Long-Term Persistent Vesicular Stomatitis Virus and Rabies Virus Infection of Cells In Vitro

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SUMMARY

BHK 21 carrier cells persistently infected with VSV Indiana for over 2 years have been shedding generally very low levels of mature infectious virus or mature T particles (averaging less than one-hundredth p.f.u./cell/day) yet most cells are producing virus antigens and are resistant to homologous superinfection. However, large amounts of biologically active T particle RNP can be recovered from cytoplasmic extracts of these carrier cells even at times when they are shedding no detectable infectious virus. This recovered cytoplasmic RNP replicates (with helper B virions) to produce mature T particles, interferes strongly after DEAE dextran-facilitated uptake and, together with B virions, allows the establishment of a persistent carrier state in exposed cells.

No 'provirus' DNA copies of the VSV RNA genome are detectable (less than 1/40 copy/cell or 1 copy per 40 cells) in carrier cells after more than 2 years of persistent infection, and all transfection attempts have failed using DNA from these VSV carriers or DNA from carrier cells persistently infected with some other negative strand RNA viruses (measles, mumps, LCM, influenza, rabies).

Infectious viruses shed after more than 1 year from carrier cells originally infected with wild-type B virions are small plaque mutants showing a slight temperature sensitivity. Cured cell populations can be obtained from the long term VSV carrier culture by cloning in the presence or absence of antiviral antibody.

INTRODUCTION

Holland & Villarreal (1974) showed that persistent non-cytocidal carrier infections of BHK 21 cells in culture with vesicular stomatitis virus (VSV) required the presence of defective interfering virus particles (DI) to modulate the virulence of the virus for these highly susceptible cells. Doyle & Holland (1973) used completely purified DI to provide prophylactic protection of mice against low virus challenge doses, and observed a slower, altered disease process with DI in the presence of higher challenge doses of virus. Holland & Villarreal (1975) found that DI can be generated and replicated entirely in vivo. These results lend credence to the speculation of Huang & Baltimore (1970) that DI generally might be
involved in alteration of acute virus disease processes and in causing some persistent virus diseases.

Kawai, Matsumoto & Tanabe (1975) recently demonstrated cyclic production of DI in rabies carrier cultures. Palma & Huang (1974) had earlier shown that VSV DI and B virions undergo cyclic replication if new susceptible cells are introduced regularly. Other factors, such as interferon (Rodriguez & Henle, 1965) and temperature-sensitive mutants of the virus, undoubtedly may play a role in virus persistence. Preble & Youngner (1973) with paramyxoviruses, Fields & Raine (1974) with reovirus, and Wagner (1974) and Stanners & Goldberg (1975) with VSV have all shown that ts mutants tend to give persistent infections in vitro and slower disease processes in vivo.

The present study presents data concerning more than 2½ years of persistent infection of BHK 21 cells in vitro with VSV, and similar observations with rabies virus. It is shown that DI play a major role in persistence and that temperature sensitivity and small plaque mutation of B virions evolve during persistence and contribute to a more stable persistent virus–cell relationship. It is also shown that biologically active DI RNP is present in persistently infected cells and that virus yields are generally very low even though all carrier cells are infected. It appears that DI and virion attenuation provide for RNA viruses the functional equivalence of lysogeny in DNA viruses. Simpson & Jinuma (1975) and Zhdanov (1975) have recently presented evidence that DNA provirus copies may be produced in carrier cultures of enveloped RNA viruses and in human lupus erythematosus. We find no evidence for DNA copies in our long-term carrier cultures of VSV.

METHODS

Cells and virus. Cells were grown at 37 °C in Eagle's minimum essential medium plus 7 % heat-inactivated calf serum. BHK 21 baby hamster kidney cells were obtained originally from Flow laboratories. The L cell mouse fibroblast line and HeLa human cervical carcinoma cell line were originally obtained from the American Type Culture Collection. VSV, measles, mumps and influenza virus plaque assays were carried out as previously described (Holland & McLaren, 1959). Rabies virus was plaque-assayed as described by Sokol et al. (1968), and LCM virus as described by Welsh, O'Connell & Pfau (1972).

Except where otherwise stated the VSV carrier culture is the BHK 21, VSV\textsubscript{int} CAR\textsubscript{4} persistently infected carrier cell line described previously (Holland & Villarreal, 1974). It was established originally using B virions and T particles of the ts G3I mutant of Pringle (1970). This carrier was twice re-exposed to ts 31 B virions at m.o.i. = 100 to ensure infection of every cell. (This was done twice within one week at 6 months after the carrier was established, and the present carrier represents the surviving cell population.) Carrier cells were passaged by trypsin treatment at regular intervals (usually 2 to 3 days) when monolayers became confluent. The generation time of these cells is less than 30 h except when cytopathic episodes slow cell growth.

Biological assays for virus ribonucleoprotein activity. A modification of the DEAE dextran-facilitated uptake method of Cartwright, Smale & Brown (1970) was used in which cell monolayers were pre-treated with 350 μg/ml of DEAE dextran, followed by addition of the RNP in Hanks' balanced salt solution (BSS) for 10 to 15 min at 25 °C. The same regimen was employed for assay of the replicating capacity and interfering ability of T particle ribonucleoprotein cores from acutely infected cells or carrier cells. T particle RNP have been shown elsewhere (Holland, Villarreal & Breindl, 1976) to be biologically active in interference tests and in more sensitive amplification tests.
Hybridization of carrier cell DNA to \(^{3}H\)-VSV virion probes. This was done as described previously (Reichmann et al. 1974) for characterization of VSV virion RNA hybridization to \textit{in vitro} mRNA. Virion RNA was labelled with iodine to a sp. act. of \(1.2 \times 10^{7}\) ct/min/\(\mu\)g as described by Commerford (1971), annealed at \(C_{a} \approx 918\) at a carrier cell DNA/probe ratio of \(2 \times 10^{6}/1\), and ribonuclease was used to determine the degree of hybridization. Because no significant annealing was observed with carrier cell DNA or normal cell DNA, the intactness of the probe after the incubation period was verified by annealing to VSV \textit{in vitro} mRNA as previously described (Reichmann et al. 1974).

Hybridization of \(^{3}H\)-VSV cDNA with unlabelled DNA and RNA from normal and carrier cells. DNA was prepared from the VSV carrier cells after 25 months of persistent infection by the urea-phosphate technique of Britten, Graham & Neufeld (1974). Carrier cell RNA was isolated from the same cells by dialysing the urea-phosphate fraction not binding to hydroxyapatite against 0.25 M-NaCl and precipitating RNA with ethanol. \(^{3}H\)-VSV cDNA was purified with reverse transcriptase and \(^{3}H\)-labelled thymidine triphosphate. A positive strand \(^{3}H\)-cDNA was selected for these experiments by reacting total \(^{3}H\)-cDNA with excess VSV virion RNA, absorbing the RNA-DNA duplex to hydroxyapatite in 0.14 M-sodium phosphate, pH 6.8, 0.2 % SDS at 60 °C and eluting with 0.3 M-sodium phosphate. This positive strand DNA was freed of RNA by alkali digestion.

