Inhibiting Rous Sarcoma Virus-induced Transformation by Preinfection with Rhabdoviruses

By MARIANNE SEMMEL* AND ANANDASURAM SATHASIVAM†
Groupe 8 du CNRS, Villejuif 94800, France and †Medical Research Institute, Colombo, Sri Lanka

(Accepted 19 September 1982)

SUMMARY

In vivo preinfection of chicks with rabies virus (RV) or vesicular stomatitis virus (VSV) ts 1026 inhibits tumour formation after superinfection with Rous sarcoma virus (RSV). The degree of inhibition depends on the titre of the infecting viruses and the interval between rhabdovirus and RSV infection. In vitro, cells preinfected with VSV ts 1026 under non-permissive conditions and superinfected with RSV, are not transformed as judged by cell morphology, serum requirement for growth or the capacity to form colonies in soft agar, all these being the same as in uninfected cells. Doubly infected cells take up less deoxyglucose than cells infected with RSV only and more than cells infected with VSV only. RSV multiplication is inhibited in doubly infected cells: the supernatant fluid of these cells contains fewer focus-forming units and less reverse transcriptase activity than that of cells infected with RSV only. Doubly infected cells contain both VSV and RSV internal antigens 15 days after infection. The supernatant fluid of cells infected with VSV and maintained under non-permissive conditions inhibits transformation by RSV and multiplication of RSV, but not of VSV. Under non-permissive conditions, the rhabdoviruses undergo at least part of the infectious cycle, but no infectious virus is produced. RV antigen can be detected in the brain of parenterally infected chicks and VSV antigen in cells infected 15 days previously. We conclude that the inhibition of RSV multiplication and expression is probably due to one or more processes linked to the persistence of rhabdovirus components and that it cannot be attributed exclusively to interferon.

INTRODUCTION

When cells are infected by more than one virus, the outcome of the infection is conditioned by the host cell and the infecting viruses. Depending on the cell–virus system, both enhancement and inhibition of the replication of the superinfecting virus have been reported. Thus, preinfection of African green monkey cells with Shope fibroma virus inhibits vesicular stomatitis virus (VSV) particle induction and amplification (Winship & Thacore, 1979). In rabbit kidney cells, normally not permissive for VSV, preinfection with Shope fibroma virus allows VSV replication (Chen & Crouch, 1978). Rabbit cornea cells can be productively infected with VSV by superinfection with vaccinia virus (Thacore & Youngner, 1975; Hamilton et al., 1980). L-cells persistently infected with VSV are resistant to superinfection with either Mengo virus or VSV. Interferon, defective interfering particles and the emergence of small-plaque temperature-sensitive (ts) mutants seem to be involved in this inhibition (Nishiyama, 1977). Poliovirus preinfection inhibits VSV mRNA translation (Ehrenfeld & Lund, 1977) and herpes simplex mRNA translation (Saxton & Stevens, 1972). Preinfection with frog virus 3 inhibits secondary transcription and genome replication of VSV (Tannenbaum et al., 1978). In some cells infected simultaneously with Mengo virus and VSV, synthesis of one or the other infecting viruses is inhibited, in other cells neither (Otto & Lenard 1980). Preinfection with most retroviruses does not influence herpes simplex type 1 replication, but preinfection with hamster
sarcoma virus does (Mouttet et al., 1980). Both influenza virus and rabies virus (RV) have been reported to inhibit Rous sarcoma virus (RSV) expression (Kravchenko et al., 1965, 1967; Rao & Shirodkar, 1978). RSV production does not decrease in cells infected first with RSV and later with VSV, but some of the progeny virions are phenotypically mixed particles (Weiss et al., 1977).

