Bot. Bull. Academia Sinica **14**: 136-150 (1973)

CYCLIC PRODUCTION OF VESICULAR STOMATITIS VIRUS CAUSED BY DEFECTIVE INTERFERING PARTICLES[1][2]

EDUARDO L. PALMA[3] and ALICE S. HUANG[3]

Channing Laboratory and Department of Medical Microbiology, Boston City Hospital

and

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts, U.S.A.

Abstract

Purified standard and defective interfering (DI) particles of vesicular stomatitis virus (VSV) were reconstituted and the mixture continuously passaged for six 24-hour growth periods on Chinese hamster ovary cells in suspension cultures The cultures were monitored for cellular viability, for intracellular virus-specific RNA, for total extracellular viral particles and for production of plaque-forming standard VSV. During the six passages, there was a cyclic, overlapping pattern of synthesis of the two viral particles; decline in the production of standard virus was always preceded by the presence of a large amount of DI particles. Intracellular virus-specific RNA synthesis reflected the pattern of production of extracellular particles. Of particular interest was the accumulation in cells of viral antigens as nucleocapsids containing the RNA of DI particles, when DI particles were being produced in large quantities. Also, under very specific conditions, infected cells survived longer than usual and were resistant to superinfection by standard VSV. Preliminary evidence indicates that this resistance was due to surface interference. These studies show that a wide variety of different virus-host interactions can occur simply by varying the total concentration of viral particles and the relative concentrations of standard and DI particles in these preparations.

Introduction

Almost every major animal virus group has been shown to be able to generate a class of defective particles which are capable of interfering with

(1) This work was supported by research grants from the United States Public Health Service and the American Cancer Society.

(2) In honor of Dr. H. W. Li on his retirement. His friendship and support of overseas Chinese biologists will remain in the hearts of all of us who had the great pleasure of associating with him.

(3) E. L. P. is a research fellow on leave from the University of Buenos Aires, Argentina. A. S. H. is a Research Career Development Awardee of the United States Public Health Service.

the growth of homologous non-defective, standard virus and at the same time be dependent on standard virus for their own multiplication (Huang, 1973). This class of viral particles has been called defective interfering (DI) particles and their presence in viral populations has been proposed as a possible determining factor in the outcome of viral diseases (Huang *et al.*, 1970a).

The best characterized viral system for investigating DI particles is vesicular stomatitis virus (VSV). Its DI particles, containing one-third of the genome (DI-T), can be purified and isolated free of the standard B particles (Huang *et al.*, 1966; Hackett *et al.*, 1967; Stampfer *et al.*, 1971). Interference with the growth of standard VSV by its DI-T particles is specific for the viral serotype and is not mediated by interferon (Huang *et al.*, 1966b; Huang *et al.*, 1972). Interference caused by DI-T particles occurs intracellularly at the stage of virion RNA replication and not during transcription (Huang *et al.*, 1972; Perrault *et al.*, 1972).

Knowing the interdependence of standard and DI particles, it has been predicted that in a system where host cells are unlimited and no other factors influence the production of virus, the production of standard and defective particles would be best described by two wave functions with overlapping frequencies (Huang, 1973). These experiments have been designed to see whether such a pattern of synthesis of viral particles is produced and whether DI particles cause the cyclic production of standard virus. Such cyclic synthesis of standard virus may provide a model for understanding the various outcomes resulting from viral infection.

The growth of VSV and the interations between its standard and DI particles in a complete host organism would be extremely complex because of multiple host defenses *in vivo*. In order to study the interaction of the two viral particles in the absence of host defenses, cells in culture were used as the host system. Under these conditions, the results show that not only was there cyclic production of standard and DI particles with an overlapping pattern for each of the particles, but that the gradual fall in titer of standard virus was always in the presence of a high concentration of DI particles. Also, depending on the relative concentration of standard and DI particles, some infected cells maintained a resistance to super-infection by VSV and other cells contained a larger amount of intracellular viral nucleoprotein antigens.

Materials and Methods

Virus and cells

Chinese hamster ovary (CHO) cells and cloned VSV of the Indiana serotype were used in all of these experiments. The growth and maintenance of suspension cultures of CHO cells were described in detail (Stampfer *et al.*, 1969).