Annealing reaction mixtures contained 0.48 M-phosphate buffer, pH 6.8, 10\(^{-4}\) M-EDTA and 10\(^{3}\) ct/min. \(^{3}H\)-cDNA and unlabelled denatured cell DNA in 0.2 to 0.4 ml total volumes. Incubation was at 68 °C, with samples being withdrawn at appropriate times and assayed by binding to hydroxyapatite (Kohn & Britten, 1971) to determine ct/min \(^{3}H\) reannealed. Optical density at 260 nm was used to follow unlabelled bulk cell DNA reassociation.

Transfection experiments with DNA from persistently infected carrier cells. Monolayers of \(2 \times 10^{7}\) target cells were pre-treated for 2 min with 350 \(\mu\)g/ml DEAE dextran in Hanks's balanced salt solution (BSS), then immediately exposed to the total undenatured DNA from between \(2 \times 10^{7}\) to \(1 \times 10^{8}\) carrier cells in 1 ml 0.15 M-NaCl. DNA was extracted from carrier cells by proteinase K treatment in 0.15 M-NaCl, 0.1 % sarkosyl, followed by vigorous shaking with equal volumes of phenol–chloroform, phase separation and repeated extraction with ether. In some experiments additional shearing was introduced by long-term chloroform–octanol shaking or by ultrasonic shearing for various times. After a 10 to 15 min uptake period at 25 °C the monolayers were rinsed with BSS and incubated for 7 days or more at 37 °C (32 °C for rabies virus), with periodic assays of the medium on other sensitive cells in attempts to detect infectious virus. Target cells were also transferred to glass coverslips or suspended in medium at intervals and examined for expression of specific virus antigens on the cell surface or in the cytoplasm by fluorescent antibody techniques described below.

Fluorescent antibody techniques; cell staining and fluorescence microscopy. Two \(\times 10^{5}\) washed cells were incubated with 20 \(\mu\)l of fluorescein-conjugated rabbit IgG antibody to VSV. After 30 min incubation at room temperature, cells were washed three times in MEM and examined for expression of virus antigens on the cell surface using a Zeiss RA fluorescent microscope with an HBO-200 (Osram) light source. An FITC filter system (Optisk Laboratorium Lyngby, Denmark) was used for most analyses. To determine cytoplasmic VSV antigens in carrier cells, the cells were grown on coverslips, fixed, handled, and viewed as described by Joseph & Oldstone, 1974, and Oldstone & Dixon, 1971).

Radiolabel assay; measurements of VSV antigens at cell surfaces. We determined virus antigens on the surfaces of infected cells using the radiolabelled antibody technique (Joseph, Perrin & Oldstone, 1976). Briefly, IgG isolated from rabbit serum containing antibodies to
VSV was labelled with $^{125}$I by the chloramine T method to a sp. act. in the range 0.7 $\mu$Ci/$\mu$g. Normal rabbit IgG was labelled with $^{131}$I by the chloramine T method to about the same level. Approx. 0.8% of the $^{125}$I-IgG antiserum preparation contained specific antibody to VSV. Radiolabelled preparations were deaggregated by centrifuging at 100,000 g for 30 min; unlabelled IgG isolates were individually deaggregated in a similar manner. Cells of each type (i.e. acute VSV, persistent VSV and uninfected BHK cells) were placed in individual glass tubes at concentrations of $5 \times 10^4$, $1 \times 10^5$ and $5 \times 10^5$ cells per tube. Unlabelled IgG was added to radiolabelled IgG at a ratio of 10:1, and a total of 200 $\mu$g was added to each tube ($^{125}$I-labelled IgG plus unlabelled antibody IgG to VSV was added to one set, and $^{131}$I-labelled IgG plus unlabelled IgG devoid of antibody to VSV were added to another). These amounts had been previously determined to saturate virus antigens expressed on surfaces of BHK cells acutely infected with VSV (12 h post infection). IgG cell mixtures were incubated for 30 min at room temperature, washed 6 times with 5 ml cold MEM containing 10% FCS, pelleted and counted in a Baird Atomic $^{125}$I/$^{131}$I paired labelled gamma counter. Counts were corrected for background and $^{131}$I crossover in the $^{125}$I window. The number of IgG molecules of antibody to VSV binding specifically per cell in each sample was determined by the following formula:

$$
\frac{(6.023 \times 10^{23}) \times (\mu g^{125}I-IgG - \mu g^{131}I-IgG \text{ fixed on the cells}) \times 10^{-6}}{\text{Number of cells} \times \text{mol. wt. of IgG}}.
$$

**RESULTS**

**VSV T particles produced during long-term persistence**

Wagner *et al.* (1963) reported that L cells infected with a small plaque mutant of VSV evolved into a persistently infected carrier state with frequent cytopathic ‘crises’. The basis for the persistence was not explored, but interferon was suggested as a possible factor. Holland & Villarreal (1974) showed that DI was a necessary component along with infectious virus for the establishment of persistence, and it was shown that a long T particle produced continuously by the carrier BHK 21 cells could lead to the establishment of persistence whenever it infected BHK 21 cells along with B virions.

We have now monitored the BHK 21 VSV$_{ind}$ CAR$_4$ carrier cells during more than 2½ years of persistent infection and have always found these cells to be producing T particles. The long T particle previously described is sometimes the sole T particle present, but other sizes are often present in addition to, or in place of, the very long T particles. Fig. 1 shows an example of some of the patterns of T particles seen after co-cultivation of the BHK 21, VSV$_{ind}$ CAR$_4$ carrier with uninfected BHK 21 cells during the period from 1½ to 2½ years after establishment of the carrier state. A variety of different sizes of T particles was produced at different times. Apparently, no single T particle can achieve continuous dominance over other types as the persistently infected cells are maintained for long periods at 37 °C. Similar unpredictable changes in the T particle population were seen in the carrier initiated with wild-type B virions plus long T particles. J. Perrault (manuscript in preparation) has characterized a variety of these different carrier T particles with regard to the secondary structure of the DI virion RNA. Some exhibit extensive or moderate levels of ‘snap back’ self-annealing (Lazzarini *et al.* 1975; Perrault, 1976); others exhibit time-dependent, concentration-dependent self-annealing; and yet others show little or no self annealing. Thus far no characteristic has been found which distinguishes carrier cell T particle varieties from a wide variety of T particles produced during acute infection.
Production of virus by carrier cultures

Between 25 months and 30 months following establishment of the carrier state, these cells have become stabilized and generally show few microscopic signs of infection. Only at rare intervals of several days during months of culture does cytopathology appear briefly. At 25 months, it was observed that no virus could be recovered in repeated attempts over several days. Thereafter, we have sampled the supernatant medium from the carrier cultures every 24 h and determined the yield of p.f.u./cell/day. Fig. 2 shows that the virus yields fluctuate in a cyclic manner. They are nearly always less than 1 p.f.u./cell/day and average less than 0.01 p.f.u./cell/day and at times the yield is below one millionth p.f.u./cell/day average (i.e. not detectable). During only one 2-day period (during a cytopathic crisis indicated by the arrow in Fig. 2) was virus produced at levels averaging greater than 1 p.f.u./cell/day. Clearly this carrier has evolved toward a non-producing state similar to that of certain LCM (Welsh et al. 1972; Staneck et al. 1972) or measles (Rustigian, 1966) carriers or SSPE brain cells (Payne, Baublis & Itabashi, 1969). Shifting this long term stabilized carrier culture to 32 °C did not increase levels of virus shed over a 3-day period.

