Replication of Reticuloendotheliosis Viruses in Cell Culture: Chronic Infection

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SUMMARY

After an initial acute infection with cell killing, chicken or duck embryo fibroblasts infected in culture with reticuloendotheliosis viruses set up a chronic infection with no cell killing or morphological transformation. Essentially all of the chronically infected cells produced virus. The virus production was not sensitive to cytosine arabinoside or mitomycin C as was virus production in an acute infection.

The chronically infected cells had a strong group-specific resistance to the c.p.e. of superinfecting reticuloendotheliosis viruses. However, they were sensitive to vesicular stomatitis virus and avian leukosis-sarcoma viruses.

After double infection, single cells produced reticuloendotheliosis virus and avian leukosis-sarcoma virus.

INTRODUCTION

Reticuloendotheliosis viruses (REV) are a newly described group of avian viruses whose virus particles contain RNA and a DNA polymerase and which replicate through a DNA intermediate (Peterson, Baxter-Gabbard & Levine, 1972; Maldonado & Bose, 1973; Purchase et al. 1973; Cooper & Temin, 1974; Kang & Temin, 1974).

A previous paper described the kinetics of the replication of REV soon after infection, which involves cell killing (acute infection), and a plaque assay for REV (Temin & Kassner, 1974). When chicken embryo fibroblasts were inoculated with spleen necrosis virus (SNV), a member of the REV group, there was approximately a 2-day interval before production of virus (Temin & Kassner, 1974). At approximately the same time that virus was first produced, there was a c.p.e. and a decrease in cell number (Temin & Kassner, 1974). With further incubation the c.p.e. disappeared, and the cells appeared to be chronically infected with REV. The present paper describes the properties of these cells chronically infected with REV.

METHODS

Cells and viruses. General descriptions have recently been published (Temin & Kassner, 1974). Fertile Muscovy and Pekin duck eggs were obtained from a local farmer, and cultures of duck embryo fibroblasts were prepared as previously described. Fertile avian leukosis virus group-specific antigen negative C/E chicken eggs were obtained from SPAFAS, and cultures of chicken embryo fibroblasts were prepared as previously described. Third and fourth passage cells were used for most experiments.

 Cultures containing $6 \times 10^6$ chicken, pheasant or duck embryo fibroblasts were prepared in 3 ml of Eagle’s minimum essential medium with 20% tryptose phosphate broth (ET
medium) in 60 mm plastic Petri dishes. After incubation overnight, cultures were inoculated with 0.2 ml of conditioned medium or virus (about 10^6 p.f.u./ml). After 40 min, 5 ml of ET medium containing 6% calf serum or 4% foetal bovine serum was added.

Chicken embryo fibroblasts were cloned in conditioned medium; clones were suspended using small stainless steel cylinders to hold trypsin; and the cells were plated in the wells of microtitre dishes (Falcon Plastics). The cloning efficiency was approx. 10^-8. The clones were then plated on duplicate 35 mm Petri dishes for infection.

Two clones of morphologically altered cells isolated from MC29 (subgroup A) leukemia virus-infected chicken embryo cells were a kind gift of Dr R. Ishizaki (Ishizaki et al. 1972).

Reticuloendotheliosis virus (strain T) (REV-T), chick syncytial virus (CSV), duck infectious anaemia virus (DIAV), Trager duck spleen necrosis virus (SNV), Rous-associated virus-61 (RAV-61), B77 virus, Schmidt-Ruppin Rous sarcoma virus (SRV), vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV) have been described (Mizutani & Temin, 1973; Temin & Kassner, 1974). Carr-Zilber-associated virus (CZAV) was a kind gift of Dr David Boettiger.

Assay of viruses. REV were assayed by production of sedimentable DNA polymerase activity, by plaque formation on duck embryo fibroblasts, or by formation of c.p.e. on chicken embryo fibroblasts as previously described (Temen & Kassner, 1974). REV formed similar plaques on Muscovy and Pekin duck embryo cells. The former were used for assay plates.

