Defective interfering influenza A virus protects *in vivo* against disease caused by a heterologous influenza B virus

Paul D. Scott, Bo Meng, Anthony C. Marriott, Andrew J. Easton and Nigel J. Dimmock

School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK

Influenza A and B viruses are major human respiratory pathogens that contribute to the burden of seasonal influenza. They are both members of the family *Orthomyxoviridae* but do not interact genetically and are classified in different genera. Defective interfering (DI) influenza viruses have a major deletion of one or more of their eight genome segments, which renders them both non-infectious and able to interfere in cell culture with the production of infectious progeny by a genetically compatible, homologous virus. It has been shown previously that intranasal administration of a cloned DI influenza A virus, 244/PR8, protects mice from various homologous influenza A virus subtypes and that it also protects mice from respiratory disease caused by a heterologous virus belonging to the family *Paramyxoviridae*. The mechanisms of action *in vivo* differ, with homologous and heterologous protection being mediated by probable genome competition and type I interferon (IFN), respectively. In the current study, it was shown that 244/PR8 also protects against disease caused by a heterologous influenza B virus (B/Lee/40). Protection from B/Lee/40 challenge was partially eliminated in mice that did not express a functional type I IFN receptor, suggesting that innate immunity, and type I IFN in particular, are important in mediating protection against this virus. It was concluded that 244/PR8 has the ability to protect *in vivo* against heterologous IFN-sensitive respiratory viruses, in addition to homologous influenza A viruses, and that it acts by fundamentally different mechanisms.

**INTRODUCTION**

Defective interfering (DI) viruses are mutants that arise spontaneously from most viruses and contain at least one major deletion in their genome. This results in their replication being dependent on complementation in the same cell by a genetically compatible (homologous) infectious helper virus to provide any missing functions. Homologous virus is usually the virus from which the DI genome was derived. DI genomes retain the ability to be encapsidated in a virus particle that is usually indistinguishable from the infectious helper virion. In cell culture, DI viruses are not only defective but also interfering, meaning that, under appropriate conditions, the presence of the DI genome reduces the amount of infectious progeny virus produced (Holland, 1990a, b; Huang & Baltimore, 1970; Nayak *et al.*, 1989; Perrault, 1981). Current thinking is that DI viruses reduce the production of helper virus in cell culture probably as a result of the smaller DI genome having a competitive advantage (Holland, 1990a, b; Huang & Baltimore, 1970; Nayak *et al.*, 1989; Perrault, 1981). There is also limited evidence that some DI viruses can protect animals from clinical disease caused by the homologous virus (Barrett & Dimmock, 1986; Dimmock, 1991, 1996; Roux *et al.*, 1991).

DI influenza A viruses were the first to be described and have been studied extensively (Nayak, 1980; Nayak *et al.*, 1985, 1989). However, most DI influenza virus preparations contain many different DI RNA sequences, so it has not been possible to determine the relationship between a sequence and its biological properties (Duhaut & Dimmock, 1998). Recently, we solved this problem with reverse genetics to produce cloned DI viruses that contained one major species of DI RNA (Dimmock *et al.*, 2008; Duhaut & Dimmock, 1998, 2000, 2002, 2003). One such influenza virus, containing the 244 DI RNA (244/PR8), has been characterized and, when inoculated intranasally, strongly protects mice from infection with several different influenza A virus subtypes (Dimmock *et al.*, 2008). The DI influenza A virus particles retain receptor specificity and hence are targeted to the natural sites of infection *in vivo*.