The growth, purification and plaque assay of standard B particles of VSV were done according to previously published procedures (Stampfer *et al.*, 1969; Stampfer *et al.*, 1971). DI-T particles of VSV were made in chick embryo cells or CHO cells by coinfection of cells with DI-T and B particles. The infection of both types of cells for the production of DI-T particles and the purification of the particles by differential and rate zonal sucrose gradient centrifugations have been described (Huang *et al.*, 1966a; Stampfer *et al.*, 1969). Tritium-labeled B particles were prepared by infecting 4×10^8 CHO cells with B particles at a multiplicity of 20. After attachment at 4°C for 30 min., the infected cells were suspended in 200 ml of medium and incubated at 34°C for 10 hrs. ^{3}H-uridine at 10 μC/ml was added after 1 hr. at 34°. VSV was harvested from the medium and purified by differential centrifugation. This gave the lowest particle to infectivity ratio (100:1) of VSV preparations made in this laboratory.

Procedures

Buffers and solutions used in these experiments have all been described in detail (Huang *et al.*, 1970b). They are: reticulocyte standard buffer (RSB), reticulocyte standard buffer without $MgCl_2$ and containing ethylenediaminetetraacetate (NEB), sodium dodecyl sulfate (SDS) buffer containing 0.5% SDS, and Earle's saline. Cytoplasmic extracts of CHO cells were made with 1% Nonidet P-40 as described (Huang *et al.*, 1972). Sucrose-gradient centrifugations were done in a Beckman L2-65B or L3-50 ultracentrifuge under the conditions specified for each experiment. Fractions of sucrose gradients were collected, precipitated with trichloroacetic acid and the radioactivity assayed as previously described (Huang *et al.*, 170b; Huang *et al.*, 1972).

Materials

Radioisotopes were obtained from New England Nuclear Corporation, Boston, Mass., with the following specific activities: uridine -5-H^3 (28.5 mCi/mM), uridine -5-6-H^3 (40.4 mCi/mM) and uridine -2-C^{14} (52.7 μCi/mM). Actinomycin D was a kind gift from Merck Sharp and Dohme, Rahway, New Jersey. Trypan blue stain was purchased from Grand Island Biological Co., Grand Island, New York.

Results

Production of virus upon continuous passage

To measure the effect of continuous viral passage on the production of standard VSV a culture of cells infected with B and DI-T particles of VSV were passaged every 24 hr by making a 1:9 mixture of infected cells and fresh uninfected cells. Just before making such mixtures an aliquot was withdrawn for VSV plaque formation on CHO cells. Figure 1 shows the gradual decline

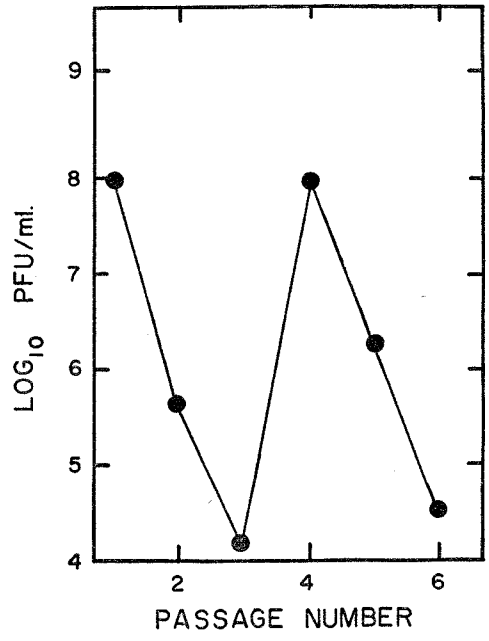

Fig. 1. Plaque assay of B particles produced during successive passages of B and DI-T particles in CHO cells. A first passage of a mixture of B and DI-T particles was made by infecting 4×10^7 CHO cells suspended in 10 ml of medium with B particles at a multiplicity of 20 and DI-T particles at an estimated multiplicity of 3. After 30 min of attachment at room temperature the cells were diluted to 100 ml and incubated at 37°C for 20 hr. Successive passages were made by resuspending 4×10^7 fresh CHO cells with 10 ml of infected cells from the previous passage. After attachment at 4°C for 30 min the cells were diluted to 100 ml and further incubated at 37°C for 20 hr. At the end of each passage, an aliquot was withdrawn, the cells pelleted by centrifugation at $600\times g$ for 7 min. and the medium assayed on monolayers of CHO cells for plaque-forming B particles.

in infectivity over three passages. At the fourth passage, however, there appeared to be a burst of synthesis of standard virus, which was again followed by a gradual decline in titer upon two more successive passages.