Most carrier cells produce VSV antigens

Fluorescent antibody staining of unfixed BHK 21 VSVind CAR4 cells for surface virus antigens between 2 and 2½ years of persistent infection always showed that over 95 % of the

Fig. 1. Diagrammatic representation of the variety of different sized T particles being shed from cells persistently infected with VSV during different time intervals in the period from 1½ to 2½ years after establishment of the carrier state. Each tube shows the position in a sucrose gradient of VSV B virions and the T particle size classes produced at each time by co-cultivation of carrier cells with normal BHK 21 cells. The longest T particles ($\beta_{90, w} = 470$) are usually, but not always, present.
Fig. 2. Yields/cell/day of infectious B virion p.f.u. from cells persistently infected with VSV for more than 2 years. At 25 months after establishment of the VSV carrier state in BHK 21 cells it was noticed that infectious virus could not be recovered from the carrier cell medium at certain times. Thereafter, the medium of carrier cells was sampled each day, plaque assayed at 32 °C, and the yield/cell/day plotted for the period from 25 months to 28 months after the establishment of persistent infection. In order to lessen the problems of interference by T particles shed into the medium, 0.3 ml samples of the medium (and dilutions of the medium) were plated on to very large monolayers of BHK 21 cells for plaque formation. Monolayers of $2 \times 10^6$ BHK 21 cells were used instead of monolayers of $2 \times 10^6$ cells. This lessens the probability of a B virion and a T particle co-infecting the same cell or cell grouping. Plaques were developed after 50 h at 32 °C under MEM with 0.4 % agarose. The arrow indicates an episode of severe cytopathic crisis.

cells were antigen positive. Fig. 3 shows a typical field with a patchy pattern of surface staining seen on some cells, and a continuous pattern on others. Furthermore, a quantitative immunoglobulin binding assay (Table 1) demonstrated that these carrier cells were expressing virus surface antigens at levels about 50 to 80 % of the level observed during acute infection. This antibody-binding assay was performed at a time when the cells were producing less than $10^{-4}$ p.f.u./cell/day. Clearly, virus antigens were being produced at significant levels in most cells, despite the paucity of infectious virus production after 2 years in the carrier state.

**Carrier cells can be 'cured' by cloning**

Walker (1964) has reviewed evidence for persistent carrier infections with enveloped RNA viruses in which prolonged antibody treatment or cloning can lead to recovery of cured cells. The BHK 21 VSV$_{ind}$ CAR$_4$ carrier studied here appears somewhat different from either of the types outlined by Walker (1964). Table 2 shows that antibody treatment of large numbers of cells for 1 month did not cure the cultures, but identical treatment of very lightly seeded monolayer cultures did cure them. Likewise, Table 2 shows that cloning in the absence of antibody did not lead to recovery of cured clones if there were more than a few clones growing in each culture plate (unless antibody was present), but it generally did
Fig. 3. Photomicrograph of BHK 21 cells persistently infected for over 2 years with VSV and incubated with monospecific fluorescnciated rabbit IgG antibody to VSV. Virus antigens are distributed over cell surfaces. These cells were not releasing detectable infectious virus at the time of immunofluorescent assay.

Table 1. $^{125}$I antibody binding assay for VSV antigens expressed on the surface of BHK 21 cells acutely infected or persistently infected for over 2 years with VSV

<table>
<thead>
<tr>
<th>BHK 21 cells employed</th>
<th>Counts bound per $10^5$ cells</th>
<th>Approximate number of antibody molecules bound/cell*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected (control)</td>
<td>3177</td>
<td>5317</td>
</tr>
<tr>
<td>Acute infections 7 h</td>
<td>3310</td>
<td>15261</td>
</tr>
<tr>
<td>Acute infection 12 h</td>
<td>3191</td>
<td>22223</td>
</tr>
<tr>
<td>Persistent carrier cells</td>
<td>3252</td>
<td>16710</td>
</tr>
<tr>
<td>Persistent carrier cells</td>
<td>4010</td>
<td>19323</td>
</tr>
<tr>
<td>Measles carrier (control)</td>
<td>3821</td>
<td>5121</td>
</tr>
</tbody>
</table>

* Data in this table were derived from duplicate samples over a range of cell concentrations ($5 \times 10^4$, $1 \times 10^5$, $5 \times 10^5$), and are corrected for background and crosstalk. Linear response was seen at the various cell dilutions. Comparable data were obtained in two additional experiments similar to the one presented. Sp. act. of the IgG was $4.5 \times 10^7$ ct/μg/30 s. Acutely infected cells were infected at m.o.i. = 3.

lead to cured clones whenever only one or a few clones grew in each culture plate. We have observed similar curing of carrier established with wild-type VSV B virions. Note that the cloning efficiency of the VSV carrier culture is rather low, but has improved from 0.03 % to better than 1 %. The above curing effect of antibody and of cloning suggests strongly that at least occasional cell to cell spread of virus is necessary to maintain the carrier state. It appears that infected cells can eliminate or outgrow infectious B virions by cell division because of the strong interfering effect of DI on virus replication. In mass monolayer cultures, cured cells are presumably soon re-infected, but in sparse cultures with antibody, or in isolated clones the cure can become permanent. As indicated in Table 2, cured cells show no virus antigen and exhibit normal plating efficiency and give normal virus yields with VSV Indiana even after 2½ years in the carrier state, during which they were resistant to homologous virus. Thus, there is no cellular genetic basis for resistance in the carrier state.
Table 2. Curing of long-term VSV carrier cells by cloning or by antibody treatment of sparsely seeded monolayers

<table>
<thead>
<tr>
<th>Treatment of carrier cells*</th>
<th>Clones/dish</th>
<th>Cloning efficiency (%)</th>
<th>Cured cells recovered?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloned in absence of antibody</td>
<td>2</td>
<td>0.03</td>
<td>Yes†</td>
</tr>
<tr>
<td>Cloned in absence of antibody</td>
<td>3</td>
<td>1.5</td>
<td>Yes</td>
</tr>
<tr>
<td>Cloned in absence of antibody</td>
<td>11</td>
<td>0.8</td>
<td>No</td>
</tr>
<tr>
<td>Cloned in presence of antibody</td>
<td>28</td>
<td>1.2</td>
<td>Yes</td>
</tr>
<tr>
<td>2 \times 10^6 cells plus antibody (30 days)‡</td>
<td>28</td>
<td>1.2</td>
<td>Yes</td>
</tr>
<tr>
<td>5 \times 10^4 cells plus antibody (30 days)</td>
<td>28</td>
<td>1.2</td>
<td>Yes</td>
</tr>
<tr>
<td>5 \times 10^4 cells, no antibody (30 days)</td>
<td>28</td>
<td>1.2</td>
<td>No</td>
</tr>
</tbody>
</table>

