A Colorimetric Assay for Quantification of Defective Interfering Particles of Respiratory Syncytial Virus

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SUMMARY

A colorimetric assay for defective interfering (DI) particles of respiratory syncytial (RS) virus was developed. This quantitative biological assay is based on neutral red dye uptake by DI particle-protected cells that survive standard virus challenge. This assay was more sensitive than the reduction of infectious yield (RIY) assay and was capable of detecting $1 \times 10^4$ to $2 \times 10^4$ DI particles/ml. The coefficient of variation for parallel, simultaneous replicates ($n = 10$) was 23%. Cell-protecting activity in the colorimetric assay appeared simultaneously with activity in the RIY assay on undiluted passage of plaque-purified virus. Both activities were particulate, were inactivated by RS virus antiserum and exhibited similar ultraviolet-inactivation kinetics. The absolute values of the slopes of dilution curves for both assays were similar, and using regression analysis both assays enabled estimation of similar numbers of active particles. These results suggest that both activities are mediated by the same DI particle. The mechanism of cell protection does not appear to involve extracellular interferon because the inclusion of interferon antibody in the assay did not diminish DI particle cell protection. Finally, the colorimetric assay was used to reveal alternating cycles of infectious and DI virus production on serial undiluted passage.

INTRODUCTION

On serial undiluted passage many animal viruses generate defective interfering (DI) particles which are characterized by the ability to interfere with the replication of the homologous standard virus from which they are derived. Decrease of infectious virus production from cells co-infected with DI and standard virus is the basis for the reduction of infectious yield (RIY) assay which has been used to quantify the DI particles of a variety of viruses (Bellett & Cooper, 1959; Huang & Baltimore, 1977). Other approaches to quantifying DI particles involve measuring reduction of infectious centre formation (Welsh et al., 1972) or reduction of standard virus RNA synthesis (Barrett et al., 1981). Yet another approach takes advantage of the ability of some DI particles to reduce standard virus cytopathology. Reduction of cytopathology is the basis for the protected focus assay described for DI lymphocytic choriomeningitis virus (Popescu et al., 1976) and adapted by Winship & Thacore (1980) for certain DI particles of vesicular stomatitis virus (VSV). Cell protection by DI particles has also been described for infectious pancreatic necrosis virus (MacDonald & Yamamoto, 1978), Sindbis virus (Kowal & Stollar, 1980), Sendai virus (Roux & Holland, 1979), and for DI VSV particles containing double-stranded RNA (Sekellick & Marcus, 1978). Cell protection is probably characteristic of most systems in which DI particles can readily establish persistent infections with minimal cell crisis.

In our initial studies characterizing the DI particles of respiratory syncytial (RS) virus we utilized the RIY assay (Treuhaft & Beem, 1982) but found it cumbersome, time-consuming and not always reliable. On observing that HEp-2 cells which were co-infected with DI and standard virus exhibited little cytopathology, we attempted to develop a protected focus assay because of its potential increased sensitivity compared to RIY assay. Unfortunately, although cells were
protected they did not readily divide to form foci of protected cells. We were successful, however, in adapting the colorimetric assay of Borden & Leonhardt (1977) for interferon to the detection and quantification of RS virus DI particles in HEp-2 cells. This colorimetric assay is based on neutral red dye uptake by cells that are protected by DI particles and survive standard virus challenge. We report here optimal conditions for a reliable and sensitive quantitative assay of cell-protecting activity in DI RS virus preparations using this colorimetric assay and demonstrate the usefulness of the method in revealing alternating cycles of infectious and DI virus production during serial undiluted passage of plaque-purified RS virus.

METHODS

Cells. HEp-2 cells (ATCC certified cell line 23) were grown at 37 °C in modified minimal essential medium with Eagle’s salts, without antibiotics and supplemented with 5% heat-inactivated foetal bovine serum (FBS).

Virus. RS standard virus (Randall strain) was grown in HEp-2 cells using previously described procedures (Treuhaft & Beem, 1982). Viral growth medium (VGM) consisted of modified minimal essential medium with Eagle’s salts supplemented with 2.5% FBS, 100 U of penicillin/ml, 100 µg of streptomycin/ml and 2 µg of amphotericin B/ml (Fungizone; E. R. Squibb & Sons, Princeton, N.J., U.S.A.). RS virus DI particle preparations were originally obtained by passage of standard virus at multiplicities of infection (m.o.i.) of 1 to 5 (Treuhaft & Beem, 1982) and subsequently replicated by undiluted passage with addition of standard virus to achieve an m.o.i. of 1 to 3. A single DI particle pool, P61, was used for optimization of assay conditions, comparison of colorimetric and RIY assays and reproducibility studies. This pool contained 2.2 x 10^6 DI particles/ml and 4.5 x 10^5 p.f.u./ml.

