Protein Synthesis by HepG2 Cells Infected with Influenza B Virus

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ABSTRACT. Reye's syndrome is an acute hepatopathy and encephalopathy affecting children during the convalescent period of a viral infection, frequently influenza B. The role of influenza B in the pathogenesis of Reye's syndrome is unknown. To investigate this relationship, an *in vitro* system was designed to examine the interaction of influenza B virus with a cell line of human hepatocytes (HepG2) which retains many specific hepatic synthetic characteristics. HepG2 was capable of supporting a productive infection by influenza B, although greater virus inputs were required than in fully permissive Madin-Darby canine kidney cells. Because of the recent association of Reye's syndrome with aspirin use, the kinetics of influenza B growth were studied in the presence of acetylsalicylic acid (25 and 100 $\mu\text{g/ml}$) and were not found to be altered. Protein synthesis by HepG2 cells was decreased by 80% in influenza B-infected cells when compared to uninfected controls. Acetylsalicylic acid, 100 $\mu\text{g/ml}$, did not affect the rate of ^{35}S -methionine incorporation by infected or uninfected HepG2 cells. The rates of synthesis of specific proteins (albumin, transferrin, and apoprotein B) by HepG2 cells were determined by immunoprecipitation of ^{35}S -methionine labeled cell lysates. After 12 h of infection, synthesis of all three plasma proteins was decreased by 40–60%. These studies describe a useful system for delineation of mechanisms by which influenza B virus interacts with host hepatocytes and the host-cell protein synthetic machinery. The studies could be pertinent to the pathogenesis of Reye's syndrome. (*Pediatr Res* 23: 334–337, 1988)

Abbreviations

MDCK, Madin-Darby canine kidney
MEM, minimum essential medium
MOI, multiplicity of infection
pfu, plaque-forming unit
PBS, phosphate-buffered saline
ASA, acetylsalicylic acid
TCA, trichloroacetic acid
BSA, bovine serum albumin
HA, hemagglutinin

Reye's syndrome is an acute hepatopathy and encephalopathy that primarily affects infants and children during the convalescent period of a viral infection. A clear epidemiologic association has been shown with influenza B virus infection (1–3).

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Inasmuch as extrapulmonary dissemination and viremia in influenza B virus infections are rare (4), it is difficult to postulate a mechanism by which the hepatic abnormalities in Reye's syndrome are induced. In rare instances, virus has been cultured from the blood or hepatobiliary tissues of children dying from Reye's syndrome, but there are no convincing data to suggest a direct inflammatory or immunologic component to the hepatic injury (5).

Using an animal model of Reye's syndrome described by Davis *et al.* (6) we recently examined the clinical and histopathologic effects of influenza B, given intranasally or intravenously, on juvenile BALB/c mice (7). Mice were observed up to 72 h after inoculation. With the exception of hepatic steatosis, no clinical, biochemical, or histopathologic features of Reye's syndrome were demonstrable. The recovery of intact influenza B virus from the liver in 25% of mice was unexpected. Inasmuch as other investigators have hypothesized a direct interaction of intact virus or a viral gene product with the hepatocyte in Reye's syndrome (8, 9), the recovery of virus from experimentally infected rats prompted the design of an *in vitro* system for direct assessment of the interaction of influenza B virus with the hepatocyte.

MATERIALS AND METHODS

Cells and viruses. HepG2 and MDCK cells were grown in Eagle's MEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), gentamicin (50 $\mu\text{g/ml}$), and amphotericin B (1 $\mu\text{g/ml}$). HepG2 cells (kindly provided by B. Knowles, Wistar Institute, Philadelphia, PA), are a continuous cell line derived from a human hepatoblastoma which secrete many serum proteins (10). MDCK cells are a permissive and sensitive cell line for influenza virus when used in the presence of trypsin (11). Monolayers were maintained in humidified, 5% CO_2 , 95% air. Influenza B/Texas/1/84, isolated from the pharynx of a child with Reye's syndrome, was grown in the allantoic fluid of 11-day embryonated eggs, purified by ultracentrifugation, and titered at $10^6/\text{ml}$.

Kinetics of virus growth in HepG2 and MDCK cells. HepG2 and MDCK cells were grown to confluence in 12 well tissue culture plates (Costar, Cambridge, MA). Monolayers were inoculated at a MOI of 0.1, 1, 10 and 100 pfu/cell and virus was adsorbed at 37° C for 1 h. After three washes with warm PBS, the cells were overlaid with 2 ml of MEM with or without 0.04% trypsin. At 4, 12, 24, and 48 h after infection, the supernatant media were removed and quick frozen at –70° C. The supernatants were assayed for infectious virus by plaque assay using MDCK cells (11) and the hemagglutinin titer was determined as described previously (12).

