Ultraviolet-irradiated Vesicular Stomatitis Virus and Defective-interfering Particles are Similar Non-specific Inhibitors of Virus Infection

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SUMMARY

The way in which ultraviolet-irradiated vesicular stomatitis virus (VSV) inhibits the early events in VSV infection has been further characterized. Comparison of several different u.v.-irradiated thermolabile, temperature-sensitive mutants before and after heat inactivation established a requirement for inhibitory activity of functional G, N and L proteins, but not M protein. Defective-interfering (DI) particles, whether irradiated or not, inhibited VSV primary transcription as efficiently as UV-VSV, suggesting that virus proteins rather than transcription products are responsible for inhibition. Addition of inhibitory UV-VSV at different times after infection established that inhibition results from an action at an intracellular site, rather than at the cell surface or in the process of internalization. A similar inhibition by UV-VSV of infection by Sendai virus, Semliki Forest virus, Sindbis virus and influenza virus suggests that UV-VSV is acting by inducing a general change in the intracellular environment.

INTRODUCTION

The infection of BHK cells by vesicular stomatitis virus (VSV) is inhibited by ultraviolet-irradiated VSV (UV-VSV) at an early stage in the infectious cycle (Miller & Lenard, 1980). Functional virus glycoprotein (G) was found to be essential for the inhibition by UV-VSV. Temperature-sensitive (ts) G protein mutants of VSV containing G protein that was selectively heat-inactivated relative to other virus proteins (Keller et al., 1978) could no longer inhibit VSV infection (Miller & Lenard, 1980). Liposomes containing virus lipid and G from wild-type (wt) VSV or from the thermolabile G protein mutants were found to be only 2 to 5% as effective as the intact UV-VSV in inhibiting VSV infection. In addition, liposomes containing mutant G protein did not show a loss of inhibition of VSV infection following heating, suggesting that the mechanism of inhibition by these liposomes was different from that of the irradiated parent virus (Miller et al., 1980). These findings suggested that a functional G protein was a necessary but not sufficient condition for inhibition by UV-VSV. Other virus components in addition to G protein must be involved.

We now report that: (i) the N and L proteins of the VSV nucleocapsid are also essential for the UV-VSV inhibition; (ii) defective-interfering (DI) particles are as effective as UV-VSV in inhibiting primary transcription on the basis of the amount of virus protein added; (iii) this inhibition occurs intracellularly; (iv) infection by other enveloped viruses can also be inhibited by UV-VSV at similar concentrations as for VSV, suggesting that inhibition is mediated by an effect on the cells rather than directly on the processes of virus replication.
METHODS

Cells and viruses. BHK cells, wt VSV and ts mutants were grown and purified as described previously (Miller et al., 1980; Miller & Lenard, 1980). Each mutant was plaque-purified and tested for temperature sensitivity before use. These mutants were originally characterized by Flamand (1969, 1970). DI VSV was grown in confluent roller bottles of BHK cells at 37 °C and separated from VSV on 15 to 30% sucrose gradients (Gillies & Stollar, 1980). The isolated DI particles visualized by negative staining in the electron microscope were uniformly about 1/3 the size of VSV particles. When incubated in the secondary RNA assay (described below), the DI particles alone did not synthesize RNA. Sendai virus and influenza virus were grown in 10-day-old embryonated hens' eggs. Sindbis virus and Semliki Forest virus (SFV) were grown in confluent monolayers of BHK cells in roller bottles.

Ultraviolet irradiation of the viruses and DI particles was performed for a total of 4.2 × 10⁵ ergs/cm² with a Sylvania G30T8 germicidal lamp (principal wavelength, 254 nm) as monitored with a J-225 short wave u.v. meter (Ultraviolet Products, San Gabriel, Ca., U.S.A.).

Assay of the inhibition of infection. VSV, SFV, Sindbis virus and Sendai virus were assayed by measuring secondary transcription. For VSV a confluent monolayer of BHK cells in a 35 mm culture dish (approx. 10⁶ cells/dish) was infected with 4 p.f.u./cell for 1 h at 37 °C in 1 ml minimal essential medium (MEM) containing 5 μg actinomycin D, 100 μg bovine serum albumin (BSA), and 50 μmol N,N-bis(2-hydroxyethyl)-2-aminoethane sulphonic acid (Miller et al., 1980). The virus was removed, and the cells were incubated for 1 to 5 h with actinomycin D and [³H]uridine (5 μCi/ml, 29 Ci/mol; Amersham). Secondary transcription of SFV, Sindbis virus and Sendai virus was assayed as for VSV.