The decline during the first three passages are reminiscent of the von Magnus phenomenon which was originally described for influenza virus (von Magnus, 1954) and subsequently also for VSV (Bellett *et al.*, 1959). The burst of synthesis at the fourth passage was first seen for this strain of VSV in CHO cells (Huang *et al.*, 1970a). It appears from Figure 1 that within six passages of 24 hr each, the titer of standard VSV can undergo one and a half oscillations.

Radioactive labeling of standard and DI particles produced during continuous passage

To determine whether the oscillations in the production of standard virus were due to the production of DI-T particles, aliquots of infected cells were treated with actinomycin and then labeled at each passage for 24 hr with H^3-uridine. Actinomycin was added to these cells in order to insure that the precursor was incorporated only into virus-specific RNA (Stampfer *et al.*, 1969). Figure 2 shows the incorporation of H^3-uridine into total virus-specific intracellular RNA and into total extracellular viral particles. Intracellular RNA synthesis appeared to reflect viral production. However, the peak of production

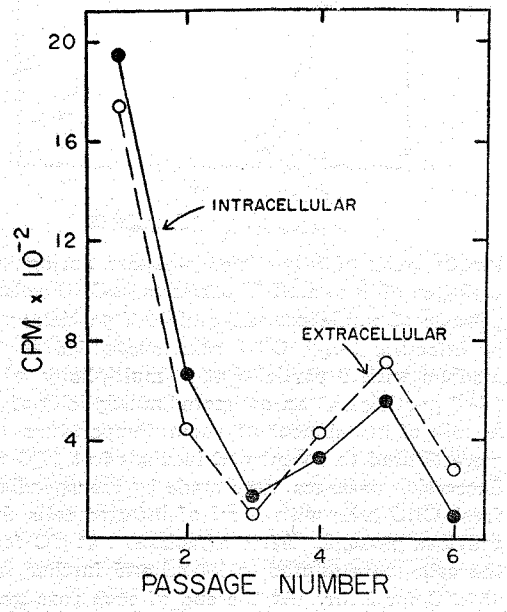

Fig. 2. Distribution of virus-specific RNA into intracellular and extracellular material during successive passages of B and DI-T particles. Aliquots of CHO cells were infected identically to the cells shown in Figure 1, except that actinomycin at a final concentration of 5 μg/ml was added to the concentrated cells (10 ml) during the attachment period and later ^{3}H-uridine at a final concentration of 10 μC/ml was added to cells at the beginning of incubation at 37°C. After each passage cytoplasmic extracts of all the cells in each aliquot were made and 1/100 of the total cytoplasmic extracts were acid-precipitated as a measure of intracellular virus-specific material. The extracellular viral particles from each passage was pelleted from the medium by centrifugation at 106,000 × g for 30 min at 4°C and resuspended in 1 ml of Earle's saline. Extracellular virus-specific material represent 100% of the total pelleted viral particles.

of total extracellular viral particles did not coincide with the production of standard virus (c. f. Fig. 1 and 2). This was best demonstrated by passages four and five where total extracellular viral particles peaked at passage five, but total plaque-forming B particles peaked at passage four. These results suggest the presence of non-plaque-forming viral particles in passage five.

To determine if the excess label was indeed in DI–T particles, the extracellular material was concentrated by centrifugation, treated with SDS and layered over sucrose density gradients. The amount of uridine-labeled standard and DI–T RNA species was estimated by their sedimentation rates as previously determined (Huang *et al.*, 1966c; Stampfer *et al.*, 1969). The relative concentrations of DI–T and standard RNA genomes reflect the relative amount of DI–T and standard particles, which is presented in Figure 3. There was an overlapping cyclic pattern for the production of both DI–T and standard particles. The decrease in synthesis of standard virus was preceded by a

