The RNAs of Defective Interfering Pichinde Virus

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SUMMARY

A Pichinde persistently infected BHK21/13S culture was established in which defective interfering (DI) virus continued to be synthesized after cessation of plaque-forming virus replication. This DI virus, concentrated from NaCl-polypeethylene glycol treated tissue culture fluids, was shown to band over a much broader range than standard virus, in either discontinuous or continuous sucrose gradients. The polyacrylamide gel profile of the RNAs extracted from standard virus contained six components with sedimentation coefficients corresponding to 31, 28, 22, 18, 15 and 4-6S. All RNAs extracted from DI virus preparations, however, did not contain the 22 and 15S species. Furthermore, a new 20S fraction was observed in DI virus taken from cultures which had been maintained for more than 175 generations after the initial infection, whereas it was absent in DI virus synthesized prior to that time.

INTRODUCTION

An increasing amount of attention has been focused on the possibility that defective interfering (DI) virus may play a regulatory role in chronic or persistent in vivo infections, as well as being useful as a probe for studying the replication of virus at the molecular level (Huang, 1973). Both lymphocytic choriomeningitis (LCM) virus, which induces the well known persistent infection in mice (Traub, 1973), and Parana virus can initiate long-term in vitro infections. These cultures progressively lose their ability to synthesize plaque-forming virus but continue to produce DI virus (Staneck et al. 1972). While the biological characteristics of the LCM (Welsh, O'Connell & Pfau, 1972) and Parana (Staneck & Pfau, 1974) DI viruses have been investigated, the biochemical basis for their defectiveness has been a matter of conjecture. The arenavirus Pichinde was chosen for the latter studies because future experimentation involving the biochemical events in arenavirus-cell interactions would require the use of highly infectious purified virus. While this is possible with Pichinde virus (Mifune, Carter & Rawls, 1971; Ramos, Courtney & Rawls, 1972) similar purification procedures with LCM virus routinely yield only 0.002 to 0.4% of the original infectivity (Pfau, 1974). As the work reported here was in progress two purification procedures were described which resulted in little loss of infectious LCM virus (Gschwender, Brummund & Lehmann-Grube, 1975). Nonetheless, another important factor in this decision was that highly concentrated virus would be used and, unlike LCM, Pichinde infection of man has thus far caused no recognizable illness (Buchmeier, Adam & Rawls, 1974).

The purification and RNA analysis of Pichinde DI virus, as well as properties of the cultures in which DI (and not plaque forming) virus replicated, are the subject of this report.

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METHODS

Viruses, cells and media. The origin of Pichinde virus, the UBC and CA1371 strains of LCM virus, vesicular stomatitis virus (VSV) and the methods of propagation of BHK21/13S and L cells may be found in previous publications (Pulkkinen & Pfau, 1970; Welsh & Pfau, 1972; Stella et al. 1974).

Assay of standard and DI Pichinde virus. Techniques for determining plaque forming unit (p.f.u.) activity of standard virus have been described (Stella et al. 1974). The procedure for measuring DI virus activity (interference with infective centre formation by standard virus) in L cells was essentially that used with LCM (Welsh et al. 1972) except that: monolayers were exposed to DI and then standard virus for 1.5 instead of 1 h intervals; all virus preparations contained 50 μg/ml DEAE-dextran (Mifune et al. 1971); DI virus was sonically treated (Ramos et al. 1972) immediately before use; BHK21/13S cells were also used except that interference was measured as a decrease in p.f.u. yield at 45 h post infection (p.i.) In this type of assay the DEAE-dextran concentration was lowered to 5 μg/ml and the challenge virus multiplicity of infection (m.o.i.) was 0.1.

Initiation of persistent infection in vitro. Early plateau-phase BHK21/13S suspension cultures were infected with Pichinde virus at an input m.o.i. of 0.5. The details of this procedure are essentially those described for the establishment of cultures persistently infected with Parana virus (Staneck et al. 1972).

