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Influenza virus protecting RNA: an effective prophylactic and therapeutic antiviral

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1 **Abstract**

2 Another influenza pandemic is inevitable, and new measures to combat this and seasonal
3 influenza are urgently needed. Here we describe a new concept in antivirals based on a
4 defined, naturally occurring defective influenza RNA that has the potential to protect against
5 any influenza A virus in any animal host. This protecting RNA (244 RNA) is incorporated
6 into virions which although non-infectious, deliver the RNA to those cells of the respiratory
7 tract that are naturally targeted by infectious influenza virus. A small intranasal dose of this
8 244 protecting virus (120 ng) completely protected mice against a simultaneous lethal (10
9 LD_{50}) challenge with influenza A/WSN (H1N1) virus. 244 virus also protected mice against
10 a strong challenge dose of all other subtypes tested (H2N2, H3N2, H3N8). This prophylactic
11 activity was maintained in the animal for at least 1 week prior to challenge. 244 virus was 10
12 to 100-fold more active than previously characterised influenza A defective viruses, and the
13 protecting activity was confirmed to reside in the 244 RNA molecule by recovering a
14 protecting virus entirely from cloned cDNA. There was clear therapeutic benefit when
15 protecting 244 virus was administered 24-48 h after lethal challenge, an effect which has not
16 been previously observed with any defective virus. Protecting virus reduced, but did not
17 abolish, replication of challenge virus in mouse lungs during both prophylactic and
18 therapeutic treatments. Protecting virus is a novel antiviral which has the potential to combat
19 influenza infections in humans, particularly when the infecting strain is not known, or is
20 resistant to antiviral drugs.

21

Introduction

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, haemagglutinin (H) and neuraminidase (N), of which there are 16 H subtypes and 9 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 1-5 million deaths from the 1957 and 1968 viruses. Currently, measures to counter human influenza include administration of killed and live vaccines and the antivirals Tamiflu and 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 Tamiflu has already been recorded in human virus isolates (13, 19) and is causing concern.

The influenza A genome comprises eight segments of single-stranded negative sense RNA that encode 9 structural and 2 non-structural 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 400-500 nt lacking around 80% of the central sequence of the template, which appears 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 8 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 non-infectious, 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 produced. Thus, virions carrying a deleted genome were known as interfering or defective-interfering (DI) viruses (15).

It has been known for some time that non-infectious 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 non-cloned 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 non-cloned populations of defective virus cannot be reproduced with certainty, and it was not possible to analyse the relationship

1 between RNA sequence and antiviral activity. Nor was it known if antiviral activity resided
2 in one defective RNA sequence or required the combined action of two or more sequences.
3 The key to analysing the mechanism(s) of interference and protection, and also to
4 clinical uses of DI viruses, is the ability to produce a DI virus containing a single, unique
5 deleted RNA species. Using reverse genetics, we have now made virus preparations that
6 contain a single defective RNA that has the ability to protect animals from serious infection
7 with influenza A viruses. We call such preparations 'protecting viruses' to distinguish them
8 from the activity of 'interfering viruses' in cultured cells (14). Our most active protecting
9 virus, described in this paper, has approximately 50-times more prophylactic activity against
10 influenza A virus in mice than non-cloned DI virus, and provides therapeutic benefit in virus-
11 infected mice that was not observed before with non-cloned virus. Protecting virus represents
12 a new concept in antivirals, and clinical trials are being planned to determine if it is effective
13 in combating human influenza A viruses. A major advantage of protecting virus is that it is
14 expected to work against any subtype or strain of influenza A virus. Viruses resistant to
15 protecting virus are unlikely to arise because the active principle, protecting RNA, uses the
16 same replication machinery as genomic RNA.
17
18
19 **Methods**
20 **Production of protecting viruses by reverse genetics.**
21 Virus was recovered from plasmids based on influenza A/PR/8/34 essentially as described
22 (32). Briefly, the DNA mix transfected into 293T cells contained 0.5 µg of each of the 8
23 A/PR8 gene segments (under PolI promoters), 0.5 µg each PB1 and PB2 expression plasmids,
24 0.1 µg PA expression plasmid, and 1 µg NP expression plasmid, using Fugene (Roche). To
25 produce protecting virus, an additional plasmid which expresses the defective RNA from the

1 PolI promoter as a negative-sense transcript (see below) was added to the mixture. Plasmids
2 which contain the gene 1 defective RNAs 220 (equine H3N8) and 317 (avian H7N7) under
3 control of PolI promoters have been described previously (11) (Table 1). In other experiments,
4 helper plasmids encoding the 8 RNA segments of A/WS/33(N) (A/WSN) or A/Victoria/3/75
5 (A/Vic) were used (22). After 24 h, the 293T cells were trypsinized, mixed with MDCK cells
6 and re-plated, and culture supernatants harvested 7 days later. Growth of virus was
7 determined by assay for viral haemagglutinin (HA). The supernatant was passaged twice in
8 embryonated chicken's eggs to make a seed stock, and then a working stock for mouse studies.
9 Virus was purified by differential centrifugation through sucrose. Stocks were resuspended in
10 PBS containing 0.1% w/v bovine serum albumin, standardized by HA titration, and stored in
11 liquid nitrogen. Optimization of the amount of defective RNA plasmid during transfection
12 (see below) and of the egg inoculum proved important in avoiding low yields of protecting
13 virus.