* Curing was carried out in glass culture flasks for 3 weeks at 37 °C. Carrier cells were trypsin-dispersed, counted and plated at various dilutions.
† Curing was verified by return of cured cell populations to full susceptibility to VSV Indiana (i.e. normal plaquing efficiency and normal virus yields); by lack of B virion or T particle shedding in cured cell cultures; and (in two cases) by failure to detect VSV antigens with the fluorescent antibody assay.
‡ Antibody treatment of carrier cell monolayers was carried out by placing 150 50% plaque reduction units of rabbit antiviral antibody in the medium over sparse (5 \times 10^4 cells/bottle) or confluent (2 \times 10^6 cells/bottle) monolayers of carrier cells for 30 days at 37 °C (with passage of the monolayers as needed to maintain confluent or sparse monolayers after passage).

Infectious centre assays

We previously reported (Holland & Villarreal, 1974) that all or nearly all carrier cells during the first months after establishment of persistent infection registered as infectious centres. This is no longer true in the stabilized carrier state after 2.5 years. In two instances, no concentration of carrier cells plated on BHK 21 cell monolayers yielded any plaques (i.e. less than 1 cell in 10^6 produced a plaque), and in a third infectious centre assay only 93 cells out of 10^5 registered as infectious centres. This is not unexpected in view of the low levels of virus being produced each day (Fig. 2).

Reduced production of mature T particles

We reported previously (Holland & Villarreal, 1974) that carrier cells were over 99% resistant to homologous VSV Indiana B virion infection and this is still true of the stabilized carrier after 2.5 years. (The yield per cell is reduced over 99.9% as compared to control normal BHK 21 cells when each are challenged with m.o.i. = 100 Indiana B virions.) The related New Jersey yield is reduced only about 90%, however, and unrelated NWS influenza virus yield are not reduced. Influenza (NWS) virus forms normal plaques on monolayers of the long-term VSV carrier cells. This strongly implicates DI and not interferon as the interfering agent. As reported above, co-cultivation of carrier cells with normal BHK 21 cells leads to production of large amounts of B virions and T particles of varying sizes. All attempts to purify visible amounts of B virions or T particles directly from the medium of large numbers of carrier cells have failed. Interference tests on the medium of carrier cultures were negative except during crisis (Table 3) indicating that very few T particles are shed into the medium at most times during the carrier state. Even a sensitive one cycle amplification test (Holland & Villarreal, 1975) was negative in 2 out of 3 tests done on different days. Therefore, there are generally less than 10^7 T particles/ml of carrier culture medium (Table 3). However, Table 3 shows that T particles are present in small numbers even when no B virions are shed, since extremely sensitive two-cycle amplification tests were positive. It is obvious that these carrier cultures generally shed only very small numbers of mature B
Table 3. *Interference and amplification tests for the presence of T particles in the medium of long-term carrier cell cultures*

<table>
<thead>
<tr>
<th>B virions shed into carrier cell medium (p.f.u./cell)</th>
<th>Interference tests for the presence of T particles in the medium (% interference)</th>
<th>Amplification tests for the presence of T particles in the medium of carrier cells†</th>
<th>One cycle</th>
<th>Two cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 × 10^-9</td>
<td>- (&lt;30 %)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>0.15</td>
<td>+(71 %)*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>None</td>
<td>- (&lt;30 %)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* This medium showing a positive interference test and one cycle amplification test was from the carrier cells at a time when they were undergoing a rare episode of cytopathology (slight c.p.e.).
† Amplification tests were as described elsewhere (Holland & Villereal, 1975; Holland et al. 1976). The two-cycle amplification test is 10^4-fold more sensitive than a single-cycle test. These assays involve infection of BHK 21 cells by B virions, and exposure to a source of T particles. This allows measurement of the T particles because each T particle is replicated about 10^4-fold at each cycle (as long as the T particle m.o.i. is less than 1.0).

Table 4. *Purification of T particles from yields of long-term carrier cells superinfected with related heterologous B virions*

<table>
<thead>
<tr>
<th>Cells challenged with B virions alone (m.o.i. = 100)*</th>
<th>Virus particles purified from yields of cells infected with VSV Indiana B virions</th>
<th>Virus particles purified from yields of cells infected with VSV New Jersey B virions</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK 21 VSV&lt;sub&gt;Ind&lt;/sub&gt; CAR&lt;sub&gt;4&lt;/sub&gt; carrier cells</td>
<td>No virus bands visible after purification†</td>
<td>B virion band plus 2 T particle bands</td>
</tr>
<tr>
<td>Cured carrier cells</td>
<td>B virion band only</td>
<td>B virion band only</td>
</tr>
<tr>
<td>Normal BHK 21 cells</td>
<td>B virion band only</td>
<td>B virion band only</td>
</tr>
</tbody>
</table>

* The challenge virus pools employed were first passage clonal pools prepared by low m.o.i. passage of wild-type virus picked directly from a plaque. They will not generate visible T particle bands on normal cells until the fourth passage (three additional undiluted passages (Holland et al. 1976).
† Following challenge of 10^6 cells of each type, the cells were incubated at 37 °C for 20 h and the virus yields purified and displayed on a sucrose gradient to visualize bands of B virions and T particles. Homologous interference was so great that no virus band was seen after challenge of the carrier cells with VSV Indiana.

virions or mature T particles, yet nearly every cell must have B virion RNP in its cytoplasm (in order to produce virus antigens) and T particle RNP (in order to resist homologous challenge). Therefore, we attempted to ‘rescue’ T particle RNP from these cells by B virion challenge.