In the course of our study of DI influenza A virus 244/PR8, we found that it also protected mice from infection with a genetically unrelated heterologous virus, pneumonia virus of mice (PVM), a member of the family *Paramyxoviridae* (Easton *et al.*, 2011). We determined that this was effected by a mechanism that is dependent on type I interferon (IFN) and other elements of the innate immune system. It is well known that dsRNAs are efficient stimulators of type 1 IFN production. This is mediated through the effector cell type I IFN receptor (IFNAR1), which is critical for the induction of IFN-stimulated genes (ISGs) and hence of innate antiviral responses. The dsRNA-dependent induction of ISGs and type I IFN production can also be induced by poly(I:C), a synthetic double-stranded RNA used as a mimic of viral dsRNA and a dsRNA binding protein (4-8-1 mobilotoxin/4-8-1MO)....
RESULTS

Lack of genetic interaction between influenza A and B viruses

Influenza B virus does not replicate influenza A virus DI RNA 244. Although influenza A and B viruses both have genomes composed of eight segments and are members of the family Orthomyxoviridae, they do not interact genetically and are classified in separate genera. The only suggestion of interaction comes from an in vitro experiment in which influenza A virus appeared to have replicated an influenza B haemagglutinin (HA) gene linked to a chloramphenicol acetyltransferase reporter (Jackson et al., 2002). However, as there is no published information on the interaction with DI RNAs, we determined first whether infectious influenza B virus was able to replicate DI 244 RNA present in 244/PR8 virus in embryonated eggs. Embryonated chicken’s eggs (three per sample) were inoculated allantoically with influenza A DI 244/PR8 serially diluted in B/Lee/40 [4 × 10^8 egg infectious units (EID_{50})]. A high B/Lee/40 dose was used to give replication of DI influenza A RNA every chance of succeeding. After incubation for 24 h, allantoic fluids were harvested and the amount of viral HA was determined. Fig. 1(a, row E) shows that B/Lee/40 grew well [10^{3.5} haemagglutinating units (HAU) ml^{-1}] and that, as expected, 244/PR8, which was non-infectious, produced no HA (Fig. 1a, row F). All mixtures of B/Lee/40 and 244/PR8 (Fig. 1a, rows A–D) gave approximately the same HA titre, showing that 244/PR8 did not interfere with the multiplication of B/Lee. RNA was extracted from allantoic fluids and subjected to RT-PCR using terminal primers for PR8 RNA segment 1. Eggs inoculated with 2000 HAU 244/PR8 without any B/Lee/40 (Fig. 1b, lane F) gave an amplicon of the size expected for 244 RNA (395 nt) and was presumably derived from residual inoculum RNA. The amplicon from the 2000 HAU dose of 244/PR8 mixed with B/Lee/40 (Fig. 1b, lane A) was similar in appearance to that derived from 244/PR8 on its own. Fig. 1(b, lane B) had a trace amount of 244 RNA, as expected from 200 HAU of 244/PR8 without amplification. There was a second amplicon of ~700 bp from eggs inoculated with B/Lee/40 alone (Fig. 1b, lane E), which was seen in all eggs receiving B/Lee/40 and presumably resulted from non-specific priming. Overall, this showed that B/Lee/40 was unable to replicate the influenza A 244 DI RNA.

As a positive control, we repeated the above experiment replacing B/Lee/40 with A/mallard/England/7277/06 (H2N3), an influenza A virus that is unrelated to 244/PR8. RT-PCR of allantoic fluid from eggs inoculated with A/mallard/England/7277/06 plus between 2 and 200 HAU 244/PR8 showed a single clear band at approximately 400 nt, as expected for 244 RNA (Fig. 1d). Thus, consistent with earlier data, we confirmed that 244 DI RNA is readily replicated by an influenza A virus that is unrelated by subtype and host species (Dimmock et al., 2008).

DI influenza A virus 244/PR8 does not interfere with influenza B/Lee/40 virus in cell culture. We used our routine assay that measures interference between DI and infectious influenza A viruses. Madin–Darby canine kidney (MDCK) cells were infected with infectious influenza A/WSN or B/Lee/40 virus and then, after allowing time for attachment, with increasing doses of influenza A DI 244/
PR8 virus. Fig. 1(c) shows that the DI virus progressively interfered with the multiplication of influenza A/WSN but not of influenza B/Lee/40 virus, and is consistent with the lack of genetic interaction between influenza A and B viruses.

