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Research Article

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Selective Reovirus Infection of Murine Hepatocarcinoma Cells during Cell Division

A Model of Viral Liver Infection

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Abstract

Reovirus type 1, strain Lang (1/L), can infect hepatocytes in vivo only after hepatocellular damage is induced by hepatotoxins, surgical trauma, resection, or profound immunosuppression. To examine the role of cell cycle and cellular differentiation on liver cell susceptibility to reovirus infection, a murine hepatocarcinoma cell line, Hepa 1/A1, was infected with reovirus and assayed for the presence of infectious virus or reovirus antigen in cells. Despite a > 95% binding of reovirus to hepatocarcinoma cells as indicated by cytometric analysis; only 10% of hepatoma cells contained infectious virus by infectious center assay. In comparison, 100% of L cells were infected. Analysis of intracellular reovirus antigen revealed its presence in dividing but not in quiescent hepatocytes. This correlation of cellular division and cell capacity to support viral replication suggests that induction of hepatocyte proliferation may be a mechanism for liver susceptibility to reovirus infection. (*J. Clin. Invest.* 1994. 94:353–360.) Key words: receptors • virus • liver regeneration • cell cycle • intestinal infection • cellular differentiation

Introduction

Reoviruses replicate in tissue culture–adapted cell lines from many different organs (1, 2). However, data obtained from studies in adult mice indicate that few types of cells are capable of supporting productive reovirus infection (3–5). A component of the diminished susceptibility of cells to infection and lack of development of acute disease in the adult mouse is immune regulated, as severe combined immunodeficient (scid)[1] mice develop progressive liver disease (6). However, the resistance to infection in adult mice also depends upon an intrinsic resistance of most cell types (3–5). Our studies have indicated that the replicative cycle of reovirus in murine intestinal epithelial cells is limited to the dividing crypt epithelial cells in the intestine; virus does not replicate in mature, differentiated villus epithelial cells (4). The cellular receptor for reovirus type 1 is expressed on both cell populations (7), however, suggesting that reovirus replication is dependent upon factors which affect virus replication subsequent to virus attachment and might be dependent upon the cellular replicative cycle. To examine whether cellular division modulates reovirus replication, we developed an in vivo model using hepatotoxins, or blunt surgical trauma to stimulate liver cell division coincident with reovirus infection (8). It was found that induction of hepatocyte proliferation correlated with a substantial increase in the number of hepatocytes expressing viral antigens.

In this report, we examine whether the in vivo effect of virus replication relative to cellular division in liver cells can be studied in a hepatocarcinoma cell line, Hepa 1/A1. The Hepa 1/A1 cell line was derived from a spontaneously arising hepatocellular carcinoma in a C57B mouse (9). Murine hepatocarcinoma cell lines, e.g., Hepa 1/A1 cells, have been shown to differentiate in the presence of low concentrations of dimethylsulfoxide (DMSO), as evidenced by an increase in albumin synthesis, an increase in contact inhibition, and a decrease in cell cycling (10–12), phenomena not seen in L cells.

We report that the cell cycle of Hepa 1/A1 cells provides control over the replication of reovirus type 1, a feature not demonstrable with L cells. In addition, the decrease in viral replication that occurs with G0 resting cells occurs at an event in the reovirus replicate cycle after cellular attachment of the reovirus to its receptor.

Methods

Virus. Reovirus serotype 1/L was initially obtained from Bernard N. Fields (Harvard Medical School, Boston, MA). Cloned virus stocks were passaged twice in L cells in Joklik's spinner minimum essential media (MEM) supplemented with 5% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD) and 1 U of penicillin per milliliter, and 1 μg of streptomycin (GIBCO BRL) per milliliter (complete L cell media). For some studies, reovirus was purified from third passage stocks in L cells by substituting ultrasonic disruption (Ultrasonic 250; Branson Sonic Power Co., Danbury, CT) for cell homogenization in a modification of previously described techniques (4, 13). The ratio of viral particles to plaque forming units (pfu) was approximately 100:1 (14).