**VSV New Jersey B virion challenge elicits production of mature T particles**

Challenge infection of the stabilized carrier cells with m.o.i. = 100 homologous Indiana strain B virions led to cell destruction but no visible B virions or T particles could be purified from the medium from 2 × 10^7 cells (Table 4). Strong homologous interference prevented significant B or T virion production. However, Table 4 shows that similar challenge of these long term carrier cells with m.o.i. = 100 VSV strain New Jersey led to release of B virions and T particles into the medium in amounts sufficient to be directly purified and visualized. Normal BHK 21 control cells infected with the same B virions of New Jersey strain yielded only B virions (visible after purification). Obviously, T particle genomes present in the carrier cells replicated (and interfered about 90 %) during replication of the challenge New Jersey strain. (This is about the same degree of heterologous interference seen in acute infection of our BHK 21 cells with our New Jersey strain B virions and Indiana T particles at high m.o.i.)
Table 5. The presence in carrier cell cytoplasmic extracts of T particle ribonucleoproteins able to replicate, interfere and re-establish a new carrier state (in cells exposed to them along with homologous VSV Indiana B virions)

<table>
<thead>
<tr>
<th>Mature B virions in medium, p.f.u./cell</th>
<th>One-cycle amplification test for mature T particles in cytoplasmic extracts (highest positive dilution)</th>
<th>Interference test for mature T particles in cytoplasmic extracts (highest positive dilution)*</th>
<th>One-cycle amplification test for T particle RNP in cytoplasmic extracts (highest positive dilution)*</th>
<th>Interference test for T particle RNP in cytoplasmic extracts (% interference)†</th>
<th>Ability of cytoplasmic RNP to establish new carrier state‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-term carrier cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>− ($)</td>
<td>− (&lt;30%)</td>
<td>+ (10⁻²)</td>
<td>+ (99.5%)</td>
<td>+</td>
</tr>
<tr>
<td>0.03</td>
<td>+ (undiluted)</td>
<td>− (&lt;30%)</td>
<td>+ (10⁻²)</td>
<td>+ (96%)</td>
<td>+</td>
</tr>
<tr>
<td>Cured carrier cells</td>
<td>−</td>
<td>− (&lt;30%)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Control normal cells</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Amplification tests for cytoplasmic particle RNP were as described (Holland et al. 1976) for cells acutely infected with B virions plus T particles. 2 × 10⁷ cells were disrupted after 1 min swelling in the 0.01 M-NaCl by five strokes of a glass tissue grinder at 0°C, and the mixture was made 0.15 M with respect to NaCl by addition of 2 M-NaCl. Nuclei were sedimented at 1000 g for 2 min and various dilutions of the cytoplasmic supernatant were absorbed to 2 × 10⁶ BHK 21 cells after DEAE dextran treatment (see Methods and Cartwright et al. 1970).

† Cytoplasmic T particle RNP were tested for interference with B virions (m.o.i. = 100) after DEAE dextran-facilitated uptake of undiluted cytoplasmic extracts. In the absence of DEAE dextran, amplification tests and interference tests were negative.

‡ Following interference tests, surviving cells were maintained at 37 °C and medium replenished at regular intervals for 30 days. Persistently infected carrier cells, when they appeared, were verified to be shedding both B virions and T particles.

§ Although this carrier culture medium was negative, a 10⁴-fold more sensitive two-cycle amplification test showed the presence of small numbers of T particles in the medium.

Biologically active T particle RNP in the cytoplasm of long term carrier cells

Cartwright et al. (1970) showed that subviral ribonucleoprotein (RNP) structures from VSV are infectious, rather efficiently so, when uptake is facilitated by DEAE dextran. We have recently extended this to show that T particle RNP (but not T particle RNA) is also biologically active after facilitated uptake with DEAE dextran (Holland et al. 1976). T particle RNP from disrupted virions or from infected cell cytoplasm caused the production of large amounts of T particles in a one-cycle amplification test in which helper B virions were added to BHK 21 cells after DEAE dextran facilitated uptake of the T particle RNP. Table 5 shows that disruption of carrier cells releases biologically active T particle RNP which replicated (and interfered with helper homologous VSV Indiana B virions) to produce large amounts of mature T particles visible after purification as major bands in sucrose gradients. Cytoplasmic extracts from carrier cells not producing detectable infectious virus yielded replicable T particle RNP. Extracts of cured carrier cells did not. The amounts of replicable RNP recoverable varied, but carrier cells often contained nearly as much biologically active T particle RNP as cells late during acute infection with B virions plus T particle (1/5000 dilutions of the latter gave positive amplification tests). These cytoplasmic T particle ribonucleoproteins from carrier cells were also able directly to facilitate the establishment of persistent infections of BHK 21 cells when adsorbed before B virion infection. The surviving cells of these B virion-carrier cell RNP double infections quickly established new
carrier cell populations which themselves shed B virions and T particles (Table 5). These results with cytoplasmic extracts provide direct evidence that carrier cells contain T particle RNP even when they are not shedding significant amounts of mature B virions or T particles, and can explain resistance to homologous superinfection in the absence of large numbers of mature T particles.

Fig. 4 graphically shows the T particles produced from carrier RNP on one occasion, and summarizes the situation with regard to T particles in the stabilized carrier cells after 2½ years in culture. It shows sucrose gradient analysis results in which no visible amount of B virions or T particles can be purified directly from the culture medium of even very large numbers of carrier cells (Tube A), but co-cultivation of carrier cells and normal BHK 21 cells yielded a visible band of B virions and two T particle bands (Tube B). Indiana challenge yielded no visible virions or DI bands (Tube C) but New Jersey challenge caused production of large numbers of the same two T particles from the carrier cells (Tube D). Finally, amplification tests (one cycle) on the medium of carrier cells generally failed to show significant levels of shed T particles (Tube E), except when B virion shedding was elevated, but amplification tests on cytoplasmic RNP were positive (Tube F). Again, the same two T particles were produced as with co-cultivation and New Jersey challenge tests done at this time. At other times in the carriers' evolution these three techniques revealed different sizes of T particles than those seen here, but at any given time the same classes of T particle are recovered from the carrier regardless of the technique used to detect their presence. We conclude that immature, biologically active T particle RNP are present in the cytoplasm of most or all of these persistently infected cells.

Fig. 4. Summary of various attempts at recovery of B virions and T particles from long-term carrier cultures. Each tube illustrates the results of a sucrose gradient analysis for the presence of virus. (A) Direct purification of the medium from 5 × 10⁶ carrier cells failed to reveal visible bands of B virions or T particles in a number of attempts. (B) Co-cultivation of carrier cells with normal BHK 21 cells always produced visible bands of T particles and B virions. (C) Super-infection of VSV<sub>Ind</sub> carrier cells with VSV<sub>Ind</sub> B virions (m.o.i. = 100) failed to produce visible bands of virus from 2 × 10⁶ cells. (D) Superinfection with VSV New Jersey serotype B virions (m.o.i. = 100) led to production of a B virion band plus two bands of T particles rescued from the carrier cells. (E) One-cycle amplification tests for the presence of mature T particles shed into the medium of carrier cells were negative. (F) Amplification tests for T particle RNP in cytoplasmic extracts of long-term carrier cells always showed the presence of biologically active T particle RNP (if DEAE dextran was used to facilitate RNP uptake into assay cells). Since all of these procedures were carried out on the carrier within 1 week, the same two T particles sizes were rescued in each case. Others were seen at other times in this carrier culture.
Table 6. Recovery of infectious RNP from the cytoplasm of long-term persistently infected carrier cells

<table>
<thead>
<tr>
<th>Infectious RNP recovered from carrier cell cytoplasm (p.f.u./cell)*</th>
<th>Infectious intact B virions released into medium by carrier cells during previous 24 h (p.f.u./cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x 10^{-3}</td>
<td>2.5 x 10^{-3}</td>
</tr>
<tr>
<td>0 (none detected)</td>
<td>0 (none detected)</td>
</tr>
<tr>
<td>2 x 10^{-4}</td>
<td>3 x 10^{-4}</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>1.6 x 10^{-3}</td>
<td>7 x 10^{-4}</td>
</tr>
</tbody>
</table>

* Infectious RNP levels were determined after DEAE dextran-facilitated uptake of RNP in dilutions of cytoplasmic extracts from carrier cells at various times between 25 and 29 months after establishment of the persistent carrier state. The subviral nature of the infectious p.f.u. was confirmed by omitting the DEAE dextran facilitation step, in which case no p.f.u. (or > thousand-fold fewer) were obtained. (See Cartwright et al. 1970; however, we cannot rule out the possibility that some of the p.f.u. measured in the RNP assays were due to mature infectious virions.)

