In vivo antiviral activity: defective interfering virus protects better against virulent Influenza A virus than avirulent virus

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A defective interfering (DI) virus differs from the infectious virus from which it originated in having at least one major deletion in its genome. Such DI genomes are replicated only in cells infected in trans with homologous infectious virus and, as their name implies, they interfere with infectious virus replication and reduce the yield of progeny virus. This potent antiviral activity has been abundantly demonstrated in cell culture with many different DI animal viruses, but few in vivo examples have been reported, with the notable exception of DI Influenza A virus. A clue to this general lack of success arose recently when an anomaly was discovered in which DI Influenza A virus solidly protected mice from lethal disease caused by A/PR/8/34 (H1N1) and A/WSN/40 (H1N1) viruses, but protected only marginally from disease caused by A/Japan/305/57 (A/Jap, H2N2). The problem was not any incompatibility between the DI and infectious genomes, as A/Jap replicated the DI RNA in vivo. However, A/Jap required 300-fold more mouse infectious units to cause clinical disease than A/PR8 and it was hypothesized that it was this excess of infectivity that abrogated the protective activity of the DI virus. This conclusion was verified by varying the proportions of DI and challenge virus and showing that increasing the DI virus : infectious virus ratio in infected mice resulted in interference. Thus, counter-intuitively, DI virus is most effective against viruses that cause disease with low numbers of particles, i.e. virulent viruses.

INTRODUCTION

With continuing problems of existing animal virus infections and newly emerging viruses, the need for antiviral measures has never been greater. One potential antiviral that has long been known, but never properly exploited, is defective interfering (DI) virus. DI virus genomes are made spontaneously by nearly all animal viruses and have one or more major deletions, acquired through premature termination and reinitiation events. DI genomes lack the genes needed for replication, but retain the cis-acting elements essential for replication and encapsidation. DI virus that packages a defective RNA is thus, by definition, non-infectious and can only be replicated by co-infection with the virus from which it was derived or a close relative. It is this interaction that results in ‘interference’, a phenomenon that eventually results in a reduction in the yield of infectious virus. DI viruses have been known for many years to have strong interfering activity in chicken embryos and cell culture (von Magnus, 1954; Huang & Baltimore, 1970, 1977; Holland, 1990) and, when there is a high ratio of DI : infectious virus, can totally abrogate detectable genome synthesis, protein synthesis and infectious virus production (e.g. Barrett et al., 1981; Barrett & Dimmock, 1985). In cell culture, there is a quantitative relationship between the amount of infectious virus and DI virus produced – initially, the infectious genome replicates both itself and the defective genome, but the small DI genome gives it a replicative advantage and eventually, when a high ratio of DI virus : infectious virus prevails, interference takes place. The resulting fall in helper virus leads to a loss of DI virus and reascendency of infectivity. This can lead to cycling, with peaks and troughs of infectious virus load (von Magnus, 1954; Roux et al., 1991).

In view of the success of DI viruses at interfering with infectious virus production in vitro, it is remarkable that there have been so few reports of DI viruses protecting adult animals from clinical disease in vivo (reviewed by Barrett & Dimmock, 1986). While negative reports are rarely published, it seems likely that this paucity of information means that the DI viruses tested failed to protect against disease in adult animal models. If the reasons for this failure could be established, a way forward might be found to establish a new generation of DI virus-based antivirals.

A notable exception with well-documented ability to protect adult animals is DI Influenza A virus. Influenza virus has a single-stranded RNA genome comprising eight separate segments (Lamb & Krug, 1996). Preparations of DI influenza
Influenza A viruses have a common replication mechanism, as shown by their ability to interact genetically and form viable recombinants (Webster et al., 1992); thus, a DI genome should be replicated by all influenza A strains, regardless of subtype, and inhibit their replication. This is potentially important, as it means that DI virus could act as a generic antiviral for Influenza A virus. However, it is reported here that a DI influenza A virus preparation that protected mice from lethal infection with the A/PR8 (H1N1) and A/WSN (H1N1) strains of influenza virus had no activity against clinical disease caused by an H2N2 virus. It transpired that this failure did not reside in any intrinsic inability of infectious virus to replicate the DI virus, or of DI genomes to interfere with infectious virus replication. Rather, the key finding was that the H2N2 virus needed a 300-fold higher mouse infectious dose to cause clinical disease than A/PR8 and that this high infectious virus: DI virus ratio prevented the interfering activity of DI virus from being expressed. This finding underlines the importance of the infectious dose rather than the disease-causing dose of virus and may help in establishing systems to study the in vivo antiviral activity of DI viruses other than influenza virus.