The inhibitory effect of one virus on a superinfecting virus is most frequently attributed to interferon (Stewart, 1979), though in some instances it has been proved that interferon is not (Winship & Thacore, 1979) or only partially responsible (Nishiyama, 1977). It seems indeed unlikely that interferon is in all cases the only intervening factor and more probable that the reduction of expression of the superinfecting virus is a consequence of many factors involving both cellular and viral products and that several events during the replication of the superinfecting virus are involved.

In this report, we describe a study of the inhibitory effect of rhabdovirus (RV and VSV) preinfection on RSV multiplication and expression. So as to be able to assess RSV expression, we chose systems which are non-permissive for the rhabdoviruses used: RV is not pathogenic for chicks and VSV ts mutant 1026 is a double mutant, non-cytopathogenic at the non-permissive temperature (Stanners et al., 1977).

**METHODS**

**Cells and viruses.** Chick embryo cells were explanted from 11-day-old, lymphomatosis-free embryonated eggs and quail embryo cells from 9-day-old embryonated eggs. Cells were cultured in plastic flasks containing Eagle's medium supplemented with 10% newborn calf serum. For passaging, cells were washed with phosphate-buffered saline lacking Ca2+ and Mg2+ and trypsinized with 0.25% trypsin in 0.002 M-EDTA in 0.1 M-Tris-HCl buffer pH 7.5.

Rabies virus (CVS) was passaged up to three times through mice by intracranial (i.c.) injection of 0.03 ml virus suspension (LD50/102). Brains were harvested 6 or 7 days after infection and suspended in 0.15 M-NaCl containing 2% rabbit serum. The LD50 was determined by i.c. inoculation of 0.03 ml suspension into 2-week-old mice (Seligman, 1973).

Stock VSV (ts mutant 1026) was grown on primary chick embryo cells at the permissive temperature (30 °C). Virus was titrated on monolayers of chick or quail embryo cells with an agar overlay. After each passage, stock virus was tested for the emergence of revertants by titration at 40 °C and was found free of revertants. The stock virus was also tested for the appearance of defective interfering particles (DIP) according to Marcus & Sekellick (1974). The virus suspensions contained less than 5% DIP. Stock virus produced on primary chick cells had titres of 107 to 108 plaque-forming units (p.f.u.) per ml. VSV (wild-type Indiana) was grown and titrated as for VSV (ts mutant 1026), but at 37 °C. The titre of stock VSV (Indiana) was 108 to 109 p.f.u./ml.

The Schmidt–Ruppin strain of RSV, subgroup D, strain London, was grown on chick embryo cells. RSV was titrated according to Temin & Rubin (1958). Stock virus had a titre of 105 to 106 focus-forming units (f.f.u.)/ml and was stored at -70 °C.

**Experimental procedures.** In vivo, 1- to 3-day-old chicks were infected by injection of 0.1 ml of either VSV 1026 or RV suspension into each wing web. In vitro, a primary or secondary cell culture was infected with VSV (ts mutant 1026) at an m.o.i. of 3 to 5. Virus was adsorbed for 1 h at 40 °C. To remove unadsorbed virus, cells were washed for 45 s with KCl-HCl buffer (0.1 M, pH 2), washed with phosphate-buffered saline lacking Ca2+ and Mg2+ and trypsinized. Two daughter cultures were established. At the same time, a parallel culture was mock-infected. Two days later, one VSV-infected culture and one mock-infected culture were superinfected with RSV at an m.o.i. of 0.1. The virus was adsorbed for 1 h at 40 °C, and fresh prewarmed medium was added. Duplicate cultures were mock-infected. All cultures were incubated at 39-5 °C to 40 °C and trypsinized when the cells reached confluence.

Deoxyglucose absorption was determined on duplicate dishes according to Hatanaka & Hanafusa (1970).

For electron microscopy, cells were fixed with 6% glutaraldehyde, washed in phosphate buffer, coloured with OsO4 and embedded in Epon. Thin sections were prepared and shaded with carbon.