Infectious B virion RNP

We next attempted to detect infectious p.f.u. in the cytoplasm of carrier cells which are producing very little or no virus, since this might be a useful way to recover virus from persistent infections such as SSPE in which virus recovery is difficult despite extensive virus antigen production (Payne, et al. 1969; Horta-Barbosa et al. 1969). Table 6 shows that infectious p.f.u. of B virion RNP were recoverable from carrier cell cytoplasmic extracts. Sometimes, more p.f.u. of infectious RNP were recoverable from these extracts of carrier cells than could be recovered from the medium of the same carrier cells. But at one time when the carrier cultures were producing no infectious mature B virions, no infectious p.f.u. of B virion RNP could be recovered from cytoplasmic extracts. The recoveries of infectious RNP in Table 6 represent significant amounts of B virion RNP in the cytoplasm of these cells since Cartwright et al. (1970) showed that VSV RNP are over 10^4-fold less infectious than intact virus from which they were prepared (even with DEAE dextran facilitation).

Failure to detect DNA 'provirus' copies of VSV RNA in long-term carrier cells

Simpson & Inuma (1975) recently reported the recovery of infectious provirus DNA from cells persistently infected with respiratory syncytial virus. The cells yielded infectious DNA capable of initiating the synthesis of complete virus in exposed cells.

Zhdanov (1975) also reported DNA provirus copies of measles virus and of other RNA viruses in cells persistently infected by these viruses. In repeated attempts, we were unable to transfect cells with DNA from the long-term VSV carrier. No induction of infectious virus occurred in recipient BHK 21 cells, HeLa cells or L cells and no induction of virus-specific antigens was detectable by immune fluorescent microscopy of recipient cell populations. Similar attempts employing DNA from cells persistently infected with lymphocytic choriomeningitis virus, measles virus, mumps virus, influenza virus, or rabies virus gave negative results. Several short term L cell carriers were included as DNA donors in the hope that the abundant endogenous retrovirus (L cell virions) in these cells might increase the likelihood of obtaining reverse copies, but these were also negative.

Table 7 shows the results of attempts to detect DNA copies of the VSV genome (or part of the genome) in the BHK 21 VSV_Inf.Car carrier cells after 2 years of persistent infection. A labelled DNA reverse transcript of the VSV genome was used as a probe. This probe...
Table 7. Hybridization of $^3$H-VSV cDNA with unlabelled DNA and RNA from long-term VSV carrier culture

<table>
<thead>
<tr>
<th>Source and type of unlabelled nucleic acid</th>
<th>Ratio of unlabelled nucleic acid to $^3$H-cDNA</th>
<th>$C_\text{ot}$</th>
<th>% $^3$H-cDNA reacted</th>
<th>% unlabelled DNA reacted</th>
<th>% annealing of $^3$H-cDNA with added VSV RNA at end of reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV-BHK 21 carrier cell DNA*</td>
<td>$(2 \times 10^7)/1$</td>
<td>741</td>
<td>5:5</td>
<td>74:9</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2457</td>
<td>7</td>
<td>86:2</td>
<td>74:2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6825</td>
<td>6:3</td>
<td>86:6</td>
<td>.</td>
</tr>
<tr>
<td>Control normal BHK 21 DNA</td>
<td>$(1:67 \times 10^7)/1$</td>
<td>424</td>
<td>5:4</td>
<td>66:8</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1404</td>
<td>6:3</td>
<td>72:7</td>
<td>72:8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3903</td>
<td>8:7</td>
<td>77</td>
<td>.</td>
</tr>
<tr>
<td>BHK 21 VSV carrier cell RNA*</td>
<td>$(3:2 \times 10^7)/1$</td>
<td>1860</td>
<td>39</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4092</td>
<td>34</td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>

* DNA and RNA were extracted from VSV carrier cells after 25 months of persistent VSV infection. The preparation of the $^3$H-cDNA probe and annealing techniques are described in Methods. A similar failure to detect DNA provirus copies was encountered with a $^3$H-labelled VSV virion probe as described in Methods.

was rendered solely of positive strand polarity by prior selection by annealing to virion RNA and hydroxyapatite chromatography. At a $C_\text{ot}$ of 6825 and a ratio of carrier cell DNA to VSV probe DNA of $2 \times 10^7$ to 1, no significant hybridization occurred even though 86:6 % of the carrier-cell DNA had reannealed as would be expected at these $C_\text{ot}$ levels. We verified that the probe was intact and still capable of specific annealing at the end of the incubation period (75 % annealed back to VSV virion RNA). We conclude that there is less than one-fortieth of a DNA provirus copy of the VSV genome per cell after 2 years of persistent infection (or less than 1 cell in 40 with a complete copy). Since we cannot verify that a DNA probe of this very high sp. act. is representative of the entire genome of VSV, we also prepared a high sp. act. – $^{131}$I-labelled VSV virion RNA probe by the method of Commerford (1971). Again, no annealing was detected at a ratio of carrier cell DNA to RNA probe of $2 \times 10^6$ to 1, and at a $C_\text{ot}$ of 918. Again the probe was able to anneal specifically (to VSV virion RNA) after termination of the incubation and this also rules out the presence of DNA provirus copies (at a level of one-sixth of a genome per cell).

These negative results show that although DNA proviruses need not be present in cells in order to achieve long-term stable, persistent infection by RNA viruses, they do not negate the possibility that DNA provirus copies may occur under certain conditions. The ability of this positive-strand DNA probe to anneal to carrier cell RNA (over one-third protected) indicates the presence in carrier cells of significant levels of virion polarity minus strand RNA. This could be B virion RNA or T particle RNA (probably mostly the latter).