The effect of aspirin on the kinetics of influenza B growth in MDCK and HepG2 cells. Preliminary experiments showed that ASA (Sigma, St. Louis, MO) concentrations up to 300 $\mu\text{g/ml}$ of

MEM had no effect on the viability and growth of MDCK and HepG2 cells for at least 96 h.

Confluent HepG2 or MDCK cells grown in ASA concentrations of 0, 25, or 100 $\mu\text{g/ml}$ were inoculated with influenza B at a MOI of 10. The virus was allowed to adsorb for 1 h at 37° C. The cells were washed with PBS three times, and MEM containing the same dosages of ASA was again added to the plates. Aliquots of media were taken at 4, 24, 48 and 72 h after inoculation and assayed for infectious virus by a MDCK plaque assay.

Electron microscopy. HepG2 monolayers infected with influenza B at an MOI = 10 were harvested at 0, 3, 6, 9, and 12 h postinoculation, immersed in 2% glutaraldehyde, osmium fixed, dehydrated in graded ethanols, stained, and examined by a Phillips 300 electron microscope.

The effect of influenza B and aspirin on total protein synthesis by HepG2 cells. HepG2 cells were grown to confluence on 100-mm dishes (Falcon, Oxnard, CA) and inoculated with influenza B (MOI = 10) or with media alone. After absorption for 1 h at 37° C, the inoculum was removed, the cells were washed with PBS, and overlaid with MEM, with or without 100 $\mu\text{g/ml}$ ASA.

Total protein synthesis was determined 1, 4, 8, 12, and 24 h after inoculation by incubating the cells in methionine-depleted (1/10 standard methionine concentration) MEM with 25 $\mu\text{Ci/ml}$ ^{35}S -methionine (New England Nuclear, Boston, MA) for 30 min at 37° C before harvesting. The cells were washed twice with ice-cold PBS and then collected with a rubber spatula, resuspended in 1 ml PBS, and centrifuged at 1600 rpm for 5 min. The pellet was resuspended in lysis buffer (10 mM Tris, adjusted to pH 7.5 with NaOH, 10 mM NaCl, and 1.5 mM MgCl_2) and incubated for 5 min at 4° C. Proteins were extracted in 10% NP-40 for 5 min at 4° C. The suspension was centrifuged at 1600 rpm for 5 min. A 200- μl aliquot of cell lysate was added to 2 ml of 20% TCA with 25 μl of 1% BSA and incubated on ice for 30 min. The precipitate was collected onto Whatman filter paper, dried, and counted by liquid scintillation.

Immunoprecipitation of hepatic secretory proteins. In a separate series of experiments, synthesis of individual proteins was examined 2, 4, 6, 9, and 12 h after infection or mock infection of HepG2 cells with influenza B. Rabbit antiserum directed against human serum albumin, transferrin, and apoprotein B were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Ten μl of antibody were added to 80 μl of cell lysate and incubated at 4° C overnight. The antigen-antibody complex was adsorbed for 20 min with 50 μl killed *Staphylococcus aureus* protein A bacteria (Boehringer Mannheim Biochemicals). The immunoprecipitate-Staphylococcal protein A complex was washed five times with PBS. Proteins were eluted and analyzed by SDS 10% polyacrylamide gel electrophoresis and an aliquot was placed directly in Aquasol (New England Nuclear) and counted in a Beckman LS-3801 scintillation counter.

RESULTS

Kinetics of influenza B growth in MDCK and HepG2 cells. HepG2 monolayers were infected at various multiplicities of infection. Release into the supernatants of virus was quantified by plaque assay and HA titer. The results, shown in Figure 1, were compared with growth in MDCK cells, a permissive cell line for influenza B. At an MOI of 0.1 pfu/cell, no influenza B growth was detected in HepG2 supernatants, whereas MDCK supernatants, after 48 h, showed more than 10^7 pfu/ml and an HA titer of 1:128. At an MOI of 1 and 10 pfu/cell influenza B growth was detected in HepG2 supernatants, however, appearance of virus was delayed by 12 h when compared with MDCK cells. In addition, viral titers were 2–4 logs less in HepG2 supernatants at 12, 24, and 48 h after inoculation than in supernatants of MDCK monolayers. HA titers were inadvertently not performed on the HepG2 supernatants at 48 h. Not shown are

kinetic curves at a MOI of 100 which did not differ substantially from curves at 10 pfu/cell.

