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Influenza Virus Protecting RNA: an Effective Prophylactic and Therapeutic Antiviral▽

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Another influenza pandemic is inevitable, and new measures to combat this and seasonal influenza are urgently needed. Here we describe a new concept in antivirals based on a defined, naturally occurring defective influenza virus RNA that has the potential to protect against any influenza A virus in any animal host. This "protecting RNA" (244 RNA) is incorporated into virions which, although noninfectious, deliver the RNA to those cells of the respiratory tract that are naturally targeted by infectious influenza virus. A 120-ng intranasal dose of this 244 protecting virus completely protected mice against a simultaneous challenge of 10 50% lethal doses with influenza A/WSN (H1N1) virus. The 244 virus also protected mice against strong challenge doses of all other subtypes tested (i.e., H2N2, H3N2, and H3N8). This prophylactic activity was maintained in the animal for at least 1 week prior to challenge. The 244 virus was 10- to 100-fold more active than previously characterized defective influenza A viruses, and the protecting activity was confirmed to reside in the 244 RNA molecule by recovering a protecting virus entirely from cloned cDNA. There was a clear therapeutic benefit when the 244 virus was administered 24 to 48 h after a lethal challenge, an effect which has not been previously observed with any defective virus. Protecting virus reduced, but did not abolish, replication of challenge virus in mouse lungs during both prophylactic and therapeutic treatments. Protecting virus is a novel antiviral, having the potential to combat human influenza virus infections, particularly when the infecting strain is not known or is resistant to antiviral drugs.

Human influenza viruses A and B are both responsible for seasonal disease in people, but only influenza A viruses cause worldwide pandemics. The last three pandemics, in 1918, 1957, and 1968, resulted from infection with the H1N1, H2N2, and H3N2 subtypes, respectively. The letters "H" and "N" in these subtypes represent the major external virion proteins, hemagglutinin (HA) and neuraminidase (NA), of which there are 16 H subtypes and nine N subtypes that probably exist naturally in all 144 possible permutations. However, the majority of influenza A viruses exist in various waterfowl, causing subclinical gut infections (5, 6, 37). Genomic studies suggest that the human pandemic viruses arose from avian viruses adapting to humans (1918) or genetically interacting with an existing human virus (1957 and 1968) (18, 27, 34) (see below). Thus, as avian viruses (such as H5N1 and H7N7) move from their natural host into domestic poultry and into close contact with humans, there has been concern that we might be seeing the early stages of an emerging new pandemic virus. However, none of these viruses transmits effectively from person to person. Highly infectious new pandemic viruses all cause widespread morbidity and mortality, with 50 million estimated worldwide deaths from the 1918 virus and 1 million to 5 million deaths from the 1957 and 1968 viruses. Currently, measures to counter human influenza include administration of killed and live vaccines and the antivirals oseltamivir (Tamiflu) and zanamivir (Relenza) (21). However, a new vaccine would be required for any new pandemic virus and would take several months before it was available for administration. Viral resistance to oseltamivir has already been recorded in human virus isolates (13, 19) and is causing concern.

The influenza A virus genome comprises eight segments of single-stranded negative-sense RNA that encode nine structural and two nonstructural proteins. All influenza A viruses appear to have a replication apparatus that allows the exchange of genome segments (reassortment) in dually infected cells, giving these viruses immense genetic flexibility (18). Such an event gave rise to the 1957 and 1968 pandemic viruses. In addition to the normal replication process, mistakes in replication occur that give rise to small RNAs of 400 to 500 nucleotides (nt) lacking around 80% of the central sequence of the template, which appear to result from the polymerase copying the initial part of the template, detaching from the template and then rejoining and copying the other terminus (14). These small defective RNAs retain the terminal replication and encapsidation signals, and their small size suggests that more copies can be made in unit time compared with the full-length RNA segment. Encapsidation of genomic RNAs appears to be an organized process so that a virion contains just one copy of each of the eight segments (25). The packaging process does not appear to discriminate between a defective and a full-length RNA, so when defective RNAs are in excess, they are preferentially encapsidated. A particle containing the deleted genome segment cannot synthesize the viral protein(s) normally encoded by that RNA and is noninfectious, although it can be replicated in *trans* when that cell is infected by an influenza A virus. Incorporation of defective RNAs into virions results in a reduction in the amount of infectious virus pro-

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TABLE 1. Derivation and nomenclature of protecting influenza RNAs and their helper viruses

Abbreviation	Defective RNA[b]	Helper virus
220/PR8[a]	RNA1_220/445_A/equine/Newmarket/7339/79 (H3N8)	A/PR/8/34 (H1N1)
317/Vic	RNA1_317/585_A/chicken/Dobson/27 (H7N7)	A/Victoria/3/75 (H3N2)
244/PR8	RNA1_244/395_A/PR/8/34 (H1N1)	A/PR/8/34 (H1N1)
244/WSN	RNA1_244/395_A/PR/8/34 (H1N1)	A/WSN (H1N1)
244/Mallard[c]	RNA1_244/395_A/PR/8/34 (H1N1)	A/mallard/England/7277/06 (H2N3)

[a] 220, protecting RNA; PR8, helper virus.
[b] Denotes, from left to right, segment of origin of defective viral RNA, breakpoint residue in the minus-sense RNA, total number of nucleotides, and virus of origin.
[c] Produced by reassortment of noninfectious 244/PR8 and infectious A/mallard in embryonated eggs (20), the others via reverse genetics.

duced. Thus, virions carrying a deleted genome were known as interfering or defective-interfering (DI) viruses (15).

It has been known for some time that noninfectious preparations of influenza A DI viruses can protect laboratory animals from a lethal challenge with homologous or heterologous influenza A viruses (20, 23, 24). However, it has not been possible to experimentally elucidate the process by which noncloned DI influenza A viruses reduce the yield of infectious virus, inhibit virus-induced cytopathology, and protect animals from clinical disease (7), because most populations of DI virus contain many different defective RNA sequences derived from different genome segments and with a variety of central deletions (11, 16). Thus, the RNA content of such noncloned populations of defective virus cannot be reproduced with certainty, and it was not possible to analyze the relationship between the RNA sequence and antiviral activity. Nor was it known if antiviral activity resided in one defective RNA sequence or if it required the combined action of two or more sequences.