Primary VSV transcription was assayed in a similar fashion except that cells were inoculated at an m.o.i. of 1000 in the presence of 10 μg/ml DEAE-dextran, and the incubation medium contained 100 μg/ml cycloheximide. To measure inhibition, 50 μl of a concentrated UV-VSV, DI, or UV-DI suspension was added to the cells 1 h prior to, or at the indicated times following, addition of the infectious virus.

Influenza virus was measured by the production of released haemagglutinin. Influenza virus (2 p.f.u./cell) was adsorbed to the cells in medium without actinomycin D. Following removal of the virus, medium without [³H]uridine or actinomycin D but containing 2% foetal calf serum was added and the cells were incubated for 20 h at 37 °C. The amount of virus released was measured by haemagglutination of an equal volume of a 0.5% suspension of human erythrocytes in phosphate-buffered saline (PBS). In all cases inhibition was measured by comparison with a standard curve of decreasing m.o.i. in the absence of inhibiting virus (Miller et al., 1980).

RESULTS

Inhibition of VSV infection by irradiated mutants

Thermolabile, ts mutants with a mutation in either the N protein [ts(IV)0100, ts(IV)0194, and ts(IV)0268] or in the L protein [ts(L)080] were irradiated and tested for their ability to inhibit VSV RNA synthesis before and after specific inactivation of the mutated protein by heating for 1 h at 45 °C. Prior to heat inactivation, all mutant UV-VSVs were effective inhibitors of normal VSV transcription requiring 1.4 to 4.1 μg UV-VSV per plate (about 10⁶ cells) for 50% inhibition. This was within half a log of the concentration of wt UV-VSV required for 50% inhibition (0.9 μg, Table 1). After heating, the inhibitory capacity of all mutant UV-VSVs was completely inactivated. Addition of up to 100 μg of thermally inactivated UV-VSV mutants had no detectable inhibitory effect on RNA synthesis arising from a wt VSV infection (Table 1). In contrast, wt UV-VSV in various experiments retained
Inhibition of VSV infection by UV-VSV

Fig. 1. Inhibition of primary (——) and secondary (—) VSV transcription by DI particles before (●) and after (○) u.v. irradiation, and comparison with UV-VSV inhibition (△). Assays were performed at 4 p.f.u./cell for secondary transcription and 1000 p.f.u./cell for primary transcription.

Table 1. Inhibition of wt VSV infection by u.v.-irradiated VSV ts mutants having thermolabile N and L proteins*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Complementation group</th>
<th>Mutant protein</th>
<th>log₁₀ Reduction in infection (thermolability)</th>
<th>Concentration for 50% inhibition (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt VSV</td>
<td>—</td>
<td>—</td>
<td>0-0.5</td>
<td>Unheated: 0.9, Heated: 1–3</td>
</tr>
<tr>
<td>ts0125</td>
<td>IV</td>
<td>N</td>
<td>1.2</td>
<td>Unheated: 1.7, Heated: &gt;100</td>
</tr>
<tr>
<td>ts0100</td>
<td>IV</td>
<td>N</td>
<td>4.1</td>
<td>Unheated: 1.4, Heated: &gt;100</td>
</tr>
<tr>
<td>ts0194</td>
<td>IV</td>
<td>N</td>
<td>3.9</td>
<td>Unheated: 4.1, Heated: &gt;100</td>
</tr>
<tr>
<td>ts0268</td>
<td>IV</td>
<td>N</td>
<td>3.6</td>
<td>Unheated: 4.7, Heated: &gt;100</td>
</tr>
<tr>
<td>ts080</td>
<td>I</td>
<td>L</td>
<td>4.2</td>
<td>Unheated: 2.0, Heated: &gt;100</td>
</tr>
</tbody>
</table>

* BHK cells were incubated with inhibitor and VSV as described in Fig. 1. The concentrations for 50% inhibition were obtained from curves such as those in Fig. 1. The thermolability of the viruses was determined by comparison of their titres obtained from growth at 31 °C before and after an initial incubation for 1 h at 45 °C.

30 to 90% of its inhibitory activity upon heating for 1 h at 45 °C. These results indicate that functional N and L proteins are required for inhibition by UV-VSV. We have previously reported similar experiments demonstrating that functional G protein is also required for inhibition, while M protein could be inactivated without loss of inhibitory activity (Miller & Lenard, 1980). These results have been confirmed in the present set of experiments (data not shown).