VSV and NDV were assayed by plaque formation, B77 virus and SRV by focus formation, and RAV-61 by formation of sedimentable DNA polymerase activity.

Chemicals. Cytosine arabinoside was a kind gift of the Upjohn Co. Mitomycin C was obtained from Sigma.

RESULTS

Replication of SNV-infected chicken cells

Cells were inoculated with SNV, and the numbers of cells in parallel infected and uninfected cultures were determined for two weeks, including one transfer (Fig. 1). At the end of the experiment, cells from an infected culture were plated on duck cells and scored for infectious centres (see Fig. 2). 50% of the cells formed infectious centres. The uninfected cells multiplied exponentially with no c.p.e. The infected cells multiplied exponentially for 2 days. From 3 to 5 days after infection, there was no increase in the number of infected cells, and a c.p.e. was seen. The cytopathology involved cells becoming refractile (sometimes with vacuolization), rounding up, and detaching from the dish. No syncytia were seen and often the majority of the cells in a culture were destroyed. Five days after infection, the infected cells multiplied at the same rate as the uninfected cells, and the c.p.e. gradually disappeared. Cells which have been inoculated with SNV and no longer show a c.p.e. are called chronically infected.

The undiluted supernatant medium of chronically infected cells was used for inoculation in the experiment described in Fig. 1 which demonstrates that the lack of a c.p.e. is not a result of interfering particles in the supernatant medium of chronically infected cells. Similar results were found with SNV cloned at high dilution.

Chicken, duck or pheasant embryo fibroblasts chronically infected with SNV also had an appearance similar to that of uninfected chicken, duck or pheasant embryo fibroblasts. The chronically infected cells were transferred several times with no appearance of a c.p.e. or of transformation. It was also found that the chronically infected chicken cells did not clone more efficiently than uninfected cells. (Although we have not seen a similar c.p.e. with most
Fig. 1. Multiplication of cells infected with SNV. 48 cultures of chicken embryo fibroblasts were inoculated with conditioned medium (CH) or SNV [CH(SNV)] as described in Methods. On the first day and succeeding day, two uninfected and two infected cultures were trypsined, and the cells were counted with a Coulter counter. The media were replaced daily with fresh ET media containing 6% calf serum. Seven days after infection, fresh cultures containing $10^6$ uninfected or infected cells were prepared. On days 9 and 13 the media were changed again.

Avian sarcoma viruses, some strains of avian leukosis virus, in particular Bryan high titre-Rous-associated virus-2, Carr-Zilber-associated virus and Rous-associated virus-61, caused an analogous early c.p.e.)

**Virus production by chicken embryo fibroblasts chronically infected by SNV: infectious centres**

To determine the percentage of chronically infected cells producing SNV, different numbers of cells were added to duplicate cultures of duck embryo cells, and the number of plaques counted. There was a linear relationship between the number of cells plated and the number of plaques, with half of the cells forming plaques (Fig. 2). Furthermore, on transferring cultures which had received approx. 1 cell and on which no plaques were seen (a blind passage), a c.p.e. appeared. (An analogous effect was found with Rous sarcoma virus; Temin, 1967.) Similar results were found with pheasant cells chronically infected with SNV.

At the same time, cells chronically infected with SNV were frozen and thawed three times, sonicated to disrupt the cells, and plated on duck cells to assay for virus associated with the cells at the time of plating. Only about 1 p.f.u. of cell-associated virus per $10^5$ cells was found, indicating that most of the chronically infected cells produced SNV after plating.

To determine if virus production by the cells from reticuloendotheliosis in chickens was analogous to virus production by the chronically infected cells, 1-day-old Cornish Craw chickens were injected with REV-T, and the spleen from a chicken that was moribund at 2 weeks was used to make cell cultures. The spleen was enlarged with splenitis and follicular
hyperplasia (H. C. Pitot, personal communication). The cells in culture were very small with little cytoplasm. They grew in clusters in suspension without agitation and could be transferred by dilution into fresh medium without use of trypsin. They did not clone efficiently in agar suspension culture.