The key to analyzing the mechanism(s) of interference and protection, and also to clinical uses of DI viruses, is the ability to produce a DI virus containing a single, unique deleted RNA species. Using reverse genetics, we have now made virus preparations that contain a single defective RNA that has the ability to protect animals from serious infection with influenza A viruses. We call such preparations protecting viruses to distinguish them from the activity of interfering viruses in cultured cells (14). Our most active protecting virus, described in this paper, has approximately 50 times more prophylactic activity against influenza A virus in mice than noncloned DI virus and provides therapeutic benefit in virus-infected mice that was not observed before with noncloned virus. Protecting virus represents a new concept in antivirals, and clinical trials are being planned to determine if it is effective in combating human influenza A viruses. A major advantage of protecting virus is that it is expected to work against any subtype or strain of influenza A virus. Viruses resistant to protecting virus are unlikely to arise because the active principle, protecting RNA, uses the same replication machinery as genomic RNA.

MATERIALS AND METHODS

Production of protecting viruses by reverse genetics. Virus was recovered from plasmids based on influenza A/PR/8/34 essentially as described previously (32). Briefly, the DNA mix transfected into 293T cells contained 0.5 μg of each of the eight A/PR8 gene segments (under PolI promoters), 0.5 μg of each PB1 and PB2 expression plasmids, 0.1 μg of the PA expression plasmid, and 1 μg of the NP expression plasmid, using Fugene (Roche). To produce protecting virus, an additional plasmid which expresses the defective RNA from the PolI promoter as a negative-sense transcript (see below) was added to the mixture. Plasmids which contain the gene 1 defective RNAs 220 (equine H3N8) and 317 (avian H7N7) under the control of PolI promoters have been described previously (11) (Table 1). In other experiments, helper plasmids encoding the eight RNA segments of A/WS/33(N) (A/WSN) or A/Victoria/3/75 (A/Vic) were used (22). After 24 h, the 293T cells were trypsinized, mixed with MDCK cells, and replated, and culture supernatants were harvested 7 days later. Growth of virus was determined by using an assay for viral HA. The supernatant was passaged twice in embryonated chicken eggs to make a seed stock and then a working stock for mouse studies. Virus was purified by differential centrifugation through sucrose. Stocks were resuspended in phosphate-buffered saline containing 0.1% (wt/vol) bovine serum albumin, standardized by HA titration, and stored in liquid nitrogen. Optimization of the amount of defective RNA plasmid during transfection (see below) and of the egg inoculum proved important in avoiding low yields of protecting virus.

Reverse transcription-PCR (RT-PCR). RNA was extracted from virus with phenol and dissolved in water. RNA from the lungs of one mouse was extracted by grinding with sterile sand and Trizol (Invitrogen). Generic segment 1-specific primers, RNA1F and RNA1R, have been described previously (8). Aliquots of 2.5 μg of total RNA (or RNA from 200 μl of virus) were reverse transcribed in 20-μl reaction mixtures for 1 h at 42°C, using RNA1F. Aliquots (1.5 or 3 μl) of the RT reaction were then amplified by PCR using *Taq* DNA polymerase (MBI Fermentas or New England Biolabs), primer RNA1F, and either RNA1R or a primer specific for the junction sequence in the 244 RNA, 244J (5′ATCCCCT CAGTCTTCTCCTG3′), in a 25-μl reaction mixture volume. RNA1F has a single mismatch to the published A/PR8 sequence, whereas RNA1R is identical to the published A/PR8 sequence. PCR consisted of 30 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 30 s. Aliquots of 10 μl of the product were analyzed by agarose gel electrophoresis.

Optimization of transfection of the 244 RNA plasmid. The 244 RNA was initially observed as a major segment 1-derived RNA of 395 nt in a preparation of A/PR8 virus, which had been recovered from plasmids as described above. The 244 RNA was amplified by RT-PCR using primers specific for the termini of A/PR8 segment 1, and the product was cloned into the PolI expression plasmid pPOLI-SapIT (32), such that a viral RNA sense transcript was expressed. Various amounts of the 244 plasmid (0 to 0.5 μg) were transfected into 293T cells along with A/WSN helper plasmids as described above. After 24 h, the 293T cells were trypsinized, mixed with MDCK cells, and replated. After 7 days, culture supernatants were harvested, and virus yield was determined by HA assay.

Infectivity titrations. Infectivity titers were determined as required by titration in cell culture, eggs, and mice. Virus was plaque assayed in MDCK cells under agar by standard procedures, or 50% tissue culture infective dose end-point titers were determined from twofold dilutions in MDCK cells after 4 days. Eggs were inoculated with limit-diluted virus and incubated for 3 days. Virus-positive eggs were identified by HA in allantoic fluid. Mouse infectivity was assayed by inoculating limit-diluted virus as described below; then, after 3 days, lungs were removed, ground lungs from individual mice were inoculated into eggs, and the presence of virus was determined by HA assay. Alternatively, mice were challenged intranasally after 3 weeks with homologous virus to determine if subclinical infection had stimulated protective immunity. Egg and mouse end-point infectivity titers were calculated according to Spearman-Kärber (17).