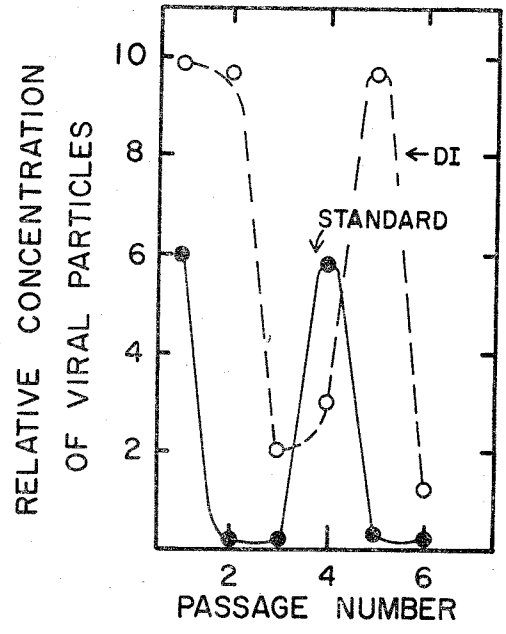

Fig. 3. Relative concentration of B and DI-T particles during successive passages. Extracellular ^{3}H-uridine-labeled viral particles from each of the passages shown in Figure 2 were deproteinized with SDS and separated on a 15-30% sucrose-SDS gradient (Stampfer *et al.*, 1969). The relative radioactivity sedimenting at 40S and at 19S representing RNA's of B particles and DI-T particles respectively are graphed here. The relative cpm in DI-T RNA was not multiplied by three and, therefore, reflects the minimum number of viral particles which contain onethird the RNA contained in B particles (Huang *et al.*, 1966 c.).

high concentration of DI-T in passages one and two. At passage three, the low concentrations of both particles prevented dual infection of cells by DI-T and standard particles and, therefore, DI-T particles were further reduced due to the lack of helper standard virus. This resulted in a situation where standard virus was no longer inhibited by DI-T because cells were infected by standard virus alone. Therefore, the interdependence between these two classes of viral particles can cause cyclic production of viral particles.

Effects of the cyclic production of viral particles on host cells

To determine whether such cyclic production of viral particles has any effect on the viability of host cells, the infected CHO cells, which had not been exposed to actinomycin, were stained at the end of each 24 hr-passage by trypan blue. Viability of the cells was determined by dye exclusion. Figure 4 shows that the percentage of viable cells at the end of each viral passage changed depending on passage number. It was surprising to find that at

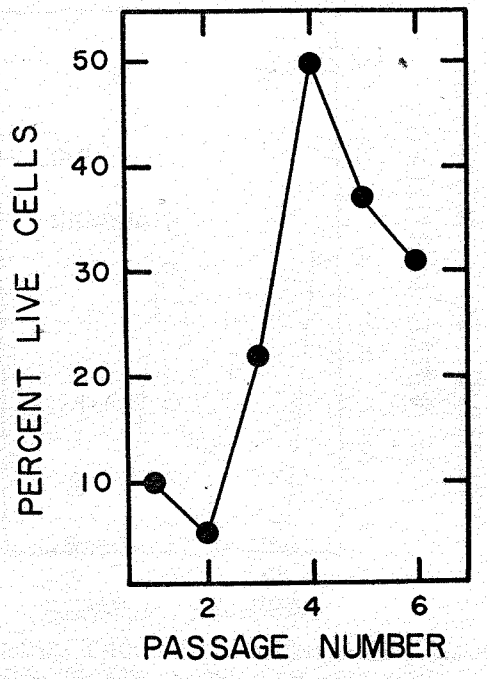

Fig. 4. Survival of host cells during successive passages of B and DI-T particles. At the end of each passage shown in Figure 1, an aliquot of cells was mixed in equal volume with undiluted trypan blue and the cells were counted in a hemocytometer chamber. Percent survival equals

$$\frac{\text{No. of unstained cells}}{\text{No. of unstained cells} + \text{No. of stained cells}} \times 100$$

The total number of cells expressed in the denominator did not vary significantly from passage to passage.

passage four, 50% of the cells excluded trypan blue when the medium surrounding the cells contained 10^8 pfu/ml of standard VSV.

To test whether these cells at passage four were all infected with VSV, they were superinfected with purified standard VSV at a multiplicity of 20 and as a measure of virus penetration and growth virus-specific RNA synthesis in these cells was monitored. Cells from passage four which were not superinfected synthesized some virus-specific RNA but not in the quantity synthesized by fresh CHO cells that were infected with VSV at multiplicity of 20 (Fig. 5).