These infected spleen cells were plated for infectious centres on duck embryo cells and, as in the experiment of Fig. 2, about half of the spleen cells formed plaques. These chronically infected spleen cells have also been used as a source of infectious REV-T DNA and of DNA which can hybridize to [3H]-labelled SNV DNA (Cooper & Temin, 1974; Kang & Temin, 1974).

Cells from spleens isolated from parallel uninfected chickens did not multiply in suspension, but grew attached to the plastic Petri dishes and persisted in culture for only one or two transfers. Exposure of these spleen cells in culture to REV-T did not cause appearance of small cells which multiplied in suspension.

**Virus production by chicken embryo fibroblasts chronically infected by SNV: inhibitors**

Chicken and duck cells chronically infected with SNV at an input multiplicity of about 0.3 p.f.u./cell produced similar amounts of progeny virus (Table 1).

It had been found previously that production of SNV from newly infected chicken embryo
REV replication in cell culture

Table 1. Virus production by chicken and duck cells chronically infected by SNV*

<table>
<thead>
<tr>
<th></th>
<th>Number of cells</th>
<th>Virus titre (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>$1 \times 10^6$</td>
<td>$1.6 \times 10^5$</td>
</tr>
<tr>
<td>Duck</td>
<td>$2.8 \times 10^6$</td>
<td>$6.2 \times 10^6$</td>
</tr>
</tbody>
</table>

* Cultures of chicken ($0.8 \times 10^6$) and duck ($1 \times 10^6$) cells chronically infected with SNV were prepared by infecting cells and transferring at 5 and 14 days after infection. 5 h later the medium was changed to 5 ml of ET medium containing 2 % calf serum and 2 % foetal bovine serum. 43 h later the medium was harvested, and the number of cells was determined. The virus titre was assayed on duck cells.

Table 2. Effect of inhibitors on virus production by cells chronically infected by SNV*

<table>
<thead>
<tr>
<th>Chemical</th>
<th>DNA polymerase activity</th>
<th>p.f.u./10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6300 ct/min/15 ml/10^6 cells</td>
<td>$2.2 \times 10^5$</td>
</tr>
<tr>
<td>Cytosine arabinoside</td>
<td>2900</td>
<td>$0.6 \times 10^5$</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>3400</td>
<td>$0.6 \times 10^5$</td>
</tr>
</tbody>
</table>

* Only the data for two days after treatment are presented. Data from the other days were similar.

Resistance of chronically infected cells to cytopathic effect of REV

To determine if the two responses to SNV, the c.p.e. and the chronic infection, were the result of a genetic heterogeneity in the chicken embryo cells, five clones of chicken embryo fibroblasts were grown and exposed to SNV, as were the two clones from Dr Ishizaki. In all cases the clones behaved like the mass cultures of chicken embryo fibroblasts. There was a c.p.e. with killing and sloughing of some of the cells. Then the c.p.e. disappeared, and the cells looked like the control cells. Therefore, genetic heterogeneity in the chicken embryo fibroblasts was sensitive to treatment with mitomycin C or cytosine arabinoside (Temin & Kassner, 1974). To determine if virus production by chronically infected chicken cells was sensitive to these inhibitors, chicken cells chronically infected with SNV were treated with cytosine arabinoside or mitomycin C, and virus production was measured. Cultures of chicken cells chronically infected with SNV were prepared by infecting cells and transferring them four times at 3-day intervals. Cultures were prepared at $10^6$ or $3 \times 10^6$ cells in 3 ml of ET medium. After incubation overnight, the media on the cultures prepared with $10^6$ cells were changed to 5 ml of ET medium containing 3 % calf serum, and the media on half of the cultures prepared with $3 \times 10^6$ cells were changed to 5 ml of ET medium containing 3 % dialysed calf serum and $2 \times 10^{-4}$ M-cytosine arabinoside. The media on the other half of the cultures prepared with $3 \times 10^6$ cells were replaced with 2 ml of ET medium containing 15 $\mu$g/ml mitomycin C. After 2 h incubation, the media containing mitomycin C were replaced with 5 ml of ET medium containing 3 % calf serum. One, two and three days later the media were harvested from two cultures in each group, and the number of cells per culture was determined. The media were assayed for sedimentable DNA polymerase activity and for p.f.u. Only a small decrease in virus production was found (Table 2), in contrast to the over 100-fold decrease in virus production found when these inhibitors were used to treat cells immediately before or during infection (Temin & Kassner, 1974).
Table 3. Virus titration on cells chronically infected with SNV*