Animal inoculation. Adult C3H/He-mg (H-2^k) mice (4 to 5 weeks old; 16 to 20 g) were inoculated intranasally under light ether anesthesia as previously described (23, 24) with a 40-μl inoculum divided between the two nares. Helper virus infectivity can be eliminated without reducing protection by a short (20-s) burst of UV irradiation at 253.7 nm because of the difference in UV target sizes—13,600 nt for infectivity and 395 nt for the protecting RNA. The lamp was calibrated by inactivating A/PR8 infectivity. Longer UV irradiation (8 min) inactivates protection and provides a preparation that controls for any immune

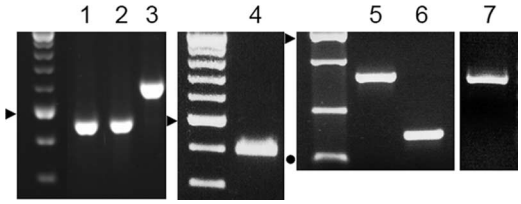

FIG. 1. RT-PCR detection of defective RNA in protecting virus preparations, amplified using primers specific for the termini of gene 1 (RNA1F and RNA1R), except in lane 6 where primers RNA1F and 244J were used. The RNA/helper virus combinations shown are as follows: lane 1, 220/Vic; lane 2, 220/PR8 (both amplicons are 445 bp); lane 3, 317/Vic (an amplicon of 585 bp); lane 4, 244/Mallard; lane 5, 244/WSN (both amplicons are 395 bp); lane 6, 244/WSN (an amplicon of 161 bp); and lane 7, 244/PR8 (an amplicon of 395 bp). DNA size markers are indicated by ▶ (500 bp) and ● (100 bp).

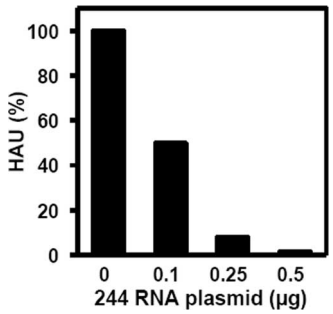

FIG. 2. Transfection of 293T cells with excess 244 protecting influenza virus RNA expression plasmid inhibits the production of viral HA by plasmids expressing infectious A/WSN. Various amounts of 244 plasmid were transfected into 293T cells together with a constant amount of plasmids encoding infectious A/WSN. One day later, these were cocultivated with MDCK cells for 7 days. Virus yield (HAU) in the culture fluid was measured.

system-stimulating or receptor-blocking effects. Irradiation did not affect HA or NA activities. Mice were given various combinations of noninfectious protecting virus, UV-inactivated protecting virus, infectious challenge virus, or diluent. Infectious challenge viruses were titrated in mice to determine a dose for each that caused comparable respiratory disease. Mice were infected with 10 50% lethal doses (LD_{50}) (100 50% infective doses) of A/WSN as determined by immunization by the intranasal route. The following higher doses of other subtypes were required to cause disease: for A/Japan/305/57 (H2N2), 3×10^5 50% egg infectious doses per mouse were used; and for 7a (H3N2; a reassortant between A/England/939/69 [H3N2] and A/PR8 [33]), 2.5×10^4 50% tissue culture infective doses per mouse were used. The health of the mice was assessed by loss of weight and by previously described clinical criteria (23). Mice were weighed as a group. Clinical criteria were scored as follows: 1 point for each healthy mouse; 2 points for a mouse showing signs of malaise, including some piloerection, a slightly changed gait, and increased ambulation; 3 points for a mouse showing signs of strong piloerection, constricted abdomen, changed gait, periods of inactivity, increased breathing rate, and sometimes rales; 4 points for a mouse with enhanced characteristics of the previous group but showing little activity and becoming moribund—such mice were killed when it was clear that they would not survive; and 5 points for a dead mouse. To allow comparison, the total clinical score was divided by the number of mice in the experimental group. All viruses caused similar clinical disease, including lung consolidation. When lung samples were taken, consolidation was estimated by viewing the percentage of the lung surface that had developed a plum-colored discoloration. Animal experiments were approved by the University's Ethical Review Committee and followed the guidelines of the United Kingdom Coordinating Committee for Cancer Research.

RESULTS

Generation of the A/PR8-derived defective RNA 244. An abundant defective RNA was found in a preparation of A/PR8 virus which had been recovered from plasmid transfection of 293T cells, and the resulting virus was found to be protective in mice (see below). RT-PCR and sequencing of RNA extracted from purified virus showed the defective RNA to be a single species 395 nt in length and comprising nt 1 to 244 and 2191 to 2341 of the A/PR8 minus-sense segment 1 RNA. The defective RNA thus retains the exact termini and the terminal sequences that contain the replication and encapsidation signals. The defective RNA was designated 244, and the virus preparation was designated 244/PR8 (Table 1). Analysis with primers specific for genome segment 1 showed that the 244 RNA was the only defective RNA present (Fig. 1, lane 7). The 244 RNA retained its sequence on passage and was not replaced or augmented by significant amounts of other defective RNAs.

Creation and propagation of cloned protecting virus. Viruses containing cloned segment 1 defective RNAs 220 (H3N8) and 317 (H7N7) were created as described previously (10) by cotransfection of 293T cells with viral and defective RNA plasmids (Table 1). The 244 RNA was also cloned into a PolI expression vector and rescued into virus using plasmids encoding the WSN strain of influenza A virus to produce 244/WSN. We found that the yield of 244/WSN was sensitive to the amount of transfected defective RNA-expressing plasmid (Fig. 2) and to the amount of virus passaged in embryonated chicken eggs (data not shown). Better virus yields were obtained by inoculating less defective RNA plasmid and passaging smaller amounts of virus in embryonated eggs. As a result, we transfected 0.1 μg of 244 expression plasmid, inoculated 100 μl of the MDCK cell supernatant into eggs to make a seed stock, and then inoculated eggs with 10 μl of seed stock to make a working stock. After purification by differential centrifugation, defective viruses were normalized to 2×10^5 hemagglutinating units (HAU) or 600 μg of virus protein per ml. For each cloned defective virus, the RNA derived from the defective RNA-encoding plasmid was the only defective segment 1-derived RNA observable by RT-PCR, as shown in Fig. 1. However, small amounts of defective RNAs derived from other genes could sometimes be observed. Such RNAs may have arisen spontaneously during virus growth in cell culture or eggs. Identity of the 244 RNA was confirmed by RT-PCR using a terminal primer and a primer specific to the unique junction sequence formed after the central deletion has occurred (Fig. 1, lanes 5 and 6) and was further authenticated by sequencing.