Infectivity assay. Infectious virus was quantified by plaque assay as previously described (Treuhaft & Beem, 1982).

Colorimetric assay. Monolayers of 1.5 x 10^5 HEp-2 cells in 16 mm diam. plastic tissue culture wells were exposed to 0.1 ml of a DI particle preparation for 2 h at 37 °C with gentle rocking. After adsorption the DI preparation was removed and 0.1 ml of standard virus at an m.o.i. of 1 or 3 was added and allowed to adsorb for 2 h at 37 °C again with gentle rocking. The virus inoculum was then removed and 1 ml VGM was added to each well. After 72 h of incubation at 37 °C, culture fluid was replaced with 0.5 ml neutral red dye (33 mg/1) in Earle’s balanced salt solution and cells were incubated for an additional 2 h at 37 °C. The dye was then removed and monolayers were washed twice with phosphate-buffered saline. Finally, the dye taken up by the monolayer was extracted with 1 ml of 50% ethanol in 0.1 M-NaH2PO4 and the quantity of dye extracted was determined spectrophotometrically at 540 nm. Cell-protecting activity was assessed by comparing the quantity of dye taken up by cells co-infected with standard and DI virus to that taken up by cells infected with standard virus alone (virus control) and to that taken up by uninfected cells (cell control).

Reduction of Infectious yield assay. Following 72 h incubation of colorimetric assay plates, culture fluid was removed, centrifuged at 1000 g for 10 min and the supernatant stored at -70 °C until analysed for infectivity by plaque assay. Yield reduction was determined by comparing titres of wells co-infected with standard and DI virus to those infected with standard virus alone.

Quantification of DI particles. Both the colorimetric and RIY assays can be used to quantify DI particles, assuming a Poisson distribution of DI particles and p.f.u., equal numbers of cells in both experimental and control cultures, and all-or-none, one-hit kinetics of interference. To ensure one-hit kinetics, relative interfering activity was plotted as a function of relative DI particle concentration. A negative linear exponential relationship with no shoulder was observed for both assays. DI particles can then be quantified by co-infecting cells with dilutions of the DI preparation and an m.o.i. of 3 of standard virus to ensure that each cell is infected with at least one standard virus. Because the dilution yielding 63% protection corresponds to a DI particle m.o.i. of 1, the number of DI particles (DIP) can be estimated as follows.

\[
\text{DIP/ml} = \frac{1}{\text{Dilution yielding 63% protection}} \times \text{Number of cells exposed} \times \frac{1}{\text{Adsorption volume}}
\]

Ultraviolet irradiation. One ml amounts of a DI particle preparation in a 35 mm diam. plastic culture dish were exposed to an intensity of 2 J/m^2/s from a 15 W germicidal lamp (General Electric) for various times at a distance of 40 cm.

Antisera. Guinea-pig and horse antisera to RS virus (Long strain) as well as pre-immune sera were obtained from Flow Laboratories. Antiserum to human α- and β-interferons (IFNs) were a generous gift from J. Vilcek (Department of Microbiology, New York University School of Medicine, N.Y., U.S.A.). Preparation, titration and specificity of the antisera were described by Panem et al. (1982). The neutralization titre, or dilution capable of neutralizing 10 international units of IFN, was 1:64000 for the antiserum to IFN-α and 1:4000 for the antiserum to IFN-β. Cross-reactivity was < 1:8 for both antisera.
Colorimetric assay for RSV DI particles

RESULTS

Optimization of conditions for colorimetric assay

Because of its potential usefulness for quantifying DI particles, we first developed an assay for standard virus at an m.o.i. of 3. We wished to identify assay conditions that would yield optimum cell protection with a virus control value (standard virus alone) of less than 0.1 absorbance unit (A) and a range (between cell control value and virus control value) which encompassed at least 1.0 A. Using these criteria, incubation of the assay with VGM containing 5% FBS was superior to that containing 2.5 or 10% FBS. Incubation of the assay at 37 °C for 3 days was superior to 2, 4 or 5 days. Replacing VGM at 2 days and incubating for another 1 or 2 days was less satisfactory than incubation for 3 days without refeeding. Using conditions of incubation with VGM containing 5% FBS for 3 days, maximum cell protection was observed when cells were exposed to DI particle preparations prior to standard virus and when both preparations were allowed to adsorb for 2 h rather than only 1 h. We had previously observed that over 90% of infectious virus is adsorbed to cells by 2 h and that little increase in virus adsorption occurs on longer incubation (data not shown); therefore, incubation times longer than 2 h were not examined in this study. Finally, the best conditions for neutral red dye uptake were found to be incubation for 2 h with a dye concentration of 33 mg/l.