Tritium labelling of BHK cells freshly infected or persistently infected with Pichinde virus. Cells were labelled before as well as after primary infection with Pichinde virus (Farber & Rawls, 1975). Prescription bottles of 32 oz were seeded with 1 x 10^7 suspension-grown BHK21/13S cells containing WPM medium (Pulkkinen & Pfau, 1970) without tryptose phosphate broth (TPB). After 24 h 0.5 mCi of 3H-uridine (25 to 40 Ci/mmol) was added to each bottle. All subsequent procedures used WPM plus TPB. Forty-eight h after seeding, the confluent monolayers were infected with 10 ml of Pichinde virus at an m.o.i. of 3. After adsorption for 1.5 h at 37 °C, the monolayers were washed three times with 5 ml volumes of medium. The final replacement medium contained 0.4 mg (5 μg/ml) of arabinosyl cytosine hydrochloride (araC). One h later 1 mCi of 3H-uridine was added to each bottle. The same procedure was used for labelling persistently infected cells, except that 48 h after seeding, monolayers were not challenged with plaque-forming Pichinde virus.

Concentration and purification of standard and DI Pichinde virus. Twenty-four h after the last addition of 3H-uridine to freshly or persistently infected BHK cultures the extracellular fluids were decanted, clarified by centrifugation (1500 g for 8 min at 4 °C) and kept at 4 °C. The monolayers were then treated for 10 min at 37 °C with 10 ml of ‘BHK’ trypsin solution (0.21 % 1:300 trypsin in WPM minus TPB and serum). The dispersed cells were then either centrifuge-washed (as above) with three 20 ml vol. of medium, or immediately combined with the extracellular fluids which were centrifuged once. The fluids containing the extracellular virus, the trypsin-released cell associated virus, or the combination were then used as the starting material for virus concentration and purification exactly as described by Carter, Biswal & Rawls (1973).

Measurement of buoyant density. The refractive index of fractions from the continuous density gradients was measured at 20 °C using an Abbe-3L refractometer (Bausch & Lomb, Rochester, New York).

RNA isolation from purified virus. Fractions from continuous 20 to 50% sucrose density gradients containing the highest amount of radioactivity were pooled (6 fractions for DI virus – 4 for standard) and used in the RNA extraction procedure described by Farber &
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Rawls (1975). Final recovery of radioactivity was about 10% of the input but this was routinely increased to 30% by addition of 100 μg of wheat germ RNA (essentially 18S and 25S) at the time of the first ethanol precipitation to the virus processed from each 32 oz prescription bottle.

Polyacrylamide gel electrophoresis (PAGE). 2.3% bis-acrylamide cross-linked gels were prepared according to the method of Bishop, Claybrook & Spiegelman (1967). The gels were then pre-run in a Bio-Rad model 300 electrophoresis cell (cooled by circulating tap water) for 30 min at 10 mA/tube using a Heathkit model IP-17A power supply. Fifteen to 100 μl of RNA (carrier plus virus recovered from one 32 oz bottle) were added to each gel and electrophoresed for 1 to 1.5 h using 10 mA/gel. The RNA samples were suspended in TNE buffer plus 0.5% SDS (Farber & Rawls, 1975), 10% glycerol, and 0.01% bromophenol blue. Following electrophoresis, gels were sliced into 2 mm fractions in a Gilson (Middletown, Wisconsin) gel fractionator, model B100/GMA/GCB. Each isotopically-labelled minced gel slice was flushed out of the apparatus into a scintillation vial with three 0.1 ml pulses of 10% Biosolv BBS-3 (Beckman Instruments, Palo Alto, California). After overnight incubation at 37°C in tightly capped vials, the fractions were analysed for radioactivity.

Radioactivity determinations. The scintillation fluid in which various samples were assayed was toluene containing TLA Fluorolloy (Beckman Instruments, Palo Alto, California) and either 3% (for 10 μl samples from sucrose density gradients) or 10% (for PAGE slices) BBS-3. The low potassium scintillation vials, containing 10 ml fluid, were counted at about 40% tritium efficiency in a Beckman LS-150 scintillation counter. The crossover of 14C into the 3H channel was 10.3% and all 3H data was corrected for this as well as background.