**Temperature-sensitive and small plaque characteristics of carrier B virions**

These long-term carrier cells were originally established with the $ts$ G31 mutant, which is leaky and revertable (Pringle, 1970). Since its establishment and maintenance at 37 °C this carrier has produced only leaky $ts$ B virions which do not revert to wild type. Presumably this non-reverting characteristic is due to additional mutations. Furthermore, these carrier B virions have mutated into small plaque variants which produce smaller plaques than the original $ts$ G31 mutant at all temperatures from 25 to 33 °C (at 32 °C, 1 to 3 mm diam. plaques are formed in 48 h; this is about half the size of $ts$ G31 plaques under these
conditions). Apparently the carrier state selects for multiple B virion mutations (ts and otherwise) which attenuate virus virulence (since any cell in which more virulent B virions are present is more likely to die or be outgrown by other cells). Another carrier cell line established with a virulent wild-type non-ts B virion (plus long T particles) illustrates this effect more clearly. The original wild-type virus averages 2 mm plaques in 27 h at 37 °C, and B virions recovered from the carrier after 2 months gave identical plaque numbers and plaque size as the input wild type at 37 and 39 °C. But after 1 to 2 years of persistent infection the virions shed by this carrier are all slightly ts, and give smaller plaques at all temperatures than did the wild-type input B virions (after 1 year the plaque size in 40 h at 37 °C averages 2 mm and these shed B virions give 3 mm plaques at 33 °C in 40 h). Clonal pools from these carrier B virion plaques still give 100 % yields (± 20 %) at 32 and 38 °C, but give essentially no yields at 40 °C after m.o.i. = 50 infection. This represents more than a 100 000-fold yield reduction whereas wild-type yield is reduced only 97 % at 40 °C and B virions shed after 2 months were reduced 98-5 % at 40 °C. Clearly the wild-type B virions are slowly evolving into ts and small plaque mutants (even though they still give normal yields at 37 °C – the temperature at which the carrier is maintained). It should be noted that this carrier established with wild-type virus shows frequent severe episodes of cytopathic crises (unlike the carrier established with the ts G31 mutant) so temperature sensitivity has an obvious stabilizing role in persistent carrier infections. Nevertheless cloned B virions from these carrier cultures have never been able to establish new carriers without DI present. They always destroy all BHK 21 cells exposed at 37 °C regardless of the ts nature of the carrier virus. In one case cloned carrier B virions did establish a new carrier but the B virion pool was found to have picked up carrier T particles during cloning and the new carrier was shedding these T particles. In their original report of a VSV carrier cell Wagner et al. (1963) showed that small plaque mutation was important. The above results confirm that small plaque and ts mutants are selected in carrier cells.

Persistent carrier state in less permissive cell lines

We have shown elsewhere (Holland et al. 1976) that our line of HeLa cells is unable to replicate VSV T particles. It was therefore of interest to determine whether they are capable of becoming persistently infected by VSV. We attempted to establish a persistent carrier state by exposure of these HeLa cells to a mixture of B virions and T particles from the long term BHK 21 carrier (at m.o.i. = 10 of B virions and 1000 of T particles). Although this B virion T particle mixture readily established a carrier state in BHK 21 cells, it failed in three separate attempts to establish persistence in the HeLa cells. In two cases all cells died and in one case several clones grew up from the survivors of 10^6 infected cells, but these clones were free of virus. In a fourth attempt, clones of surviving cells grew up only after 2 months to form a carrier population. Since then (for 25 days) these persistently infected cells have continuously shed B virions and T particles (so cells able to replicate DI have been selected in establishing this persistent infection).

Since these HeLa cells are severalfold to tenfold less efficient in replicating VSV B virions we examined several other less permissive cell lines. L cells did establish a persistent carrier state with VSV for several months, but two independent L cell VSV carrier cell lines ‘cured’ spontaneously after 3 months of shedding B virions and T particles, and no further virus could be recovered thereafter. RK19 rabbit cells, which are very non-permissive for VSV Indiana, exhibited an initial cytopathology followed by outgrowth of normal cells which shed no virus.

C. Blifeld & J. Holland (unpublished data) have failed to find mature T particles in a
Table 8. Ability of clonal virus pools and high passage rabies virus pools to establish persistent carrier infections of BHK 21 cells in culture

<table>
<thead>
<tr>
<th>Virus inoculum pool</th>
<th>m.o.i.</th>
<th>Degree of c.p.e. at days p.i.</th>
<th>T particles present in yield at</th>
<th>Persistent carrier state established by surviving cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonal†</td>
<td>100</td>
<td>2+</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Clonal†</td>
<td>0.01</td>
<td>2+</td>
<td>3+</td>
<td>Yes</td>
</tr>
<tr>
<td>3rd undiluted passage†</td>
<td>100</td>
<td>0</td>
<td>2+</td>
<td>Yes</td>
</tr>
<tr>
<td>3rd undiluted passage†</td>
<td>0.01</td>
<td>1+</td>
<td>3+</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Following extensive c.p.e. (4+) nearly all cells degenerated and only a small percentage of cells survived to repopulate the culture when they were maintained at 37 °C and medium was replenished regularly beginning on day 8 p.i. at 32 °C. By 30 days all cultures had resumed vigorous growth and were shedding B virions and T particles.

† The clonal virus pool was the first passage pool of rabies HEP Flury virus prepared by low (m.o.i. = 0.001) multiplicity passage of virus recovered directly from a clone and harvested at day 6. The undiluted 3rd passage pool was the same clonal pool passaged 2 more times on BHK 21 cells at m.o.i. = 100 and harvested each time on day 7.

Drosophila melanogaster cell line persistently shedding small amounts of virus after infection with VSV B virions alone or B virions plus T particles. These insect cells do not exhibit cytopathology when infected with VSV at high m.o.i., and they generate multiple mutant progeny of input B virions (Mudd et al. 1973). Thus, it appears that certain cells are not able to establish persistent infection with, or without, DI, whereas at least some insect cells are able to do so without generating detectable levels of mature T particles. Eaton (1975) recently showed that Semliki Forest virus DI do not interfere or replicate well in mosquito cells.

Generation of carrier cultures by cloned rabies virus

Wiktor & Clark (1972) showed that rabies virus establishes persistent infection of cells in culture. Crick & Brown (1974) demonstrated that rabies virus generates T particles and Kawai et al. (1975) and Holland & Villarreal (1975) have recovered DI from chronically infected rabies carrier BHK 21 cells. We have attempted to determine whether rabies T particles are required to establish persistence by infecting cells with cloned B virion pools alone (or with the addition of purified rabies T particles). Table 8 shows that clonal pools of rabies virus generated a persistently infected carrier state in BHK 21 cells whether or not T particles were added. In the presence of added T particles, cytopathology was lessened and surviving cells resumed growth within 10 days. In the absence of added T particles the clonal rabies B virion pool caused extensive cytopathology, and most of the cell degenerated. However, the surviving cells eventually resumed growth and established a persistent carrier state in which both B virions and T particles were continuously shed into the medium. This ability of clonal pools of rabies virus to generate carrier cultures alone seemed to differ from the results seen with VSV in which T particles are required. However, this apparent discrepancy may be explained (Table 8) by the finding that even clonal pools of our HEP Flury rabies virus contain (or quickly generate) T particles. Perhaps rabies virus generated DI more readily than VSV, but in any case the 7-day period of plaque formation and the additional 5 days required to prepare a pool from cloned rabies virus allows ample time for DI generation. Regardless of the reasons, our clonal pools of rabies virus show only B virions visible during the first 4 days following infection of BHK 21 cells, but in the days thereafter a variety of T particles appear (Holland et al. 1976). Differing abilities to generate
DI may help explain the varying capacities of different RNA viruses (or different strains) to establish persistent infection. It has been found that even clonal pools of VSV have already generated T particles even though it takes several additional high multiplicity passages to visualize them directly (Holland et al. 1976); so merely cloning does not assure freedom from DI or their biological effects. Crick & Brown (1974) also reported that their rabies virus pools showed interfering activity after passages at m.o.i. less than 1.0.