Virus infection of Hepa 1/A1 and L cells. L cells were grown in Joklik's spinner MEM as above. Hepa 1/A1 cells were grown in MEM supplemented to contain 10% FCS, 2 mM L-glutamine, 1 U of penicillin per milliliter, and 1 μg of streptomycin per ml (complete Hepa 1/A1 media). For infection of Hepa 1/A1 and L cells, 2×10^5 to 2.5×10^6

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1. *Abbreviations used in this paper:* BrdU, 5-bromo-2′-deoxyuridine; MOI, multiplicity of infection; pfu, plaque forming units; scid, severe combined immunodeficient.

cells were seeded into T-25 tissue culture flasks (Costar Corp., Cambridge, MA). In some experiments, complete L cell or Hepa 1/A1 cell media was supplemented with 2 or 3% dimethyl sulfoxide (DMSO) for 24 to 48 h before infection with reovirus. Cells were adsorbed with reovirus type 1/L, at a multiplicity of infection (MOI) of five plaque forming units (pfu) per cells, for 1 h at 37°C. The cells were washed once and the appropriate media added for each cell type and experimental condition. Cells were maintained at 37°C in 5% CO_2 for the duration of the experiment.

Viral binding and flow cytometry analysis. Viral binding was assayed by flow cytometry as previously described (15). Briefly, cells were centrifuged and were washed twice in 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS), pH 7.4/0.1% NaN_3 (FAC wash), and 5×10^4 cells in 50 μl were distributed in 2% BSA/PBS/0.1% NaN_3 plates. Each cell type (Hepa 1/A1 or L) was incubated with reovirus type 1, at 5×10^4 particles per cell, for 1 h in 50 μl of ice cold FAC wash, washed twice and re-suspended in a 1:20 dilution of rabbit anti-reovirus antibody in 100 μl of FAC wash for 1 h. The wash procedure was repeated and cells were re-suspended in 1:50 dilution of FITC-labeled goat anti–rabbit antibody (Sigma Chemical Co., St. Louis, MO) for 30 min, washed twice and were fixed in 500 μl of 1% formaldehyde (Electron Microscopy Sciences, Ft. Washington, PA). Fluorescence intensity was determined by flow cytometry. To determine the quantity of infectious virus bound to cells, Hepa 1/A1 and L cells, at 10^4 per ml, were incubated with reovirus type 1, MOI = 5, suspended in 50 μl of RPMI 1640 for 1 h at 4°C. Cells were washed twice in PBS and lysed by three cycles of freezing and thawing prior to virus titration on L cell monolayers for plaque assay.

Virus plaque assay. The concentration of virus was determined by plaque assay as previously described (4), with a modification of the methods of diluting the samples to be assayed. Briefly, all dilutions for these assays were made in microtiter plates using 180 μl per well of saline containing gelatin with a multichannel pipetter and 20 μl of sample or dilutant. All samples were diluted serially in 10-fold increments with changes in the microtiter tips between sample wells. Duplicates of each sample to be titered were made by skipping the adjacent columns of wells on the plate (1 and 3 for sample 1, etc.), thereby four or six samples were diluted per 96-well plate. The use of every other column of wells for samples allows for simplified addition of the replicate samples to the L cell monolayers by using two channels of the multichannel pipetter, which proceeds from the wells containing the most dilute sample to the wells containing the most concentrated samples that were titered. By simultaneously doing tube dilutions of the same sample, the above method was found to be as accurate as the tube dilution method. To add virus to monolayers, plates were inverted over ice to remove medium and quickly covered while inverted to maintain sterility. The ice decreased splashing of media back into the plate. Plates were manipulated to evenly distribute the virus over the monolayer and incubated for 1 h at 37°C before adding nutrient medium in agar. At 6 d, wells were overlayed with neutral red, at .02%, containing media in agar, and plaques were counted 24 h later.

Reovirus infectious center assay of Hepa 1/A1 and L cells. Infectious cell assay was performed as previously described (16). Reovirus type 1/L was adsorbed to either L or Hepa 1/A1 cells for 1 h at 4°C, cells were washed twice with PBS, re-suspended in either complete L cell or Hepa 1/A1 cell media. To evaluate internalization of virus, cells were incubated at 37°C in complete media for 1 h after virus absorption, then washed twice in saline containing gelatin and treated with anti-reovirus antibody (1:10 dilution) for 1 h at 23°C. After an additional two washes to remove excess antibody in saline containing gelatin, cells were allowed to bind to tissue culture plates in limiting dilution followed by the addition of L cells sufficient to form monolayers (7.5×10^5 cells). The number of infectious centers was scored by counting plaques as indicated in the methods for a viral plaque assay and dividing the number of plaques obtained by the total number of cells plated. The total release of reovirus present in infected Hepa 1/A1 or L cells was determined by allowing either cell type treated as above to adhere to tissue culture plates for 72 h, after which cells were removed from the plates by treatment of the monolayers with trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) in PBS. The dispersed cells were either plated as above for an infectious center assay or exposed to three cycles of freezing then thawing prior to virus titration on L cell monolayers.