To determine reverse transcriptase activity and the titre of RSV, fresh medium was added to semiconfluent cultures which were then incubated overnight. Reverse transcriptase activity was assayed according to Ross et al. (1971). F.f.u. were determined as described above for the titration of stock RSV.

To study cell morphology and for immunofluorescence, cells were grown on coverslips in Leighton tubes. For cell morphology studies, the coverslips were fixed in methanol, stained with May–Grünwald–Giemsa stain and mounted on slides. Photographs were taken with a Zeiss photomicroscope 3.
Transformation inhibited by rhabdoviruses

For the determination of VSV and RSV antigens, an indirect immunofluorescence test was used. Cells were fixed in methanol for 10 min, mounted on slides, rehydrated with HEPES-buffered saline, reacted with the pertinent antiserum for 30 min, washed three times with buffer, reacted with the corresponding fluorescent antispecies antiserum, washed three times in buffer, and dried. Controls were reacted with preimmune antisera and the correspondent fluorescent antispecies antisera.

**Materials.** White Leghorn chicks (Hyline) came from Three Acres Farm (Colombo, Sri Lanka). Brown Leghorn chicks came from Institut G. Roussy (Villejuif, France) as did the embryonated chicken eggs. Rabies virus (CVS) was a gift from Dr Atanasiu (Institut Pasteur, Paris, France). VSV (ts mutant 1026 and wild-type Indiana) were a gift from Dr N. Genty (Orsay, France). RSV, Schmidt–Ruppin, subgroup D, strain London, came from Dr P. Vigier’s laboratory (Orsay, France). Anti-VSV immunoglobulins were a gift from Dr J. Závada (Virologicki Lister, Bratislava, Czechoslovakia). Anti-RSV serum came from the NIH (Bethesda, Md., U.S.A.). FITC-conjugated antirabies serum was purchased from BBL (Baltimore, Md., U.S.A.). FITC antispecies sera were purchased from Nordic Laboratories. Preimmune sheep, rabbit and turkey sera were obtained by bleeding animals purchased from Institut G. Roussy. Media and newborn calf serum were purchased from either Eurobio or Fllobio. All chemicals were either Merck or Carlo Erba purest grade. Radioactive precursors were purchased from New England Nucare. May–Grünwald and Giemsa stains were purchased from Fluka (Buchs, Switzerland).

**RESULTS**

**Effect of preinfection with rhabdovirus on Rous sarcoma virus infection in vivo**

When newly hatched chicks are infected with VSV, ts mutant 1026, RV, strain CVS, and then superinfected with RSV (either Schmidt–Ruppin or Bryan strain), tumour formation was inhibited. As shown in Table 1, this inhibition was more pronounced for RV than when VSV was used for preinfection. It was most pronounced when the interval between infections was 48 h, and when low amounts of RSV are used for superinfection. When chicks preinfected with rhabdovirus developed tumours, the appearance of the tumours was delayed in comparison to chicks infected with RSV only. None of the uninfected chicks or of the chicks infected with either RV or VSV developed tumours.

When RV (CVS) was injected into the wing web of newly hatched chicks, some infectious virus could be found for 3 days after the infection at the injection site. The brains of these chicks were not infectious. The chicks showed no clinical symptoms for at least 90 days after parenteral injection of RV, but their brains reacted positively with anti-RV serum, though the inclusion bodies were not very numerous. VSV (ts mutant 1026) injected into the wing web of newly hatched chicks did not cause disease for at least 64 days.

**Effect of preinfection with VSV (ts mutant 1026) on RSV expression and multiplication in vitro**

Fig. 1 shows that chick embryo cells were not transformed morphologically if they were preinfected with VSV and superinfected with RSV. Two weeks after RSV infection, doubly infected cells or cells infected with VSV alone resembled uninfected controls, whereas cells infected with RSV alone showed rounded cells and criss-cross patterns.

In medium containing 10% calf serum, uninfected cells, cells infected with VSV only and doubly infected cells multiplied, though more slowly than cells infected with RSV only (Fig. 2a).