14 **RT-PCR**

15 RNA was extracted from virus with phenol and dissolved in water. RNA from the lungs of
16 one mouse was extracted by grinding with sterile sand and Trizol (Invitrogen). Generic
17 segment 1-specific primers, RNA1F and RNA1R, have been described previously (8).
18 Aliquots of 2.5 μg total RNA (or RNA from 200 μl virus) were reverse-transcribed in 20 μl
19 reactions for 1 h at 42°C, using RNA1F. Aliquots (1.5 or 3 μl) of the reverse transcription
20 reaction were then amplified by PCR using *Taq* DNA polymerase (MBI Fermentas or New
21 England Biolabs) and primer RNA1F and either RNA1R, or a primer specific for the junction
22 sequence in the 244 RNA, 244J (5'ATCCCCTCAGTCTTCTCCTG3'), in a 25 μl reaction
23 volume. RNA1F has a single mismatch to the published A/PR8 sequence whereas RNA1R is
24 identical to the published A/PR8 sequence. PCR consisted of 30 cycles of 94°C for 20 s,

1 50°C for 30 s and 72°C for 30 s. Aliquots of 10 µl of the product were analysed by agarose
2 gel electrophoresis.

3 **Optimization of transfection of the 244 RNA plasmid**

4 244 RNA was initially observed as a major segment 1-derived RNA of 395 nt in a preparation
5 of A/PR8 virus, which had been recovered from plasmids as described above. The 244 RNA
6 was amplified by RT-PCR using primers specific for the termini of A/PR8 segment 1, and the
7 product was cloned into the PolI expression plasmid pPOLI-SapIT (32), such that a vRNA-
8 sense transcript was expressed. Varying amounts of the 244 plasmid (0-0.5 µg) were
9 transfected into 293T cells along with A/WSN helper plasmids as described above. After 24
10 h, the 293T cells were trypsinized, mixed with MDCK cells and re-plated. After 7 days,
11 culture supernatants were harvested, and virus yield determined by HA assay.

12 **Infectivity titrations**

13 Infectivity titres were determined as required by titration in cell culture, eggs, and mice.
14 Virus was plaque assayed in MDCK cells under agar by standard procedures or $TCID_{50}$ end-
15 point titres were determined from 2-fold dilutions in MDCK cells after 4 days. Eggs were
16 inoculated with limit-diluted virus and incubated for 3 days. Virus-positive eggs were
17 identified by HA in allantoic fluid. Mouse infectivity was assayed by inoculating limit-
18 diluted virus as described below, then after 3 days lungs were removed, and ground lungs
19 from individual mice were inoculated into eggs, and the presence of virus was determined by
20 HA assay. Alternatively, mice were challenged intranasally after 3 weeks with homologous
21 virus to determine if subclinical infection had stimulated protective immunity. Egg and
22 mouse end-point infectivity titres were calculated according to Spearman-Kärber (17).

23 **Animal inoculation**

24 Adult C3H/He-mg ($H\text{-}2^k$) mice (4 to 5 weeks-old; 16-20 g) were inoculated intranasally under
25 light ether anaesthesia 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 minutes) inactivates protection and provides a preparation that controls for any immune system-stimulating or receptor-blocking effects. Irradiation did not affect HA or neuraminidase (NA) activities. Mice were given various combinations of non-infectious 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 LD_{50} (100 ID_{50}) of A/WSN as determined by immunization by the intranasal route. Higher doses of other subtypes were required to cause disease: for A/Japan/305/57 (H2N2), 3 x 10^5 EID_{50} per mouse were used; for 7a (H3N2; a reassortant between A/England/939/69 (H3N2) and A/PR8, (33)), 2.5 x 10^4 $TCID_{50}$ per mouse were used. The health of 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, 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 sometime râles; 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 eye as 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 UK 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, comprising nt 1-244 and 2191-2341 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 as 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). 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 (10) by co-transfection 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