Cell protection assay using anti-reovirus antibody. 100 μl of L cells or Hepa 1/A1 cells, at 1×105 cells per ml, were plated in 96-well plates in RPMI 1640 media (GIBCO BRL) containing 10% FBS, 2 mM L-glutamine, and 1 U penicillin per ml and 1 μg streptomycin per milliliter. Media was removed at 24 h and rows of cells were absorbed with serial twofold dilutions of reovirus type 1, MOI = 160 in highest virus dilution, in 100 μl of media for 1 h at 4°C followed by incubation at 37°C to allow for internalization of virus. Monolayers of cells were washed twice with PBS, and then maintained in 100 μl of fresh complete RPMI 1640 media. In some rows of L cells and Hepa 1/A1 cells the media was supplemented with a 1:50 dilution of rabbit anti-reovirus type 1 antibody. To demonstrate that the quantity of antibodies used was sufficient to neutralize virus in both cell types, dilutions of reovirus were pre-incubated with anti-reovirus antibodies for 1 h before adsorption of virus-antibodies mixture on L-cell or Hepa 1/A1 cell monolayers. Plates were incubated at 37°C for 4 d, media was removed by inversion, cells were washed twice with PBS, fixed with 100% ethanol, and stained with gentian violet.

Cell cycle analysis. To determine the phase of the cell cycle for Hepa 1/A1 or L cells, cells were incubated with 5-Bromo-2′-deoxyuridine (BrdU), 10 μM, (Sigma Chemical Co.) for 2 h and then washed with PBS, pH 7.4. Reovirus, at a MOI of 5, was absorbed to cells for 1 h at 37°C, then washed twice with PBS, and maintained in complete MEM supplemented with Apidicholin, 1 μg/ml (17). Cells were suspended in FACS buffer after removal from the tissue culture plates by trypsin/EDTA in PBS. The membrane was solubilized with acetone after a 10-min incubation with 4 N HCl, and then cells were incubated with a murine fluorescein conjugated anti-BrdU monoclonal antibody (Becton-Dickinson, Mountain View, CA), diluted 1:10, and sorted for fluorescent (BrdU) containing and BrdU negative populations of cells by FAC. Fluorescent positive and negative populations of sorted cells were cytospun onto glass slides and incubated sequentially with rabbit anti-reovirus antibodies, avidin-peroxidase labeled goat anti-rabbit antibodies, and a mixture of DAB (50 μg/ml), nickel sulfate (80 mg/ml) and H_2O_2 (0.12 μl/ml) (18). Cells that were immunohistochemically stained were counted without knowledge of sorted population and reported per 100 cells counted. At least 300 cells were examined from each sample to determine the presence of reovirus antigen. In other experiments, Hepa 1/A1 cell cycle was determined following DNA synthesis inhibition and reovirus infection by substituting propidium iodine for BrdU labeling.

Statistical analysis. Statistical analysis utilized Student *t* test. Statistical significance was achieved at $P < 0.05$.

Results

Receptors for reovirus type 1 are expressed on Hepa 1/A1 cells. Previous experiments with various cells suggested that a major factor in the capacity of reovirus to replicate is the presence of cellular receptors that allow viral attachment (15, 19, 20). To assess whether reovirus type 1/L can bind to Hepa 1/A1 cells, reovirus type 1/L was adsorbed at 4°C, and virus binding was examined by indirect immunofluorescence using flow cytometry. We found that reovirus type 1/L binding to Hepa 1/A1 cells occurs with a fluorescent intensity similar to the binding of reovirus type 1/L to L-cells (Fig. 1). In addition, similar titers of reovirus were recovered from L cells (4.2×10^4 per 10^4 cells) and Hepa 1/A1 cells (4.3×10^4 per 10^4 cells) after binding of virus to cells at 4°C indicating that infectious virus was not bound out of proportion to total virus particles for either cell type. Therefore, Hepa 1/1A cells contain reovirus receptors which bind infectious and noninfectious virus.