In medium containing only 2% calf serum, uninfected cells, cells infected with VSV only and doubly infected cells remained stationary, whereas cells singly infected with RSV multiplied (Fig. 2b).

In soft agar, cells preinfected with VSV formed fewer colonies than cells infected with RSV only. This difference decreased with an increase in time, indicating that the non-transformed cells gradually overgrew the transformed cells (Table 2).

Deoxyglucose absorption is increased in transformed cells (Hatanaka & Hanafusa, 1970). We found that deoxyglucose absorption was increased in cells infected with RSV only and decreased in cells infected with VSV only, whereas intermediate values were obtained for doubly infected cells.
RSV-specific antigens could be detected by immunofluorescence in doubly infected cells, as could VSV antigens (Fig. 3). Both VSV and RSV antigens persisted for as long as 15 days in doubly infected cells. Most of the doubly infected cells seemed to contain less antigen than the cells infected with only one of the viruses, and most of the antigen found in doubly infected cells appeared to be internal.

Electron microscopy showed that doubly infected cells contained very few or no C-type particles 9 to 12 days after RSV infection, whereas cells infected with RSV alone contained many such particles. Cells infected with VSV alone and doubly infected cells contained masses of microfibrils (Fig. 4).

Reverse transcriptase activity significantly higher than control levels could not be detected in doubly infected cells, but was detected in cells infected with RSV only (Table 3).
Transformation inhibited by rhabdoviruses

Fig. 2. Growth of cells in different media. (a) Eagle's medium supplemented with 10% newborn calf serum. Cells were seeded 10 days after VSV infection on Falcon plastic dishes, and counted from 1 to 5 days after seeding. (b) Eagle's medium supplemented with 2% newborn calf serum. Cells were seeded 10 days after VSV infection on Falcon plastic dishes in medium containing 10% newborn calf serum. One day later the medium was replaced with Eagle's medium containing 2% newborn calf serum, and cells were counted from day 1 to day 5 after the medium change. ○, Uninfected cells; ▲, cells infected with VSV only; ■, cells infected with RSV only; ○, doubly infected cells.

Table 1. Effect of preinfection with rhabdovirus on tumour formation in vivo*

<table>
<thead>
<tr>
<th>Rhabdovirus</th>
<th>Titre</th>
<th>RSV titre</th>
<th>Delay between infections (days)</th>
<th>Tumour-bearing chicks/infected chicks</th>
<th>Tumour appearance (mean days post-infection)</th>
<th>Duration of experiment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV</td>
<td>$10^7$</td>
<td>$10^3$</td>
<td>2</td>
<td>6/9</td>
<td>14</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>$10^3$</td>
<td>-</td>
<td>9/9</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>RV</td>
<td>$10^{5-5}$</td>
<td>$10^4$</td>
<td>2</td>
<td>0/8</td>
<td>-</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>$10^4$</td>
<td>2</td>
<td>8/8</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>RV</td>
<td>$10^4$</td>
<td>$10^5$</td>
<td>0</td>
<td>8/9</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>$10^5$</td>
<td>0</td>
<td>5/5</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>RV</td>
<td>$10^4$</td>
<td>$10^5$</td>
<td>2</td>
<td>4/8</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td>RV</td>
<td>$10^4$</td>
<td>$10^5$</td>
<td>7</td>
<td>6/9</td>
<td>15</td>
<td>30</td>
</tr>
</tbody>
</table>

* Chicks were infected by inoculation in to the wing web, first with rhabdovirus and later with RSV (or mock-infected with 0-15 M-NaCl). The titre of RV is expressed as LD_{50}/0.03 ml, that of VSV in p.f.u./ml and that of RSV as f.f.u./ml.

