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Autointerference in Infectious Hematopoietic Necrosis Virus of Salmonid Fish[1] (37906)

P. E. McAllister and K. S. Pilcher

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331

Autointerference in virus replication due to the use of undiluted inocula was first described for influenza virus (1). Studies of vesicular stomatitis virus (VSV) have indicated that a transmissible, morphologically distinct particle was associated with autointerference. The autointerfering particle was presumptively identified as a truncated, bullet-shaped virion approximately one-third the length of the infectious particle (7). These truncated virions were less dense than the infectious ones and were readily separated from the latter by centrifugation on sucrose density gradients and visualized in negatively stained films by electron microscopy. Partially purified preparations of these particles were found to interfere with the production of infectious virus (8, 9).

Reports concerning the morphology and ultrastructure of some salmonid rhabdoviruses have intimated that, based on the occasional observation of truncated bullet-shaped or oval particles and analogy to VSV, the autointerference phenomenon may occur with some of these agents (3, 5). To date, however, no experimental evidence has been reported. The purpose of the present study was to determine whether autointerference is demonstrable with infectious hematopoietic necrosis virus (IHNV) of salmonid fish, and if so, whether it influences the morphology of the virions.

Materials and Methods. Preparation of stock virus. The virus used in these experiments was the Oregon sockeye salmon strain (2) of infectious hematopoietic necrosis virus (IHNV). Stock virus was prepared by inoculation of monolayer cultures of a heteroploid cell line (CHSE-214) derived from chinook salmon embryos (*Oncorhynchus tshawytscha*), with a 1:1000 dilution of an earlier stock virus preparation. The cell cultures were propagated in Eagle's minimum essential medium (MEM) containing 100 U penicillin and 100 μg streptomycin/ml and 10% fetal bovine serum (FBS) (6). At the time of virus inoculation, the serum content was reduced to 5%. The inoculated cultures were incubated at 18° for 96 hr, when the culture fluid was harvested and centrifuged to remove cell debris. It was dispensed in small Pyrex tubes and stored at −60°. The infectivity titer was determined on thawed samples by the monolayer plaque assay in cultures of CHSE-214 cells (10). The stock virus used in this study had a titer of 1.57×10^7 plaque forming units (PFU)/ml.

Serial passage of diluted and undiluted virus. The series of culture passages employing undiluted virus inoculum was initiated by exposing a monolayer of CHSE-214 cells in a 32-oz prescription bottle to 4 ml of undiluted stock virus. This represented a multiplicity of infection of approximately 1.2. The series of cultures employing diluted virus inoculum was started in the same manner, except that the cells were exposed to 4 ml of a 1:1000 dilution of the stock virus. In both series the virus inoculum was allowed to adsorb for 2 hr at 18° after which it was removed and replaced with 50 ml of MEM containing 5% FBS. The infected cultures were incubated at 18° for

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96 hr, when the virus containing fluid was harvested and centrifuged at 2200*g* for 15 min. An aliquot from each culture was immediately assayed for infectious virus. Four milliliters of the fluid without dilution was used to inoculate each of the next group of cultures in the undiluted series, while the same volume of the fluid diluted 1:1000 was the inoculum for each culture in the diluted virus series. The method for adsorption of virus, replacement of culture medium, and incubation was the same as described for the first cultures in each series.

Preparation of thin sections for electron microscopy. For studying the morphology of virions formed in cells infected with a dilute virus inoculum, monolayers of CHSE-214 cells were exposed to a 1:1000 dilution of stock virus and incubated at 18° for periods of 24, 48, or 72 hr. Then the cells were washed with 0.125 *M* phosphate buffer of pH 7.0 and fixed in 4.0% glutaraldehyde in the same buffer. They were scraped from the glass, suspended in buffer, centrifuged, and the cell pellet embedded in 2% aqueous Ionagar No. 2. The agar was diced into 1- to 2-mm cubes, stained with 1% osmic acid in buffer, dehydrated by exposure to 30, 50, and 70% acetone and stained in a saturated solution of uranyl acetate in 70% acetone. After final dehydration in 100% acetone, the specimens were infiltrated with and embedded in a plastic mixture composed of 35 ml of Araldite 6005, 12 ml of Epon 812, and 53 ml of dodecenyl succinic anhydride. The specimens were cured, and ultrathin sections were cut and stained with lead citrate. Sections were examined with the Philips EM300 electron microscope. Preparations of cells infected with undiluted virus inoculum were made by exposing CHSE-214 monolayers to undiluted stock virus and incubating for 18 hr at 18°. They were then fixed, stained, and sectioned as described above.