We also examined conditions to maximize the sensitivity of the assay for detecting low levels of DI particles. Various m.o.i. of standard virus, ranging from 0.1 to 5, were examined at 2, 3 and 4 days and at 3, 4 and 5 days following refeeding at 2 days. A standard virus m.o.i. of 1 combined with an incubation period of 3 days without refeeding gave the most reproducibly sensitive assay.

Comparison of colorimetric and RIY assays

Both the colorimetric and RIY assays were performed simultaneously on twofold dilutions of DI particle preparation P61 and the results using a standard virus m.o.i. of 3 are presented in Fig. 1. For the colorimetric assay (Fig. 1a) log10 A was a linear function of log dilution over a 32-fold dilution range. For the RIY assay (Fig. 1b), log10 infectious virus yield was a linear function of log dilution over a 16-fold dilution range. The correlation coefficients for both assays over their linear range were high (r = 0.98 for colorimetric and r = 0.99 for RIY assay) and the absolute values of the slopes for the two assays were very similar (0.82 for colorimetric and 0.92 for RIY assay). Estimation of the dilution yielding 63% protection was determined by linear regression analysis and was found to vary less than twofold for the two assays (1:4.4 for colorimetric and 1:8 for RIY assay), suggesting that both assays are measuring the same interfering particle. The colorimetric assay is the more sensitive, because it was able to detect interference at a 1:128 dilution while the RIY assay did not detect interference beyond a 1:16 dilution. We estimate that the colorimetric assay using a standard virus m.o.i. of 3 can detect as few as 1 × 10^4 to 2 × 10^4 DI particles/ml.

RIY and colorimetric assay both measure DI particles

Interfering activity in both assays is mediated by RS virus interfering particles because it was inactivated by RS virus antisera (Table 1) and because it was particulate. Over 90% of both activities was removed from the supernatant and over 75% was recovered from the pellet following centrifugation at 150,000 g for 45 min. It is unlikely that these interfering activities are due to temperature-sensitive (ts) virus mimicking the action of DI particles, because plaque assay of DI preparations showed no evidence of ts virus able to grow at 32 °C, but not at 37 or 39 °C.

The most compelling evidence suggesting that the RIY and colorimetric assays measure the same interfering particle comes from the observation that activity in both assays appeared simultaneously on the fifth undiluted passage of plaque-purified virus (data not shown). Supportive evidence comes from the observations reported above that the absolute values of the slopes of dilution curves for the two assays are similar and that the estimates of the number of interfering particles in a DI particle preparation, determined by 63% protection, were similar.
Fig. 1. Colorimetric (a) and RIY (b) assays for RS virus DI particles. Twofold dilutions of the same DI particle preparation were analysed in quadruplicate for interfering activity, using a standard virus m.o.i. of 3. The RIY assay utilized culture fluid harvested at 72 h from the colorimetric assay culture wells. Arrows indicate 63% protection.

Table 1. Effect of RS virus antisera on interference assays

<table>
<thead>
<tr>
<th>Interfering preparation</th>
<th>Antiserum</th>
<th>Colorimetric assay</th>
<th>RIY assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$A \times 10^3$</td>
<td>$\log_{10}$ of P61 A</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>15 ± 14*</td>
<td>3</td>
</tr>
<tr>
<td>P61</td>
<td>None</td>
<td>523 ± 16</td>
<td>100</td>
</tr>
<tr>
<td>P61</td>
<td>Guinea-pig pre-immune</td>
<td>425 ± 51</td>
<td>81</td>
</tr>
<tr>
<td>P61</td>
<td>Guinea-pig immune</td>
<td>21 ± 6</td>
<td>4</td>
</tr>
<tr>
<td>P61</td>
<td>Horse pre-immune</td>
<td>449 ± 101</td>
<td>86</td>
</tr>
<tr>
<td>P61</td>
<td>Horse immune</td>
<td>14 ± 2</td>
<td>3</td>
</tr>
</tbody>
</table>

* Mean of four replicates ± s.D.  
† Mean of triplicate wells of four replicates ± s.D.