In cell culture systems, proteolytic cleavage of influenza virus HA facilitates viral penetration of the host cell membrane into the cytoplasm (13). In the absence of 0.04% trypsin in the cell culture medium, viral growth in both HepG2 and MDCK cell lines was decreased by 10^3 – 10^4 pfu/ml (data not shown).

The effect of aspirin on the kinetics of influenza B growth in MDCK and HepG2 cells. The consumption of aspirin by children with viral illness is known to affect the epidemiology of Reye's syndrome (14–17). The addition of 25 or 100 μg ASA/ml of growth medium to HepG2 cells infected with influenza B (MOI = 10) did not appear to affect the growth of influenza B when compared to control cells. These data are shown in Table 1 and are expressed as the ratio of influenza B growth in the presence of ASA to growth in the absence of ASA. Similar data were derived from studies in MDCK cells (Table 1).

Electron microscopic studies of influenza B-infected HepG2 cells. Viral particles were first seen 9 h after inoculation at a MOI of 10 pfu/cell. Twelve h after inoculation, large numbers of tubular and spherical forms of influenza B virus were seen in the extracellular space surrounding HepG2 cells (Fig. 2). Because titrations of the supernatant media at 12 h did not detect any infectious virus, the morphologically characteristic particles seen at this time were noninfectious virus that may represent incomplete virus or defective interfering particles. No consistent HepG2 cell ultrastructural abnormalities were noted.

The effect of influenza B and aspirin on total protein synthesis by HepG2 cells. The total protein synthesis by influenza B-

Fig. 1. Kinetics of influenza B growth in MDCK and HepG2 cells. MDCK (—) and HepG2 (---) monolayers were inoculated at MOI = 0.1, 1.0, and 10. Influenza B virus titers were determined as described in "Materials and Methods." HA titers are shown in parentheses.

Table 1. Influenza B titers* after exposure to ASA

ASA ($\mu\text{g/ml}$)	Time (h)	Ratio of growth with ASA/ growth without ASA	
		HepG2 cells	MDCK cells
25	4	0.8	1.0
	24	1.8	1.0
	48	1.3	0.60
	72	1.2	1.7
100	4	0.8	1.0
	24	1.1	1.0
	48	0.8	0.72
	72	0.9	0.83

* Influenza B virus titers (pfu/ml) are expressed as the ratio of growth with ASA/growth without ASA.

infected and mock-infected HepG2 cells was determined by ^{35}S -methionine incorporation into TCA-precipitable protein. Figure 3 illustrates a representative experiment with the mean of duplicate samples shown. The initial (1 h) burst of protein synthesis in influenza-infected cells was repeatedly demonstrated and was not seen in mock-infected cells. Subsequently, however, protein synthesis was consistently reduced in influenza B-infected HepG2 cells. At 24 h, protein synthesis by uninfected cells was nearly twice that seen at 1 h. In sharp contrast, after 24 h of infection, the rate of protein synthesis in infected cells was 20% of that seen in uninfected cells. Three additional experiments demonstrated comparable results, with protein synthesis in infected cells ranging from 10–42% of that seen in uninfected cells.

The effect of ASA on protein synthesis by uninfected and influenza B-infected HepG2 cells was examined from 1 to 24 h after inoculation. The presence of ASA did not appreciably change the rate of protein synthesis in uninfected cells at any time studied. Similarly, inhibition of protein synthesis by influenza B-infected HepG2 cells was not significantly changed by the addition of ASA to the media (data not shown).

Synthesis of specific secretory proteins. Cell lysates prepared

Fig. 2. Electron photomicrograph 12 h after inoculation of HepG2 cells with influenza B virus. Typical spherical and tubular influenza virus forms are seen ($\times 17,000$).

Fig. 3. ^{35}S -methionine incorporation into influenza B infected and mock-infected HepG2 cells. Cell lysates were prepared from infected (○) or mock-infected (●) monolayers after ^{35}S -methionine pulse labeling at the time indicated (see "Materials and Methods").

from infected and mock-infected HepG2 cells were immunoprecipitated to determine the relative rates of albumin, transferrin, and apoprotein B synthesis. The rate of protein synthesis was determined at time intervals from 1 to 12 h after inoculation. The cells were infected at a MOI = 10, an inoculum that resulted in no detectable virus growth at 12 h, but recovery of approximately 10^3 pfu/ml by 24 h. As demonstrated in experiments on total protein synthesis, the initial rate of albumin, transferrin, and apoprotein synthesis was markedly stimulated 1 h after inoculation with influenza B when compared with controls (data not shown). Figure 4 shows that the subsequent rate of protein synthesis of each of the three proteins was substantially decreased as early as 6 h postinoculation. Albumin synthesis was decreased by approximately 50%, and transferrin and apoprotein B synthesis were decreased by 11 and 19% respectively. After 12 h of infection, albumin synthesis was decreased 60%, and transferrin and apoprotein B synthesis rates were decreased by approximately 40%. These data indicate that influenza B virus is capable of rapid inhibition of specific host HepG2 protein synthesis.