Since these defective viruses differ from infectious viruses only by the deletion of part of one genome segment, it is not possible to separate the two types of particles physically. However, UV irradiation targets nucleic acids in proportion to size and rapidly inactivates the infectivity of helper virus (genome, 13,600 nt), whereas the defective RNA (approximately 400 to 600 nt) is little affected by this dose. Inoculation of MDCK cells, embryonated eggs, and mice (intranasally, followed by culture of homogenized lungs in embryonated eggs) showed no residual infectivity (data not shown). Prolonged UV irradiation destroyed the mouse-protecting activity of defective virus (see below).

Verification that mouse-protecting activity resides in RNA 244. As trace amounts of other defective RNAs were present in

TABLE 2. Comparison of the prophylactic activity in mice mediated by various defined protecting viruses against infectious influenza virus

Total amt of protecting virus (HAU) per mouse (mass of virus protein)[a]	Prophylactic activity of[b]:			
	244/PR8	244/WSN	220/PR8	317/Vic
4,000 (12 μg)	++++	++++	++++	++++
400 (1.2 μg)	++++	++++	+	+++
40 (0.12 μg)	++++	++++	+	−
4 (0.012 μg)	++	++	ND	ND
None[c]	−	−	−	−
Minimum dose required for solid protection[d]	0.12 μg[e]	0.12 μg[e]	12 μg	1.2 μg

[a] Given as a single intranasal dose under light anesthesia simultaneously with 10 LD_{50} of A/WSN challenge virus.
[b] The scale ranges from complete protection from weight loss and clinical disease (++++) to no difference to the controls given UV-inactivated protecting virus plus challenge virus (−). ND, not done. Groups of five to seven mice were used; this experiment is representative of two to four independent experiments.
[c] Mice were given 4,000 HAU of UV-inactivated protecting virus.
[d] Defined as the smallest dose of protecting virus effecting +++ protection or better.
[e] Total virus protein inoculated per mouse.

244/PR8, it was important to verify that the antiviral activity of 244/PR8 in mice resided in RNA 244, rather than in a combination of 244 and another defective RNA. To this end, we generated cloned 244 RNA entirely from plasmids. In a parallel titration, the resulting defective 244/WSN virus had the same protecting activity as 244/PR8 (complete protection with 120 ng per mouse and at least 10-fold higher than that of other defective viruses) (Table 2), confirming that RNA 244 was responsible for prophylaxis. This also demonstrates the ease with which a defective RNA can be transferred to a new helper virus (from A/PR8 to A/WSN). Finally, the experiment demonstrates for the first time that a defective virus containing a single defective RNA can protect mice from infection.

Prophylactic protection of mice from influenza. These experiments were designed to show the efficacy with which defective viruses protected mice from influenza. Mice were inoculated intranasally with either noninfectious defective virus or with defective virus whose potential protecting activity had been destroyed by prolonged UV irradiation. The latter retains full HA and NA activities and serves as a control for immunogenicity and a cell receptor blockade. In the first experiments, mice were inoculated simultaneously with a single dose of 244/PR8 defective virus (400 HAU or 1.2 μg) and mouse-pathogenic infectious A/WSN. Mice that received UV-inactivated defective virus plus A/WSN suffered from weight loss and clinical disease, and all died (Fig. 3a and b). This was identical to the disease in mice receiving infectious virus alone (data not shown). In comparison, mice receiving protecting virus plus A/WSN continued to gain weight, as did the mock-infected control animals, and showed no sign of disease (Fig. 3a and b). A 10-fold dilution of protecting virus (to 40 HAU or 120 ng per mouse) kept major clinical disease and death at bay, although there was slight, transient weight loss and some malaise, which resolved by day 10 (Fig. 3d and e). Finally, 4 HAU (12 ng) of protecting virus per mouse slowed the onset of clinical signs and weight loss and increased survival from 0 to 60% (Fig. 3g and h). Thus, defective virus exerts strong mouse protection that titrates out and hence is referred to as protecting virus.

The same minimum dose (40 HAU or 0.12 μg per mouse) of 244/PR8 gave solid protection from infectious virus challenge with five independent preparations, attesting to the reproducibility of the production and the action of protecting virus. This was equivalent to 120 ng of virus protein or approximately 400×10^6 virus particles per mouse. Three other protecting viruses containing one or the other of two previously described defined segment 1 protecting RNAs, which were produced, HAU normalized, and tested in exactly the same way, were 10- to 100-fold less active than 244/PR8 (Table 2). These had the same relative ability to protect against A/PR8, showing that the differences were not challenge virus specific (data not shown). Finally, the highest dose of 244/PR8 completely prevented clinical disease caused by a 10-fold higher A/WSN challenge dose (100 LD_{50}) and converted 1,000 LD_{50} A/WSN into a transient disease with only mild clinical signs (data not shown).

Protecting virus prevents clinical disease but allows adaptive immunity to the challenge virus to develop. Three weeks after mice were protected from 10 LD_{50} of A/WSN, they were rechallenged with a much-higher dose of A/WSN (10,000 LD_{50}). This dose was used because it swamps even undiluted protecting virus (data not shown) and thus allows an assessment of A/WSN-specific B- and T-cell immune responses. Figure 3 (panels c, f, and i) shows that all groups of surviving mice were completely immune to the rechallenge. As animals given 400 or 40 HAU (1.2 or 0.12 μg) of protecting virus showed no sign of disease during the primary challenge, their abilities to survive the second virus challenge show that the mice had developed protective immunity, and therefore that protecting virus had effectively converted the initial LD of virulent virus into a subclinical live vaccine. Counterintuitively, mice receiving the highest dose of protecting virus (4,000 HAU or 12 μg) (Table 3) were less well protected from the second challenge, suggesting that virus replication and antigen production are so severely suppressed in this situation that the resulting infection is only weakly immunogenic.

