PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **175**, 84–87 (1984)

Viral Enhancement and Interference Induced in Cell Culture by Hepatitis A Virus: Application to Quantitative Assays for Hepatitis A Virus (41771)

WILLIAM M. HURNI, WILLIAM J. MILLER, WILLIAM J. MCALEER, PHILIP J. PROVOST, AND MAURICE R. HILLEMAN

Division of Virus & Cell Biology Research, Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

Abstract. Hepatitis A virus (HAV) growing in human diploid lung fibroblast (MRC5) monolayers can either interfere with or enhance the cytopathic effect of Newcastle Disease virus (NDV) challenge. Enhancement of NDV occurred if HAV-infected monolayers were challenged with a low multiplicity of infection of NDV and incubated at 35°C. Interference occurred if HAV-infected monolayers were given a high NDV multiplicity of infection and incubated at 32°C. These phenomena were applied to assays for quantifying HAV and may be useful in providing new insights into viral interference and enhancement.

The growth of human hepatitis A virus (HAV) in cell cultures was reported in 1979 (1). This work was confirmed and extended in a number of laboratories. Viral infection is transmissible not only to monkey kidney cell cultures but also to human embryonic diploid lung fibroblasts (2, 3). Infectivity titers ranging from 10^6 to 10^8 50% infectious doses per ml of cell culture fluids were readily achieved. However, incubation periods are prolonged in other than low-passage monkey kidney cell cultures, and evidence of cytopathic activity of HAV in cell culture has not been provided to date. Both of these factors contribute to relatively tedious titration assays for HAV infectivity in human diploid cell cultures (MRC5), requiring 35 days incubation and subsequent assay of culture vessels for HAV antigen by radioimmunoassay (RIA) (1, 4). Seeking possible alternative assay systems, we have found both viral interfering and enhancing activity associated with HAV infection in MRC5 cells that permit detection and quantitation of the virus in cell culture. Preliminary observations of these phenomena are described.

Materials and Methods. *Viruses. HAV* variants adapted to growth in MRC5 cells (human embryonic diploid lung fibroblasts) were prepared in the course of our live virus vaccine development program (3). Several of these variants, adapted to growth in MRC5 at 32 and 35°C, were used in the present work. The data reported here were obtained using a particular variant with a history of 31 passages in marmoset monkeys plus 25 passages in fetal rhesus kidney cell cultures at 35°C and 4 passages in MRC5 cell cultures at 35°C. The virus preparation consisted of a homogenate of cell monolayer plus overlay fluids prepared by two freeze–thaw cycles and sonication. It was stored at −70°C. *Newcastle Disease virus* (NDV, California strain) was a chick embryo allantoic fluid preparation. It had a titer of 10^{10} $TCID_{50}$ per ml based on assay for cytopathology in MRC5 cells incubated at 35°C, and was stored at −70°C.

Cells and culture media. For use in the interference and enhancement assays, MRC5 cell suspensions at 200,000 cells per ml were made in Basal medium Eagle supplemented with 10% fetal calf serum, 2 m*M* glutamine and 50 μg/ml neomycin. After planting, cells were maintained with Williams Medium E (Flow) containing 0.5% fetal calf serum, glutamine, and neomycin as above (maintenance medium).

Cell culture assays. Addition of HAV and MRC5 cells to microtiter plates. HAV was diluted in maintenance medium in 10-fold steps from 10^{-2} through 10^{-8}. Seventy-five microliters of the sequential dilutions was added to each of 12 wells in seven rows of a Falcon Microtiter II plate. For control, the eighth row was filled with 75 μl/well of maintenance medium alone. Seventy-five microliters of MRC5 cell suspension was then added to all wells. The plates were incubated at 32°C in a humidified 5% CO_2 incubator for 2 days to bring about cell attachment and growth. Medium

0037-9727/84 $1.50
Copyright © 1984 by the Society for Experimental Biology and Medicine.
All rights reserved.

