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Absence of Interference During High-Multiplicity Infection by Clonally Purified Vesicular Stomatitis Virus

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Stocks of vesicular stomatitis virus free of defective interfering particles were produced by serial clonal isolation. High-multiplicity infections with these stocks led to no interference or formation of defective interfering particles. Defective interfering particles were generated by three successive passages at high multiplicity.

Replication of the Indiana serotype of vesicular stomatitis virus (VSV) produces standard plaque-forming B particles and defective interfering (DI) particles (5, 8, 11). The major DI particle found in our stocks is one-third the length of standard B particles and is referred to below as DI-T rather than "T particles" as has been the previous convention (8). Previous work has shown that the sucrose gradient pattern of ribonucleic acid (RNA) species synthesized by cells infected with VSV varies, depending upon the amount of DI-T present (12). In the absence of interfering particles, the predominant effects are synthesis of B particles and of group I RNA species (mainly 28*S* and 13*S* RNA). When cells are co-infected with DI-T and B, synthesis of DI-T and group II RNA (mainly 19*S* and 6*S*) predominates. High-multiplicity infections with B particles purified by rate zonal centrifugation will also yield group II RNA and DI-T.

Production of DI-T and group II RNA species at high multiplicities could be either an intrinsic property of cells multiply infected by B particles or the result of contamination by undetectable amounts of DI-T in B preparations leading to co-infection of cells by DI-T and its helper, standard virus. To distinguish between these possibilities, several clonal plaque isolations of VSV have been made. Because initial passages appeared to be free of DI particles, successive high-multiplicity passages were analyzed for the production of DI particles. To detect DI yields in this study, we used radioactive labeling and separation on sucrose gradients, as well as analysis of the species of RNA produced in infected cells. A typical clonal isolation of B particles and the initial detection of DI particles are presented here. Repetition of this procedure has led to similar results.

To isolate cloned preparations of VSV, a monolayer of Chinese hamster ovary (CHO) cells infected with 6 plaque-forming units (PFU) of VSV was overlaid with agar as previously described (12). A well isolated plaque of approximately 1.5 mm in diameter was picked by inserting a capillary tube into the agar directly above the plaque, withdrawing the capillary tube, and blowing the occluded agar into 1 ml of medium. After disrupting the agar by using a vortex supermixer, the solution was diluted and assayed for plaques on fresh, CHO cell monolayers. This plaque isolation was repeated four successive times.

To build up an initial stock of this plaque-purified VSV with minimal cross-infection of the cells, four CHO cell monolayer cultures on 60-mm plastic petri dishes were each infected with 200 PFU of virus from the fifth successive isolation. After 1 day, when most of the plaques were not overlapping, virus was harvested by collecting the agar overlay from each of the four plates. Each plate was then rinsed with 1 ml of medium. This medium and 5 ml of fresh medium were combined with the agar, and the suspension was centrifuged at 27,000 $\times$ *g* at 4 C for 10 min in the Sorvall RC2-B centrifuge. This procedure concentrated the agar, and a total of 15 ml of supernatant was collected which contained 10^8 PFU/ml. From this initial stock, another passage of VSV was made by infecting suspended CHO cells at a multiplicity of 0.4 PFU per cell and harvesting the virus at 7.5 hr after infection. This diluted passage was then used for the following infections at high multiplicities. Details

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of VSV infections and growth of CHO cells have been described (12).

To determine the species of viral RNA and types of VSV particles synthesized at high multiplicities, suspended CHO cells were infected for three successive passages at multiplicities of 100, 50, and 50, respectively. After each infection, labeled viral particles from the medium and viral RNA from the cytoplasm were analyzed by sucrose gradient centrifugation as previously described (12). Under these conditions of infection, incorporation of radioactive uridine into virus-specific RNA was complete by 5 hr after infection, and production of viral particles ceased by 6 to 7 hr after infection. Unlabeled infected cells, harvested in parallel with the labeled cells, provided virus for the subsequent passage. The first high-multiplicity passage yielded 1.5×10^9 PFU/ml, the second 7.3×10^8 PFU/ml, and the third 7.8×10^7 PFU/ml. The decrease in VSV titer after the third successive high-multiplicity passage shows the interference phenomenon which was first described by Bellett and Cooper in 1959 (2).