**METHODS**

**Viruses.** Infectious virus stocks of influenza virus A/PR/8/34 (A/PR8, H1N1), A/WSN/33, and A/japan/305/77 (A/jap, H2N2) were produced by low-multiplicity infection [approx. 10^4 median egg infectious dose (EID_{50})] of the allantoic cavity of 10-day-old embryonated chicken eggs. After 2 days incubation, virus was harvested, clarified and stored at −70°C.

**Infectivity titrations.** Cell culture, embryonated chicken egg and mouse infectivity titres were determined for each virus. Virus was plaque-assayed in MDCK cells under agar by standard procedures and plaques were counted after 4 days incubation. Eggs were inoculated with limited-diluted virus and incubated for 3 days. Virus-positive eggs were identified by haemagglutination by allantoic fluids. Mice were inoculated as described above with limited-diluted virus. Three days later, mice were killed and ground lungs from individual mice were inoculated into eggs. The presence of virus was determined by haemagglutination with chicken red blood cells. Egg and mouse infectivity titres were calculated according to Spearman–Kärber (Kärber, 1968).

**DI virus.** The DI virus used in this study was originally produced from A/equine/Newmarket/7339/79 (A/EQV, H3N8) by three high-multiplicity, overnight passages of infectious virus in embryonated chicken eggs [approx. 10^6 haemagglutination units (HAU) per egg]. DI virus was purified by differential centrifugation, standardized by haemagglutination titration and stored in liquid nitrogen. Such preparations contain several different DI RNAs from different genomic segments and protect mice from lethal infection with various subtypes of Influenza A virus (Noble & Dimmock, 1994, 1995; Duhaud & Dimmock, 1998; Noble et al., 2004). However, sequencing studies showed that the population of DI RNAs in egg-grown virus differed markedly from that found in the lungs of mice infected with that DI virus (Duhaud & Dimmock, 1998), suggesting that not all DI RNAs replicated well in the mouse. As it seemed logical that only DI RNAs that were replicated in the mouse were likely to be antiviral, a mouse-adapted A/EQV DI virus was made by passing DI and helper A/EQV three times in mice via undiluted lung homogenates. Eggs were then inoculated with the final lung homogenate to amplify these DI RNAs. Whilst this was successful, there was still a problem with poor A/EQV growth so, to improve yields, third mouse-passage A/EQV DI RNAs were rescued by using a high-growing A/PR8 strain, also in mice. Before inoculation, the EQV DI virus preparation was rendered non-infectious by irradiation with a critical dose (40 s) of UV light. The UV inactivates infectivity that would confuse the infectious dose given to the mice. DI RNA and activity are not affected significantly, as it has a smaller UV target size (approx. 400 nt) than that of the infectious genome (13600 nt). The lamp was calibrated by inactivating A/PR8 infectivity. Lung homogenate was then passaged in eggs three times at high m.o.i. The resulting passage 3 virus was confirmed as a reassortant by the presence of A/PR8, but not A/EQV haemagglutinin (HA), and the presence of A/EQV DI RNAs as determined by RT-PCR. This was designated mouse-passaged DI A/EQV(PR8) virus. This DI virus strongly protected mice from a lethal dose of A/PR8 or A/WSN influenza virus (see Results), whereas DI A/PR8 produced by passing A/PR8 in parallel was only weakly protective (data not shown). Finally, DI virus was purified by differential centrifugation and pelleting through 10% sucrose and was resuspended at 10^6 HAU ml^{-1} in PBS containing 0.1% (w/v) BSA.

**Animal inoculation.** C3H/He-mg (H-2b) mice were inoculated intranasally as described previously (Noble & Dimmock, 1994; Noble et al., 2004), with interfering but non-infectious DI virus, which was produced by UV irradiation for 40 s as described above. To control for any immune system-stimulating or receptor-blocking effects, other mice were inoculated in parallel with a ‘dead’ non-interfering preparation produced by prolonged UV irradiation (8 min). This inactivates both infectivity and interfering activity, but does not affect haemagglutination or neuraminidase activities. Specifically, mice (4-week-old; 16–20 g) were lightly anaesthetized with ether and a 20 µl...
inoculum was divided between the two nares. Inocula comprised active, non-infectious DI virus containing a defined dose of infectious influenza virus, UV-kill DI virus, active DI virus alone, or diluent. Infectious viruses were titrated in mice to determine a dose for each that caused comparable respiratory disease. Morbidity was assessed according to loss of weight and by previously described clinical criteria (Noble & Dimmock, 1994). Clinical criteria were scored quantitatively as follows: 1 point for each healthy mouse; 2 points for each mouse showing signs of malaise, including slight piloerection, slightly changed gait and increased ambulation; 3 points for each mouse showing signs of strong piloerection, constricted abdomen, changed gait, periods of inactivity, increased breathing rate and sometime rales; 4 points for each 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 achieve parity, the total clinical score was divided by the number of mice in the experimental group. All viruses caused similar clinical disease, including lung consolidation. Experiments followed the guidelines of the UK Co-ordinating Committee for Cancer Research.

