Rescue of Rous Sarcoma

Virus from Virogenic Mammalian Cells Associated with Chicken Cells and Treated with Sendai Virus

By J. SVOBODA

Institute of Experimental Biology and Genetics,
Czechoslovak Academy of Sciences, Prague, 6, Czechoslovakia

AND R. DOURMASHKIN

Imperial Cancer Research Fund, Burtonhole Lane, London, N.W. 7

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SUMMARY

The rescue of Rous sarcoma virus in mixed cultures of vireogenic Chinese hamster cells transformed by RSV (RSCh cells) and chick fibroblasts treated with Sendai virus was studied. When the ratio of RSCh to chick fibroblasts was 1:64, or less, the amount of rescued Rous sarcoma virus decreased proportionately with the dilution of RSCh cells. Over this range treatment with Sendai virus increased rescue of Rous sarcoma virus 100 times as compared with untreated cell mixtures. About 600 RSCh cells were necessary for obtaining 1 f.f.u. of Rous sarcoma virus under these conditions. No infectious virus was found in tissue culture fluid or extracts from RSCh cells when both were incubated with chick embryo cells which afterwards underwent treatment with Sendai virus.

Using different conditions of incubation after treatment of cell mixtures with Sendai virus, a varying frequency of heterokaryon formation was obtained. There was good correlation between heterokaryon formation and rescue of Rous sarcoma virus measured in parallel cultures. The effect of Sendai virus on mixtures of chick embryo and RSCh cells under various conditions was examined by electron microscopy.

INTRODUCTION

In a previous report (Svoboda, Hložánek & Machala, 1968) the assay system for rescue of Rous sarcoma virus (RSV) from vireogenic mammalian cells was described. The assay was based on the enumeration of RSV foci in mixed culture of irradiated vireogenic Chinese hamster cells transformed by RSV (RSCh), and non-irradiated chick embryo cells treated with Sendai virus. In the present experiments this assay was used for the quantitative estimation of the proportion of vireogenic cells giving rise to RSV production in mixed cultures. In addition, data are presented concerning the relationship between RSV rescue and heterokaryon formation, electron microscopy of cells treated with Sendai virus, and absence of mature RSV particles in RSCh cells.
METHODS

Cells. Lines of virogenic Chinese hamster cells (RSCh) transformed with the Schmidt-Ruppin strain of Rous sarcoma virus (sr-RSV) (Hložánek, Donner & Svoboda, 1966) and chick embryo fibroblasts derived from the Brown Leghorn strain from Mill Hill, London, were used.

Treatment with u.v.-inactivated Sendai virus, the focus assay for measuring RSV rescue from virogenic cells and tissue culture procedures were reported previously (Svoboda et al. 1968). The only difference was that cells treated with Sendai virus were incubated at 37° for only 30 min. At 10 min. intervals they were shaken for 1 min.

RSV focus assay. One million chick embryo cells in 4 ml. medium were placed in a 6 cm. Petri dish with the test material. Otherwise the procedure for focus assay described by Svoboda et al. (1968) was followed.

Extracts. Extracts from RSCh cells were prepared by freezing (in a mixture of solid CO₂ + acetone) and thawing three times at 37° the cells obtained from tissue culture. Destroyed cells were centrifuged for 10 min. at 5000 g in the cold and the supernatant fluid was filtered through a Millipore membrane (pore size 0.45 μm.) using a Swinny filter.

Culture fluid from RSCh cells was collected from fully grown cultures and filtered in the same way.

Labelling of RSCh cells with [³H]thymidine. Two million cells were seeded on 10 cm. Petri dishes and 20 ml. culture fluid containing 4 μc [³H]thymidine (specific activity: 20 C/m-mole) was added. The next day the medium was changed and fresh medium containing the same amount of [³H]thymidine was added. On the third day, the labelled cells were harvested and used for the experiment. During the incubation with Sendai virus 1000-fold excess cold thymidine was added to the cell mixture. A portion of the cell mixture was plated for focus assay, and a portion in amounts of 10⁶ cells was seeded on 6 cm. Petri dishes with two coverslips. After 24 hr the coverslips were fixed according to Birnie & Simons (1967), processed for autoradiography using Ilford Nuclear Research emulsion in gel form (type K 5), exposed for 2 to 3 weeks and stained with May–Grünwald–Giemsa. Using this procedure no unlabelled nuclei were found in 500 cells inspected.