In addition, u.v. inactivation studies of the interfering activities revealed very similar one-hit kinetics for the two assays (Fig. 2) suggesting that the two assays are mediated by the same or similar sized segments of an interfering genome.

Finally, these RS virus interfering particles are defective because the ability of preparations with high interfering activity to p.f.u. ratios to replicate interfering activity for both assays depended on co-infection with additional standard virus.

Reproducibility

Reproducibility of the colorimetric assay using standard virus m.o.i. of 3 and 1 was examined with 10 replicates performed on the same day (intraday experiment) and 11 replicates performed on different days (interday experiment). The same DI particle preparation (P61) was used in all assays. The reproducibility of the assays was assessed by determining the cell protection produced by twofold dilutions, using regression analysis to estimate the dilution that would yield 63% protection and evaluating the variation in this estimated dilution.

Dilution curves for the colorimetric assay and RIY assay using an m.o.i. of 1 were similar to those shown in Fig. 1 for an m.o.i. of 3 in that $\log_{10} A$ (Fig. 1a) and $\log_{10}$ infectious virus yield (Fig.1b) were linear functions of log dilution (data not shown).
**Colorimetric assay for RSV DI particles**

As shown in Table 2, the colorimetric assay using an m.o.i. of 1 estimated a higher 63% protection dilution than did the assay using an m.o.i. of 3, demonstrating that the lower m.o.i. did result in a more sensitive assay. Using a t-test for the intraday comparisons, this difference was significant at the 0.0005 level and using a matched pair t-test for the interday variation data, this difference was also significant at the 0.0005 level.

For both m.o.i. of 1 and 3 the intraday variation was less than the interday variation. The extreme dilution values varied less than twofold for assays performed on the same day but varied almost fivefold for assays performed on different days. Therefore, comparison of colorimetric assay values will be most reliable when determinations are done on the same day.

**Mechanism of cell protection in colorimetric assay**

As demonstrated above, cell-protecting activity in the colorimetric assay is mediated by a particle which is inactivated by RS virus antisera. Because the incubation period for this assay was relatively long (72 h) we considered the possibility that DI particles effected cell protection by inducing IFN. As shown in Table 3, when antisera to human IFN-α and IFN-β were included in the VGM during the 72 h incubation, no decrease in cell protection or reduction of infectious virus yield was observed. Similar results were observed when 10-fold higher concentrations of antisera, sufficient to neutralize 640 units of IFN-α and 200 units of IFN-β, were used.

**Alternating cycles of infectious and DI virus production on undiluted passage**

The usefulness of the colorimetric assay for detecting DI particles during undiluted passage of plaque-purified virus is demonstrated in Fig. 3. Two parallel series of undiluted passages were initiated from a single plaque. Infectivity and interfering activity were followed for 15 passages. For both passage series a drop in infectious titre occurred at passage 5 and coincided with the appearance of interfering activity. This interfering activity was particulate on centrifugation at 150,000 g for 45 min. Alternating cycles of infectious and DI virus production were observed.
Table 2. Reproducibility of colorimetric assay for estimating dilution giving 63% cell protection*

<table>
<thead>
<tr>
<th>Test comparisons</th>
<th>Standard virus m.o.i.</th>
<th>No. of replicates</th>
<th>Mean dilution ± S.D.</th>
<th>Extreme dilution values</th>
<th>Coefficient of variation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday</td>
<td>3</td>
<td>10</td>
<td>0.82 ± 0.19‡</td>
<td>0.74–1.08‡</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>1.75 ± 0.32</td>
<td>1.25–2.44</td>
<td>18</td>
</tr>
<tr>
<td>Interday</td>
<td>3</td>
<td>11</td>
<td>1.50 ± 0.97</td>
<td>0.76–4.35</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11</td>
<td>2.81 ± 1.01</td>
<td>1.92–5.37</td>
<td>36</td>
</tr>
</tbody>
</table>

* For each colorimetric assay twofold dilutions of the same DI particle pool were evaluated for cell-protecting activity.
† Coefficient of variation (%) = standard deviation divided by the mean, ×100.
‡ Reciprocal of the dilution.