Sucrose density gradient centrifugation of virus. The methods used for partial purification and density gradient centrifugation were similar to those used successfully with rabies and vesicular stomatitis viruses (8, 12). Fluid from cultures infected by either undiluted or diluted inoculum was partially purified by a single cycle of differential centrifugation (2200*g* for 30 min; 55,000*g* for 60 min) and the resuspended pellet treated consecutively with chymotrypsin and with a mixture of ribonuclease and deoxyribonuclease. The virus was again pelleted and resuspended in 1% of the original volume of a solution (TEB buffer) containing 0.05 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4, containing 0.025 *M* ethylenediaminetetraacetate (EDTA) and 0.1% bovine serum albumin (BSA).

Two milliliters of the resulting virus was layered on 20.0 ml of a linear 15–55% sucrose gradient containing 0.025 *M* EDTA and 0.1% BSA. After centrifugation at 4° for 2 hr at 60,000*g*, 1 or sometimes 2 visible bands were present. They were withdrawn separately from the top of the tube with a needle and syringe.

Sucrose was removed from samples representing one of the bands by either of 2 methods. Some samples were dialyzed at 4° against 0.05 *M* Tris buffer, pH 7.4, containing 0.13 *M* NaCl and decreasing concentrations of sucrose, from 30% at the start to 0 at the end of a 24-hr period. The volume was then reduced to about 0.2 ml by further dialysis against 30% polyethylene glycol 4000. Alternatively, the sample containing the band was brought to a volume of 20 ml by addition of TEB buffer. The virus was then pelleted at 60,000*g* for 60 min and the pellet resuspended in 0.2 ml of the buffer.

After removal of sucrose, the concentrated sample was fixed at 4° by adding an equal volume of 1% glutaraldehyde and holding for 20 min. A drop of the fixed specimen was placed on a carbon-coated Formvar grid, allowed to remain 5 min, and the excess fluid removed with filter paper. It was stained for 5 sec with 1% phosphotungstic acid, pH 6.8, and excess fluid again removed.

Results and Discussion. Autointerference in the replication of a virus may be demonstrated by a reduction in the yield of infectious virus in a series of cultures when undiluted virus or culture fluid is used as inoculum, compared with the yield in a parallel series where a dilute inoculum is used (1, 4). A comparison of this type was

TABLE I. Autointerference in the Serial Passage of Infectious Hematopoietic Necrosis Virus in CHSE-214 Cells.

	Culture series initiated and maintained using	
	Diluted virus inoculum	Undiluted virus inoculum
Titer of virus to which cells in first passage were exposed	1.54×10^4 (PFU/ml)	1.54×10^7 (PFU/ml)
Final virus titer in culture fluids of		
First-passage cultures[a]	1.71×10^7	2.32×10^6
Second-passage cultures[a]	2.59×10^7	1.43×10^5
Third-passage cultures[a]	1.92×10^7	6.86×10^5

[a] After 96-hr incubation at 18°.

made with IHNV in monolayer cultures of CHSE-214 cells. The results are shown in Table I. It is evident that virus titers of culture fluids in the series where undiluted virus was used as inoculum were very significantly lower than in parallel cultures where the inoculum was diluted virus. It seems clear that the differences noted are due to autointerference.