Reagents. Acrylamide, bis-acrylamide, ammonium persulphate, and TEMED were purchased from Bio-Rad, Richmond, California. DEAE-dextran (mol. wt. approx. 500000) was obtained from Pharmacia, Uppsala, Sweden. SDS (BDH-specially pure) was purchased from Gallard-Schlesinger, Carle Place, New York. PEG 6000 was obtained from Union Carbide Corp., New York, N.Y. 2-14C-uridine (43 mCi/mmol) was from Schwarz/Mann, Orangeburg, New York. AraC was a Nutritional Biochemicals Corp. (Cleveland, Ohio) product. 5-3H-uridine was purchased from New England Nuclear, Boston, Massachusetts or Amershams/Searle, Arlington Heights, Illinois.

RESULTS

Progression of Pichinde virus infection in cell culture

Suspension cultures of BHK21/13S cells were followed for over 150 generations after infection with Pichinde virus (Fig. 1). The initial peak in extracellular plaque-forming Pichinde virus, as well as total infective centre expression, was reached four generations p.i. While the previously studied cultures, persistently infected with LCM or Parana viruses, ceased to produce p.f.u. by about 50 cell generations after infection (Staneck et al. 1972) the present culture did not (Fig. 1a). The cell densities of the former cultures were controlled by dilution with centrifuge-washed cells but the latter culture was maintained by simple dilution. The centrifugation technique effecting a virtual complete medium change was indeed necessary for p.f.u. turn-off. This was shown with the Pichinde culture in the following way: a fresh culture was started from cells frozen at the 35th generation p.i. Until the 59th generation the cells were centrifuge-washed prior to dilution. At this time another culture was started from these cells and maintained by dilution alone. Shut-down of
Fig. 1. Establishment of Pichinde virus persistent infection in a BHK21/13S suspension culture. 
(a) Shows two types of cultures. One (—) was infected with virus and maintained uninterrupted
for 60 cell generations. The other (—) was started from the above cells that were stored in liquid
nitrogen at the 35th generation p.i. The percentage of the cell population scoring as infective
centres (●) and the extracellular p.f.u./cell (△) were determined in these cultures at specific intervals.
The initial culture was maintained by dilution alone ( ), while in the second culture this was
accomplished by centrifuge-washing the cells prior to dilution ( ). (b) Shows the continuation of
the culture started at the end of the time period represented in (a). (c) Shows the characteristics
of the same culture as in (b) but maintained only by the dilution method.

p.f.u. synthesis was achieved only by the centrifuge-washed cells (Fig. 1 b). Eight generations
after this the culture maintained by dilution still displayed a pattern of cycling p.f.u.
synthesis (Fig. 1 c).

Characteristics of persistently infected BHK21/13S cells

When Pichinde-BHK cells (persistently infected cells once infective centre and extra-
cellular virus activity could no longer be detected) were substituted for normal cells in our
plaque assay procedure (Pulkkinen & Pfau, 1970) the efficiency of plating (EOP) of standard
Pichinde virus and the UBC and CA1371 strains of LCM virus was found in each instance
to be less than 10-4. This homotypic and heterotypic virus exclusion was consistent with the
properties of other arenavirus persistently infected cells (Staneck et al. 1972). However, the
Pichinde-BHK cells did appear different from the other two persistently infected cell lines
in the following ways: although standard virus could not be detected by direct assay in the
medium from the LCM- or Parana-BHK cells, re-cultivation of cells stored in liquid nitrogen
or infection of normal cells with the cell-free medium would initiate p.f.u. synthesis
(Staneck et al. 1972). Neither technique would elicit p.f.u. from Pichinde-BHK cells. Even
though haemocytometer counts indicated that infection of a culture with Pichinde virus
did not alter the cell division rate, a more rapid drop in pH combined with extensive cell
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Fig. 2. Density gradient centrifugation of \( ^{14}\text{C}-\text{uridine} \) standard- and \( ^{3}\text{H}-\text{uridine} \) DI-Pichinde-virus. Partially purified virus (1.0 ml) was layered on top of a 40 ml 20 to 50 % continuous sucrose gradient. The virus was centrifuged in a Spinco SW39 rotor at 35000 rev/min for 2 h at 4 °C. □--□, plaque-forming virus; ○--○, DI virus; △--△, density.

debris (not seen with LCM- or Parana-BHK cells) suggested that the division rate might have been greater, but due to a fortuitous balance in cell lysis, it appeared the same as in control cultures.