Table 3. Effect of interferon antiserum on interfering assays

<table>
<thead>
<tr>
<th>Interfering preparation</th>
<th>Antiserum</th>
<th>Colorimetric assay</th>
<th>RIY assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A x 10²</td>
<td>% of P61 A</td>
<td>Infecivity (log₁₀ p.f.u./ml)</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>47 ± 7*</td>
<td>6.87 ± 0.02†</td>
</tr>
<tr>
<td>P61</td>
<td>None</td>
<td>892 ± 33</td>
<td>5.39 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>α and β†</td>
<td>926 ± 32</td>
<td>5.46 ± 0.09</td>
</tr>
<tr>
<td>P61</td>
<td>α</td>
<td>903 ± 47</td>
<td>5.24 ± 0.03</td>
</tr>
<tr>
<td>P61</td>
<td>β</td>
<td>794 ± 18</td>
<td>5.53 ± 0.08</td>
</tr>
</tbody>
</table>

* Mean of four replicates ± S.D.
† Mean of triplicate wells of four replicates ± S.D.
‡ Anti-IFN-α was used at a dilution sufficient to neutralize 64 international units/ml and anti-IFN-β was used at a dilution sufficient to neutralize 20 international units/ml.
Fig. 3. Infectious virus and DI particle production on undiluted passage of plaque-purified virus. The same plaque-purified virus was used to initiate parallel passage series A and B. Flasks containing $2 \times 10^6$ HEp-2 cells were infected with 1 ml of undiluted culture fluid supernatant from the preceding passage and then 10 ml VGM containing 2.5% FBS was added. After 72 h at 37°C, the supernatant (centrifuged at 1000 g for 10 min) was frozen until determination of infectious virus by plaque assay (●) and interfering activity by colorimetric assay using a standard virus m.o.i. of 1 (○).

throughout the entire 15 passages for series B and up to passage 10 for series A, after which the two types of virus appeared to cycle together.

**DISCUSSION**

This study describes a new approach to quantifying biologically active DI particles, based on neutral red dye uptake by protected cells which survive standard virus challenge. This assay should be useful in future studies evaluating the role of DI particles in acute and persistent RS virus infections. In addition, this approach should be applicable to other virus–host cell systems in which DI particles provide cell protection.

Factors other than DI particles which have been shown to interfere with standard virus replication and which might be present in this system are IFN and $ts$ virus. The presence of IFN in these preparations has not yet been analysed. However, the particulate nature of the interfering activity for RS virus described here suggests that if IFN is present, it is not active under the conditions of either the colorimetric or the R1Y assay. Recent studies indicate that some $ts$ viruses can interfere with standard virus replication (Youngner & Quagliana, 1976; Ahmed et al., 1980) and that $ts$ viruses can be generated on undiluted passage (Youngner et al., 1981). However, no evidence of $ts$ virus was found in the DI particle preparation used in this study.
How DI particles provide cell protection from standard RS virus challenge is not known. The cell protection induced by VSV DI particles of the snap-back type was shown to be mediated by IFN induced by double-stranded RNA of the DI genome (Sekellick & Marcus, 1978). However, extracellular IFN does not appear to be involved in RS virus DI particle cell protection because the presence of IFN antiserum during incubation of the colorimetric assay had no effect on dye uptake. The presence and relevance of intracellular IFN remains to be evaluated. Although RS virus does not shut off host macromolecular synthesis early in its replication (Levine et al., 1977), disruption of host synthetic processes undoubtedly occurs late in replication when cell death and syncytium formation are evident. Protection from cytopathology may simply be a by-product of the inhibition of standard virus replication. If the RS virus DI particles described here, like many other negative-strand RNA DI particles, are non-transcribing and interfere with standard virus replication by competing for the RNA replicase, then cell protection may result from reduced synthesis and translation of standard virus mRNA and consequently reduced viral protein synthesis.

DI particle-mediated cell protection may be a significant component in DI particle modulation of viral pathogenesis in natural infections. Reduction of tissue damage during infection is an obvious consequence. In addition, restriction of infectious virus genomes to cells releasing little infectious virus, but expressing viral antigens on their surface, may allow more efficient eradication of the infection by natural killer cells, antibody-dependent cytotoxic T cells and immune T cells. Alternatively, if protected cells express little viral antigen, then cell protection may be the first step in establishing persistent infections. Intermittent expression of viral antigen could result in chronic tissue damage either by viral destruction of the host cell or by immune mechanisms.

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REFERENCES


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