Duration of prophylactic protection exerted by protecting virus. To determine the duration of prophylaxis, mice were given a single intranasal dose of noninfectious protecting virus or control UV-inactivated protecting virus (400 HAU or 1.2 μg). This had no apparent deleterious effect, with animals remaining completely healthy and gaining weight at the expected rate (Fig. 4a and c). Mice were challenged with infectious virus 1 week later by the intranasal route; those animals that had received protecting virus were completely protected (Fig. 4c and d), but those given UV-inactivated protecting virus succumbed to the infection (Fig. 4a and b). A separate group of mice challenged 2 weeks after treatment with protecting virus were susceptible to the same challenge infection, showing

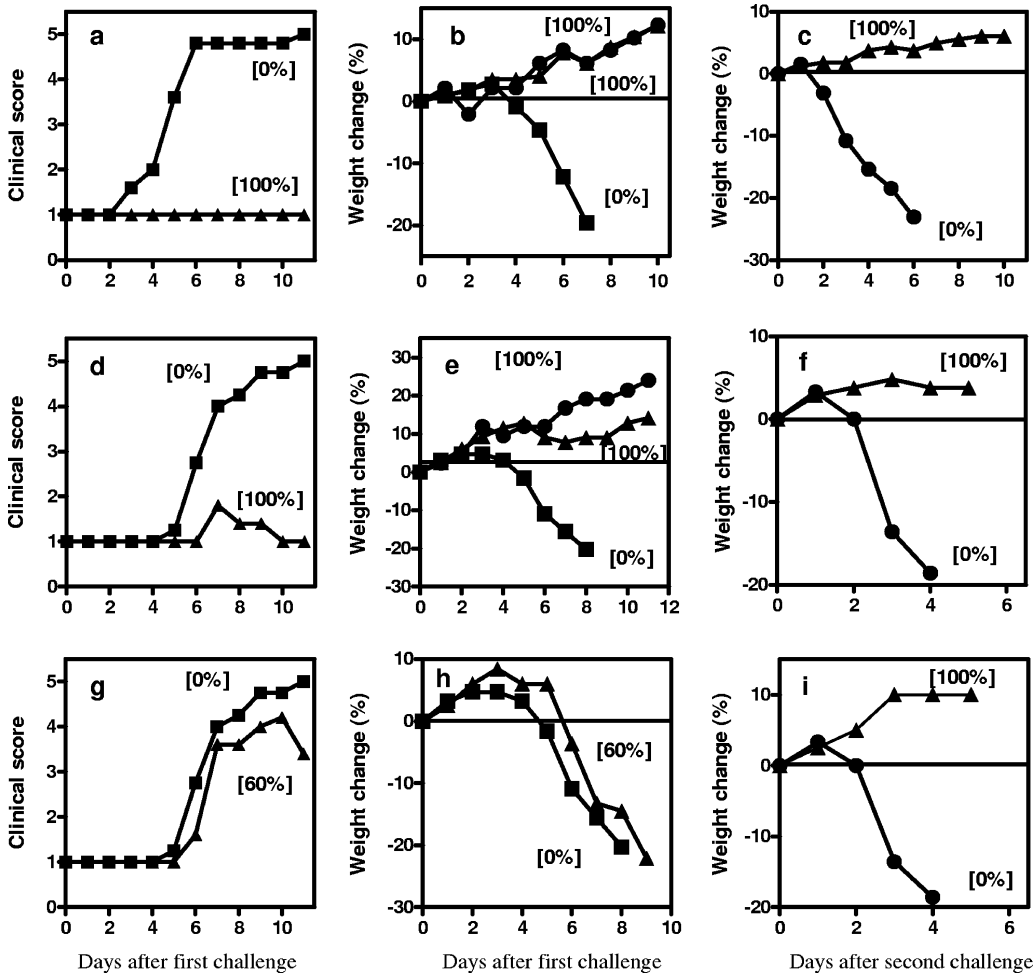

FIG. 3. Prophylactic activity mediated by protecting virus 244/PR8 in mice against infectious A/WSN, as monitored by clinical disease and body weight change. All mice were inoculated intranasally. Mice received 400 (a, b, and c), 40 (d, e, and f), and 4 HAU (g, h, and i) of 244/PR8 protecting virus (12, 1.2, and 0.12 μg, respectively) mixed with 10 LD_{50} A/WSN. The figure shows clinical scores (a, d, and g) and weight changes (b, e, and h). Percent survival is shown in brackets. Symbols denote the inocula given in panels a, d, and g, as follows: ■, UV-inactivated protecting virus plus 10 LD_{50} A/WSN; ▲, protecting virus plus 10 LD_{50} A/WSN; and ●, diluent. Panels c, f, and i show the result (change in weight) when survivors were challenged with 10,000 LD_{50} A/WSN, at 3 weeks after the first infection. This very large dose of A/WSN abrogates protection even by the highest dose of protecting virus (not shown), and hence tests for the development of adaptive immunity.

that protection had decayed and also that the mice had not mounted an adaptive immune response (not shown). The conclusion that protecting RNA persists in the murine respiratory tract was tested by RT-PCR using RNA extracted from the lungs of mice that had been inoculated with a 10-fold-higher dose of protecting virus. The inoculum of 4,000 HAU (12 μg) was used in this case, since RNA was not reproducibly detected in lungs from mice inoculated with 400 HAU of protecting virus. Figure 5 shows that protecting RNA did persist and could be detected for up to 3 weeks. Mice given this dose of protecting virus were completely protected from an infectious challenge given up to 6 weeks later (data not shown). This dose of protecting virus appeared to be around the 50% immunizing dose, as in some experiments adaptive immunity developed.