Biological activity of Pichinde DI virus

As shown with the LCM (Welsh et al. 1972) or Parana (Staneck & Pfau, 1974) systems, Pichinde DI virus had no effect on the synthesis of heterologous (vesicular stomatitis) virus. However exposure of L cells to Pichinde DI virus prior to infection with standard homotypic virus markedly reduced both infective centre number (measured immediately after the virus adsorption period) and the 24 h extracellular yield. In all experiments control cultures not receiving DI virus were mock-infected with media containing the same concentration of DEAE-dextran (50 μg/ml) as used in the DI, as well as standard virus preparations. Without DEAE-dextran little biological activity of the DI virus could be demonstrated. Tissue culture fluid from persistently infected cells was clarified by low speed centrifugation and used as the source of DI virus. In some experiments DI virus was concentrated by high speed centrifugation (Welsh et al. 1972). An as yet unexplained phenomenon was the invariable finding that the biological activity of DI virus was enhanced by removing it from the harvested tissue culture medium, i.e. by high speed centrifugation and resuspension of virus with fresh medium to the original volume.

Buoyant density of Pichinde DI virus

After PEG precipitation of DI or plaque-forming Pichinde virus from equal volumes of tissue culture fluid, the resuspended pellets were placed on the top of discontinuous 20 to 50 % sucrose gradients. After centrifugation, a single band was visible with a midpoint
Fig. 3. Biological activity of Pichinde DI virus after velocity sedimentation through a continuous sucrose gradient. To each 0.3 ml fraction was added 0.7 ml of growth medium containing 5 μg DEAE-dextran. These served as inocula for a plaque-forming virus interference assay using BHK21/13S cells. In a separate experiment various concentrations of sucrose were mixed with a sucrose-free DI preparation that would lower the 45 h post-challenge yield from $6.1 \times 10^7$ to $1.6 \times 10^4$ p.f.u./ml. While the highest concentration of sucrose used, 15%, had only a slight effect on control p.f.u. yield (a 20% reduction) its presence in the DI preparation reduced the challenge virus titre to only $2.1 \times 10^5$ p.f.u./ml. Sucrose concentration was then plotted against the factor necessary to correct the observed inhibition to that obtained with sucrose-free DI virus. A factor was then extrapolated for each gradient fraction. •—•, actual data; □—□, corrected data.

PAGE analysis of Pichinde standard virus RNA

A reference profile of plaque-forming virus RNA was established by electrophoresing in 2.3% polyacrylamide gels for 1 h. Five distinct bands were observed (Fig. 4). Assuming our

2 mm below the original interface between the two sucrose solutions. The midpoint of each band was about 8.0 mm from the bottom of the tubes but the widths were different – 2 mm for plaque-forming virus and 5 mm for DI virus or the combination of both viruses. Bands from $^3$H-DI virus and $^{14}$C-plaque-forming virus were collected from these gradients, and mixed prior to addition to the final continuous sucrose gradient. No visible band was apparent after centrifugation. As can be seen in Fig. 2, the profile of the radioactivity from the DI virus preparation was very broad and encompassed the peak formed with the standard virus. Although biological activity of DI virus could not be detected in these $^3$H-labelled preparations, a well defined profile (Fig. 3) could be demonstrated when extracellular and cell-associated virus was harvested and concentrated from a 50-fold greater quantity of persistently infected cells. Even though sucrose was shown to mask DI activity, all measurements from the gradient fractions had to be performed in its presence since further pelleting of the virus led to complete loss of activity. When a masking factor which became greater with increasing sucrose concentration was applied to the DI activity in each gradient fraction, the profile became broader (Fig. 3) and more closely resembled that determined by radioactivity.
Fig. 4. PAGE analysis of $^3$H-uridine-labelled RNA from plaque-forming Pichinde virus preparations. RNA was extracted from the virus by treatment with sodium lauryl sarcosinate and phenol in the presence of dithiothreitol. The $^{14}$C-marker RNA used in all PAGE analyses presented here was extracted from normal BHK21/13S cells according to the procedure of Farber & Rawls (1975).