1 chicken's eggs (data not shown). Better virus yields were obtained by inoculating less
2 defective RNA plasmid, and passaging smaller amounts of virus in embryonated eggs. As a
3 result, we transfected 0.1 µg 244 expression plasmid, inoculated 100 µl of the MDCK cell
4 supernatant into eggs to make a seed stock, and then inoculated eggs with 10 µl of seed stock
5 to make a working stock. After purification by differential centrifugation, defective viruses
6 were normalised to 2 x 10^5 haemagglutination units (HAU) or 600 µg virus protein per ml.
7 For each cloned defective virus, the RNA derived from the defective RNA-encoding plasmid
8 was the only defective segment 1 derived RNA observable by RT-PCR, as shown in Fig. 1.
9 However, small amounts of defective RNAs derived from other genes could sometimes be
10 observed. Such RNAs may have arisen spontaneously during virus growth in cell culture or
11 eggs. Identity of the 244 RNA was confirmed by RT-PCR using a terminal primer and a
12 primer specific to the unique junction sequence formed after the central deletion has occurred
13 (Fig. 1, lanes 5 and 6), and was further authenticated by sequencing.
14 Since these defective viruses differ from infectious viruses only by deletion of part of
15 one genome segment, it is not possible to separate the two types of particles physically.
16 However, UV irradiation targets nucleic acids in proportion to size, and rapidly inactivates the
17 infectivity of helper virus (genome 13,600 nt), whereas the defective RNA (approximately
18 400-600 nt) is little affected by this dose. Inoculation of MDCK cells, embryonated eggs, and
19 mice (intranasally, followed by culture of homogenized lungs in embryonated eggs) showed
20 no residual infectivity (data not shown). Prolonged UV irradiation destroyed the mouse-
21 protecting activity of defective virus (see below).
22
23 **Verification that mouse-protecting activity resides in RNA 244**
24 As trace amounts of other defective RNAs were present in 244/PR8, it was important to verify
25 that the antiviral activity of 244/PR8 in mice resided in RNA 244, rather than 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 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 non-infectious 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 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 weight loss and clinical disease, and all died (Fig. 3a, 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, 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 a slight, transient weight loss and some malaise, which resolved by day 10 (Fig. 3d, e). Finally, 4 HAU (12 ng) of protecting virus per mouse slowed the onset of

1 clinical signs and weight loss and increased survival to from 0 to 60% (Fig. 3g, h). Thus,
2 defective virus exerts strong mouse protection that titrates out, and is referred to as 'protecting
3 virus'.

4 The same minimum dose (40 HAU or 1.2 µg per mouse) of 244/PR8 gave solid
5 protection from infectious virus challenge with 5 independent preparations, attesting to the
6 reproducibility of production and action of protecting virus. This was equivalent to 120 ng of
7 virus protein or approximately 400 x 10^6 virus particles per mouse. Three other protecting
8 viruses containing one or other of 2 previously described defined segment 1 protecting RNAs,
9 which were produced, HAU normalized, and tested in exactly the same way, were 10- to 100-
10 fold less active than 244/PR8 (Table 2). These had the same relative ability to protect against
11 A/PR8, showing that the differences were not challenge virus-specific (data not shown).
12 Finally, the highest dose of 244/PR8 completely prevented clinical disease caused by a ten-
13 fold higher A/WSN challenge dose (100 LD_{50}), and converted 1000 LD_{50} A/WSN into a
14 transient disease with only mild clinical signs (data not shown).
15

16 **Protecting virus prevents clinical disease but allows adaptive immunity to the challenge**
17 **virus to develop**

18 Three weeks after mice were protected from 10 LD50 of A/WSN, they were re-challenged
19 with a much higher dose of A/WSN (10,000 LD_{50}). This dose was used because it swamps
20 even undiluted protecting virus (data not shown), and thus allows assessment of A/WSN-
21 specific B and T cell immune responses. Figure 3 (c, f, i) shows that all groups of surviving
22 mice were completely immune to the re-challenge. As animals given 400 or 40 HAU (1.2 or
23 0.12 µg) of protecting virus showed no sign of disease during the primary challenge, their
24 ability to survive the second virus challenge shows that the mice had developed protective
25 immunity, and therefore that protecting virus had effectively converted the initial lethal dose

1 of virulent virus into a subclinical live vaccine. Counter intuitively, mice receiving the
2 highest dose of protecting virus (4000 HAU or 12 μg; Table 3) were less well protected from
3 the second challenge, suggesting that virus replication and antigen production are so severely
4 suppressed in this situation that the resulting infection is only weakly immunogenic.
5
6 **Duration of prophylactic protection exerted by protecting virus**
7 To determine the duration of prophylaxis, mice were given a single intranasal dose of non-
8 infectious protecting virus or control UV-inactivated protecting virus (400 HAU or 1.2 μg).
9 This had no apparent deleterious effect, with animals remaining completely healthy and
10 gaining weight at the expected rate (Fig 4a, c). Mice were challenged with infectious virus 1
11 week later by the intranasal route: those animals that had receiving protecting virus were
12 completely protected (Fig. 4c, d), but those given UV-inactivated protecting virus succumbed
13 to the infection (Fig. 4a, b). A separate group of mice challenged 2 weeks' after treatment
14 with protecting virus were susceptible to the same challenge infection, showing that
15 protection had decayed and also that the mice had not mounted an adaptive immune response
16 (not shown). The conclusion that protecting RNA persists in the murine respiratory tract was
17 tested by RT-PCR using RNA extracted from lungs of mice that had been inoculated with a
18 10-fold higher dose of protecting virus. The inoculum of 4000 HAU (12 μg) was used in this
19 case since RNA was not reproducibly detected in lungs from mice inoculated with 400 HAU
20 protecting virus. Fig. 5 shows that protecting RNA did persist, and could be detected for up to
21 3 weeks. Mice given this dose of protecting virus were completely protected from an
22 infectious challenge given up to 6 weeks later (data not shown). This dose of protecting virus
23 appeared to be around the 50% immunizing dose as in some experiments adaptive immunity
24 developed.
25