**244/PR8 protects mice from serious disease caused by a simultaneous challenge with B/Lee**

Mice were inoculated intranasally with a mixture of influenza A DI 244/PR8 virus (12 μg virus protein per mouse) and the heterologous challenge virus influenza B/Lee/40 (~10 LD₅₀ per mouse). Controls received a mixture of UV-inactivated DI influenza A virus and challenge virus. Parallel groups of mice were inoculated simultaneously with DI virus and a homologous challenge virus, influenza A/WSN. Fig. 2 shows that DI 244/PR8 virus protected mice from weight loss and clinical disease caused by A/WSN virus as expected but also protected mice from weight loss and clinical disease caused by B/Lee/40. None of the mice given UV-inactivated DI virus was protected from either of the challenge viruses: all became seriously ill and the majority had to be called. A lower dose of 1.2 μg DI virus protein per mouse gave some protection, but there was none with a dose of 0.12 μg (not shown).

**Pre-treatment of mice with 244/PR8 increases the efficacy of protection from B/Lee/40**

244/PR8 or UV-inactivated 244/PR8 virus (1.2 or 0.12 μg per mouse) was administered intranasally to mice 24 h before infection with B/Lee/40 virus or as a mixture. Fig. 3 (e, f) shows that mice given 1.2 μg 244/PR8 and B/Lee/40 became sick simultaneously and lost weight transiently, although they were protected from the more serious disease and death that occurred in mice inoculated with UV-inactivated 244/PR8 + B/Lee/40. By comparison, pre-treatment with 244/PR8 prevented virtually all weight loss and all clinical disease (Fig. 3a, b). Pre-treatment with a
lower dose of 244/PR8 (0.12 mg; Fig. 3c, d) attenuated clinical disease and prevented death, although there was transient weight loss. There was no weight loss or clinical disease with DI virus alone (Fig. 3g, h). Surviving mice were challenged with a high dose of B/Lee/40 at 3 weeks after infection to determine whether mice that had not suffered overt clinical disease (Fig. 3a, b) had developed a conventional immunity to B/Lee/40. All were completely protected, suggesting that they had undergone a silent respiratory infection and been immunized (data not shown).

Reduction in influenza B virus lung infectivity in mice pre-treated with 244/PR8

The amount of infectious B/Lee/40 in the lungs of mice inoculated with 244/PR8 at 24 h before infection with B/Lee/40 was lower than in control groups that received inactivated 244/PR8 + B/Lee/40 (Table 1). The sparing effect of 244/PR8 was greatest in the early stages of infection, and was more than 34-fold lower at day 1 after inoculation of B/Lee/40. The disparity was evident at all times examined, although the effect gradually lessened.

Duration of prophylaxis afforded by 244/PR8

We showed earlier that mice treated with 244/PR8 were protected from an infectious influenza A virus challenge for several weeks (Dimmock et al., 2008). Here, preliminary experiments demonstrated that the strong heterologous protective response evident with 244/PR8 administered 1 day before infection with B/Lee/40 had declined virtually to zero when 244/PR8 was administered 7 days before infection (data not shown). We then looked at intermediate times. Fig. 4(a, b) shows that 244/PR8 given intranasally at day 1 afforded excellent protection with no sickness or death, with only a small and transient weight loss during days 8 and 9, whereas mice given B/Lee/40 alone all died. There was less protection when 244/PR8 was administered 4 days before infection, although disease onset (weight loss and clinical signs) was delayed by 1 day, and 60% of mice survived. Control mice given UV-inactivated 244/PR8 + B/Lee/40 or 244/PR8 + A/WSN; $\bullet$, 244/PR8 alone. The percentage of mice surviving is indicated in parentheses. In (a) and (c), the DI alone symbols (●) all have a value of 1 and are not visible. Data are representative of two experiments with five mice per infected group and three per control group.