Prophylaxis extends to different subtypes of influenza A virus. One of the problems in combating influenza is that there may be 144 distinct A virus subtypes, as well as the progressive drift variation that viruses undergo in humans, and each subtype and significant drift variant requires its own vaccine. However, intranasally administered 244/PR8 protecting virus protected mice from clinical disease caused by human strains of H3N2 (7a), H2N2 (A/Japan/305/57), and the antigenically distinct H1N1 viruses (A/PR/8/34 and A/WSN) and the equine strain H3N8 (A/Newmarket/7339/79). Figure 6 shows protection data for H2N2 and H3N2 viruses. Mice given H2N2 virus and control UV-inactivated protecting virus all became ill by day 5 and lost 24% of their starting weight by day 8; four of five animals recovered. However, noninfectious protecting virus prevented any H2N2-infected animal from becoming ill or losing significant weight (Fig. 6a and b). The disease caused by the H3N2 infection was rapid and more severe (Fig. 6c and d); all mice given simultaneous H3N2 virus and UV-inactivated protecting virus became ill by day 2 and experienced significant weight loss; most (four of five mice) were dead by day 6. Protecting virus prevented virtually all clinical disease; early and transient weight loss was reversed after day 3. There were

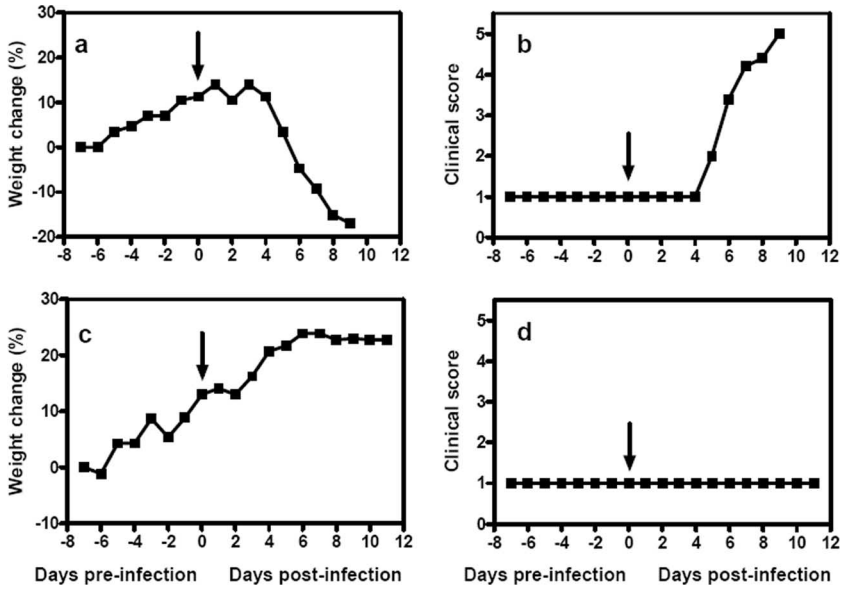

FIG. 4. Duration of prophylactic activity of 244/PR8 protecting virus. A single dose of protecting virus (c, d) or UV-inactivated protecting virus (a, b) (400 HAU or 1.2 μg) was administered intranasally at 1 week before infection (arrow). Mice were challenged with 10 LD_{50} A/WSN on day 0 and were monitored by percent weight change (a, c) and average clinical score (b, d). Healthy mice were given a score of 1 and dead mice were given a score of 5.

no deaths. All control groups given protecting virus alone or saline showed steady weight gain and no clinical disease. Thus, protecting virus affords broad protection that does not appear to be limited by the HA and NA surface antigens. In addition, it did so even though both subtypes (H2N2 and H3N2) required 2 to 3 orders of magnitude more infectious virus to cause overt disease in mice than did A/WSN. 244/PR8 is thus more active than noncloned protecting virus, which failed to prevent disease mediated by the same H2N2 virus in a previous study, although the noncloned protecting virus interfered with the multiplication of a smaller H2N2 virus dose (8). Furthermore, 244 RNA can be rescued by reverse genetics using A/WSN as the helper or by reassortment (20) using an avian H2N3 strain (A/mallard/England/7277/06) as the helper (Fig. 1). This suggests that protecting virus can be replicated by a variety of helper virus subtypes.

TABLE 3. The highest dose of protecting virus provides only a weak vaccine effect[a]

First challenge		Second challenge		
Dose of protecting virus (HAU)	No. of dead mice/no. of infected mice	Weight loss	No. of ill mice/no. of challenged mice (%)	No. of dead mice/no. of challenged mice (%)
4,000 (12 μg)	0/7	Yes	5/7 (71)[b]	4/7 (57)[c]
	0/4	Yes	4/4 (100)	2/4 (50)
	0/4	Yes	4/4 (100)	3/4 (75)
400 (1.2 μg)	0/4	No	0/4	NA
	0/4	No	0/4	NA
40 (120 ng)	0/5	No	0/5	NA
	0/4	No	0/4	NA
	0/4	No	0/4	NA
4 (12 ng)	2/5	No	0/2	NA
None[d]	5/5	NA	NA	NA

[a] Mice were intranasally inoculated with a mix of protecting virus and 10 LD_{50} challenge virus A/WSN (first challenge: columns 1 and 2) and then 3 weeks later inoculated with 10,000 LD_{50} A/WSN alone (second challenge). This latter experiment tests adaptive immunity and not residual protecting virus activity, as the higher dose of A/WSN completely overcomes protecting virus when given simultaneously (not shown). Data from 3 separate experiments are shown. NA, not applicable.
[b] Mean, 87% ill.
[c] Mean, 60% dead.
[d] Mice were given 4,000 HAU of UV-inactivated protecting virus.

Protecting virus has therapeutic benefit. Previous work with noncloned interfering virus showed no therapeutic effect, but because of the strong prophylactic action of defined protecting virus, this experiment was revisited. Mice were infected with 10 LD_{50} of A/WSN as before and treated intranasally 24 and 48 h later with a single dose of noninfectious protecting virus 244/

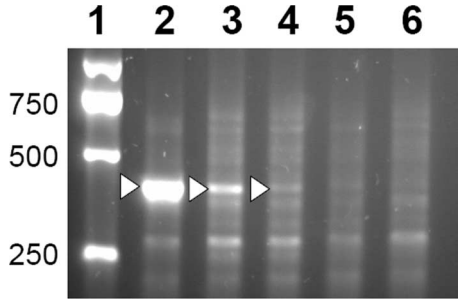

FIG. 5. Persistence of protecting RNA 244 (395 nt) in mouse lung in the absence of infectious virus, as demonstrated by RT-PCR with primers RNA1F and RNA1R. Mice were inoculated intranasally with 4,000 HAU (12 μg) of protecting virus. Lane 1, DNA size markers (bp); lanes 2 to 6, amplicons from mouse lungs. RNA for lanes 2 to 5 was extracted 1 day, 9 days, 21 days, and 42 days, respectively, after inoculation; lane 6, mock inoculation with saline.

