One Defective Interfering Particle per Cell Prevents Influenza Virus-mediated Cytopathology: an Efficient Assay System

By L. McLAin, S. J. ARMSTRONG AND N. J. DIMMOCK*
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

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SUMMARY

The titre of defective interfering (DI) influenza virus measured by an assay based on the inhibition of cytopathology caused by A/WSN (H1N1) influenza virus in MDCK cells was 320000-fold greater than titres measured by inhibition of infectious centre formation. Interference was less in other types of cell. By electron microscopy, we have shown that the ratio between physical particles and DI units in preparations of the DI virus was approximately unity, which suggested that one or few DI particles is/are required to confer resistance of a MDCK cell to viral cytopathology. This human DI virus interfered heterotypically with an avian H7N1 influenza virus.

The observation that the particle : infectivity ratio of influenza virus increases on passage at high m.o.i., by several orders of magnitude, while the overall production of virus particles remains constant (von Magnus, 1951) led to the recognition of what is now called defective interfering (DI) virus. Physical studies demonstrated that populations of such particles had a reduced content of RNA (Ada & Perry, 1955) and it was later shown that there was a reduction in the three largest genomic RNA segments with the generation of several presumptive DI RNAs, usually smaller than the segments that constitute the genome of the standard (infectious) virus (Crumpton et al., 1978; Nayak et al., 1978; Janda et al., 1979). More recently, presumptive DI RNAs have been sequenced and found to be highly variable in sequence and origin (Jennings et al., 1983; Nayak & Sivasubramanian, 1983). By comparison, understanding of the biology of DI influenza virus is incomplete, although it has been demonstrated that DI virus can protect mice from lethal infection (Bernkopf, 1950; von Magnus, 1951; Dimmock et al., 1986) and prevent plaque formation in cell culture (Janda et al., 1979). Interference is associated with small viral ribonucleoproteins (Janda & Nayak, 1979), and DI influenza virus can give rise to persistent infections in vitro (De & Nayak, 1980). Furthermore, interference is stable in the absence of virus multiplication in dividing cells; in MDCK cells its minimum half-life is 25 days (Cane et al., 1987). We report on the number of aspects relating to the biology of DI influenza virus and describe a simple, highly efficient assay based on the ability of DI virus to inhibit the cytopathology produced by standard virus.

The original seed stock of influenza virus A/WSN (H1N1) was cloned for the experiments described herein by picking plaques on chicken embryo fibroblast (CEF) cells (Morser et al., 1973) and re-plaquing consecutively ten times, during which time the p.f.u. : haemagglutination (HA) ratio increased to 10^6.4. Stocks of standard infectious virus were prepared by inoculating approx. 10^3 p.f.u. into the allantoic cavity of fertile 10 day old chicken eggs, and harvesting after a 2 day incubation at 33 °C. DI virus was prepared in the same way except that about 10^6 p.f.u. was inoculated per egg and allantoic fluids were harvested after a 16 h incubation. After three serial high multiplicity passages the p.f.u. : HA ratio fell to 10^2.6. DI viruses were stored in liquid nitrogen and standard virus at −70 °C. Viruses were assayed by their ability to agglutinate red blood cells, using the conventional assay with doubling dilutions in phosphate-buffered saline.
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Interference by DI virus was measured by a new assay which detects its ability to prevent the c.p.e. caused by standard virus in MDCK cells. In its optimized form, we use 96-well flat-bottom plastic trays (Sterilin) seeded with 2 x 10^4 cells/well in 200 µl DMEM containing 10% newborn calf serum (Flow Laboratories). After rinsing twice with PBS, monolayers were infected with 10 p.f.u./cell standard virus in 20 µl for 30 min at 37 °C. Monolayers were rinsed again and inoculated with 20 µl of 0-5 log_10 dilutions of DI virus, as before. The inoculum was removed and monolayers were incubated in DMEM at 37 °C for 60 min, to allow attached, non-internalized virus to elute. Monolayers were rinsed and then incubated in 200 µl DMEM containing 0-1% (w/v) bovine serum albumin for 30 h. Cells were fixed in formol–saline and stained with 1% (w/v) crystal violet (Fig. 1). The dilution of DI virus that gave 63% protection of the monolayer was determined by interpolation.