It was of interest to determine whether the morphology of virions formed under conditions of autointerference would be significantly altered by these conditions, as was found to be the case with vesicular stomatitis virus, another of the rhabdovirus group (7). Experiments were first performed with virus purified by differential ultracentrifugation, treatment with ribonuclease and deoxyribonuclease, and rate zonal centrifugation on sucrose density gradients. On such gradients, virus from cultures infected with undiluted inoculum gave a visible band not seen in parallel preparations from cultures receiving dilute inoculum. It was of lower density than the band shown in earlier experiments to contain the most infectious virus. However, repeated attempts to compare these bands by electron microscopy of negatively stained specimens were unsatisfactory, as only a few intact virus particles could be found. Most of the particles observed were fragmented or distorted. Hence, it was not possible to study the effect of autointerference on morphology of the virions using this approach. Other workers have also noted that various strains of this virus are very fragile and easily disrupted by the physical forces accompanying purification (3, 5). No attempt was made to compare the biological activities of the two bands.

Alternatively, the morphology of the virions was studied in ultrathin sections of CHSE-214 cells exposed to undiluted stock virus and incubated for 18 hr at 18°, and in

TABLE II. Dimensions of Three Types of IHNV Virions and Their Distribution in CHSE-214 Cells Inoculated with Undiluted or Diluted Virus.

		Dimensions (nm)				Numbers observed in cultures inoculated with	
		Length		Diameter			
Type of viron	Number measured	Mean	Range	Mean	Range	Diluted virus	Undiluted virus
B	89	188	(157–213)	70	(60–78)	71	18
LT	31	118	(100–142)	69	(60–78)	2	29
T	52	81	(64–96)	66	(57–82)	0	52
						73	99