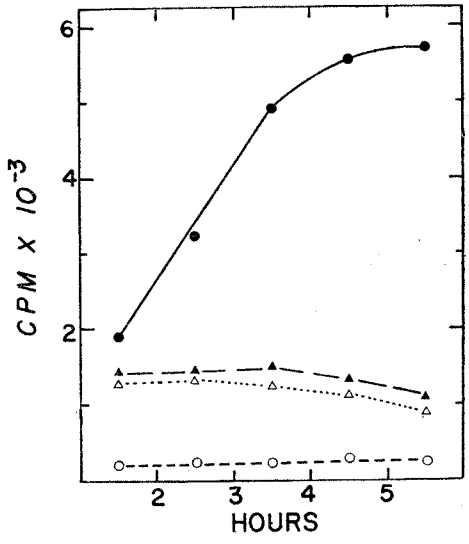

Fig. 5. Actinomycin-resistant RNA synthesis by viable cells from passage 4. Infected CHO cells from passage 4 shown in Figure 1 were washed 1× with PBS-EDTA and 3× with medium by repeated centrifugation at 300 ×g for 3 min at 4°C. The resultant pellet of cells contained 95% viable cells when measured by exclusion of trypan blue. Two 1 ml aliquots of these viable cells, each containing 4×10^6 cells, were prepared. One aliquot was superinfected with B particles at a multiplicity of 20. Two other 1 ml aliquots of 4×10^6 fresh CHO cells were prepared under the same conditions as the cells from passage 4 and one was infected with B particles at a multiplicity of 20. All 4 samples were treated with 5 μg of actinomycin and incubated at 22°C for 30 min. Then another ml of medium containing 0.6 μC of ^{14}C-uridine was added and the incubation temperature elevated to 37°C. Aliquots of 0.1 ml were removed at hourly intervals beginning at 30 min after incubation at 37°C and assayed for acid-precipitable radioactivity as previously described (Huang *et al.*, 1972). (△—△) Incorporation by cells from passage 4; (▲—▲) incorporation by cells from passage 4 superinfected with B particles; (○—○) incorporation by fresh CHO cells; (●—●) and incorporation by fresh CHO cells infected with B particles.

Superinfection of the cells from passage four did not show an increase of viral RNA synthesis over that produced by the cells without superinfection (Fig. 5). These results suggest that not only were all of the cells at the end of passage four infected, but that they were also resistant to superinfection with VSV. Further incubation of these resistant cells from passage 4 resulted in eventual cell death.

Alteration of cell surfaces by standard and DI particles to superinfection

The previous experiment may indicate a true resistance of cells to superin fection or the resistance may be due to nonspecific cytoxicity exerted by VSV during exposure of these cells to VSV for 24 hours. To test whether infection of cells with standard and DI-T particles can produce cells which become resistant to superinfection, fresh CHO cells were infected with DI-T and standard particles at a ratio of 2:3 and their resistance to superinfection was measured by determining whether superinfecting, labeled virus, became cell-associated or not. Figure 6 shows that within 1.5 hr after infection with standard and DI-T particles, the cell surface was altered enough to prevent 50% of the labeled virus from attaching and penetrating at 37°C. Although the resistance to superinfection was not complete, this experiment indicates that at certain concentrations of DI-T and standard particles, cells develop resistance to superinfection by homologous standard virus.

Production of DI-T particles is related to build-up of intracellular nucleocapsids

It has been shown previously that production of DI–T particles alters the pattern of VSV-specific RNA synthesized intracellularly from the group I RNA species to the group II RNA species (Stampfer *et al.*, 1969). In summary, production of DI–T particles is correlated with the intracellular accumulation of 19S RNA, which is the size of the RNA found in DI–T particles. Because in our passages there are two instances where DI–T particles were produced in abundance, it was of interest to examine the intracellular patterns of RNA species during each of these passages (Fig. 7). At passage one there was production of both group I and II RNA species, giving a rather undistinguished pattern (Fig. 7a). At passages three, four and six, there was too little intracellular radioactivity to give any pattern except for material at the top of the gradients (Fig. 7c, d & f). Passages two and five gave the clearest group II patterns (Fig. 7b & e) and it was at these passages that there was noticeable production of DI–T particles (c. f. Fig. 3).