Fig. 2. Influenza A DI 244/PR8 virus protects mice from disease caused by simultaneous intranasal inoculation of influenza B/Lee/40 (a, b) or A/WSN (c, d) virus. Control mice received 244/PR8 that had been UV inactivated for 8 min (244/PR8). 244/PR8 or 244/PR8 virus (12 μg virus protein per mouse; filled arrows) and infectious challenge viruses (open arrows) were mixed and administered simultaneously on day 0 to lightly anaesthetized mice by the intranasal route. Clinical disease assessment is shown in (a) and (c), whilst (b) and (d) show the percentage change in mean weight recorded up to the first death. ■, 244/PR8 + B/Lee/40 or 244/PR8 + A/WSN; ▲, i244/PR8 + B/Lee/40 or 244/PR8 + A/WSN; ●, 244/PR8 alone.
Fig. 3. Pre-treatment of mice with influenza A DI 244/PR8 virus increases the efficacy of protection from influenza B/Lee/40 virus compared with simultaneous intranasal inoculation. Control mice received the same amount of UV-inactivated 244/PR8 (i244/PR8). Mice were pre-treated intranasally with 244/PR8 virus (■) or i244/PR8 DI virus (▲) at 24 h (filled arrows) before intranasal infectious challenge (a–d), or simultaneously treated (filled arrow) and infected (open arrow) by the intranasal route (e, f). The virus-alone controls (g, h) received saline on day −1 and influenza B virus on day 0. Clinical disease assessment is shown in the left-hand graphs, whilst the right-hand graphs show the percentage change in mean weight recorded up to the first death. The amount of virus protein per mouse was 1.2 μg 244/PR8 or i244/PR8 in (a), (b), (e) and (f), and 0.12 μg 244/PR8 or i244/PR8 in (c) and (d). Control groups of mice (g, h) received DI 244/PR8 virus alone (●), diluent (○) or challenge virus alone (◇). Mice receiving no DI virus were given diluent instead. The percentage of mice surviving is also shown. Other data are as in Fig. 2.
244/PR8-mediated protection from disease caused by influenza B virus is reduced in IFN receptor-null mice

Previously, we found that the heterologous protection mediated by 244/PR8 from the paramyxovirus PVM resulted in part from induction of type I IFN (Easton et al., 2011). Accordingly, we compared protection from B/Lee/40 in wild-type 129SvEvBrd/Bkl (129Sv) mice and knockout mice that lacked a functional type I IFN receptor (129Sv-IFNα/βR−/−). Wild-type mice treated with 244/PR8 alone on day −4 and diluent on day 0 showed a reduced clinical score and weight loss compared to control mice inoculated with 1.2 μg 244/PR8 alone on day −4 and diluent on day 0. Control groups receiving 1.2 μg 244/PR8 alone on day −4 and diluent on day 0, or diluent on both occasions, showed a similar clinical score and weight loss as control mice receiving 1.2 μg 244/PR8 alone on day −4 and diluent on day 0. Clinical signs and percentage weight change were recorded as before. A † denotes a death, with remaining mice being weighed on subsequent days. Other data are as in Fig. 2.

Table 1. Treatment with influenza A DI 244/PR8 virus reduces influenza B/Lee/40 virus infectivity in mouse lungs

Mice were anaesthetized and inoculated intranasally with 1.2 μg 244/PR8 or UV-inactivated 244/PR8 (i244/PR8) 1 day before infection with B/Lee/40. Lungs were removed at the times shown and assayed for B/Lee/40 infectivity in MDCK cells. Titres are given as log10 f.f.u. per lung, and values are geometric means. There was no lung infectivity in control mice inoculated with 1.2 μg 244/PR8 alone. The differences in infectivity were significant (P<0.012) at all time points tested (Mann–Whitney U test).