This assay system was used to investigate the effect of different types of cell upon interference. We found that the ability of WSN to multiply in CEF, L929, BHK-21 and MDCK cells varied greatly, but all gave sufficient progeny to allow measurement of interference which varied considerably with cell type (Table 1). There was no detectable inhibition in CEF and BHK-21 cells; interference was increased nearly 100-fold in L929 and over 10^5-fold in MDCK cells.

Table 2 shows that the inhibition of cytopathology assay (ICA) for DI WSN is 320000-fold more sensitive than the infectious centre reduction assay (ICRA) of Janda et al. (1979) (as well as being less labour-intensive and quicker to perform). Tables 1 and 2 show that sister preparations of DI virus can vary 10-fold in the amount of DI virus present.

The assay was used to demonstrate that the interfering activity of DI WSN was sensitive to inactivation by β-propiolactone (BPL). BPL is an alkylating and acylating agent which inactivates virus infectivity by reacting primarily with nucleic acid (Fraenkel-Conrat, 1981) and would thus be expected to inactivate DI virus-mediated interference which operates through the activity of the DI virus genome (Perrault, 1981). Incubation of DI WSN with BPL (Sigma), under conditions described previously (Barrett et al., 1984), completely inactivated interference, even though neither the HA titre nor the neuraminidase enzyme activity was affected by BPL (data not shown).

In general, DI viruses only interfere with the strain of standard virus from which they originated (homotypic interference). However, exceptions include the interference between DI particles of the Indiana strain of vesicular stomatitis virus and the New Jersey serotype (Prevec & Kang, 1978; Schnitzlein & Reichmann, 1976), between DI Semliki Forest virus and Sindbis virus (Barrett & Dimmock, 1984) and between subtypes of human influenza virus (De & Nayak, 1980). We examined interference between human DI WSN (H1N1) and the avian strain A/FPV/Rostock/34 (H7N1). DI WSN interfered with the production of c.p.e. by FPV, although the extent of interference was reduced by about 100-fold from the homologous titre of 10^7.2 to 10^5.2 DI units (DIU)/ml.

Since WSN-induced cytopathology in MDCK cells is so sensitive to DI virus, we were interested to know how many physical particles (PP) were required to protect a cell. This was determined by comparing the concentration of PP with a known concentration of latex beads (Agar Aids, Stansted, U.K.) using a JEOL JEM-1005 transmission electron microscope (Table 3). Infectious or DI virus was purified by velocity centrifugation through a gradient of 15% to 45% sucrose and the peak of virus, as determined by HA, was pooled and pelleted. Both viruses peaked at approximately the same position in the gradient. Resuspended virus was stained with 1% sodium silicotungstate (Agar Aids) pH 6.5. The mean PP : DI particle (DIP) ratio, estimated using the interference titre measured by the ICA, was 1:15. Thus, by inference, approximately 1 DIP/cell protects against infection with 10 p.f.u. (equivalent to about 1000 PP) of standard virus. We do not understand the mechanism of what appears to be a very efficient process of interference.

Although highly efficient, this assay for DI influenza virus will detect a minimum of 6.3 x 10^5 DIP/ml, which compares unfavourably with the highly sensitive 'negative plaquing' techniques.
Fig. 1. Assay for the inhibition of cytopathogenicity by DI influenza virus. MDCK cells were inoculated with WSN (m.o.i. 10) followed by 0.5 log₁₀ dilutions of DI WSN. After 30 h at 37 °C, monolayers were fixed and stained with crystal violet. CC, mock-infected cells; VC, cells inoculated with standard WSN virus only.