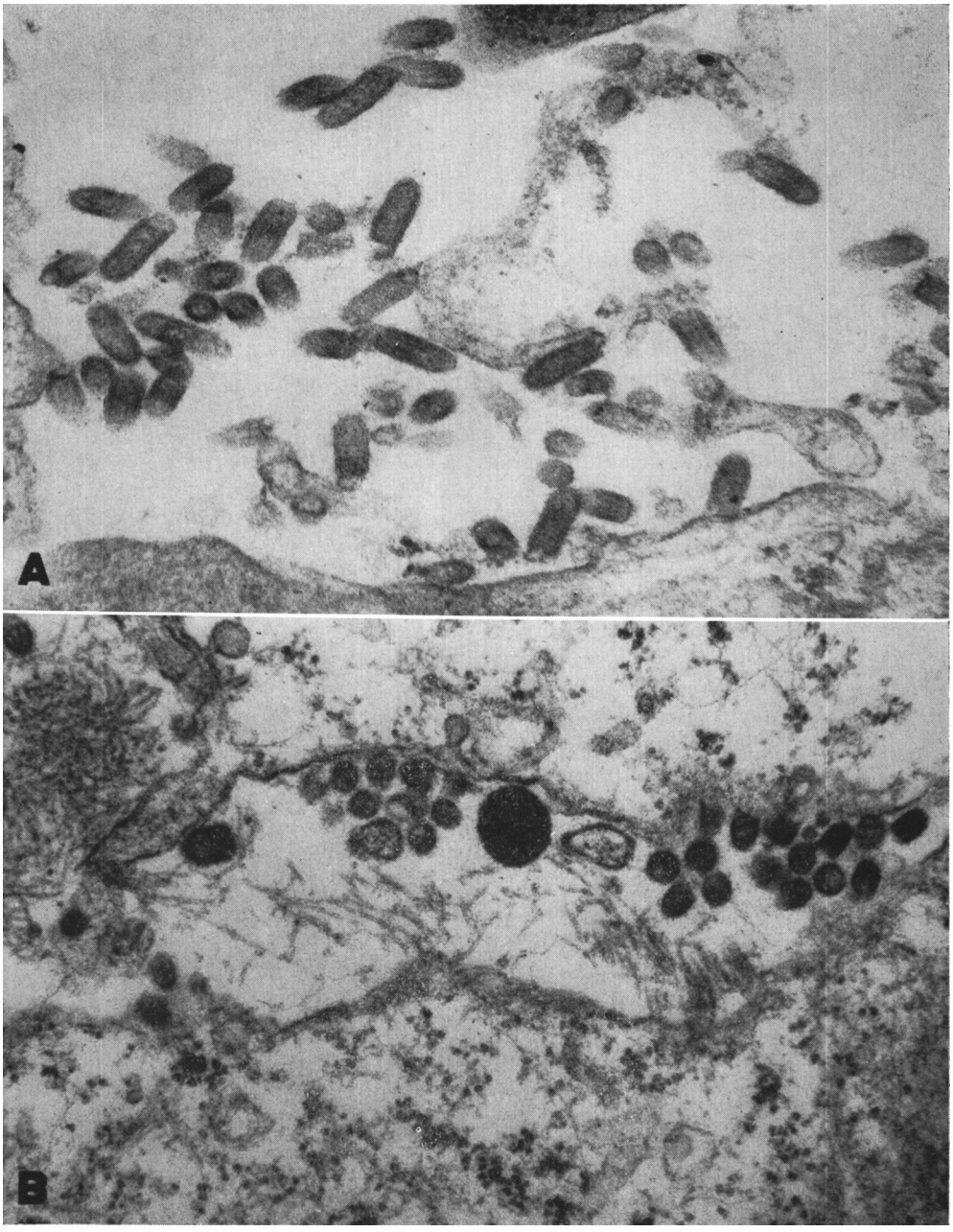

FIG. 1A. Monolayer culture of CHSE-214 cells inoculated with 1:1000 dilution of IHNV stock virus. Most of the virions are the B type, averaging 188 nm in length. A few appear to be cross-section or diagonal-section views. They are extracellular, lying adjacent to the surface membrane of an infected cell.

FIG. 1B. Monolayer culture of CHSE-214 cells inoculated with undiluted IHNV stock virus. Eight of the truncated or T type bullet-shaped virions averaging 81 nm in length may be seen, as well as several cross-section views of particles with round or oval outline. To the left of center is one of the long truncated or LT type virions. All appear to be extracellular, in a space between two infected cells.

other cultures inoculated with a 1:1000 dilution of stock virus and incubated for 72 hr at 18°. The longer incubation period was required with the dilute inoculum to give an adequate concentration of virions for electron microscopy. Three morphological types were observed in sections of cells infected with undiluted virus. Their dimensions and the relative numbers of each type are shown in Table II. The B type virion was bullet shaped, with mean dimensions of 188 × 70 nm. Almost all of the virions found in cells inoculated with dilute virus were of this type. A typical view of these virions may be seen in Fig. 1A. The LT type virion differed from the B type only in its length, which had a mean value of 118 nm. Only 2 of these were found among 73 virions classified in cells inoculated with dilute virus; one may be seen to the left of center in Fig. 1B. About 29% of the virions in cells inoculated with undiluted virus were in this category. The third morphological type, referred to as T virions in Table II, were both shorter and narrower than the others, averaging 81 nm in length and 66 nm in width. These were found only in cells inoculated with undiluted virus, where they constituted about 52% of the virus particles classified. They still possess the bullet shape, as may be seen in Fig. 1B, where a number of these particles appear.

These results indicate that when this virus replicates under conditions that produce autointerference, the morphology of the virions is significantly altered. Under such conditions, truncated bullet-shaped particles were abundant, and only about 18% of the usual full-length particles were found. When a dilute inoculum was used, few if any truncated virions were found, and the full-length virions were found in abundance.

In a study of the Indiana serotype of VSV, three morphological types of virions were found (11). The longest of these, the B particle, was reported to be infectious, while a T particle about one-third the length of the B, and a long T, about half of that length, were considered to be defective, and presumably autointerfering particles. The evidence presented here seems to indicate an analogous situation for IHNV and suggests that the full-length or B type virion may be the infectious one and the other two possibly defective virions.

Summary. When serial passage of infectious hematopoietic necrosis virus was carried out in cultures of a salmon cell line, using undiluted virus inoculum at each transfer, the resulting yield of infectious virus was very significantly lower than in parallel cultures prepared with dilute virus inoculum. Under these conditions of autointerference, the morphology of the progeny virions was altered. Three types were observed, with mean lengths of 188, 118, and 81 nm respectively. Roughly 80% fell into the two shorter categories. When a dilute virus inoculum was used, almost all progeny virions belonged to the class with a mean length of 188 nm.

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