Neuraminidase treatment. Cells were first agglutinated with 1000 haemagglutinating units (HAU) of Sendai virus at 4° for 20 min.; 100 units of purified neuraminidase were then added to group no. 2 (see Table 2). Groups 3 and 4 were incubated for 2 and 30 min. at 37° respectively, before the addition of neuraminidase. Group 1 was treated in the same way as group 4 but no Sendai virus was added. The total length of the incubation with neuraminidase was 2 hr at 4° (cells were thoroughly stirred three times with a pipette at 60 min. intervals). During this period almost all visible cell clumps produced by Sendai virus were released. In the final step of the procedure, the cell mixtures were washed twice with medium at 4° and plated.

Electron microscopy. (a) Suspensions of cells were prepared for electron microscopy by the method of Schneeberger & Harris (1966). Fixation by glutaraldehyde was effected by adding 5 ml. cold 1.5 % glutaraldehyde in 0.15 M-phosphate buffer to the suspension of cells. After 2 hr fixation at 4° the cells were washed in 0.15 M-phosphate buffer and set in an agar pellet. The agar pellet was then chopped into small blocks in Palade's OsO₄ fixative and embedded in Araldite.
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(b) Pellets were prepared from the medium of cell culture (TCF pellets) first by low-speed centrifugation (1500 g for 10 min.) and then by high-speed centrifugation (100,000 g for 60 min.) of the resulting supernatant. The pellets were fixed for 2 hr in 1.5% buffered glutaraldehyde, washed in buffer and then postfixed for 1 hr in Palade's OsO4. Thin sections were cut on a Porter-Blum microtome, mounted on grids, stained with aqueous saturated uranyl acetate at 60° for 1 hr, and then stained with Reynold's lead citrate for 1 to 5 min. Afterwards they were coated with carbon and examined in a Siemens Elmiskop I electron microscope. In order to determine whether virus particles were present in TCF pellets, several blocks were sectioned from each pellet.

![Graph](image)

**Fig. 1.** Relationship between the number of RSCh cells in mixtures with chick cells, and the number of foci arising in dishes seeded with 20% of the cells from each mixture. • •, Number of foci in mixed cultures treated with 1000 HAU of Sendai virus; ▲ ▲, number of foci in untreated mixed cultures; -----, the broken line represents a slope of 1. Each point on the graph represents the number of foci counted in one dish. The four points for each dilution represent the foci in two dishes, from each of two independent experiments.

**RESULTS**

*Quantitative dependence of RSV rescue on the number of virogenic cells in the mixture with chicken cells*

RSCh cells, diminishing from $3.1 \times 10^6$ to $3.1 \times 10^4$ by half-log. decrements, were mixed with $2 \times 10^7$ chicken fibroblasts and treated with 1000 HAU Sendai virus. Focus formation was measured by plating $4 \times 10^6$ cells of each mixture/dish. The following relationship was obtained. With $3.1 \times 10^5$ virogenic cells, or fewer, the number of foci decreased proportionately (Fig. 1). Since only 20% of the cells given on the graph
were plated on the test dish, about 600 RSCh cells were necessary to obtain the rescue of one transforming RSV unit under these conditions. When more than \(3 \times 10^6\) of virogenic cells were introduced in the mixture with chick embryo cells, the relationship of focus formation to the number of RSCh cells was no longer linear.

The effect of treatment with Sendai virus on RSV rescue can be estimated from comparison of the curves from treated and untreated cell mixtures in Fig. 1. From this comparison it follows that treatment with Sendai virus increased RSV rescue about 100 times in mixed cultures of virogenic and sensitive cells.

Foci which appeared in control dishes were isolated and subcultured on feeder-layers of chick embryo cells. All of them retained their transformed character and produced infectious RSV.

\[
\text{Table 1. Tests for the presence of RSV in RSCh cells and culture fluid}
\]

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>0.5 ml.</th>
<th>3.0 ml.</th>
<th>7.5 ml.</th>
<th>20.0 ml.</th>
<th>56 \times 10^6 cells</th>
<th>145 \times 10^6 cells</th>
<th>210 \times 10^6 cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>NT</td>
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<tr>
<td>1</td>
<td>0</td>
<td>0(\dagger)</td>
<td>0(\dagger)</td>
<td>0(\dagger)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0(\dagger)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>3</td>
<td>0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* In this experiment chick embryo cells were incubated with cell extracts for 3 min. For the Sendai virus treatment, 5000 HAU of virus were used.

\(\dagger\) In addition, the culture fluid was injected in doses of 0.5 to 1.0 ml. into five 10-day-old chicks. No tumours occurred during 2 months of observation.

\[\dagger\] One dish from the second passage was infected with 2.5 \times 10^3 f.f.u. sr-RSV. Six days later, culture produced 5.7 \times 10^3 f.f.u. of RSV.