<table>
<thead>
<tr>
<th>Superinfecting virus</th>
<th>Uninfected</th>
<th>SNV-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNV</td>
<td>$10^5$</td>
<td>$&lt; 5$</td>
</tr>
<tr>
<td>B77 virus</td>
<td>$10^7$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>SRV</td>
<td>$8 \times 10^5$</td>
<td>$5 \times 10^3$</td>
</tr>
<tr>
<td>NDV</td>
<td>$10^7$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>VSV</td>
<td>$10^7$</td>
<td>$10^5$</td>
</tr>
</tbody>
</table>

* Chicken cells chronically infected with SNV were prepared as described in the footnote to Table 1. They and parallel uninfected cells were used to assay by standard techniques the viruses listed. Since SNV plaques in chicken cells were often not too distinct, an end-point assay was used for SNV. NDV and VSV were assayed on cultures containing four times as many cells as the other assay plates and were overlaid with agar immediately after infection.

† Virus titre (p.f.u./ml) or focus forming units (f.f.u./ml).

Table 4. Assay of SNV and VSV on chronically infected chicken cells*

<table>
<thead>
<tr>
<th>Original virus</th>
<th>SNV</th>
<th>VSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$5 \times 10^4$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>CSV</td>
<td>$&lt; 5$</td>
<td>$1.5 \times 10^5$</td>
</tr>
<tr>
<td>DIAV</td>
<td>$&lt; 5$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>SNV</td>
<td>$&lt; 5$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>B77 virus</td>
<td>$5 \times 10^4$</td>
<td>ND†</td>
</tr>
<tr>
<td>CZAV</td>
<td>$5 \times 10^4$</td>
<td>ND</td>
</tr>
<tr>
<td>RAV-6t</td>
<td>$5 \times 10^4$</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Chicken cells were exposed to the viruses listed in the first column, transferred three times until the c.p.e. disappeared or transformation was complete, and used for titration of SNV and VSV. Since SNV plaques in chicken cells were often not too distinct, an end-point assay was used. Resistance to plaque formation by SNV and sensitivity to plaque formation by VSV was also found with duck cells chronically infected with the four REV.

† Not done.

cells is apparently not the explanation for the two responses, acute and chronic, to the SNV infection.

To determine the specificity of the resistance in response to SNV infection, uninfected and chronically infected chicken embryo fibroblasts were exposed to several different viruses (Table 3). No resistance was found to plaque formation by NDV and VSV or focus formation by B77 virus and SRV, indicating that the resistance was not general, but was related to the c.p.e. of SNV.

To determine whether this resistance was group- or subgroup-specific, chicken cells chronically infected with a variety of REV and avian leukosis-sarcoma viruses were prepared, and SNV and VSV were assayed on them (Table 4). All of the cells chronically infected with REV were resistant to plaque formation by SNV and were sensitive to plaque formation by VSV. CSV also caused no c.p.e. on cells chronically infected with SNV, indicating that the resistance to the c.p.e. of SNV was group-specific.

These experiments and previous results (Halpern et al. 1973) also show that there is no interference between REV and avian leukosis-sarcoma viruses.