**DISCUSSION**

The above results further implicate DI as a major factor in long-term, persistent infections by VSV. Amplification assays for DI RNP now allow DI detection in persistent infections where DI and even infectious virus may otherwise be difficult to recover and where their presence may hitherto have been overlooked.

The role of *ts* mutants in resistance is more difficult to evaluate. Our results show that wild-type non-*ts* VSV will establish carriers in the presence of DI but not in their absence. Even the slightly *ts* small plaque mutants (from the wild-type carrier cells) and frank *ts* mutants require T particles to establish persistent infection. We have not as yet achieved a persistent carrier state with rhabdoviruses in which the carrier cells were not producing DI. On the other hand, the carrier persistently infected with the *ts* G31 mutant (plus DI) is much more stable and undergoes fewer and less severe episodes of cytopathology than wild type carriers, so temperature sensitivity is a stabilizing factor in this balanced host-virus relationship. This is reinforced by the fact that the virus carrier originally established with wild-type virus has shed only virus that is slightly *ts* after more than a year of persistent infection. Temperature-sensitive mutations are not the only ones which occurred during persistence, since the shed virus is a small plaque mutant even at low temperatures. It would be surprising if a variety of mutations did not occur during persistent infection by an RNA virus. Some of these must be selected for the attenuating effect they have on virus virulence (since any carrier cell in which virulent virus is present or arises by mutation is more likely to die or be outgrown).

It should be emphasized that the BHK 21-VSV system is one which is normally very virulent, with up to 20000 p.f.u./cell being produced in acutely infected cells in the absence of DI. Less susceptible cells may show different modes of persistence or failure to achieve long-term persistence (as reported for *Drosophila* cells or RK13 cells alone). Since insects, for example, are natural vectors for some rhabdoviruses, their cellular resistance to VSV cytopathology (Mudd et al. 1973) may allow limited replication and persistent shedding of (mainly mutant) virus.

The ability of cloning (or antiviral antibody on light monolayers) to ‘cure’ cells from carrier cultures in which all or nearly all cells were producing large quantities of virus antigen suggests that DI RNP must be able to completely out-replicate B virion RNP at times in carrier cells. This could lead directly to ‘curing’, or indirectly by cell division diluting out all B virion RNP in some daughter cells. In any case the ‘cured’ cells behave as fully susceptible normal BHK 21 cells so the carrier state cannot be attributed to cellular mutations which lead to virus resistance. This tendency for DI to overwhelm B virions is well illustrated by the data of Table 5 in which large numbers of biologically active DI were recovered at one time period during which no infectious B virions or infectious RNP could be recovered. This can explain why curing might occur with fairly high probability. In the absence of antibody, ‘cured’ cells should form a clone of cells which are highly susceptible to re-infection by virus from neighbouring cells once all of their DI RNP are degraded or diluted. When
such cured clones represent only a small percentage of the total carrier monolayer, their re-
reinfection would lead to only low virus yields and minor or undetectable cytopathology,
especially if some DI RNP remain in some cells of the clone (or arrive in the clone at about
the same time as infectious virions). A larger percentage of ‘cured’ cells with no DI RNP
could give high yields of virus sufficient to trigger overt cytopathology. It is obvious from
the very low virus levels in the carrier medium at any time (Fig. 3) that only a tiny proportion
of cells in the carrier culture is ever cured of B virion RNP and of T particle RNP. If only
0.1% of the cells in the carrier culture were fully susceptible and became infected simulta-
neously, the amount of infectious virus released into the medium from these cells alone
would approximate 10 p.f.u./cell (since cured cells and normal BHK 21 cells yield 10000 to
20000 p.f.u./cell). Because this amount of virus is very rare in the carrier cell medium this
situation must occur only infrequently, probably because DI spread from cell to cell at
least as frequently as B virions.

All of the above discussion is based on the supposition that our plaque assay can detect
most infectious B virions released by the carrier culture. We cannot rule out the possibility
that non-cytopathic mutant virions are released from the carrier culture, or even that they
are a predominant component of the carrier virus population. In any event, the ability of
isolated cells or ‘antibody-protected’ cells to cure spontaneously helps to explain why per-
sistent infection in vivo is not a commonplace event at least at a level of tissue involvement
that leads to overt disease. Such ‘curing’ may also be important in recovery from acute
virus disease. The ability of antibody to cause capping and modulation of virus antigens
under normal conditions at the cell surface (Joseph & Oldstone, 1974, 1975) could increase
the probability of cell ‘curing’ in vivo by preventing T cell destruction or complement lysis
until ‘curing’ is achieved. However, cells which divide infrequently in vivo or not at all (e.g.
neurons) may be much more resistant to ‘curing’, and tend to provide foci of persistent
infection more readily than would dividing cell populations in which cured cells could
out-compete the infected population.

The accompanying paper (Villarreal & Holland, 1976) shows that VSV virion RNA replica-
tion is usually undetectable in carrier cells and VSV mRNA synthesis is very low. This helps
to explain how curing can occur at a detectable frequency in carrier cells. We conclude
that the VSV carrier state studied here is due to constantly changing levels of B virions and
T particles (and their intracellular RNP). Although the yields of virus and DI are much
lower than in the rabies carrier cells of Kawai et al. (1975) the mechanisms seem to be very
similar. In both cases there are cyclic variations in virus and DI yields. These are probably
explainable by the cycling model of DI replication (Palma & Huang, 1974), with the added
complexity of carrier cell survival and even curing, so that carrier cell cycling may involve
RNP cycles within single cells as well as at the cell population – mature virion – mature DI
level. Thus, DI (and evolution of attenuated B virion mutants) provide for RNA viruses
the functional equivalent of lysogeny in DNA viruses.

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Note added at proof

Youngner & Quagliana (1976) recently interpreted VSV persistence in L cells as being
due to ts mutant B virion interference with wild-type VSV. Their interference tests are
difficult to evaluate since they used wild-type virus at m.o.i. = 0.1 and t-mutant at m.o.i. = 1.0. However, cloned B virions from our carrier cells after 21 years showed no interference with wild-type VSV when both were infected at equal m.o.i. (1 or 100) and when verified to be free of DI.

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VSV persistence


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