1 **Prophylaxis extends to different subtypes of influenza A virus**

2 One of the problems in combating influenza is that there may be 144 distinct A virus subtypes,
3 as well as the progressive drift variation that they all undergo in humans, and each subtype
4 and significant drift variant requires its own vaccine. However, intranasally administered
5 244/PR8 protecting virus protected mice from clinical disease caused by human strains of
6 H3N2 (7a), H2N2 (A/Japan/305/57), and the antigenically distinct H1N1 viruses (A/PR/8/34
7 and A/WSN and the equine strain H3N8 (A/Newmarket/7339/79). Fig. 6 shows protection
8 data for H2N2 and H3N2 viruses. Mice given H2N2 virus and control UV-inactivated
9 protecting virus all became ill by day 5 and lost 24% of their starting weight by day 8; 4/5
10 animals recovered. However, non-infectious protecting virus prevented any H2N2 infected
11 animal from becoming ill or losing significant weight (Fig. 6 a, b). The disease caused by the
12 H3N2 infection was rapid and more severe (Fig. 6 c, d): all mice given simultaneous H3N2
13 virus and UV-inactivated protecting virus became ill by day 2 and experienced significant
14 weight loss; most (4/5) were dead by day 6. Protecting virus prevented virtually all clinical
15 disease; an early and transient weight loss was reversed after day 3. There were no deaths.
16 All control groups given protecting virus alone or saline showed a steady weight gain and no
17 clinical disease. Thus, protecting virus affords broad protection that does not appear to be
18 limited by the HA and NA surface antigens. In addition it did so even though both subtypes
19 (H2N2 and H3N2) required 2-3 orders of magnitude more infectious virus to cause overt
20 disease in mice than did A/WSN. 244/PR8 is thus more active than non-cloned protecting
21 virus which failed to prevent disease mediated by the same H2N2 virus in a previous study,
22 although the non-cloned protecting virus interfered with the multiplication of a smaller H2N2
23 virus dose (9). Furthermore, 244 RNA can be rescued by reverse genetics using A/WSN as
24 the helper or by reassortment (20) using an avian H2N3 strain (A/mallard/England/7277/06)

as helper (Fig. 1). This suggests that protecting virus can be replicated by a variety of helper virus subtypes.

Protecting virus has therapeutic benefit

Previous work with non-cloned 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 non-infectious protecting virus 244/PR8 or control UV-inactivated protecting virus (4000 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 7 c, d). 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% death in the group treated with UV-inactivated protecting virus.

244/PR8-mediated inhibition of virus multiplication and lung pathology during prophylaxis and therapy

In this section we determined the effect of protecting virus on the multiplication of challenge virus infectivity and on consolidation of the lungs. Fig. 7a shows that lung virus infectivity titres 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 non-infectious protecting virus reduced, lung infectivity by more than 10-fold on days 3 and 5, and by day 7 virus titres in both treated groups (and in the group inoculated with virus alone – 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

1 euthanized. Survivors made a slow recovery. Infected animals treated with active protecting
2 virus showed virtually no sign of disease (Fig. 7c) or weight loss (not shown). These
3 differences were reflected in the observed consolidation which after 5 days extended to the
4 most of the lung tissue in mice treated with UV-inactivated protecting virus, but was
5 negligible when protecting virus was administered (Fig. 7b). The difference in extent of
6 consolidation on day 5 was over 100-fold.

7 Similarly, in mice treated therapeutically with the control UV-inactivated protecting
8 virus at 24 h after infection with A/WSN, lung infectivity peaked at 3 days. Treatment with
9 protecting virus reduced lung infectivity on day 3 by more than 40-fold, and on day 5 by 6-
10 fold. Infectious titres fell from day 5 (Fig. 7d). All infected mice treated with UV-inactivated
11 protecting virus became severely ill and died or were euthanized. Therapy with protecting
12 virus ameliorated clinical disease and weight loss (not shown) and the majority of animals
13 (80%) recovered (Fig. 7f). In line with this protecting virus reduced lung consolidation by
14 factor of 2 to 3-fold compared with controls receiving UV-inactivated protecting virus (Fig.
15 7e).
16
17
18 **Discussion**

19 Intranasally administered cloned and non-cloned (7, 20, 24) protecting influenza viruses give
20 excellent prophylactic activity against a strong infectious virus challenge in both mouse and
21 ferret models – the latter closely mimicking human disease. However, the best cloned
22 protecting virus (244/PR8) is approximately 50-fold more active than any of our other
23 protecting viruses (24) , and also protects mice for far longer than non-cloned protecting virus.
24 Further, only defined protecting virus has therapeutic activity, which is probably a function of
25 its overall higher activity. As already noted, different protecting viruses vary in the

1 magnitude of their antiviral activity when normalized to total HAU, and a quantitative (Q)RT-
2 PCR specific for the defective RNA is needed to develop a better interpretation of what this
3 means. QRT-PCR will also inform our understanding about how a protecting virus exerts its
4 antiviral activity. As a rough estimate, assuming one defective RNA molecule per virion, the
5 fully protective dose of 40 HAU of 244/PR8 virus contains approximately 2×10^8 copies of
6 the 244 RNA molecule.