<table>
<thead>
<tr>
<th>Days after infection with B/Lee/40</th>
<th>Lung infectivity in mice inoculated with:</th>
<th>Reduction in lung infectivity (a/b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i244/PR8 + B/Lee/40 (a)</td>
<td>244/PR8 + B/Lee/40 (b)</td>
</tr>
<tr>
<td>1</td>
<td>1.0 × 10^3</td>
<td>≤2.9 × 10^1</td>
</tr>
<tr>
<td>3</td>
<td>1.2 × 10^6</td>
<td>8.0 × 10^4</td>
</tr>
<tr>
<td>5</td>
<td>1.1 × 10^6</td>
<td>5.1 × 10^5</td>
</tr>
<tr>
<td>7</td>
<td>6.0 × 10^4</td>
<td>9.1 × 10^3</td>
</tr>
</tbody>
</table>

Fig. 4. Duration of protection afforded by influenza A DI 244/PR8 virus against influenza B/Lee/40. Mice were inoculated as in Fig. 3 with 1.2 μg 244/PR8 (■), 1.2 μg UV-inactivated 244/PR8 (i244/PR8) (▲) or diluent (●) at day −1 (a, b) or day −4 (c, d) (filled arrows) before infection with B/Lee/40 on day 0 (open arrows). Control groups received 1.2 μg 244/PR8 alone on day −4 and diluent in place of virus (●), or diluent on both occasions (○). Clinical signs and percentage weight change were recorded as before. A † denotes a death, with remaining mice being weighed on subsequent days. Other data are as in Fig. 2.
PR8 at the same time as being infected with B/Lee/40 were modestly protected, as shown by a delay in the onset of clinical disease of 1 day, and a 3-day delay in progression of disease in all mice in the group, compared with the control group treated with UV-inactivated 244/PR8 (Fig. 5g, h). Pre-treating wild-type mice at 1 day before infection with B/Lee/40 improved protection markedly, as shown by a delay in the onset and progression of clinical disease, and survival of 80% (compared with 0% surviving in the inactivated 244/PR8 control group) (Fig. 5c, d). There was also a corresponding sparing of weight loss (Fig. 5c, d). In the 129Sv-IFNα/β R−/− mice, simultaneous inoculation of 244/PR8 + B/Lee/40 gave no protection by any of our criteria (Fig. 5e, f). However, in knockout mice pre-treated with 244/PR8 1 day before infection, there was reduced but still significant protection. Clinical signs developed earlier than in wild-type mice, there was more disease and the survival rate was more than halved to 40% (Fig. 5a, b). Thus, we concluded that the protection stimulated by 244/PR8 is in part due to the induction of type I IFN, but that there are additional protective responses from other elements, possibly also originating from the innate immune system. In support of this conclusion, we showed that the multiplication of infectious B/Lee/40 was indeed sensitive to mouse type I IFN-α and -β in vitro, and was inhibited 57- and 118-fold, respectively (Fig. 6). The differences in virus titre obtained between IFN-α or -β and no IFN, or between IFN-α and -β were all significant (P<0.05).

**DISCUSSION**

A DI genome is only replicated when complemented in the same cell by the replication machinery of a homologous virus and, at certain high ratios of DI genome : infectious virus, there is interference, when fewer infectious genomes and infectious progeny are produced (Holland, 1990a, b; Huang & Baltimore, 1970; Nayak et al., 1989; Perrault, 1981). Hence, a DI virus was believed to interfere only with the homologous virus from which it arose. Exceptions were the highly double-stranded snapback and copyback DI RNAs of vesicular stomatitis virus and Sendai virus, which efficiently induce type I IFN synthesis in cell culture (Marcus & Gaccione, 1989; Marcus & Sekellick, 1977; Sekellick & Marcus, 1982; Strahle et al., 2006). There are no data available for protection from infection in vivo with snapback and copyback DI RNAs. All known DI influenza virus RNAs, including 244 RNA, have no major double-stranded structure and were not therefore expected to be potent inducers of type I IFN.