**RT-PCR.** RNA was extracted from the lungs of one mouse by grinding with sterile sand in 4 ml TRIzol reagent (Invitrogen) and dissolved in 100 μl water. Aliquots of 5 μg RNA were reverse-transcribed in 20 μl reactions for 1 h at 42 °C, using a generic type A influenza RNA 1-specific primer (RNA1F, 5'-AGCGAACGAGGTCAATATA-3'), complementary to the 3' terminus of the viral RNA (vRNA). RNA 1 encodes the PB2 protein component of the viral replicase. Aliquots (1-5 μl) of the reverse transcription reaction were then amplified by PCR using Taq DNA polymerase (MBI Fermentas) and generic primers specific for RNA 1 of Influenza A virus, RNA1F and RNA1R (5'-AGTAGAAAACGGCGTTTCTTTA-3', complementary to the 3' terminus of the cRNA), or primers specific for RNA 1 of A/EQV virus EQV1F (5'-CAAATATATTCAATATGGAG-3'), complementary to nt 14–33 of vRNA) and EQV1R (5'-GGTCGGTTTTTAAAACAAATCT-3', complementary to nt 12–31 of the cRNA). EQV1F and EQV1R have mismatches compared with the equivalent sequences in A/PR8 and A/Jap.

**RESULTS**

**DI virus protects mice from A/PR8 and A/WSN, but not A/Jap**

A single dose of mouse-passaged DI A/EQV(PR8) virus given simultaneously with the infecting dose completely protected mice from loss of weight, clinical disease and death resulting from infection with 10 LD50 of A/PR8 or A/WSN, whereas all control mice receiving UV-inactivated, non-interfering DI virus died. In contrast, infection of mice with A/Jap followed the same disease course irrespective of prophylactic treatment with DI virus or inactivated DI virus (data not shown). In a second set of experiments, we attempted to maximize protection against A/Jap by reducing the dose of virus inoculated to 1 LD50 and increasing the applied DI virus to two doses, the first given 2 h before infection and the second given simultaneously with the infecting dose. This DI virus regimen has been shown to increase protection (Dimmock et al., 1986). The dose of A/PR8 was kept at 10 LD50. The progress of infection was monitored by daily weighing and clinical assessment (Fig. 1). Data show that mice infected with 10 LD50 A/PR8 were completely protected by DI virus, whereas those given UV-inactivated DI virus were all dead by day 10 (Fig. 1a–c). The same scenario was seen in mice infected with 10 LD50 WSN (data not shown). However, Fig. 1(d–f) shows that, despite the lower dose of A/Jap and increased application of DI virus, only marginal protection was observed: a slight sparing effect in the clinical scores of infected mice given DI treatment (Fig. 1f) and a reduction in the mortality between the infected groups treated with DI virus (2/11 or 18%) or with inactivated DI virus (5/11 or 45%) (Fig. 1e). The difference in weight loss between the infected groups treated with DI virus or with inactivated DI virus did not differ statistically (Fig. 1d). These data were highly reproducible and similar results were obtained with other preparations of DI virus. The lack of protection against A/Jap was unexpected, as influenza A viruses have a common replication mechanism (Webster et al., 1992) and should thus replicate all influenza A DI genomes and be inhibited by them. Possible explanations for the low inhibition of A/Jap by DI virus are that (a) A/Jap does not replicate the applied DI RNAs in mouse lung, (b) A/Jap replicates DI virus in the lung, but is insensitive to DI virus-mediated interference or (c) infection of mice by A/Jap requires a larger dose of infectious virus than infection with the other influenza viruses and thus interference by DI virus is ineffective.

