraminidase-minus mutant of influenza virus and its rescue by cloned neuraminidase genes. *Virology* **194**:403–407.
20. Liu, C., M. C. Eichelberger, R. W. Compans, and G. M. Air. 1995. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. *J. Virol.* **69**:1099–1106.
21. Luytjes, W., M. Krystal, M. Enami, J. D. Pavin, and P. Palese. 1989. Amplification, expression, and packaging of foreign gene by influenza virus. *Cell* **59**:1107–1113.
22. Lyles, D. S. 2000. Cytopathogenesis and inhibition of host gene expression by RNA viruses. *Microbiol. Mol. Biol. Rev.* **64**:709–724.
23. Mozdzanowska, K., K. Maiese, M. Furchner, and W. Gerhard. 1999. Treatment of influenza virus-infected SCID mice with nonneutralizing antibodies specific for the transmembrane proteins matrix 2 and neuraminidase reduces the pulmonary virus titer but fails to clear the infection. *Virology* **254**:138–146.
24. Muster, T., B. Ferko, A. Klima, M. Purtscher, A. Trkola, P. Schulz, A. Grassauer, O. G. Engelhardt, A. García-Sastre, P. Palese, et al. 1995. Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. *J. Virol.* **69**:6678–6686.
25. Nedyalkova, M. S., F. G. Hayden, R. G. Webster, and L. V. Gubareva. 2002. Accumulation of defective neuraminidase (NA) genes by influenza A viruses in the presence of NA inhibitors as marker of reduced dependence on NA. *J. Infect. Dis.* **185**:591–598.
26. Neumann, G., A. Zobel, and G. Hobom. 1994. RNA polymerase I-mediated expression of influenza viral RNA molecules. *Virology* **202**:477–479.
27. Neumann, G., and G. Hobom. 1995. Mutational analysis of influenza virus promoter elements in vivo. *J. Gen. Virol.* **76**:1709–1717.
28. Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. USA* **96**:9345–9350.
29. Neumann, G., T. Watanabe, and Y. Kawaoka. 2000. Plasmid-driven formation of influenza virus-like particles. *J. Virol.* **74**:547–551.
30. Palese, P., and R. W. Compans. 1976. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA): mechanism of action. *J. Gen. Virol.* **33**:159–163.
31. Palese, P., F. Zavala, T. Muster, R. S. Nussenzweig, and A. García-Sastre. 1997. Development of novel influenza virus vaccines and vectors. *J. Infect. Dis.* **176**:S45–S49.
32. Percy, N., W. S. Barclay, A. García-Sastre, and P. Palese. 1994. Expression of a foreign protein by influenza A virus. *J. Virol.* **68**:4486–4492.
33. Piedra, P. A., L. Yan, K. Kotloff, K. Zangwill, D. I. Bernstein, J. King, J. Treanor, F. Munoz, M. Wolff, I. Cho, P. M. Mendelman, J. Cordova, and R. B. Belshe. 2002. Safety of the trivalent, cold-adapted influenza vaccine in preschool-aged children. *Pediatrics* **110**:662–672.
34. Romano, G., P. Micheli, C. Pacilio, and A. Giordano. 2000. Latest development in gene transfer technology: achievements, perspectives, and controversies over therapeutic applications. *Stem Cells* **18**:19–39.
35. Saito, R., H. Oshitani, H. Masuda, and H. Suzuki. 2002. Detection of amantadine-resistant influenza A virus strains in nursing homes by PCR-restriction fragment length polymorphism analysis with nasopharyngeal swabs. *J. Clin. Microbiol.* **40**:84–88.
36. Schultz-Cherry, S., and V. S. Hinshaw. 1996. Influenza virus neuraminidase activates latent transforming growth factor β . *J. Virol.* **70**:8624–8629.
37. Shiraishi, K., K. Mitamura, Y. Sakai-Tagawa, H. Goto, N. Sugaya, and Y. Kawaoka. 2003. High frequency of resistant viruses harboring different mutations in amantadine treated children with influenza. *J. Infect. Dis.* **188**:57–61.
38. Strobel, I., M. Krumbholz, A. Menke, E. Hoffmann, P. R. Dunbar, A. Bender, G. Hobom, A. Steinkasserer, G. Schuler, and R. Grassmann. 2000. Efficient expression of the tumor-associated antigen MAGE-3 in human dendritic cells, using an avian influenza virus vector. *Hum. Gene Ther.* **11**:2207–2218.
39. Wahl, S. M. 1992. Transforming growth factor beta (TGF- β) in inflammation: a cause and a cure. *J. Clin. Immunol.* **12**:61–74.
40. Watanabe, T., S. Watanabe, G. Neuman, H. Kida, and Y. Kawaoka. 2002. Immunogenicity and protective efficacy of replication incompetent influenza virus-like particles. *J. Virol.* **76**:767–773.
41. Wickham, T. J. 2003. Ligand-directed targeting of genes to the site of disease. *Nat. Med.* **9**:135–139.
42. Wright, P. F., and R. G. Webster. 2001. Orthomyxoviruses, p. 1533–1579. *In* D. M. Knipe and P. M. Howley (ed.), *Fields virology*, 4th ed. Lippincott/The Williams & Wilkins Co., Philadelphia, Pa.
43. Zheng, H., P. Palese, and A. García-Sastre. 2000. Antitumor properties of influenza virus vectors. *Cancer Res.* **60**:6972–6976.
44. Zhou, Y., M. König, G. Hobom, and E. Neumeier. 1998. Membrane-anchored incorporation of a foreign protein in recombinant influenza virions. *Virology* **246**:83–94.