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

14 As non-cloned protecting virus populations contain a rich assortment of defective
15 RNAs (11), it is not possible to determine how any one RNA molecule exerts protection, or
16 indeed if protection requires more than one RNA sequence. Such a study is now both feasible
17 and timely. One possibility is that the copying of an RNA genome is proportional to its size,
18 so that a protecting RNA that is 5 times smaller is replicated 5 times faster. Thus, starting
19 from equal numbers of defective and infectious genomes in a cell, over 90 and 99% of
20 genomes would be defective after 3 and 5 rounds of replication, respectively. Under these
21 conditions, assuming that influenza RNA packaging is an organized process (25) and that the
22 defective RNA and its full-length counterpart are packaged with equal efficiency, the majority
23 of progeny particles will contain a defective RNA and be non-infectious. In addition to this
24 reduction in infectious progeny, defective virions would transmit protecting RNA to
25 neighbouring cells and make them resistant to infection. Defective RNA may also compete

1 with its non-defective counterpart for limiting amounts of viral or cell constituents, induce
2 alpha/beta interferon (28, 31), or induce an antiviral siRNA response from defective RNA –
3 although the latter is only known so far from plant and invertebrate systems (30, 36). Indeed,
4 such mechanisms might work in concert. It would be of great interest to determine if
5 protecting virus is still able to exert its protective effects in interferon-knock-out mice.
6 Current research in our laboratory is aimed at elucidating which of these mechanisms
7 contribute to the observed protective effect.

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

19 We believe that the *in vivo* data presented here justify human trials to determine how
20 effective protecting virus is in people. Here, protecting virus would probably be administered
21 by a nasal spray, as used for live influenza vaccine (2). We do not anticipate problems with
22 toxicity because apart from having one smaller RNA segment, protecting virus has the same
23 composition as the infectious influenza virus that everyone is exposed to naturally. However,
24 we will have to ensure that protecting virus delivers protecting RNA to the same cells in the
25 respiratory tract that 'wild' influenza virus normally infects, i.e., both use the same cell

1 receptors (29). The defective influenza RNAs described here arose naturally, and human
2 beings are probably exposed to them during normal infection.

3 Protecting virus potentially offers a number of advantages over vaccines or existing
4 drugs in combating pandemic influenza. Influenza vaccines are exquisitely specific for the
5 virus strain of the day, and it can take several months to a year to select a new strain, produce
6 and test a vaccine, and distribute and administer it to a significant section of the world's
7 population. Vaccine-induced immunity takes approximately 3 weeks to mature, and the
8 elderly may be incapable of mounting an effective immune response. In contrast, protecting
9 virus exerts its full effect immediately, is relatively long-lived, and should be active against
10 any strain of influenza A. Its activity resides in the viral genome rather than the host response,
11 so protection should also be effective in the elderly. A major limitation of anti-viral drugs is
12 the rapidity with which resistance occurs, and human influenza isolates resistant to Tamiflu
13 have already been isolated (13, 19). However, protecting RNAs are dependent on the highly
14 conserved replication machinery of normal virus, so resistance is unlikely to arise.
15
16
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25

References

1. **Aronsson, F., B. Robertson, H. G. Ljunggren, and K. Kristensson.** 2003. Invasion and persistence of the neuroadapted influenza virus A/NWS/33 in the mouse olfactory system. Viral Immunology **16:**415-423.
2. **Belshe, R. B., K. L. Nichol, S. B. Black, H. Shinefield, J. Cordova, R. Walker, C. Hessel, I. Cho, and P. M. Mendelman.** 2004. Safety, efficacy, and effectiveness of live attenuated, cold-adapted influenza vaccine in an indicated population aged 5-49 years. Clinical Infectious Diseases **39:**920-927.
3. **Cane, C., and N. J. Dimmock.** 1990. Intracellular stability of the gene encoding influenza virus haemagglutinin. Virology **175:**385-390.
4. **Cane, C., L. McLain, and N. J. Dimmock.** 1987. Intracellular stability of the interfering activity of a defective interfering influenza virus in the absence of virus multiplication. Virology **159:**259-264.
5. **Cox, N. J., and K. Subbarao.** 2000. Global epidemiology of influenza: past and present. Annual Review of Medicine **51:**407-421.
6. **Cox, N. J., and K. Subbarao.** 1999. Influenza. The Lancet **354:**1277-1282.
7. **Dimmock, N. J.** 1996. Antiviral activity of defective interfering influenza virus *in vivo*, p. 421-445. *In* S. Myint and D. Taylor-Robinson (ed.), Viral and Other Infections of the Respiratory Tract. Chapman and Hall, London.
8. **Dimmock, N. J., and A. C. Marriott.** 2006. In vivo antiviral activity: defective interfering virus protects better against virulent influenza A virus than avirulent virus. J. Gen. Virol. **87:**In press.
9. **Doherty, P. C., D. J. Topham, and R. A. Tripp.** 1996. Establishment and persistence of virus-specific $CD4^+$ and $CD8^+$ T cell memory. Immunological Reviews **150:**23-44.