The uptake of ^{35}S -methionine into infected or mock-infected HepG2 monolayers was determined after 15, 30, and 45 min of incubation. The uptake rates were not different indicating that the observed differences in protein synthesis rates are not due to alterations in the membrane permeability or uptake of ^{35}S -methionine (data not shown).

Fig. 4. Inhibition of individual protein synthesis by influenza B virus infection of HepG2 cells. Cell lysates were prepared and specific proteins were immunoprecipitated as described in "Materials and Methods." The ordinate represents the percent inhibition of protein synthesis in influenza B virus infected cells as compared with mock-infected controls. Each data point represents an average of two experiments.

DISCUSSION

Many animal models of Reye's syndrome have been proposed and studied (18), however, the pathogenesis of this disorder remains incompletely understood in part because the models do not fully mimic the human disease. The association of a prodromal viral illness with Reye's syndrome has prompted speculation regarding a direct interaction of viruses or specific viral gene-products with the host hepatocyte protein synthetic apparatus (8, 9). The intracellular mechanisms for such a pathogenetic sequence would not be easily demonstrated using an animal model. Consequently, a cell culture system was established for study of the interaction of influenza B with a human liver cell line.

Our study demonstrates that influenza B virus is capable of a productive infection in a human hepatoma cell line. However, when compared with growth kinetics in a standard influenza B cell line (MDCK), a greater virus input was required for detection of replication in HepG2 cells. For example, no virus was detected in HepG2 supernatants up to 48 h after inoculation at a MOI = 0.1 while growth in MDCK supernatants was early (12 h) and brisk (10^7 pfu/ml at 48 h). At greater virus inputs, the amount of virus detectable in supernatants was 10^2 – 10^4 pfu/ml less in HepG2 than in MDCK cells. Proteolytic cleavage of viral surface HA has been shown to be necessary for optimal penetration of influenza B virus through host cell membranes (13). This appeared to be particularly true for HepG2 cells as no virus growth was detectable in the absence of added trypsin except at 48 h after an input of 100 pfu/ml. The apparent differences in influenza B growth curves for HepG2 and MDCK cells could be accounted for by several mechanisms, none of which was further addressed herein. These include diminished virus attachment and penetration of HepG2 cells, a decreased rate of viral replication or reassembly of viral particles, assembly of defective viral particles or a decrease in the rate of virus release. In our studies, electron photomicrographs demonstrated large numbers of typical tubular and spherical forms of influenza B virus in the extracellular space of HepG2 cells by 12 h after inoculation although no infectious virus was detected at this time. These data suggest that influenza B infection of HepG2 cells generates many noninfectious, incomplete, or defective interfering particles. Similar data were recently reported in a mouse model of influenza B induced Reye's-like syndrome in which a nonpermissive influenza B infection of the liver and brain of infected animals was demonstrated (9). In contrast to a previous study with varicella (19), in our studies ASA did not enhance growth of influenza B in either HepG2 or MDCK cells.

The ability of influenza B virus to alter host HepG2 cell protein synthesis was examined. After an initial burst of protein synthesis at 1 h, total protein synthesis by influenza B-infected HepG2 cells was markedly inhibited. This inhibition was not enhanced by ASA. Three specific proteins were also studied: apoprotein B, albumin, and transferrin. An initial burst of protein synthesis with a subsequent rapid decrease in the synthesis of apoprotein B, albumin, and transferrin was demonstrated. The rate of albumin synthesis appeared to decrease at a faster rate than transferrin and apoprotein B. Although the initial 1 h burst of host protein synthesis in infected cells has not been previously described, the differential virus-directed suppression of host protein synthesis at subsequent times has been described with other viruses in other cell lines (15–17).

Herein the data indicate that influenza B virus can establish a productive but less permissive infection in a human hepatoma cell line and rapidly alter host cell protein synthesis. Although this *in vitro* system is clearly not a model for Reye's syndrome, further delineation of the mechanisms by which influenza B virus-encoded proteins inhibit hepatocyte protein synthesis may provide insight into mechanisms important in the pathogenesis of Reye's syndrome. Identification of a specific virus-encoded protein responsible for the inhibition of protein synthesis in cell culture systems may provide a method of probing for a similar protein in hepatic tissues in animal models or patients with Reye's syndrome.

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