$^{14}$C-uridine BHK cell ribosomal RNA had sedimentation constants of 28 and 18S and mol. wt. of 1.7 and 0.7 × 10^6, the size of the virus RNA components was calculated by using the linear relationship between the logarithm of the mol. wt. of the RNA and the relative electrophoretic mobility (Bishop et al. 1967). These were 31, 28, 15–18 and 4–6S. If electrophoresis was carried out for 1.5 h, instead of 1 h, so that the 4–6S RNA migrated off the gels, two distinct peaks were observed at 15 and 18S (data not shown). Standard as well as DI virus for these experiments was routinely obtained from cells treated with araC at the time of infection. While this antimetabolite caused the BHK21/13S cells to swell to 2 to 3 times their normal size, it had no effect on the synthesis of the virus (for LCM virus data see Campbell et al. 1968). Under these conditions uridine incorporation into Pichinde virus, as measured in the extracted RNA, increased by about fivefold. Furthermore, the radioactivity in extracted RNA of cell-associated virus was double that of extracellular virus. The RNA profiles of cell-associated-, extracellular-, and araC grown-virus were essentially the same (Dutko, 1975).

**PAGE analysis of Pichinde DI virus RNA**

None of the DI RNA preparations analysed under the same conditions as above contained two of the components seen in the standard virus preparations, namely the 22 and 15S species (Table 1, Fig. 5 and 6). However, two types of profiles were found (Table 1). The first was from DI virus synthesized up to the 175th cell generation after the initial
TABLE I. The RNAs of Pichinde virus as a function of the number of cell generations after infection

<table>
<thead>
<tr>
<th>Fig. no.</th>
<th>Cell generations p.i. activity*</th>
<th>Sedimentation constants (S) of RNAs*</th>
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<tr>
<td></td>
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<td>Cell Sedimentation constants (S)</td>
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<td>4</td>
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<td>185</td>
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<tr>
<td>6</td>
<td>205</td>
<td>- - + - - + + + + + + + + + + +</td>
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* + = present; - = absent; ? = questionable.

Fig. 5. PAGE analysis of 3H-uridine-labelled RNA extracted from DI Pichinde virus synthesized in a BHK21/13S culture that had been maintained for 165 generations.

infection, and appeared to be almost the same as that of standard virus except for the two missing RNAs stated above. The second type of RNA profile was from DI virus synthesized after the 175th cell generation. This RNA seemed to progressively lose the 31S species (Table I) and acquire a heretofore unrecognized 20S RNA (Fig. 6). No increase in resolution of the various RNA fractions was noted when any of the above DI RNA preparations were electrophoresed for longer periods so that the 4–6S RNA migrated off the gels.

**DISCUSSION**

Establishment of the Pichinde persistently infected BHK cultures (Pichinde-BHK cells) appeared to proceed in a manner similar to Parana virus (Staneck et al. 1972). The gradual decrease in plaque-forming activity seen with both these Tacaribe complex viruses was markedly different from the steep peaks and troughs of p.f.u. titre observed in the progression of the LCM long-term cultures (Staneck et al. 1972). The disappearance of plaque-forming
Pichinde DI virus RNAs

Fig. 6. PAGE analysis of $^3$H-uridine-labelled RNA extracted from DI Pichinde virus synthesized in a BHK21/13S culture maintained for 205 generations after infection with plaque-forming virus.

The Pichinde DI virus found in the medium surrounding the persistently infected BHK suspension cells behaved like standard virus in its density, and requirement of DEAE-dextran for efficient adsorption to cells (Mifune et al. 1970).