1 10. **Duhaut, S. D., and N. J. Dimmock.** 2003. Defective influenza A virus generated
2 entirely from plasmids: its RNA is expressed in infected mouse lung and modulates
3 disease. Journal of Virological Methods **108:**75-82.
4 11. **Duhaut, S. D., and N. J. Dimmock.** 1998. Heterologous protection against a lethal
5 human H1N1 influenza virus infection of mice by a H3N8 equine defective interfering
6 virus: comparison of defective RNA sequences isolated from the DI inoculum and
7 mouse lung. Virology **248:**241-253.
8 12. **Eichelberger, M. C., M. Wang, W. Allan, R. G. Webster, and P. C. Doherty.** 1991.
9 Influenza virus RNA in the lung and lymphoid tissue of immunologically intact and
10 CD4-depleted mice. Journal of General Virology **72:**1695-1698.
11 13. **Hayden, F. G.** 2006. Antiviral resistance in influenza viruses - implications for
12 management and pandemic response. New England Journal of Medicine **354:**785-788.
13 14. **Holland, J. J.** 1990. Generation and replication of defective viral genomes, p. 77-99.
14 *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
15 15. **Huang, A. S., and D. Baltimore.** 1970. Defective viral particles and viral disease
16 processes. Nature (London) **226:**325-327.
17 16. **Jennings, P. A., J. T. Finch, G. Winter, and J. S. Robertson.** 1983. Does the higher
18 order of the influenza virus ribonucleoprotein guide sequence rearrangements in
19 influenza viral RNA. Cell **34:**619-627.
20 17. **Karber, G.** 1968. p. 118. *In* A. J. Rhodes and C. E. van Rooyen (ed.), Textbook of
21 Virology, 5th ed. Williams and Wilkins Co., Baltimore.
22 18. **Lamb, R. A., and R. M. Krug.** 1996. *Orthomyxoviridae*: the viruses and their
23 replication, p. 1353-1395. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.),
24 Fields Virology, 3rd ed, vol. 1. Lippincott-Raven Publishers, Philadelphia.

1 19. **Le, Q. L., M. Kiso, K. Someya, Y. Sakai, N. D. Nguyen, K. L. H. Nguyen, N. D.
2 Pham, H. H. Ngyen, S. Yamada, Y. Muramoto, T. Horimoto, A. Takada, H. Goto,
3 T. Suzuki, Y. Suzuki, and Y. Kawaoka.** 2005. Isolation of drug-resistant H5N1 virus.
4 Nature (London) **437:**1108.
5 20. **Mann, A., A. C. Marriott, S. Balasingam, R. Lambkin, J. S. Oxford, and N. J.
6 Dimmock.** 2006. Interfering vaccine (defective interfering influenza A virus) protects
7 ferrets from influenza, and allows them to develop solid immunity to reinfection.
8 Vaccine **24:**4290-4296.
9 21. **Mayor, S.** 2006. Review says oseltamivir and zanamivir should be kept for epidemics
10 of flu. British Medical Journal **332:**196.
11 22. **Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D.
12 R. Perez, R. Donis, G. Hobom, and Y. Kawaoka.** 1999. Generation of influenza A
13 viruses entirely from cloned DNAs. Proceedings of the National Academy of Sciences
14 of the United States of America **96:**9345-9350.
15 23. **Noble, S., and N. J. Dimmock.** 1994. Defective interfering type A equine influenza
16 virus (H3N8) protects mice from morbidity and mortality caused by homologous and
17 heterologous subtypes of type A influenza virus. Journal of General Virology
18 **75:**3485-3491.
19 24. **Noble, S., L. McLain, and N. J. Dimmock.** 2004. Interfering vaccine: a novel
20 antiviral that converts a potentially virulent infection into one that is subclinical and
21 immunizing. Vaccine **22:**3018-3025.
22 25. **Noda, T., H. Sagara, A. Yen, A. Takada, H. Kida, R. H. Cheng, and Y. Kawaoka.**
23 2006. Architecture of ribonucleoprotein complexes in influenza A virus particles.
24 Nature (London) **439:**490-492.

1 26. **Park, C. H., K. Matsuda, Y. Sunden, A. Ninomiya, A. Takada, H. Ito, T. Kimura,**
2 **K. Ochiai, and T. Umemura.** 2003. Persistence of viral RNA segments in the central
3 nervous system of mice after recovery from acute influenza A virus infection.
4 Veterinary Microbiology **97:**259-268.

5 27. **Russell, C. J., and R. G. Webster.** 2005. The genesis of a pandemic influenza virus.
6 Cell **123:**368-371.

7 28. **Sekellick, M. J., and P. I. Marcus.** 1982. Interferon induction by viruses. VIII
8 Vesicular stomatitis virus: [$^{+/-}$] DI-011 particles induce interferon in the absence of
9 standard virions. Virology **117:**280-285.

10 29. **Shinya, K., M. Ebina, S. Yamada, M. Ono, N. Kasai, and Y. Kawaoka.** 2006.
11 Influenza virus receptors in the human airway. Nature (London) **440:**435-436.