In contrast to the in vitro situation, there are limited data on DI viruses exerting antiviral activity in vivo (Barrett & Dimmock, 1986; Dimmock, 1991, 1996) and, as there may or may not be inhibition of the production of infectious progeny, it is not entirely clear how this is brought about (Barrett & Dimmock, 1984; Barrett et al., 1984; Dimmock et al., 1986). Previously, we have reported that non-cloned DI (Dimmock, 1996; Dimmock et al., 1986; Duhaut & Dimmock, 1998; Morgan & Dimmock, 1992; Noble & Dimmock, 1994; Noble et al., 2004) and cloned (Dimmock et al., 2008) DI influenza A viruses protect animals from clinical disease caused by various subtypes of influenza A virus. This is homologous interference/protection, as influenza A viruses have a common genetic system, as shown by the reassortant phenomenon in which two co-infecting viruses can exchange any of their eight segments of genomic RNA. However, as we have reported above, influenza A DI 244/PR8 virus also affords solid protection in mice against heterologous viruses — influenza B virus (as reported above) and PVM, a member of the genus Pneumovirus belonging to the family Paramyxoviridae (Easton et al., 2011). Specifically, we showed that B/Lee/40 did not replicate influenza A DI 244 RNA, and thus that DI 244 RNA cannot interfere directly with influenza B virus replication. Rather, the observed heterologous interference was dependent in part on the generation of type I IFN, as evidenced by a reduction in protection in knockout mice that had no type I IFN receptor. This is consistent with the stimulation of type I IFN in the lung by intranasally inoculated 244/PR8 (in the absence of infectious virus), which we showed previously (Easton et al., 2011). However, it appears that additional protective responses from other elements, possibly from the innate immune system, are also generated. This might involve type III IFN-λ, which has recently been shown to be important in protection against influenza A and B viruses in vivo (Jewell et al., 2010; Mordstein et al., 2008, 2010). The improved efficacy of protection, seen when mice were pre-treated with DI virus (Fig. 3), is also consistent with the time needed to stimulate the production of IFN and the establishment of an antiviral state in surrounding cells. This contrasts with the ability of 244/PR8 to protect mutant mice that have no type I IFN receptor from an influenza A virus challenge (Easton et al., 2011) and suggests that homologous and heterologous protection operate largely through different mechanisms. The type I IFN that protects wild-type mice from influenza B may directly inhibit virus replication by establishing an intracellular antiviral state or may act on other elements of the immune system, such as by increasing the activity of natural killer cells (Gidlund et al., 1978). The diminution of such ‘downstream’ responses may be responsible, at least in part, for the loss of protection seen in type I IFN receptor-null mice.

The work described here and elsewhere has shown that the cloned influenza A DI 244/PR8 virus protects from serious clinical disease in vivo caused by a number of different respiratory viruses, including influenza A and B viruses and paramyxoviruses (Dimmock et al., 2008; Easton et al., 2011). DI influenza A virus thus exerts a far broader antiviral action than was previously thought.

**METHODS**

**Viruses and cells.** Mice were infected with influenza B/Lee/40 or influenza A/WSN (H1N1). We also used A/mallard/England 7277/06
(H2N3). All viruses were grown in embryonated chickens eggs. The identity of B/Lee/40 was confirmed by reaction with influenza B virus nucleoprotein-specific antibody; there was no cross-reaction with influenza A virus. B/Lee/40 and A/WSN were used at approximately 10 LD$_{50}$ per mouse. Cloned DI 244/PR8 influenza A virus, which carries the 244 395 nt DI segment 1 RNA, was prepared as described previously from plasmids encoding 244 RNA and infectious A/PR/8/34 virus by transfection of 293T cells and co-cultivation with MDCK