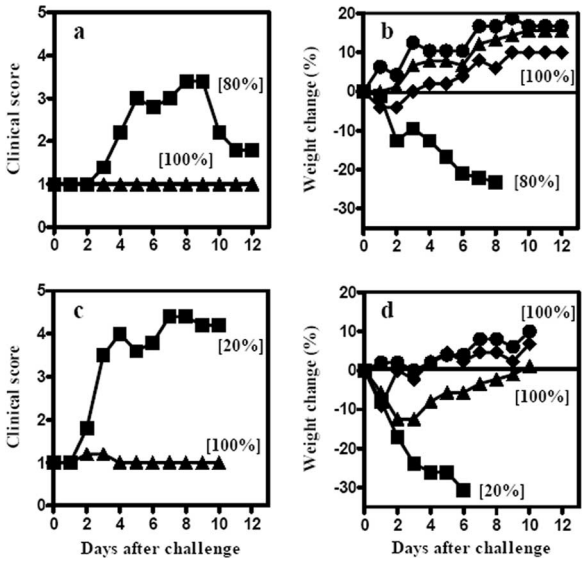

FIG. 6. Protecting virus 244/PR8 prevented clinical disease in mice infected with an H2N2 virus (A/Japan/305/57) (a, b) or an H3N2 virus (7a) (c, d). The experiment was conducted in the same way as the initial phase shown in Fig. 3. Mice (five per group) were inoculated simultaneously with a mixture of challenge virus and protecting virus (4,000 HAU or 12 μg) (▲) or challenge virus and UV-inactivated protecting virus (4,000 HAU or 12 μg) (■). Clinical scores (a, b) and weight changes (b, c) are shown, with percent surviving mice shown in brackets. Weight changes in noninfected control groups (two mice) given protecting virus alone (◆) or saline (●) are also shown. None of these became ill.

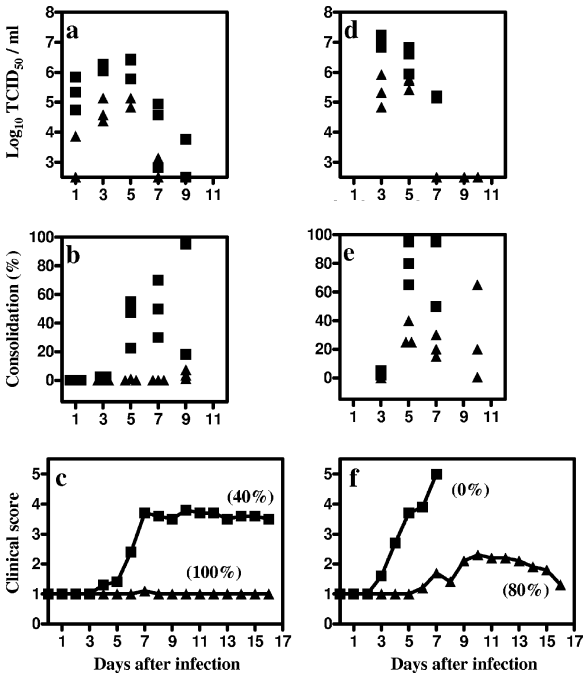

FIG. 7. Virus infectivity in the lungs of A/WSN-infected mice treated with protecting virus (▲) or UV-inactivated protecting virus (■). For prophylaxis (a, b, and c), a mixture of 400 HAU (1.2 μg) of protecting virus 244/PR8 or UV-inactivated protecting virus and A/WSN were inoculated on day 0. For therapy (d, e, and f), 4,000 HAU (12 μg) of protecting virus 244/PR8 or UV-inactivated protecting virus were inoculated intranasally into mice 1 day after intranasal infection with A/WSN. Mice (three per group) were killed and their lungs were removed. Lungs were frozen and later ground with sand, clarified, and end-point assayed in MDCK cells for infectivity (a, d). Each point represents one animal. Lung consolidation is shown (b, e) and is an average of the values for the left and right lung of each mouse shown. Clinical scores for groups of animals (n = 10) treated in parallel are also shown (b, e). No infectivity or consolidation was detected in controls inoculated with protecting virus alone or diluent, and these animals remained healthy for the duration of the experiment (data not shown).

PR8 or control UV-inactivated protecting virus (4,000 HAU or 12 μg). While all control mice died, therapy in this experiment with protecting virus at 24 h completely prevented clinical disease, weight loss, and death. In repeat experiments, therapy reproducibly protected the majority of animals (e.g., Fig. 7e and f). Therapy at 48 h after infection was less effective, although illness was delayed. All mice became ill and 33% recovered (Table 4), compared with 100% dying in the group treated with UV-inactivated protecting virus.

244/PR8-mediated inhibition of virus multiplication and lung pathology during prophylaxis and therapy. We then determined the effect of protecting virus on the multiplication of challenge virus infectivity and on consolidation of the lungs. Figure 7a shows that lung virus infectivity titers in mice inoculated prophylactically with simultaneous UV-inactivated control protecting virus and A/WSN challenge virus peaked on days 3 and 5 after infection. However, prophylactic noninfectious protecting virus reduced lung infectivity by more than 10-fold on days 3 and 5, and by day 7, virus titers in both treated groups (and in the group inoculated with virus alone) (data not shown), were resolving. Clinical disease was severe in infected animals given UV-inactivated protecting virus, and the majority of mice (60%) died or were euthanized. Survivors made a slow recovery. Infected animals treated with active protecting virus showed virtually no sign of disease (Fig. 7c) or weight loss (not shown). These differences were reflected in the observed consolidation, which after 5 days extended to most of the lung tissue in mice treated with UV-inactivated protecting virus but was negligible when protecting virus was administered (Fig. 7b). The difference in the extent of consolidation on day 5 was over 100-fold.