The VSV particles produced during the three high multiplicity passages and the species of viral RNA which were synthesized are shown in Fig. 1. In the first passage at a multiplicity of 100, only the group I cytoplasmic RNA species were detected (Fig. 1a), and only labeled standard B virions were made (Fig. 1d). This shows that high-multiplicity infections can be carried out with purified B preparations in the absence of detectable DI production. In the second passage, at a multiplicity of 50, again only group I RNA species were present in the cytoplasm (Fig. 1b), and labeled B particles were found in the supernatant (Fig. 1e) along with some B aggregates sedimenting ahead of the main peak.

During the third successive passage, at a multiplicity of 50, extensive production of group II RNA species, as well as some group I species, was observed (Fig. 1c). Also, RNA between 19S and 28S was present which had not been seen previously (12). Labeled particles produced during the third passage gave a heterogeneous distribution with peaks in the regions expected for standard B, as well as what appears to be shorter defective particles (Fig. 1f). If, during the third successive passage, co-infection of cells was reduced by using a multiplicity of one or less, the results resembled those seen during the first high-multiplicity passage. Therefore, it is most likely that DI particles are detectable by the third high-multiplicity passage because of coinfection of cells with standard virus and DI particles which were presumably generated in undetectable amounts during the first and second high-multiplicity passages. However, we cannot rule out the possibility that the third high-multiplicity passage differs in some indeterminate way from the first and second by its ability to generate large amounts of DI.

The heterogeneity of the viral particles produced after the third high-multiplicity passage and the markedly reduced yield in virus titer indicated that DI particles were generated. Although these results and similar results obtained by Crick et al. (4) do not yield an absolute number for the frequency of appearance of DI particles among a population of VSV, the rate of generation of DI particles is similar to reovirus where DI particles are detected upon

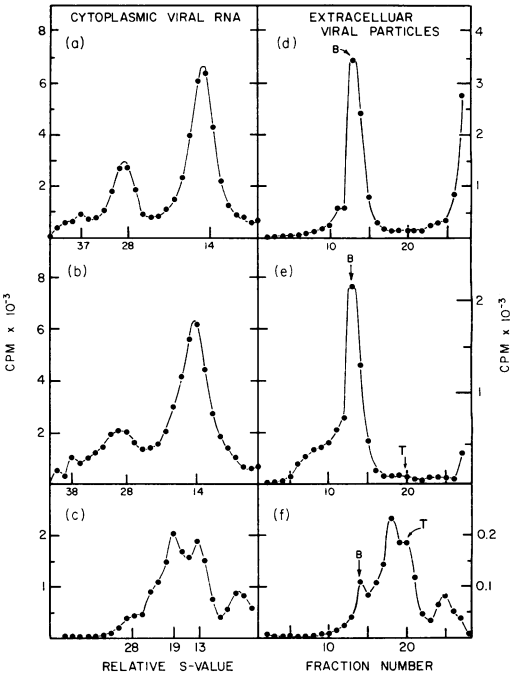

FIG. 1. *Sucrose gradient patterns of viral RNA species and types of VSV particles synthesized in three successive high-multiplicity passages with clonally purified VSV. Suspensions of CHO cells at 4×10^6/ml were infected with purified B, and 10 μg/ml actinomycin D was added. After a 30-min attachment period, cells were diluted twofold and exposed to 0.3 μCi of ^{14}C-uridine/ml (New England Nuclear Corp., Boston, Mass., 55.6 mCi/mM). At 7.5 hr postinfection the cells were harvested to obtain the cytoplasmic extract and the released viral particles. For panels (e) and (f), viral particles were obtained by centrifugation of the medium as previously described (10). For panel (d), the cell-free medium was layered directly on the sucrose gradient. The data in each panel represent acid-precipitable radioactivity from 10^7 cells. (a) and (d) First passage at a multiplicity of 100 PFU/cell; (b) and (e) second passage at a multiplicity of 50 PFU/cell; (c) and (f) third passage at a multiplicity of 50 PFU/cell.*

the third or fourth successive high-multiplicity passages after clonal isolations (10). In contrast, poliovirus requires 18 successive high-multiplicity passages to generate detectable levels of DI particles (C. N. Cole, D. Smoler, E. Wimmer, and D. Baltimore, J. Virol., *in press*). However, it should be noted that in certain cells successive high-multiplicity passages do not lead to progressive interference for influenza (3), VSV (6), or Sendai virus (9).

Data from the first passage show that a high-multiplicity passage per se with VSV does not result in the production of large amounts of DI particles and that the stock used to initiate the high-multiplicity passages was free of detectable DI particles. Thus, biochemical experiments (7) can be performed with VSV without any interference at high multiplicities if the first few passages from clonally purified virus are used. Furthermore, very high titer VSV stocks (1) can be grown using virus from the early passages.

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