### Table 1. Interference by DI WSN in various types of cell as measured by interference with the development of cytopathology

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. cells/monolayer*</th>
<th>Interference titre (log₁₀ DIU/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEF</td>
<td>1.0 × 10⁵</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>L₂₀</td>
<td>2.0 × 10⁴</td>
<td>2.52</td>
</tr>
<tr>
<td>BHK-21</td>
<td>5.0 × 10⁴</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>MDCK</td>
<td>1.0 × 10⁴</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* In 96-well plates. Monolayers were inoculated with 10 p.f.u. WSN/cell and subsequently with 0.5 log₁₀ dilutions of DI virus (allantoic fluid) after incubating for 30 min at 37 °C. Plates were fixed and stained 30 h later.
  † DIU were calculated by interpolation from the dilution of DI virus that gave 63% inhibition of cytopathology.

### Table 2. Relative efficiencies of bioassays for interference by DI WSN influenza virus in MDCK cells

<table>
<thead>
<tr>
<th>Assay</th>
<th>DIU/ml</th>
<th>DIP/ml*</th>
<th>Efficiency of assay†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICRA</td>
<td>NA</td>
<td>10⁻⁷⁻⁵</td>
<td>1</td>
</tr>
<tr>
<td>ICA</td>
<td>10⁻²</td>
<td>10⁻³⁻⁶</td>
<td>3.2 × 10⁵</td>
</tr>
</tbody>
</table>

* The number of DI particles was calculated according to the Poisson distribution and the number of cells/monolayer in each assay.
  † Relative to the ICRA.
  ‡ NA, Not applicable.

### Table 3. Ratios of physical to biologically active particles in purified defective interfering and standard A/WSN influenza virus preparations

<table>
<thead>
<tr>
<th>Virus</th>
<th>PP/ml*</th>
<th>HAU†/ml</th>
<th>PP:HAU</th>
<th>DIP/ml†</th>
<th>PP:DIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>4.59 × 10¹⁰</td>
<td>2.0 × 10⁵</td>
<td>2.3 × 10⁷</td>
<td>≤6.30 × 10⁵</td>
<td>≥2.9 × 10⁴</td>
</tr>
<tr>
<td>DI</td>
<td>8.56 × 10¹⁰</td>
<td>2.5 × 10⁵</td>
<td>3.4 × 10⁷</td>
<td>9.95 × 10¹⁰</td>
<td>0.86§</td>
</tr>
</tbody>
</table>

* The PP concentration was estimated by electron microscopy. At least 100 particles were counted.
  † HA units.
  ‡ DIP were estimated by the ICA in MDCK cells.
  § With different DI and standard virus preparations this value was 1.45, giving a mean of 1.15 PP:DIP.
used with DI lymphocytic choriomeningitis virus (Popescu et al., 1976), DI vesicular stomatitis virus (Winship & Thacore, 1980) and DI rabies virus (Kawai & Matsumoto, 1982), all of which detect around 10 DIU/ml. All available evidence indicates that the interfering activity was mediated by the DI virus nucleic acid, because interference was sensitive to BPL and to u.v. irradiation and has a genome about 20-fold smaller than that of infectious virus (data not shown). These observations are consistent with known attributes of DI viruses (Perrault, 1981). The ability of DI influenza virus to interfere heterotypically in vitro, not only between human subtypes (De & Nayak, 1980) but between human and avian subtypes as shown above, makes it essential to examine whether this property extends to the situation in vivo. As DI virus-mediated interference in vitro operates at the level of macromolecular synthesis (Perrault, 1981), heterotypic interference in vivo should not be limited by the antigenic variations of influenza virus. However, protection of mice from lethal WSN pneumonia by DI WSN is not accompanied by any demonstrable alteration in virus multiplication and may be mediated by an entirely different mechanism (Dimmock et al., 1986).

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REFERENCES


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