Because it has been suggested that interference by DI particles of measles virus results in intracellular accumulation of nucleocapsids (Nakai *et al.*, 1969), infected cells from passage 5 were examined for their content of viral nucleocapsids. Figure 8 shows that when RNA was not completely deproteinized, almost all of the 19S RNA seen in Fig. 7e was found in structures sedimenting

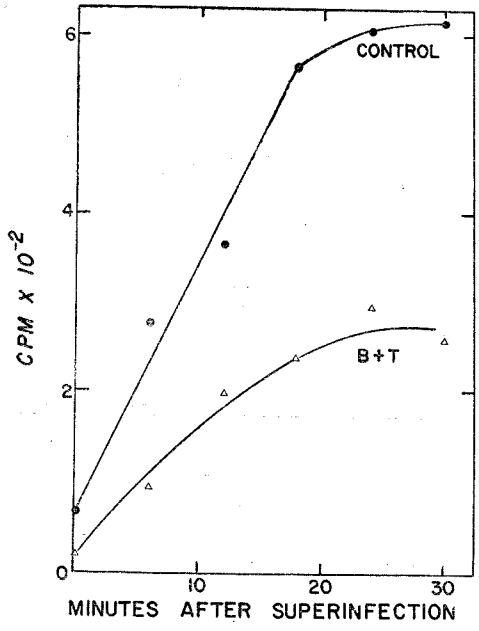

Fig. 6. Effect of pre-infection of cells with B and DI-T particles on the attachment of ^{3}H-uridine-labeled B particles. Two-1 ml aliquots containing 4×10^{6} CHO cells were prepared and one was infected with B and DI-T particles at equivalent multiplicities of 10 and 6 respectively. After attachment at 4°C for 30 min, cells were diluted to 2 ml with medium and incubated at 37°C for 1.5 hr. Then ^{3}H-uridine-labeled B particles (10^{-3} cpm/PFU) were mixed with both aliquots of cells and 0.1 ml samples were removed at the indicated times. The withdrawn samples were diluted 40× with cold Earle's saline and the cells washed 3× with Earle's saline, after which cell-associated radioactivity was collected on filters and the radioactivity measured as described (Huang *et al.*, 1972). (●—●) association of labeled B particles to CHO cells, (△—△) association of labeled B particles to CHO cells previously infected with B and DI-T particles.

at 80S. They were identified as DI-T nucleocapsids (TNC) by their resistance to ribonuclease. Because nucleocapsids are known to constitute inclusion bodies, similarly infected cells were fixed and stained with Giemsa. Diffuse granular pink staining appeared in the cytoplasm of infected cells, but no obvious inclusions were observed.

Discussion

These studies show that the interdependence between standard and DI particles of VSV resulted in an apparent cyclic production of both types of