12 30. **Simon, A. E., M. J. Roossinck, and Z. Havelda.** 2004. Plant virus satellite and
13 defective interfering RNAs: new paradigms for a new century. Annual Review of
14 Phytopathology **42:**415-437.

15 31. **Strahle, L., D. Garcin, and D. Kolakofsky.** 2006. Sendai defective-interfering
16 genomes and the activation of interferon-beta. Virology **351:**101-111.

17 32. **Subbarao, K., H. Chen, D. Swayne, L. Mingay, E. Fodor, G. Brownlee, X. Xu, X.**
18 **Lu, J. Katz, N. Cox, and Y. Matsuoka.** 2003. Evaluation of a genetically modified
19 reassortant H5N1 influenza A virus vaccine candidate by plasmid-based reverse
20 genetics. Virology **305:**192-200.

21 33. **Sweet, C., and H. Smith.** 1980. Pathogenicity of influenza virus. Microbiological
22 Reviews **44:**303-330.

23 34. **Taubenberger, J. K., A. H. Reid, R. M. Lourens, R. Wang, G. Jin, and T. G.**
24 **Fanning.** 2005. Characterization of the 1918 influenza virus polymerase genes.
25 Nature (London) **437:**889-893.

1 35. **Wang, M., and R. G. Webster.** 1990. Lack of persistence of influenza virus genetic
2 information in ducks. Archives of Virology **111:**263-267.
3 36. **Wang, X.-H., R. Aliyari, W.-X. Li, H.-W. Li, K. Kim, R. Carthew, P. Atkinson,**
4 **and S.-W. Ding.** 2006. RNA interference directs innate immunity against viruses in
5 adult Drosophila. Science **312:**452-454.
6 37. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.**
7 1992. Evolution and ecology of influenza A viruses. Microbiological Reviews **56:**152-
8 179.
9

1 **Table 1: Derivation and nomenclature of protecting influenza RNAs and their**
2 **helper viruses.**

Abbreviation[a]	Defective RNA [b]	Helper virus
220/PR8	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	As above	A/WSN (H1N1)
244/mallard [c]	As above	A/mallard/England/7277/06 (H2N3)

3

4 [a] 220, protecting RNA; PR8, helper virus.

5 [b] Denotes from left to right: segment of origin of defective viral RNA, breakpoint residue in
6 the minus-sense RNA, total number of nucleotides, virus of origin.

7 [c] Produced by reassortment of non-infectious 244/PR8 and infectious A/mallard in
8 embryonated eggs (20), the others via reverse genetics.

9

10

1 **Table 2: Comparison of the prophylactic activity in mice mediated by various defined**

2 **protecting viruses against infectious influenza virus.**

3

Total protecting virus per mouse (HAU and mass of virus protein)[a]	**244/PR8**	**244/WSN**	**220/PR8**	**317/Vic**
4000 (12 µg)	++++ [b]	++++	++++	++++
400 (1.2 µg)	++++	++++	+	+++
40 (0.12 µg)	++++	++++	+	-
4 (0.012 µg)	++	++	nd	nd
0 [c]	-	-	-	-
Minimum dose required for solid protection [d]	0.12 µg [e]	0.12 µg [e]	12 µg	1.2 µg

4

5 [a] Given as a single intranasal dose under light anaesthesia simultaneously with 10 LD_{50} of

6 A/WSN challenge virus.

7 [b] The scale ranges from complete protection from weight loss and clinical disease (++++) to

8 no difference to the controls given UV-inactivated protecting virus plus challenge virus (-).

9 [c] Mice were given 4000 HAU of UV-inactivated protecting virus.

10 [d] Defined as the smallest dose of protecting virus effecting +++ protection or better.

11 [e] Total virus protein inoculated per mouse

12 Nd, not done; groups of 5-7 mice were used; this experiment is representative of 2-4

13 independent experiments.

1 **Table 3: The highest dose of protecting virus provides only a weak vaccine effect [a].**

2

3

First challenge		Second challenge		
Dose of protecting virus (HAU)	**Number dead/ number infected**	**Weight loss**	**Number ill /number challenged**	**Number dead /number challenged**
4000 (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%)	¾ (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
0 [d]	5/5	na	na	na

4

5 [a] Mice were intranasally inoculated with a mix of protecting virus + 10 LD_{50} challenge virus
6 A/WSN (first challenge: columns 1 and 2); and then 3 weeks later inoculated with 10,000
7 LD_{50} A/WSN alone (second challenge). This latter experiment tests adaptive immunity and
8 not residual protecting virus activity, as the higher dose of A/WSN completely overcomes
9 protecting virus when given simultaneously (not shown). Data from 3 separate experiments
10 are shown.

11 [b] Mean = 87% ill.

12 [c] Mean = 60% dead.

13 [d] Given 4000 HAU of UV-inactivated protecting virus.