in all wells was replaced with 150 μl of maintenance medium on Days 2 and 16. Incubation was at 32°C throughout whether testing 35°C-adapted or 32°C-adapted HAV variants. NDV challenge was done on Day 30. *NDV challenge.* On Day 30, NDV was diluted in maintenance medium to desired level and 150 μl were added to each well following removal of fluid. The section on results shows the optimal NDV doses and incubation temperatures to produce enhancement or interference of NDV cytopathic effect (CPE). *Enhancement effect.* One hundred and fifty microliters of NDV at 10^4 $TCID_{50}$/ml was added per well to the plates that had been incubated for 30 days. This represented about 0.03 $TCID_{50}$ per cell. The plates were incubated at 35°C for 5 days and then examined by light microscopy for wells with evidence of CPE typical of that caused by NDV. CPE, where it occurred, was distinct, consisting of partial destruction of the cell sheet and the presence of characteristic rounded, refractile bodies. *Interference effect.* One hundred and fifty microliters of NDV at 10^6 $TCID_{50}$/ml was added to each well of the plates that had been incubated for 30 days. This represented about 3.0 $TCID_{50}$ per cell. The plates were then incubated at 32°C for 5 days and examined by light microscopy for evidence of NDV CPE in individual wells.

Radioimmunoassay. Plates carried through the 30-day HAV incubation period plus the 5-day NDV challenge period were frozen and thawed to release cell-associated HAV. The well contents were removed and assayed by RIA (1, 4) to determine the presence or absence of HAV antigen.

Results. Table I shows the findings in a study of the effects of NDV dose and incubation temperature on CPE patterns produced by NDV in wells of control and HAV-infected (30 days) MRC5 cells. The 35°C-adapted HAV variant described under the Materials and Methods section was used. A CPE-enhancement phenomenon, dependent on NDV dose, was noted when cultures were incubated at 35°C post-NDV challenge. Too great a dose of NDV (10^5 $TCID_{50}$/ml) produced CPE in both control and HAV-infected wells over the 5-day incubation period. At 1/10 this dose (10^4 $TCID_{50}$/ml), however, CPE was produced in HAV-infected wells and not in control wells over the 5-day period. A lower dose of NDV (10^3 $TCID_{50}$/ml) produced a slight degree of CPE in HAV-infected wells while controls remained normal. Thus, an NDV challenge of 10^4 $TCID_{50}$/ml with incubation at 35°C for 5 days appeared optimal to demonstrate the NDV CPE enhancement phenomenon.

In contrast, by incubating at 32°C for 5 days after NDV challenge, we noted an inhibition of CPE development in HAV-infected MRC5 cells. This phenomenon depended on the use of sufficient doses of NDV (10^5, 10^6 $TCID_{50}$/ml) to produce CPE in control wells. Under these conditions, HAV infection inhibited or interfered with CPE development. Lesser NDV doses were not useful in demonstrating this phenomenon. Doses of NDV greater than 10^6 were not tested. Thus, an NDV challenge dose of 10^6 $TCID_{50}$/ml followed by incubation for 5 days at 32°C appeared optimal to demonstrate the CPE inhibition phenomenon.

Table II shows the content of HAV, as measured by RIA, in wells which had exhibited either positive or negative effects in the enhancement and interference assays. Nearly total agreement was obtained between the presence or absence of the enhancement and interference effects and the presence or absence of HAV antigen. The few discrepancies noted could probably be eliminated with further refinement of the assays.

Table III shows the utility and comparable sensitivities of the enhancement and interference assays in estimating the infectivity of a single HAV preparation. The assays were done on three separate occasions. HAV was the 35°C variant described under the Materials and Methods section. Each time the assays were compared, there was minimal difference between the two methods.

Discussion. The phenomenon of viral interference has been widely described with numerous pairs of viruses and may be due to the elaboration of interferon or to cell-associated factors (5). Viral enhancement is less well described in the literature. Closest to the present work are the descriptions of NDV enhancement by certain noncytopathic, enveloped RNA viruses (6, 7). The present work is the first description of either enhancement or interference ascribed to HAV, and it would appear particularly unique in that *both* en-

TABLE I. COMPARATIVE EFFECTS OF NDV DOSE AND INCUBATION TEMPERATURE ON THE DEVELOPMENT OF NDV CPE IN HAV-INFECTED AND CONTROL MRC5 CELL CULTURES

		NDV CPE in		
NDV dose ($TCID_{50}$/ml)	Incubation temperature post-NDV addition	HAV-infected cells	Control cells	Interpretation of HAV effect
10^5	35°	Positive	Positive	No HAV effect[a]
10^4	35°	Positive	Negative	Enhancement of NDV CPE
10^3	35°	Weakly positive	Negative	Slight enhancement of NDV CPE
10^6	32°	Negative	Positive	Interference with NDV CPE
10^5	32°	Negative	Positive	Interference with NDV CPE
10^4	32°	Negative	Negative	No HAV effect[b]

[a] NDV dose overwhelming. HAV effect obscured.
[b] Insufficient NDV to produce CPE under test conditions.

hancement and interference can be demonstrated with appropriate combinations of HAV and NDV. It also appears to be the first description of enhancement induced by a noncytopathic picornavirus.