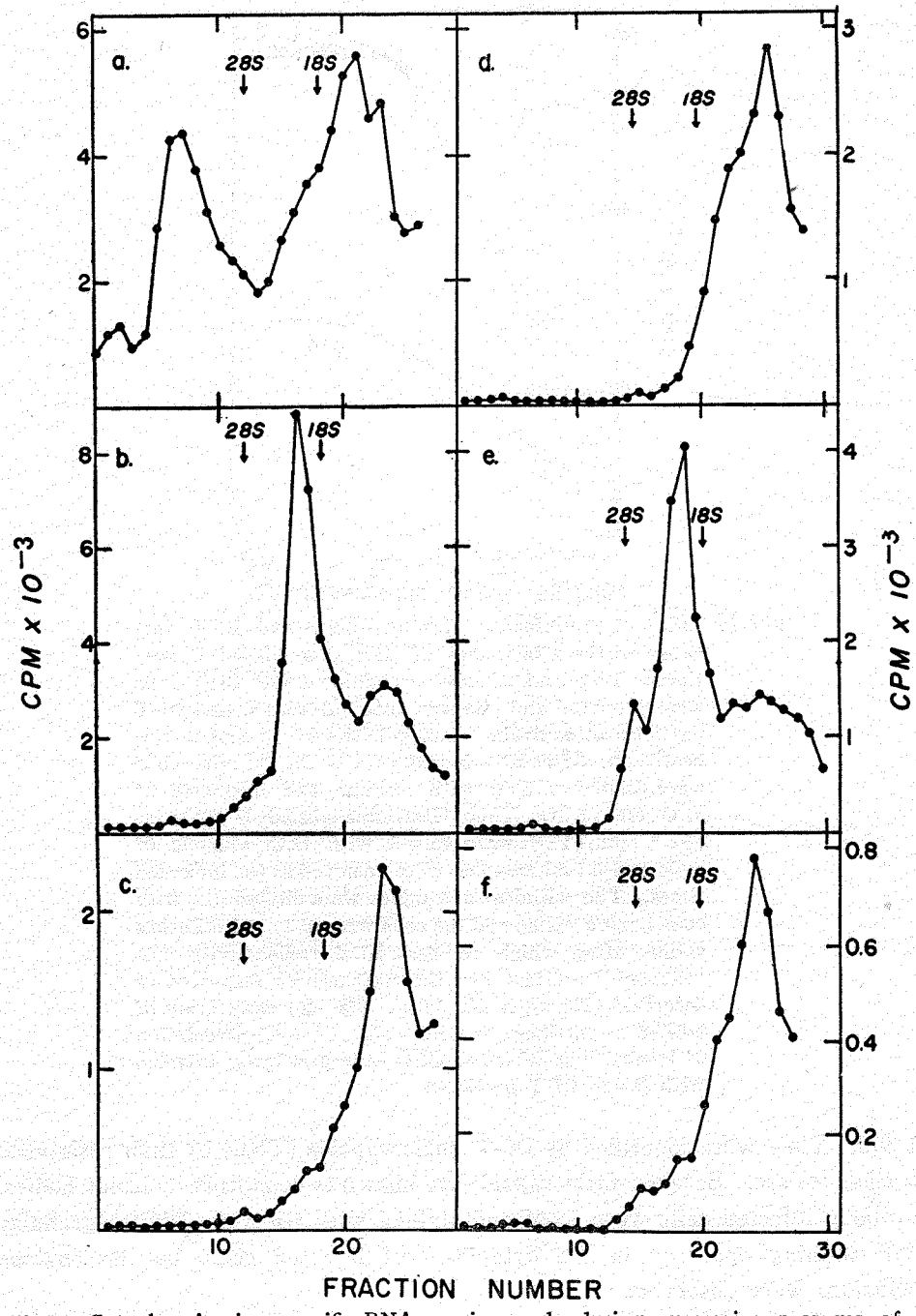

Fig. 7. Cytoplasmic virus-specific RNA species made during successive passages of B and DI-T particles in CHO cells. After each passage shown in Figure 2, 0.2 ml of the cytoplasmic extracts were treated with SDS to a final concentration of 0.5% and layered on 15–30% sucrose gradients containing 0.5% SDS. Centrifugation was in the Beckman SW 41 rotor at 78,000 × g for 16 hr at 22°C. Acid precipitable radioactivity from (a) passage 1, (b) passage 2, (c) passage 3, (d) passage 4, (e) passage 5 and (f) passage 6.

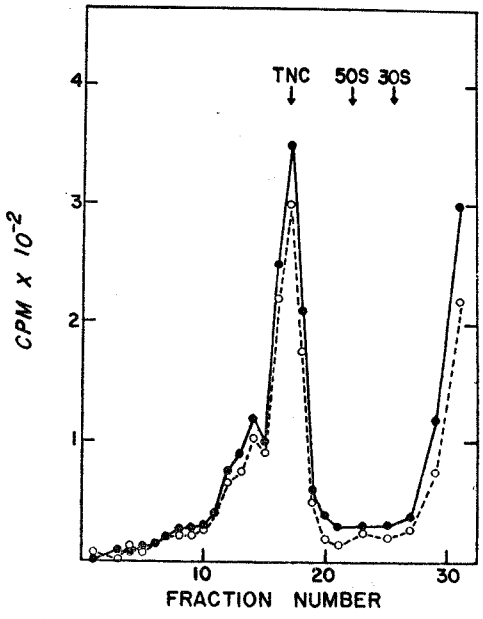

Fig. 8. Presence of nucleocapsid material in the cytoplasm of cells infected during the 5th successive passage of B and DI-T particles. 4×10^7 CHO cells were mixed with 10 ml of infectious culture from passage 4 containing 50 μg of actinomycin D. After 30 min at 37°C, 90 ml of medium containing 200 μCi of ^{3}H-uridine were added and the cells incubated further for 20 hr at 37°C. Then a cytoplasmic extract was made and sodium deoxycholate was added to a final concentration of 0.5%. The extract was layered over a 15–30% sucrose-NEB gradient and centrifuged for 6.5 hr at 96,300 × g at 4°C in the Beckman SW 27 rotor. After collecting the gradient each fraction was divided in half and one-half digested with ribonuclease as previously described. (Stampfer *et al.*, 1969). (●) acid-precipitable ^{3}H-uridine; (○) ribonuclease-resistant acid-precipitable ^{3}H-uridine

viral particles in cell culture. A rise in production of DI particles was promptly followed by a decrease in the synthesis of standard VSV. During the six continuous viral passages there was a changing dynamic interaction between 3 populations: standard virus, DI particles and host cells. The type of cellular response to infection depended not only on the total concentration of viral particles but also on the relative ratio of DI to standard particles.