**Attempt to detect infectious RSV in RSCh cells**

A series of control experiments was made in which chick embryo cells were incubated with culture fluid or with extracts from RSCh cells and then treated with Sendai virus. The purpose of these experiments was to determine whether mature RSV particles containing complete virus genome, but incapable of penetrating into the chicken cell without Sendai virus treatment, were present in RSCh cells. Culture fluid or cell extracts from RSCh cells were incubated with 20 \times 10^8 chick embryo cells at 37°C for 45 min. with shaking, centrifuged, treated with 1000 HAU Sendai virus for 20 min. at 4°C and plated on two 10 cm. Petri dishes. Cultures were split weekly in the ratio 1:2 and the culture fluid from each passage was assayed at least once for the presence of RSV by focus assay. Each RSV assay was set up on at least three dishes with 0.1, 0.2 and 1.0 ml. of culture fluid tested. Repeated experiments performed along this line always gave negative results.

**Electron microscopic examination of cells and media for virus particles**

Four samples of mixtures of RSCh and chick embryo cells, one sample of RSCh cells, and three samples of chick embryo cells were examined. Pellets from tissue culture fluid of three samples of RSCh cells, and one sample of chick embryo cells were examined by electron microscopy. Several blocks from each sample were sectioned in
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searching for virus particles in both the cell pellets and the TCF pellets. Neither the RSCh cell media nor media from chick embryo cells showed any virus particles of the avian leucosis-RSV group in any of the samples examined. Particles resembling mycoplasma were occasionally found. Similarly, none of the samples of RSCh cells or chick embryo cells showed avian leucosis-RSV particles. The RSCh cells could usually be distinguished by their difference in size and also by the presence of large numbers of pinocytotic vacuoles in the cytoplasm of the chick embryo cells (Pl. 2).

Morphologically detectable changes in the interaction of virogenic and sensitive cells induced by Sendai virus and RSV rescue

Okada, Murayama & Yamada (1966) found that incubation at 37° was necessary for cell fusion by Sendai virus. We used this finding for obtaining different frequencies of heterokaryon formation under various conditions of incubation; the frequency was correlated with simultaneously measured RSV rescue. The mixtures, consisting of 3 × 10⁶ RSCh cells labelled with [³H]thymidine and 2 × 10⁷ chick embryo cells, were first agglutinated in the cold with 1000 HAU Sendai virus. Some samples were then allowed to stand at 4°, while others were incubated for 4 or 30 min. at 37°. After the

Table 2. Correlation between RSV rescue and heterokaryon formation in mixed cultures of RSCh and chick embryo cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1 No. of Foci†</th>
<th>2 No. of Foci†</th>
<th>3 No. of Foci†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group*</td>
<td>37° Incubation time (min.)</td>
<td>Heterokaryons‡</td>
<td>Heterokaryons‡</td>
</tr>
<tr>
<td>1</td>
<td>0 30</td>
<td>1 1</td>
<td>2 0</td>
</tr>
<tr>
<td>2</td>
<td>1000 HAU 0</td>
<td>6 1</td>
<td>12 1</td>
</tr>
<tr>
<td>3</td>
<td>1000 HAU 2</td>
<td>37 16</td>
<td>63 14</td>
</tr>
<tr>
<td>4</td>
<td>1000 HAU 30</td>
<td>66 579</td>
<td>100 105</td>
</tr>
</tbody>
</table>

The coefficient of rank correlation between focus and heterokaryon formation is 0.89; P<0.05.

* Details concerning the procedure of treatment of different groups are given in the methods.
† Average number of foci from two dishes.
‡ Number of heterokaryons on one coverslip.

incubation, the cells were treated with neuraminidase for 2 hr at 4°, which released most of the cellular clumps. Four million cells from each sample were then plated for calculating RSV rescue and 1 × 10⁶ cells were plated in the dishes with two coverslips for the counting of heterokaryons formed. We made three independent experiments (Table 2). There was good correlation between the degree of RSV rescue and the occurrence of heterokaryons. The number of heterokaryons was, however, obtained from the evaluation of only about 1/20 of cells which were used for measurement of RSV rescue, as estimated from the surface area of one coverslip and the number of cells plated. Two examples of heterokaryon formation are given in Pl. 1a, b. Pl. 1a represents a heterokaryon containing the labelled nucleus from a virogenic cell, and two unlabelled nuclei from chicken fibroblasts. The nuclei of the virogenic cells are clearly bigger than the nuclei of fibroblasts. In Pl. 1b the heterokaryon contains two labelled nuclei from virogenic cells and three unlabelled nuclei from chicken cells.
The formation of cytoplasmic bridges was studied by electron microscopy in the separate groups given in Table 2. The disappearance of the profile of the cell membrane at the point of contact of two closely apposed cells was interpreted as 'cytoplasmic bridges'. Since the cells were fixed in suspension, it was possible to avoid artifically linked cells, as might result from the fixation of a centrifuged cell pellet. Occasionally, because of tangential sectioning of the cell membrane, the space between the two cells remained visible, giving the impression of discontinuity of the cell membrane. In spite of the finding of cytoplasmic bridges, the character of the cytoplasm of the connected cells remained discrete (Pl. 3). Only bridges found between RSCh and chick embryo cells are considered in the discussion.