Inhibition by defective-interfering particles

To investigate the role of the UV-VSV genome in this inhibition, wt DI particles with and without u.v. irradiation were compared with complete UV-VSV. DI particles do not undergo primary transcription except for synthesis of a positive-strand leader sequence (Colonno & Banerjee, 1976). DI particles are replicated during secondary transcription and, at this stage, compete with VSV to produce classical virus interference (for review, see Huang, 1973; Reichmann & Schnitzlein, 1979).

DI particles, whether irradiated or not, inhibited primary RNA transcription equally as well as did UV-VSV when inhibition was standardized to the amount of virus protein added...
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Fig. 2. Inhibition of primary (---) and secondary (——) VSV transcription by UV-VSV added at different times post-infection. (a) 12 p.f.u./cell (secondary transcription) or 1000 p.f.u./cell (primary transcription) were adsorbed to BHK monolayers for 1 h at 5 °C. Serial dilutions of UV-VSV were added in 50 μl amounts either at the same time as infectious VSV (—0.5 h) or at the indicated times post-infection and harvested at 5 h post-infection. Final concentrations (μg/ml/plate) of UV-VSV were as follows: O, 1.6; □, 6.2; △, 25; ■, 50; ●, 100. (b) Plot of log UV-VSV concentration against time at which that concentration gave 50% inhibition of secondary transcription (determined graphically; see arrow in a).

(Fig. 1). In contrast, unirradiated DI particles were almost 700 times as effective in inhibiting secondary transcription as were either UV-VSV or UV-DI particles (Fig. 1). The effect of unirradiated DI particles on secondary transcription clearly represents classical virus interference, while the inhibition of both primary and secondary RNA production by irradiated DI particles constitutes a distinctly different type of inhibition. Since irradiated DI particles and VSV inhibit both primary and secondary transcription to the same extent, much of the virus genome appears to be unnecessary for the observed inhibition.

**UV-VSV inhibition occurs inside the cell**

While it was previously reported that inhibition persisted for over 20 h after addition of UV-VSV to cells (Miller & Lenard, 1980), it was not determined whether this inhibition resulted from an intracellular action. Consequently, UV-VSV was added to cells at various times after infection by VSV and washing to remove unbound particles. The UV-VSV could be added as late as 3 h post-infection and could still induce partial inhibition in a secondary transcription assay (Fig. 2a). Complete inhibition could be obtained with a high enough UV-VSV dose as long as 30 min post-infection, long after the infectious virus had already been internalized (Miller & Lenard, 1980). It was found that the greater the amount of UV-VSV added to the cells, the later could be its time of addition after infection to obtain a certain amount of inhibition (Fig. 2a). Thus, 50% inhibition was obtained by the addition of 1.6 μg UV-VSV at 1 h post-infection while the same inhibition required the addition of 50 μg UV-VSV at 3 h post-infection. Similar results were seen with primary VSV transcription assays. Fifty μg UV-VSV inhibited primary transcription at an m.o.i. of 1000 with a similar time course as 1 μg UV-VSV inhibited secondary transcription at an m.o.i. of 15, i.e. at a similar ratio (about 15 to 20) of inhibitory to infectious virions. The inhibition of wt VSV infection by UV-VSV followed pseudo first-order kinetics (Fig. 2b). Thus, inhibition can be attained by the addition of UV-VSV either at a higher concentration or at an earlier time. This suggests that cellular processing is required to deliver a certain amount of inhibitory virus to an intracellular site with a constant time scale of movement.

This internal inhibition by UV-VSV did not occur by a limitation of the accessibility of the [3H]uridine into the nucleotide pool used for RNA synthesis. Experiments were performed in which both UV-VSV and [3H]uridine were added to the cells at different times after infection.
Inhibition of VSV infection by UV-VSV

Fig. 3. Inhibition by UV-VSV of virus infection by Sendai (RU strain) (Δ), influenza (WSN strain) (□), VSV (■), Sindbis virus (○) and SFV (●). Plates were incubated with the indicated concentrations of UV-VSV for 1 h at 37 °C followed by addition of the virus for 1 h at 37 °C. Incubations were for an additional 4 h for VSV, Sindbis virus and SFV, 10 h for Sendai virus, and 22 h for influenza virus. Concentrations added were: 20 haemagglutinating units/plate of Sendai virus; 2 p.f.u./cell of influenza virus; 4 p.f.u./cell of VSV; 2 p.f.u./cell of Sindbis virus; 1 p.f.u./cell of SFV.