These results may explain the bewildering variety of observations made on virus-cell interactions, especially when the interaction is a long term one. Persistent infections of cells in culture have been established with a variety of lytic viruses (Walker, 1968). These interactions are characterized by occasional detection of plaque-forming virus accompanied by cell destruction

or a continual low level of virus production without massive cell destruction. Many of these infected cells have intracellular viral antigens which do not elicit neutralizing antibodies. Persistent or chronic viral infection of animals can also result in occasional bursts of detectable viral particles or infectious virus, as well as continuous production of virus (Mims *et al.*, 1966; Hotchin, 1962; Rowe, 1954). Cells isolated from persistently infected animals contain no infectious virus, but complement fixing antigens. These cells also exhibit a specific resistance to infection by homologous virus.

Although variations in host response to viral infection have been postulated to be due to the immunologic capabilities of the host or to interferon production, our studies with VSV suggest that the interdependence between DI and standard particles could account for much of the observed variations. For instance, in passage 2, cell death was rapid and a large amount of intracellular nucleocapsids accummulated. In passage 3, cell death also occured, but no nucleocapsids were detected. In contrast, passage 4 had many viable cells with very little intracellular accummulation of nucleocapsids whereas passage 5 contained relatively large numbers of viable cells, containing viral nucleocapsids. Although VSV is a fairly cytotoxic virus, cells from passage 4 indicate that a state of cellular resistance to superinfection can occur under these circumstances.

Our observations were made *in vitro* in the absence of interferon and antibodies. In the whole animal, induction of antibodies or interferon by viruses and subsequent viral inhibitory properties of antibodies and interferon can result in a similar interdependent relationship with standard VSV.

Unlike DI-T particles, antibodies and host immune responses inhibit the production of all viral particles in the animal and do not discriminate between DI particles and standard virus. It has been well documented, however, that viral antigens and viral lesions occur in the presence of high levels of neutralizing antibodies (ter Meulen *et al.*, 1972; Hotchin, 1973; Johnson *et al.*, 1972). Also, interferon protected cells have not been found to contain large amounts of viral antigens. For these reasons, it is tempting to postulate that, even *in vivo*, DI particles play a major role in modulating the effects of viral infections.

There are, however, host factors which differentially affect the production of standard and DI particles. Cells from different tissues, from different genetically inbred mice or from different species differ in their ability to support interference by DI particles (Huang, 1973). In fact, this cellular ability to differentiate between the production of DI and standard particles can be very specific for one group of viruses and not others. It is postulated that the temperature of incubation may also differentially affect the synthesis of DI and standard virus (Huang *et al.*, 1970a). With VSV in CHO cells there appears

to be no marked effect on the rate of synthesis of DI particles between 31°C to 39°C (Palma & Huang, unpublished observations). This parameter, together with others such as pH, age of the host and hormonal action, should be tested for their effect on the synthesis of DI particles among a variety of viruses.

A particularly interesting cellular interaction with standard and DI particles is the one in which the infected cells remained viable for relatively long periods and contained little virus-specific RNA (Fig. 7d). These cells were resistant to superinfection by standard VSV. The result suggests that the block to superinfection resides at an early step in the virus-cell interaction. It is possible that DI particles in these cells have inhibited almost all virus-specific RNA synthesis except primary transcription by standard virions (Huang *et al.*, 1972; Perrault *et al.*, 1972; Stampfer *et al.*, 1969). Primary transcription would be enough to lead to virus-specific protein synthesis which could in turn alter the surface properties of infected cells. The result would be resistance of these infected cells to superinfection by standard VSV. Although these results on cellular surface resistance to superinfection are extremely preliminary, they do suggest that previous examples of viral interference at cell surfaces should be re-examined in relation to DI particles.

Acknowledgment

We thank Ms. Louise Chu for excellent technical support.

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