To determine whether a single cell could produce both an avian leukosis virus and a reticuloendotheliosis virus, cells chronically infected with SNV were superinfected with
Table 5. Cells producing SNV and SRV

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number positive</th>
<th>SNV</th>
<th>SRV</th>
<th>Only SNV</th>
<th>Only SRV</th>
<th>SNV unsure SRV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number examined</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>26/96</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>5/24</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>21/60</td>
<td>16</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>15/40</td>
<td>12</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>24/40</td>
<td>19</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>91/260</td>
<td>53 (78%)</td>
<td>10</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

SRV and transferred three days after infection. At seven days after infection, when most of the cells appeared transformed, the cells were released from the culture dishes with trypsin, counted, diluted, and distributed in 0.05 ml of ET medium at 0.5 cell/well of a microtitre dish. Then $7 \times 10^8$ to $10^4$ uninfected chicken embryo fibroblasts were added in 0.15 ml ET medium. After incubation overnight, foetal bovine serum was added to give a final concentration of 5%. After a few days of incubation, each well was examined microscopically for transformation or c.p.e. The media in many wells with transformation or c.p.e. (positive wells) were assayed on chicken and duck cells for SNV and SRV. In some cases, the assay plates were transferred or the assay was repeated. When there was a great deal of SNV, the c.p.e. made it difficult to see transformation by SRV (SNV, unsure SRV). Approx. 35% of the wells were positive for c.p.e. or transformation, in good agreement with the expected distribution of 0.5 cell/well. About 80% of the virus-producing cells produced both viruses (Table 5). Therefore, cells can produce SNV and SRV. Other studies, using an assay for infectious virus DNA, have shown that the DNA intermediates of SNV and SRV are not linked in these doubly infected cells (Cooper & Temin, 1974).

DISCUSSION

After the initial acute infection, chicken, pheasant or duck embryo fibroblasts infected in culture with REV and spleen cells from chickens infected with REV-T set up a chronic infection. Essentially all of the chronically infected cells produced virus, as shown by the experiments in Fig. 2 and Table 5. These cells multiplied with no c.p.e. and, except possibly for the cells from the chicken with reticuloendotheliosis, with no sign of morphological transformation.

Virus production from these chronically infected cells apparently no longer required DNA synthesis or cell division (Temin & Kassner, 1974), since it was not inhibited by treatment with cytosine arabinoside or mitomycin C. A virus DNA intermediate has been shown to be present and activated in the cells chronically infected with SNV (Cooper & Temin, 1974; Kang & Temin, 1974). These characteristics are thus like those of avian leukosis-sarcoma virus replication (Temin, 1974).

There was a strong (over $10^4$-fold) group-specific resistance in these chronically infected cells to c.p.e. of superinfecting REV. This resistance did not appear to result from a genetic heterogeneity in the chicken embryo cells. It was apparently not mediated by interferon, since no resistance to other viruses, for example, vesicular stomatitis virus, was found.

However, because of the absence of suitable genetic markers, it was not determined if the absence of c.p.e. was a result of interference with infection and replication of the super-infecting virus or to replication of the superinfecting virus with no c.p.e.
No intergroup interference was found between REV and avian leukosis-sarcoma viruses. Single cells were, therefore, infected and produced SNV and SRV.

Somewhat similar types of virus–cell interactions have been observed for other viruses, for example, lymphocytic choriomeningitis virus (Lehmann-Grube, Slenczka & Tees, 1969) and feline syncytium-forming virus (G. T. Todaro, personal communication). The lymphocytic choriomeningitis virus may be an example of a regulated or a self-limited infection (Walker, 1964; Hotchin, 1973). However, the fundamental question of what the difference in the virus–cell complex between the acute and chronic infections is has not been resolved in any of these systems. There probably is more than one type of difference, since lymphocytic choriomeningitis virus does not replicate through a DNA intermediate.

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