In the following experiments cells from the groups 1 to 4 (Table 2) were fixed for electron microscopy before the addition of neuraminidase. In group 1, where RSCh and chick embryo cells were incubated without Sendai virus, very few cytoplasmic bridges could be found. In the groups 2 to 4, where incubation with Sendai virus was progressively increased, cytoplasmic bridges were found. Cytoplasmic bridge formation could not be quantified by electron microscopy because of the few cells that can be examined by this method. However, it appeared that there was an increase of cytoplasmic bridges with more prolonged Sendai virus incubation. In groups 2 to 4, after neuraminidase treatment, cell bridges were found resembling those seen before treatment with the enzyme (Pl. 4).

DISCUSSION

The study of the relationship between the number of RSCh cells in the mixture with chick embryo cells and RSV rescue showed that the amount of rescued virus is proportional to the dilution of virogenic (RSCh) cells when the ratio of RSCh to chick embryo cells is 1:64 or less. Within this range the assay system can be used for a quantitative detection of RSV genome. With an increasing ratio of RSCh:chick embryo cells less RSV was rescued than was expected from the slope of the curve. This is unlikely to be due to inaccuracy of focus counting owing to overlapping of foci, at least up to counts of 200 (Dougherty & Simons, 1962). However, with more RSCh cells the probability also increases of homokaryon formation, which does not contribute to RSV rescue.

Sendai virus treatment was also used in experiments designed to determine whether or not mature RSV, though incapable of penetrating into the chick embryo cells, is present in mammalian cells. Piraino (1967) showed that RSV is adsorbed even to resistant cells but does not penetrate them. Enders, Holloway & Grogan (1967) found that poliovirus could replicate in chicken cells when these cells were first incubated with poliovirus and then treated with Sendai virus. However, repeated experiments in which chicken cells were incubated with the culture fluid and extracts from RSCh cells, and then treated with Sendai virus, did not reveal the presence of RSV, just as electron microscopy of cells and high-speed sediments from the culture fluids failed to show virus particles. Although, theoretically, RSV formation cannot be excluded in more than $10^8$ RSCh cells, the cell association combined with Sendai virus treatment allows a 100-fold more sensitive detection of RSV than isolation of RSV from the destroyed cells.

The mechanism of action of Sendai virus on RSV rescue has not yet been clarified. Our experiments show that there is a correlation between the amount of rescued virus
(a) Heterokaryon containing $[^3]H$thymidine-labelled nucleus from a virogenic cell and two unlabelled nuclei from chicken fibroblasts.

(b) Heterokaryon containing two $[^3]H$thymidine-labelled nuclei from virogenic cells and three unlabelled nuclei from chicken fibroblasts.
Low power electron micrograph of cells from Table 2, group 4, showing RSCh cells (A) and BLEF cells (B). A point of contact between the two types of cell is enclosed in a rectangle, and is shown enlarged in Pl. 3.

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A high magnification of the area enclosed in Pl. 2. The cell membranes (-----) of both cells are seen to disappear, forming a cell bridge. However, the cytoplasmic features of the cells remain distinct.

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Cell bridges (-----) between two cells in Table 2, group 2, which remained after treatment with neuraminidase.

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and the frequency of the heterokaryon formation. Heterokaryon formation may, however, considerably exceed the amount of rescued virus. Thus heterokaryon formation need not always lead to the rescue of RSV; and it still remains to be demonstrated whether cytoplasmic bridges alone may allow the passage of RSV genome from virogenic to sensitive cells.

The results obtained in our experiments support the original concept according to which the association of virogenic and sensitive cells leads to the formation of intercellular cytoplasmic bridges and cell fusion, and thus allows the transfer of RSV genetic material from virogenic to sensitive cells where this genome can express all its functions (Svoboda et al. 1963; Svoboda, 1964; Šimkovič, 1964).

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REFERENCES


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