Fig. 5. Protection by influenza A DI 244/PR8 virus against influenza B/Lee/40 in knockout mice that lack a functional type I IFN-α/β receptor is reduced but not eliminated. Wild-type mice (129Sv) (c, d, g, h), and IFN type I receptor knockout mice (129Sv−IFNα/βR−/−) (a, b, e, f) were treated (filled arrows) with 1.2 μg 244/PR8 (■) or 1.2 μg UV-inactivated 244/PR8 (i244/PR8) (▲) either 1 day before infection with B/Lee/40 (a–d) (open arrows) or simultaneously with B/Lee/40 (e–h). Other control groups received 1.2 μg 244/PR8 alone and diluent in place of virus (●), or diluent on both occasions (○). Clinical signs and percentage weight change were recorded as before. A † denotes a death, with remaining mice being weighed on subsequent days. Other data are as in Fig. 2.
Mouse L929 cells were seeded into 96-well plates and treated at the same time with 0, 0.16, 0.8, 4, 20 or 100 U IFN-α or -β per well in triplicate. After incubation overnight at 37 °C, the medium was removed and the cells rinsed and infected with B/Lee/40 in maintenance medium. Plates were incubated overnight at 33 °C and the B/Lee/40 infectivity titre in the presence or absence of IFN was determined as log_{10} f.f.u. ml^{-1}.

**RT-PCR.** RNA was extracted from virus with phenol and amplified using the primers RNA1F and RNA1R specific for the termini of segment 1 of influenza A virus (Dimmock & Marriott, 2006; Dimmock et al., 2008).

**Interference assay.** MDCK cell monolayers in 96-well plates were infected with B/Lee/40 (600 f.f.u. per well) or an equivalent amount of A/WSN. After virus had attached, the inoculum was replaced with 244/PR8 in a fivefold titration series in triplicate. After attachment had taken place, the plates were treated as for the infectivity assay, except that the conjugate was detected with an alkaline phosphatase substrate (Sigma) in diethanalamine buffer (Pierce). The EC_{50} for interference was calculated by reading plates at 405 nm with an ELISA reader and plotting non-linear regression curves using Prism software (Graphpad Software).

**Interferon sensitivity of B/Lee.** Mouse L929 cells were seeded into 96-well plates in DMEM with 10% FBS, l-glutamine, penicillin and streptomycin. Cells were treated at the same time with fivefold dilutions of mouse IFN-α or IFN-β (from 100 to 0.16 U per well; R&D Systems) in triplicate, or without IFN. After incubation overnight at 37 °C, the medium was removed and the cells rinsed and infected with B/Lee/40 in maintenance medium. Plates were incubated at 33 °C for 24 h and the infectivity titre was determined.

**Animals.** Inbred C3H/He-mg mice of both sexes were supplied by the Small Animal Unit at the University of Warwick and used at approximately 4–6 weeks of age and at a weight of 16–20 g. Males and females were equally susceptible to infection with influenza viruses. Mice were housed in single-sex cages, generally in groups of four to six animals, at 19–23 °C and 45–65 % relative humidity. Food and water were available continuously. Mice were inoculated intranasally with DI and/or infectious virus, after light ether anaesthesia, with a 40 μl volume split between the two nostrils. After infection, mice were assessed daily, scored clinically and the group weight determined. A quantitative clinical scoring system was used 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, constipated abdomen, changed gait, periods of inactivity, increased breathing rate and sometimes 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. In addition, we used inbred 129sv mice and a knockout strain lacking a functional type 1 IFN-α/β receptor (129sv-IFNα/β R^{-/-}) (both male; B&K Universal Ltd).

The LD_{50} values of B/Lee/40 and A/WSN were determined in C3H/He-mg mice. For the DI protection experiments, mice received 550 f.f.u. B/Lee/40 and 640 f.f.u. A/WSN. Mice that were fully protected by DI virus showed no evidence of clinical disease, but if they had been silently infected they would have established an adaptive protective immunity. Thus, mice were given a second dose of the same challenge virus (−10 000 LD_{50}) 3 weeks after infection to determine their original infection status. Virus infectivity in lungs was titrated in MDCK cells by an F.I.u. assay as above. The right lung from each mouse was removed and stored at −70 °C. Lungs were then thawed, homogenized with sand and PBS containing 0.1 % BSA (1 ml per lung) and centrifuged to remove debris before titrating.

**ACKNOWLEDGEMENTS**

We thank the UK Medical Research Council for financial support, Sam Dixon and Lesley Harvey-Smith for providing expert technical help, and Ian Brown (VLA, UK) for providing us with A/mallard/England/7277/06.
REFERENCES