Fig. 4. Inhibition of (a) SFV (-----) and VSV (---) and (b) the Z strain of Sendai virus infection by UV-VSV added at various times after infection. Cells were adsorbed for 1 h at 5 °C with 1.5 p.f.u./cell SFV or 20 haemagglutinating units/plate Sendai virus, washed, and incubated at 37 °C for an additional 5 h (SFV or VSV) or 12 h (Sendai virus) before harvesting. Fifty μl amounts of UV-VSV were added at the indicated times. Final UV-VSV concentrations (μg/ml/plate) were as follows: □, 1.6; Δ, 6.2; ○, 25; ●, 100.

The amount of inhibition due to UV-VSV added at any particular time after infection remained unchanged relative to controls without UV-VSV addition even though the total number of counts obtained was reduced with the later additions of the [3H]uridine (results not shown).

UV-VSV inhibition of infection by other enveloped viruses

The inhibitory effect of UV-VSV was also observed in cells infected with Sendai virus, SFV, Sindbis virus and influenza virus (WSN). Infection by all these viruses was inhibited by similar concentrations of UV-VSV when the UV-VSV was added 1 h prior to the heterologous infectious virus (Fig. 3). Three strains of Sendai virus with different fusogenic capacities, high (Z), medium (RU), and low (Obayashi), gave similar results (not shown), indicating that the inhibition had little correlation with the ability of the virus to fuse with the plasma membrane.

SFV and the Z strain of Sendai virus were selected for further comparison with VSV. To cells that had been infected with either virus, UV-VSV was added at successive times after infection and compared with UV-VSV added to VSV (Fig. 4a). SFV and Sendai virus RNA
production could both be inhibited by UV-VSV added several hours post-infection, but this inhibition was not as effective as with the VSV (Fig. 4). This suggests that, for at least these two other viruses, VSV had an intracellular locus of inhibition.

**DISCUSSION**

Aspects of a similar inhibition to that described here have been recognized in a variety of contexts for both UV-VSV (Huang & Wagner, 1965; Wagner & Huang, 1966; Wertz & Youngner, 1972; Marcus & Sekellick, 1975; Bablanian, 1975; Baxt & Bablanian, 1976a; Dubovi & Youngner, 1976a) and DI particles (Huang & Wagner, 1965; Doyle & Holland, 1973; Baxt & Bablanian, 1976a, b; Dubovi & Youngner, 1976b). Huang & Wagner (1966) reported that incubation of Krebs-2 tumour cells with about 0.6 μg UV-VSV/10^6 cells [estimated from data provided in their paper, assuming a particle/p.f.u. ratio of 20:1 with 5.8 × 10^{-16} μg protein/particle (Miller & Lenard, 1980)] produced a 50% inhibition of VSV infection. More recently, Dubovi & Youngner have shown that similar concentrations of UV-VSV (Dubovi & Youngner, 1976a) or DI particles (Dubovi & Youngner, 1976b) inhibited the replication of VSV as well as that of pseudorabies. The concentrations of inhibitor virus used in these studies were similar to those used in the present report.

It is important to note that, while inhibition of RNA synthesis has been measured in this paper, the inhibition need not be of RNA synthesis itself. Inhibition could equally well arise from an effect at some earlier step in the infection process, e.g. virus penetration or uncoating. By measurement of RNA synthesis it has been possible to determine that the inhibitory particles act at an early state, up to or including primary RNA synthesis.

The inhibition of VSV transcription might be mediated either by input virus proteins, or by leader RNA synthesized from input UV-VSV or DI particles (Colonnio & Banerjee, 1976; Leppert & Kolakofsky, 1980). Leader synthesis is largely unaffected by u.v. inactivation at the levels employed in these studies (Abraham & Banerjee, 1976; Ball & White, 1976). Our data do not permit us to distinguish unequivocally between these two possibilities. It is striking, however, that inhibition by UV-VSV and DI particles is quantitatively identical when expressed as a function of the amount of input virus protein (Fig. 1). Since the population of DI particles used in this study is about one-third the size of complete particles, it contains about three times as many DI genomes, and hence three times as many sites for leader synthesis for each microgram of protein compared with UV-VSV. If inhibition were a result of leader production, then DI particles might be expected to be considerably more effective inhibitors than UV-VSV; in fact, this was not observed. We suggest, therefore, that virus inhibition by UV-VSV and DI particles is mediated by the input virus proteins rather than by any aspect of function of the input virus genome. The fact that several different enveloped viruses, exhibiting quite diverse replication strategies, are all inhibited by similar concentrations of UV-VSV suggests that UV-VSV may affect the cellular milieu rather than specific virus processes.

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REFERENCES


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