**DI RNA is replicated by A/Jap**

RT-PCR of RNA extracted from the lungs of mice showed a band of approximately 370 bp amplified by generic RNA 1-specific primers only when mice had been co-inoculated with DI virus and A/Jap (Fig. 2, lane 4). Further, reaction of the same RNA with A/EQV RNA 1-specific primers strongly amplified a smaller band of 340 bp, suggesting that the major 370 nt DI RNA band identified in lane 4 is derived specifically from A/EQV and not from A/PR8 or A/Jap (Fig. 2, lane 5). Thus, the replication systems of A/Jap and the DI virus are compatible and failure to replicate DI genomes is not the reason for the lack of protection from A/Jap-mediated disease. Finally, although only the presence of defective RNAs derived from RNA 1 is shown, it is likely that DI RNAs originating from other virus RNAs were also present (Duhaut & Dimmock, 1998).

**Infectivity titres of A/Jap and A/PR8 in eggs and mice**

The viruses used here are egg-adapted and, as eggs give the highest infectivity titres, they were used here to assay infectivity. Table 1 shows that A/Jap and A/PR8 had similar egg infectivity titres. The HAU reflects the total number of physical virus particles present and allows the calculation of specific infectivity (egg infectivity : HAU ratio). The ratios for A/Jap and A/PR8 were also very similar (Table 1). Infectivity was also determined by end-point titration in mice and, by this measure, A/Jap had fourfold-lower specific mouse infectivity than A/PR8 (Table 1). However, A/PR8 had a higher specific lethality than A/Jap. With this information, the level of infectivity required by A/Jap and A/PR8 to cause disease in mice was calculated and it was found...
that a lethal dose of A/Jap contained 2000-fold more egg infectious units and 300-fold more mouse infectious units than A/PR8 (Table 2). This suggests that DI virus did not protect mice against A/Jap because there is an unfavourably high infectious virus : DI virus ratio. The fact that a 10-fold dilution of DI virus abrogates protection against PR8-induced disease (Table 4) supports the notion of the importance of the DI virus : infectious virus ratio (Table 4).

**DI virus inhibits multiplication in vivo of a low infectious dose of A/Jap**

The conclusion above predicted that the DI virus should interfere with a 300-fold lower infectious dose of A/Jap. This was tested by inoculating mice with a low dose of A/Jap (6 MID₅₀) together with DI virus or the UV-inactivated DI-virus control. The dose of DI virus used was the same amount that prevented disease caused by A/PR8 (Fig. 1) or A/WSN (data not shown). As the dose of A/Jap was too low to cause disease, lung virus infectivity was the read-out. Lung samples were taken at 24 h post-infection when A/Jap infectivity first reached a plateau (data not shown). Table 3 shows that A/Jap lung infectivity was reduced by 100-fold in
mice given active DI virus compared with those given UV-inactivated control DI virus. These data indicate that DI virus can interfere with multiplication of A/Jap in mice, provided that there is an appropriately high ratio of DI virus : infectious virus, a putative critical ratio.

Further test of the critical-ratio hypothesis: a decreased dose of DI virus protects mice when the infecting dose is also low

End-point titration of virus infectivity in mice revealed that 10 LD$_{50}$ of WSN contains only 6 MID$_{50}$, meaning that an infectious dose is the same as the lethal dose (data not shown). This compares with 200 MID$_{50}$/10 LD$_{50}$ for A/PR8 and 60 000 MID$_{50}$/10 LD$_{50}$ for A/Jap (Table 2). WSN is thus the most virulent of the viruses used in this study. This situation allowed us to test the prediction that one-tenth of the DI virus dose used above should protect mice against the low amount of infectious A/WSN present in 10 LD$_{50}$. This indeed proved to be the case and Table 4 shows that 20% of mice given A/WSN plus active DI virus developed clinical disease, compared with 100% of mice given A/WSN and inactivated DI virus. However, as predicted from the critical-dose hypothesis, the same low dose of DI virus did not protect mice against the 33-fold-higher infectious dose of A/PR8 that is required to cause disease (Table 4). Nonetheless, as expected, the low DI virus dose interfered significantly with the multiplication in the lung of a 100-fold-lower dose of A/PR8 (data not shown).

DISCUSSION

These studies were predicated on the fact that the amount of infectivity required to cause disease in one animal host varies from virus to virus and the data shown above indicate that DI virus-mediated protection of animals from influenza virus infection depends upon the size of the infectious inoculum and not the disease-causing dose. This leads to the conclusion that the ratio of DI virus (probably DI virus genomes) : infectious virus (probably infectious genomes) determines DI virus-mediated protection in vivo. Thus, it appears that in vivo, as in vitro, an excess of infectious virus replicates both its own RNA and that of defective virus, without infectious virus suffering any interference. Only when the ratio of defective genomes : infectious genomes favours DI virus, either as a result of increasing the DI virus dose or decreasing the infectious virus dose, is there interference in the production of infectious progeny and protection of the animal from disease. Such protection is unlikely to require DI virus to inhibit replication of infectious virus completely, but rather a sufficient reduction in infectious load swings the virus/host-defence system balance in favour of the host, rendering it subclinical or ameliorating disease symptoms (Noble et al., 2004). The rather surprising conclusion is that DI virus is most effective against virulent virus, i.e. virus that infects with the least number of virus particles.