Table 2. Colony formation in soft agar

<table>
<thead>
<tr>
<th>Infected with</th>
<th>Days after RSV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV</td>
<td>VSV + RSV</td>
</tr>
<tr>
<td>20*</td>
<td>2</td>
</tr>
<tr>
<td>119</td>
<td>18</td>
</tr>
<tr>
<td>177</td>
<td>42</td>
</tr>
</tbody>
</table>

* Number of colonies per $10^4$ cells. Colony formation was determined by the method of McPherson & Montagnier (1964).
Fig. 3. Immunofluorescence of cells infected with VSV and RSV 15 days after VSV infection. Magnification × 190. (a) Infected with VSV, anti-VSV antiseraum 1/40 and FITC-conjugated anti-sheep serum 1/40; (b) infected with RSV, anti-RSV serum 1/20 and FITC-conjugated anti-turkey serum 1/40; (c) infected with VSV and RSV, anti-VSV serum 1/40 and FITC-conjugated anti-turkey serum 1/40; (d) infected with VSV and RSV, anti-RSV serum 1/20 and FITC-conjugated anti-sheep serum 1/40; (e) uninfected, anti-VSV serum 1/40 and FITC-conjugated anti-sheep serum 1/40; (f) uninfected, anti-RSV serum 1/20 and FITC-conjugated anti-turkey serum 1/40.

Table 3. Reverse transcriptase activity in the supernatant of 10^5 cells preinfected with VSV and superinfected 48 h later with RSV

<table>
<thead>
<tr>
<th>Infection with</th>
<th>Time (days)</th>
<th>Ct/min incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV RSV</td>
<td>after VSV infection</td>
<td></td>
</tr>
<tr>
<td>- -</td>
<td>9</td>
<td>147</td>
</tr>
<tr>
<td>+ -</td>
<td>9</td>
<td>181</td>
</tr>
<tr>
<td>- +</td>
<td>9</td>
<td>8650</td>
</tr>
<tr>
<td>+ +</td>
<td>9</td>
<td>280</td>
</tr>
</tbody>
</table>
Table 4. Presence of infectious RSV in the supernatant of cells preinfected with VSV and superinfected 48 h later with RSV

<table>
<thead>
<tr>
<th>Infection with</th>
<th>Time (days) after RSV infection</th>
<th>Titre (f.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV</td>
<td>RSV</td>
<td>9</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>15</td>
</tr>
</tbody>
</table>

* Results corrected for cell numbers.

Table 5. Effect of supernatant of cells infected with VSV ts 1026*

<table>
<thead>
<tr>
<th>Growth in soft agar (number of colonies per 10⁴ cells 10 days after RSV infection)</th>
<th>Control</th>
<th>VSV</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcriptase activity (ct/min/10⁵ cells 10 days after RSV infection)</td>
<td>23</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Plaque formation (2 days after VSV infection)</td>
<td>22,813</td>
<td>9,894</td>
<td>43</td>
</tr>
</tbody>
</table>

* Cells were infected at an m.o.i. of 3 to 5 and maintained at 40 °C for 48 h. The supernatant medium was filtered through a 22 filter. Fresh cells were treated for 24 h with the supernatant and infected with either RSV or VSV (Indiana). Controls were treated with supernatant from untreated cells.

Nine days after RSV infection, the supernatant of doubly infected chick cells contained 50-fold fewer f.f.u. than the supernatant of chick cells infected with RSV only, and eightfold less 15 days after RSV infection (Table 4).

The supernatant medium from VSV-infected cells, maintained at 40 °C for 48 h and then filtered on a 22 μm Millipore filter in order to eliminate any residual virus, inhibited both transformation and RSV multiplication in cells infected with RSV. The same supernatant had no effect on VSV replication (Table 5).