Both viral interference and enhancement are poorly understood, and the present work, being preliminary in nature, did not attempt to provide new understanding. What is clear from this work, however, is that low multiplicities of NDV infection coupled with higher NDV incubation temperature (35°C) give rise to CPE enhancement in HAV-infected cells; whereas, by contrast, higher multiplicities of NDV infection coupled with lower incubation temperature (32°C) give rise to inhibition of NDV CPE in HAV-infected cells. Of possible importance is an observation we made that was not presented in the section on results. We have noted that HAV variants that had been adapted to growth at 32°C by serial passage at this temperature, and which do not grow well at 35°C, did not cause enhancement (at 35°C). However, these 32°C-adapted HAV variants were active in the interference process. This may indicate that active HAV replication is required to produce the enhancement phenomenon.

Thus the present work describes new methods for detecting HAV growth in cell cultures. HAV strains adapted to growth in MRC5 cells at 35°C can be assayed by either the enhancement or interference method, while HAV variants which grow well in MRC5 cells only at 32°C can only be assayed by the interference method. Both the interference and enhancement assays offer alternatives to conventional RIA assays of HAV, are less labor intensive,

TABLE II. CORRELATION OF POSITIVE AND NEGATIVE NDV CPE ENHANCEMENT AND INTERFERENCE EFFECTS WITH THE PRESENCE OR ABSENCE OF HAV ANTIGEN DETECTED BY RIA

	No. of wells		HAV assays of corresponding wells		
Effect measured	Positive	Negative	RIA positive	RIA negative	Percentage agreement
Enhancement	19		18	1	95
		8	0	8	100
Interference	24		24	0	100
		23	4	19	83

TABLE III. COMPARATIVE HAV TITERS OBTAINED IN REPLICATE TESTS OF A SINGLE HAV SPECIMEN USING THE NDV CPE ENHANCEMENT AND INTERFERENCE ASSAYS

Test no.	HAV titers[a] determined by: Enhancement assay	Interference assay
1	$10^{6.4}$	$10^{5.9}$
2	$10^{6.3}$	$10^{6.4}$
3	$10^{5.8}$	$10^{5.6}$

[a] Titers expressed as $TCID_{50}$/ml.

require less sophisticated apparatus, and are readily adaptable to microtiter plates. The preliminary observations described in the present paper show promise for providing not only new assay procedures for HAV infectivity, but also new insights into the phenomena of viral interference and viral enhancement.

We thank P. Giesa for preparing the Newcastle Disease virus.

1. Provost PJ, Hilleman MR. Propagation of human hepatitis A virus in cell culture *in vitro*. Proc Soc Exp Biol Med **160**:213–221, 1979.
2. Provost PJ, McAleer WJ, Hilleman MR. *In vitro* cultivation of hepatitis A virus. In: Szmuness W, Alter H, Maynard J, eds. Viral Hepatitis. 1981 International Symposium. Philadelphia, Franklin Inst Press, p21, 1982.
3. Provost PJ, Banker FS, Giesa PA, McAleer WJ, Buynak EB, Hilleman MR. Progress toward a live, attenuated human hepatitis A vaccine. Proc Soc Exp Biol Med **170**:8–14, 1982.
4. Purcell RH, Wong DC, Moritsugu Y, Dienstag JL, Routenberg JA, Boggs JD. A microtiter solid-phase radioimmunoassay for hepatitis A antigen and antibody. J Immunol **116**:349–356, 1976.
5. Marcus PI, Carver DH. Intrinsic interference: a new type of viral interference. J Virol **1**:334–343, 1967.
6. Kumagai T, Shimizu T, Ikeda S, Matumoto M. A new *in vitro* method (END) for detection and measurement of hog cholera virus and its antibody by means of effect of HC virus on Newcastle Disease virus in swine tissue culture. I. Establishment of standard procedure. J Immunol **87**:245–256, 1961.
7. Hermodsson S. Inhibition of interferon by an infection with parainfluenza virus type 3 (PIV-3). Virology **20**:333–343, 1963.

Received May 18, 1983. P.S.E.B.M. 1984, Vol. 175.
Accepted October 3, 1983.