PAGE analysis of standard Pichinde virus revealed six species of RNA with sedimentation coefficients of 31, 28, 22, 18, 15 and 4–6S. The number of species isolated agrees with those reported for Pichinde virus by Rawls and co-workers (Carter et al. 1973; Farber & Rawls, 1975). The proportionality between the species, however, did not fit any published pattern. Even though the significance of such comparison remains obscure, it is worth noting the spectrum of results thus far obtained. Using PAGE analysis of standard Pichinde virus RNA preparations, Carter (1972) initially found no 31 or 15S RNAs. In 1973 Carter et al. reported a 31S RNA in Pichinde virus confirming the results of Pedersen (1971) who had found a 32S RNA in LCM virus preparations. Later Farber & Rawls (1975) detected the presence of the 15S species. Our gel patterns showed a relatively small amount of radioactively labelled 31S RNA (as did the DI virus RNA profiles) and a large amount of 15S RNA. The cells were then checked for contamination, since comparison of PAGE profiles of uridine-labelled RNA from normal and mycoplasma-infected tissue cultures can clearly demonstrate the presence of 23 and 16S mycoplasma RNAs (Schneider, 1975). That the 22 and 15S RNAs (Fig. 4) were virus-specific was shown both by negative PPLO culture tests (Zgorniak-Nowosielska et al. 1967) of virus stocks and persistently-infected as well as normal cell lines; and the absence of 23 and 16S species in ribosomal marker RNA prepared from virus-free cell lines. Preliminary experiments (F. Dutko,
unpublished observations) indicate that the amount of 15S RNA observed in Pichinde preparations depends on the number of passages of the virus in specific cell types. Other factors possibly involved in the degree of labelling of this and other RNA species may depend on the input m.o.i., and the time of addition of label, i.e. whether the various species of virus-specific RNAs are made at different times. With respect to the latter point, it is still not resolved whether the genome of Pichinde (or LCM) is segmented, or broken during extraction at specific weak points to give the observed species.

The synthesis of DI particles has been reported for most viruses, with all those biochemically studied lacking non-random genome segments (Huang, 1973). Since the interfering activity of LCM and Parana DI virus was much more resistant to U.V. inactivation than standard virus (Welsh et al. 1972; Staneck & Pfau, 1974), it was not unexpected that Pichinde DI virus would have one or more species of RNA missing. In fact two classes of particles were found (Table 1). While both lacked the 22 and 15S RNAs, there was a progressive loss, as the culture aged, of the highest mol. wt. virus-specific 31S piece. Relevant to this may be the suggestion of Frenkel et al. (1975) that continued passage of defective herpes simplex virus populations would lead to the selection of ‘terminal’ defective molecules containing only those features necessary for their replication. Since none of the species of Pichinde virion-associated RNAs that were presumably ribosomal in origin were missing in either class of DI preparations, it is possible that functional DI particles require ribosomes in order to interfere with the synthesis of standard virus. On the other hand it could be that any piece of virus-specific RNA within the budding virus particle controls the incorporation of the ribosomes. If the genome of Pichinde virus is indeed segmented, the RNA profile of its DI virus does not exactly fit the pattern established with the known viruses in this category. Defective interfering reovirus (9 segments) and influenza virus (5 to 6 segments) both lack the largest piece of RNA (Nonoyama & Graham, 1970; Pons & Hirst, 1969); i.e. the largest piece of the Pichinde genome is lost but it is the last of three pieces to disappear.

Welsh et al. (1975) were also able to purify, with retention of biological activity, LCM DI virus harvested from persistently infected cultures. They confirmed the presence of 31, 28, 23, and 18S RNA in the standard virus (Pedersen, 1971) but could find little, if any, difference in the RNA profile of the DI virus. The less stringent control of plaque-forming virus synthesis in LCM-(Staneck et al. 1972) compared with Pichinde- persistently infected cells possibly might have led to preferential incorporation of isotope into a small amount of standard virus that usually escapes detection unless injected into neonatal mice (Welsh et al. 1972).

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REFERENCES


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