When chick embryo cell cultures were infected with VSV (ts mutant 1026) and maintained at the non-permissive temperature, virus did not persist for more than 15 h and could be rescued for up to 48 h. In the infected cultures, cell morphology was the same as in uninfected controls (Fig. 1), and no virus was found in electron micrographs of these cells (Fig. 4). VSV antigen could be detected in these cells by immunofluorescence as late as 15 days after VSV infection (Fig. 3). In some experiments (not shown), we found that the supernatant medium of cells infected with VSV (ts mutant 1026) and maintained at the non-permissive temperature contained a small-plaque revertant as well as defective interfering particles. The titre of the revertant was \(10^5\) p.f.u./ml. At low virus dilutions, no plaques could be seen, and this was shown to be due to the presence of defective interfering particles, whose presence was demonstrated after centrifugation on a velocity gradient (Stanners et al., 1977) (not shown).

**DISCUSSION**

We find that preinfection with either rhabdovirus inhibits tumour formation in vivo. When such an inhibition was reported before (Kravchenko et al., 1965, 1967), it was attributed to interferon action, as RV in particular is known to be a potent interferon inducer (Marcus, 1979). But it seems unlikely that the inhibition we observe is solely due to interferon, since chicks infected with RV 7 days before they were infected with RSV developed tumours more rarely than did the controls, even though interferon is reputed to be no longer present 7 days after infection. VSV, a poor interferon inducer, (Stewart, 1979), inhibits tumour formation in vivo. The effect of VSV is less pronounced and comparable to that of RV when the challenge was made 7 days after RV infection.

Our results *in vitro* suggest that a minor proportion of the cell population is unaffected by VSV and therefore responds to RSV infection as do control cells, but that the majority of the cell population is affected by the VSV infection and shows only a partial response to RSV infection. Modification of morphology, growth in serum-deprived medium, the capacity to form colonies...
in soft agar and RSV production are severely inhibited, but the amount of RSV-specific antigens is about half that of the cells infected with RSV only.

The mechanism of inhibition is at present unknown. As regards interferon action in vitro, we could not find any interferon in the supernatant of VSV-infected cells. Supernatants of these cells did not reduce plaque formation by VSV, whether treated at pH 2 or not. But this supernatant was capable of inhibiting transformation and RSV replication, though to a lesser
Transformation inhibited by rhabdoviruses

degree than VSV infection. To our knowledge, plaque reduction is the most sensitive assay available for the detection of interferon (Carver & Marcus, 1967), though an antiviral state of cells infected with VSV ts mutant 1026 has been postulated (Francoeur et al., 1980), and this antiviral state has been linked to persistent infection with VSV (Sekellick & Marcus, 1979).

It is possible that our doubly infected cells were persistently infected with VSV. Though infectious virus disappeared after 15 h at the non-permissive temperature and could not be rescued after 48 h, VSV antigens persisted for at least 15 days and these cells contained microfibril formations (Fig. 4) similar to those described by Zajac & Hummeler (1970) in VSV-infected cells. It is also possible that revertants appeared in our cells, but that their detection was masked by the concomitant production of defective interfering particles, and their number so low as to be undetectable by electron microscopy (Fig. 4), or that VSV 1026, described as a mutant deficient in viral transcriptase (Weiss et al., 1977) does in fact transcribe part of the viral genome at the non-permissive temperature.

If interferon is not the only factor mediating RSV inhibition other mechanisms must be considered, such as a general or selective slowdown of cellular metabolism in the cells preinfected with VSV. This could be mediated by VSV soluble antigens: it is known that VSV-infected cells shed surface antigens (Little & Huang, 1978) and that these antigens inhibit cellular macromolecular biosynthesis (McSharry & Choppin, 1978). In order to determine the mechanism(s) of the observed inhibition, it will be necessary to analyse the VSV- and RSV-specific components in doubly infected cells: this work is currently in progress.

We would like to thank Dr Jacques Harel for revision of this paper, Ms Eugenie Chiric for the electron microscope work and M B. M. W. Jayaratne for technical assistance with the rabies virus work.

REFERENCES


(Received 23 June 1982)