Similarly, in mice treated therapeutically with the control UV-inactivated protecting virus at 24 h after infection with A/WSN, lung infectivity peaked at 3 days. Treatment with protecting virus reduced lung infectivity on day 3 by more than 40-fold and on day 5 by sixfold. Infectious titers fell from day 5 (Fig. 7d). All infected mice treated with UV-inactivated protecting virus became severely ill and died or were euthanized. Therapy with protecting virus ameliorated clinical disease and weight loss (not shown), and the majority of animals (80%) recovered (Fig. 7f). In line with this, mice receiving protecting virus had reduced lung consolidation by a factor of two- to threefold compared with controls receiving UV-inactivated protecting virus (Fig. 7e).

DISCUSSION

Intranasally administered cloned and noncloned (7, 20, 24) protecting influenza viruses give excellent prophylactic activity

TABLE 4. Therapeutic benefit of protecting virus in mice[a]

Time of therapy	UV-inactivated protecting virus		Protecting virus	
	% of sick mice	% of recovered mice	% of sick mice	% of recovered mice
24 h p.i.	100 (by day 5)	0 (died during days 5–7)	0	NA
48 h p.i.	100 (by day 5)	0 (died during days 5–7)	100 (during days 6–16)	33

[a] Infected with 10 LD_{50} A/WSN and treated postinfection (p.i.) at the times shown with UV-inactivated protecting virus or protecting virus (4,000 HAU or 12 μg virus protein). All inoculations were intranasal with light anesthesia. Groups of five to seven mice were used; this experiment is representative of three independent experiments. NA, not applicable.

against a strong infectious virus challenge in both mouse and ferret models, the latter closely mimicking human disease. However, the best cloned protecting virus (244/PR8) is approximately 50-fold more active than any of our other protecting viruses (24) and also protects mice for far longer than noncloned protecting virus. Further, only defined protecting virus has therapeutic activity, which is probably a function of its overall higher activity. As already noted, different protecting viruses vary in the magnitude of their antiviral activities when normalized to total HAU, and a quantitative RT-PCR specific for the defective RNA is needed to develop a better interpretation of what this means. Quantitative RT-PCR will also increase our understanding of how a protecting virus exerts its antiviral activity. As a rough estimate, assuming one defective RNA molecule per virion, the fully protective dose of 40 HAU of 244/PR8 virus contains approximately 2×10^8 copies of the 244 RNA molecule.

We reported earlier the persistence of influenza A virus RNAs in cultured cells under conditions where the virus was not replicating (3, 4). Both defective RNAs present in naturally nonreplicating virus and the HA gene from infectious virus that had been critically UV irradiated to just remove infectivity persisted for several weeks. However, the persistence of protecting RNA in vivo described here was unexpected and deserves further study, since influenza A virus RNAs are not generally thought to persist in immunocompetent animals, although there are exceptions (1, 9, 12, 26, 35).

As noncloned protecting virus populations contain a rich assortment of defective RNAs (11), it is not possible to determine how any one RNA molecule exerts protection, or indeed, if protection requires more than one RNA sequence. Such a study is now both feasible and timely. One possibility is that the copying of an RNA genome is proportional to its size, so that a protecting RNA that is five times smaller is replicated five times faster. Thus, starting from equal numbers of defective and infectious genomes in a cell, over 90 and 99% of genomes would be defective after three and five rounds of replication, respectively. Under these conditions, assuming that influenza virus RNA packaging is an organized process (25) and that the defective RNA and its full-length counterpart are packaged with equal efficiencies, the majority of progeny particles will contain a defective RNA and be noninfectious. In addition to this reduction in infectious progeny, defective virions would transmit protecting RNA to neighboring cells and make them resistant to infection. Defective RNA may also compete with its nondefective counterpart for limiting amounts of viral or cell constituents, induce alpha/beta interferon (28, 31), or induce an antiviral small interfering RNA response from defective RNA, although the latter is only known so far from plant and invertebrate systems (30, 36). Indeed, such mechanisms might work in concert. It would be of great interest to determine if protecting virus is still able to exert its protective effects in interferon knockout mice. Current research in our laboratory is aimed at elucidating which of these mechanisms contribute to the observed protective effect.

Protecting concentrations of cloned and noncloned protecting viruses attenuate the virulent virus infection in mice and ferrets (20, 24). There is no clinical disease, but there is evidently enough antigen produced by the virulent virus to stimulate an adaptive immunity that renders these animals resistant to reinfection with homologous virus (Table 3). Counterintuitively, immunity was weakest after treatment with the highest concentration of protecting virus, presumably because antigen formation is suppressed to an almost subimmunogenic level. The data presented here also show that protecting virus reduces, but does not abolish, challenge virus multiplication in mouse lungs, and this progeny virus is presumed to stimulate subsequent adaptive immunity. Consolidation, the response of the host's immune responses to newly synthesized viral antigens associated with the lung, was also diminished by protecting virus.

We believe that the in vivo data presented here justify human trials to determine how effective protecting virus is in people. Here, protecting virus would probably be administered by a nasal spray, as used for live influenza virus vaccine (2). We do not anticipate problems with toxicity, because apart from having one smaller RNA segment, protecting virus has the same composition as the infectious influenza virus that everyone is exposed to naturally. However, we will have to ensure that protecting virus delivers protecting RNA to the same cells in the respiratory tract that "wild" influenza virus normally infects, i.e., that both use the same cell receptors (29). The defective influenza virus RNAs described here arose naturally, and human beings are probably exposed to them during normal infection.

Protecting virus potentially offers a number of advantages over vaccines or existing drugs in combating pandemic influenza. Influenza vaccines are exquisitely specific for the virus strain of the day, and it can take several months to a year to select a new strain, produce and test a vaccine, and distribute and administer it to a significant section of the world's population. Vaccine-induced immunity takes approximately 3 weeks to mature, and the elderly may be incapable of mounting an effective immune response. In contrast, protecting virus exerts its full effect immediately, is relatively long-lived, and should be active against any strain of influenza A virus. Its activity

resides in the viral genome rather than the host response, so protection should also be effective in the elderly. A major limitation of antiviral drugs is the rapidity with which resistance occurs, and human influenza virus isolates resistant to oseltamivir have already been isolated (13, 19). However, protecting RNAs are dependent on the highly conserved replication machinery of normal virus, so resistance is unlikely to arise.

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