14 Na, not applicable.

1 **Table 4: Therapeutic benefit of protecting virus in mice [a].**

	UV-inactivated protecting virus		**Protecting virus**	
Therapy	**Sick**	**Recovered**	**Sick**	**Recovered**
24 h p.i.	100% (by day 5)	0% (died days 5-7)	0%	100%
48 h p.i.	100% (by day 5)	0% (died days 5+7)	100% (during days 6-16)	33%

2

3 [a] Infected with 10 LD_{50} A/WSN and treated post infection (p.i.) at the times shown with UV-

4 inactivated protecting virus or protecting virus (4000 HAU or 12 µg virus protein). All

5 inoculations were intranasal with light anaesthesia. Groups of 5-7 mice were used; this

6 experiment is representative of 3 independent experiments.

1 **Figure legends**

2 Figure 1. RT-PCR detection of defective RNA in protecting virus preparations, amplified
3 using primers specific for the termini of gene 1 (RNA1F and RNA1R), except in lane 6 where
4 primers RNA1F and 244J were used. RNA/helper virus combinations shown are: lane 1
5 220/Vic; lane 2 220/PR8 (both amplicons 445 nt); lane 3 317/Vic (amplicon of 585 nt); lane 4
6 244/Mallard; lanes 5 (both amplicons 395 nt) and 6 244/WSN amplicon xxx nt); lane 7
7 244/PR8 (amplicon 395 nt). DNA size markers are indicated by ► 500 bp, ● 100 bp.
8
9 Figure 2. Transfection of 293T cells with excess 244 protecting influenza RNA expression
10 plasmid inhibits the production of viral HA by plasmids expressing infectious A/WSN.
11 Various amounts of 244 plasmid were transfected into 293T cells together with a constant
12 amount of plasmids encoding infectious A/WSN. One day later these were cocultivated with
13 MDCK cells for 7 days. Virus yield (HAU) in the culture fluid was measured.
14
15 Figure 3. Prophylactic activity mediated by protecting virus 244/PR8 in mice against
16 infectious A/WSN, as monitored by clinical disease and body weight change. All mice were
17 inoculated intranasally. Mice received 400 (a, b, c), 40 (d, e, f), and 4 HAU (g, h, i) of
18 244/PR8 protecting virus (12, 1.2 and 0.12 μg respectively) mixed with 10 LD_{50} A/WSN.
19 The figure shows clinical scores (a, d, g) and weight changes (b, e, h). Percentage survival is
20 in parenthesis. Symbols denote the inocula given in panels a, d, g: ■, UV-inactivated
21 protecting virus + 10 LD_{50} A/WSN; ▲, protecting virus + 10 LD_{50} A/WSN; ●, diluent.
22 Panels c, f, i show the result (change in weight) when survivors were challenged with 10,000
23 LD_{50} A/WSN, at 3 weeks after the first infection. This very large dose of A/WSN abrogates
24 protection even by the highest dose of protecting virus (not shown), and hence tests for the
25 development of adaptive immunity.

1

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

8

9 Figure 5. Persistence of protecting RNA 244 (395 nt) in mouse lung in the absence of
10 infectious virus, as demonstrated by RT-PCR with primers RNA1F and RNA1R. Mice were
11 inoculated intranasally with 4000 HAU (12 µg) of protecting virus. Lane 1, DNA size
12 markers (bp); lanes 2-6 amplicons from mouse lungs: RNA for lanes 2-5 was extracted 1 day,
13 9 days, 21 days and 42 days respectively after inoculation; lane 6, mock-inoculated with
14 saline.

15

16 Figure 6. Protecting virus 244/PR8 prevented clinical disease in mice infected with an H2N2
17 virus (A/Japan/305/57) (a, b) or an H3N2 virus (7a) (c, d). The experiment was conducted in
18 the same way as the initial phase of Figure 3. Mice (5 per group) were inoculated
19 simultaneously with a mixture of challenge virus and protecting virus (4000 HAU or 12 µg)
20 (▲) or challenge virus and UV-inactivated protecting virus (4000 HAU or 12 µg) (■).
21 Clinical scores (a, b) and weight changes (b, c) are shown, with surviving mice in parenthesis.
22 Weight changes in non-infected controls groups (2 mice) given protecting virus alone (♦) or
23 saline (●) are also shown. None of these became ill.

24

1 Figure 7. Virus infectivity in the lungs of A/WSN-infected mice treated with protecting virus
2 (▲), or UV-inactivated protecting virus (■). For prophylaxis (a, b, c) a mixture of 400 HAU
3 (1.2 μg) protecting virus 244/PR8 or UV-inactivated protecting virus and A/WSN were
4 inoculated on day 0. For therapy (d, e, f), 4000 HAU (12 μg) protecting virus 244/PR8 or
5 UV-inactivated protecting virus were inoculated intranasally one day after intranasal infection
6 with A/WSN. Mice (3 per group) were killed and lungs removed. Lungs were frozen and
7 later ground with sand, clarified and end-point assayed in MDCK cells for infectivity (a, d).
8 Each point represents one animal. Lung consolidation is shown (b, e), and is an average of
9 the values for the left and right lung of each mouse shown. Clinical scores for groups of
10 animals (n=10) treated in parallel are also shown (b, e). No infectivity or consolidation was
11 detected in controls inoculated with protecting virus alone or diluent, and these animals
12 remained healthy for the duration of the experiment (data not shown).
13
14
15