Thus far, we have been unable to determine the protective ratio of defective genomes: infectious genomes, as spontaneously occurring preparations of DI Influenza A virus contain a large and varied population of defective RNA sequences with different deletion break points, which can originate from all eight segments of RNA (Noble & Dimmock, 1995; Duhaut & Dimmock, 1998). A further complication is that different DI RNA sequences may not interfere with the same efficiency (Thomson et al., 1998). What is needed for quantification is a cloned DI/infectious influenza virus preparation that contains a single, identifiable DI RNA. A prototype system has been described, but

**Table 1. Properties of A/PR8 and A/Jap**

<table>
<thead>
<tr>
<th>Strain</th>
<th>EID$_{50}$ (ml$^{-1}$)*</th>
<th>HAU (ml$^{-1}$)*</th>
<th>EID$_{50}$:HAU</th>
<th>MID$_{50}$ (ml$^{-1}$)*</th>
<th>MID$_{50}$:HAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR8 (H1N1)</td>
<td>9·7</td>
<td>4·7</td>
<td>5·0</td>
<td>8·5</td>
<td>3·8</td>
</tr>
<tr>
<td>A/Jap (H2N2)</td>
<td>9·5</td>
<td>4·3</td>
<td>5·2</td>
<td>7·5</td>
<td>3·2</td>
</tr>
</tbody>
</table>

*End-point titres as log$_{10}$.

**Table 2. Amount of A/PR8 and A/Jap infectivity required to cause disease in mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>10 MLD$_{50}$ (20 μl)</th>
<th>EID$<em>{50}$ : 10 MLD$</em>{50}$</th>
<th>MID$<em>{50}$ : 10 MLD$</em>{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR8 (H1N1)</td>
<td>4·5*</td>
<td>3·5</td>
<td>2·3</td>
</tr>
<tr>
<td>A/Jap (H2N2)</td>
<td>1·0 (3000-fold)</td>
<td>6·8 (2000-fold)</td>
<td>4·8 (300-fold)</td>
</tr>
</tbody>
</table>

*Values shown are end-point titres as log$_{10}$. 

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needs further development to make it a practical proposition (Duhaut & Dimmock, 2003). The varied nature of DI genome sequences in uncloned preparations also makes it difficult to determine how DI virus exerts protection. A recent review of the mechanism of action of DI RNAs points out that not all DI RNAs are protective and that protecting DI RNAs may act by competing for transactivating factors, sequestering a limited amount of virus product such as polymerase, prevention of protein–protein interactions or production of small interfering RNAs (Simon et al., 2004).

This study underlines the fundamental point that different virus strains have a unique infectious dose for each in vivo or in vitro assay system used and thus the infectious dose: disease-causing dose ratio in vivo is likely to be strain-unique. A priori, evolution probably disfavours any mutant that requires a large infectious dose, as infection is unlikely to produce a sufficiently high virus titre in body fluids and/or to transmit a large amount of virus to a new host. Thus, viruses would be expected to evolve a small infectious dose. This would be good news for any proposed DI-mediated prophylaxis, as only a minimal dose of DI virus would be required to abort clinical disease successfully.

Our manipulation of the DI virus used in this study is of some interest. This is the first time that DI virus has been produced that is enriched for DI RNAs that are preferentially replicated by an animal and the first time that defective RNAs have been incorporated into a new helper virus. No selection pressure was available, but none was needed. Additionally, there seemed to be no problem in transferring defective RNA from a parent virus of one subtype (H3N8) to that of a virus of a different subtype (H1N1). This was all achieved by using simple, non-recombinant DNA technologies.

This study supports the hypothesis that DI Influenza A virus has antiviral activity against all influenza A viruses and further defines this relationship. On this basis, it is suggested that this generic activity should be considered as an additional measure to counter newly emerging and potentially catastrophic pandemic influenza A virus strains (Webster, 1997). Finally, our conclusion concerning the critical ratio of DI virus : infectious virus required for protection of animals may explain why so few in vivo models for the antiviral activity of DI viruses have been described and may help in devising new model systems for studying the prevention of virus diseases.

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REFERENCES