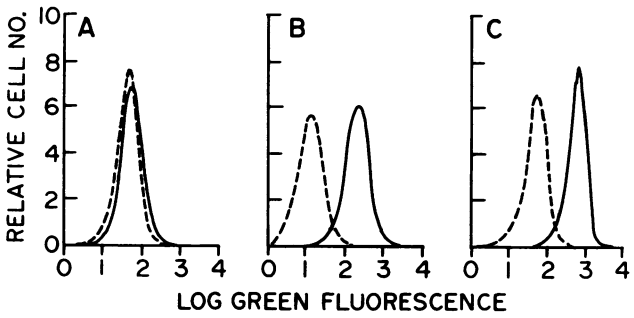

Figure 1. Reovirus binding to Hepa 1/A1 cells is similar to virus binding to L cells. Reovirus binding to Hepa 1/A1 cells and L cells assayed by flow cytometry. *A* demonstrates that rabbit anti-reovirus antibodies do not bind to cells in the absence of virus. The dashed (– – –) line denotes populations of Hepa 1/A1 cells without primary or secondary antibodies whereas the solid (——) line denotes cells exposed to primary and secondary antibodies in the absence of reovirus. The dashed line in *B* and *C* indicates populations of cells that were exposed to anti-reovirus antibodies and fluorescein-conjugated goat anti-rabbit IgG in the absence of virus (control), whereas the solid line represents populations of L cells (*B*) or Hepa 1/A1 cells (*C*) in which reovirus was adsorbed before the addition of rabbit anti-reovirus antibodies and immunofluorescent reagents.

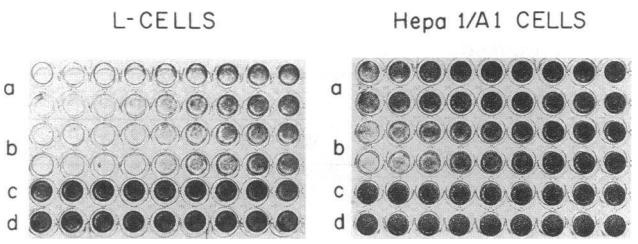

Figure 3. Anti-reovirus antibody protects Hepa 1/A1 cells, but not L cells, from viral spread. Rows of Hepa 1/A1 cells or L cells were incubated with twofold dilutions of reovirus type 1 at 4°C for 1 h, then washed and maintained for 4 d in complete RPMI 1640 at 37°C. The highest titer of virus is present in the left-most column of wells. Some rows of cells also had a 1:50 dilution of rabbit anti-reovirus antibody added to the entire row. Surviving cells were washed with PBS, fixed in 100% ethanol and stained with gentian violet. The proportionate decreases in stained cells is consistent with increases in lytic infection of the monolayer. Duplicate rows are shown for (*a*) reovirus infected cells incubated with anti-reovirus antibodies for 1 h; and (*b*) reovirus infected cells not incubated with anti-reovirus antibodies; Single rows of cells are shown for (*c*) reovirus pre-incubated with anti-reovirus antibodies for 1 h before absorption; and (*d*) non-infected cells.

A limited number of Hepa 1/A1 cells replicate reovirus type 1. To determine the number of reovirus infected Hepa 1/A1 cells present after virus adsorption, infectious center assays were performed. The titers of reovirus 1/L-bound-to-cells after treatment with anti-reovirus antibodies were nondetectable if internalization did not occur (data not shown). Reovirus replication was found to occur in 10% of the plated Hepa 1/A1 cells and virtually 100% of the L cells (Fig. 2). In other experiments, anti-reovirus antisera was used to inhibit replication of non-adsorbed virus as above, but infectious centers were then determined at the termination of a 72-h infection in either L cells or Hepa 1/A1 cells (Fig. 2). In these experiments, ~ 40% of the Hepa 1/A1 cells were found to contain reovirus, compared to virtually all of the L cells. Controls consisting of similarly treated cells which were not incubated at 37°C to deter internalization had no detectable infectious centers.

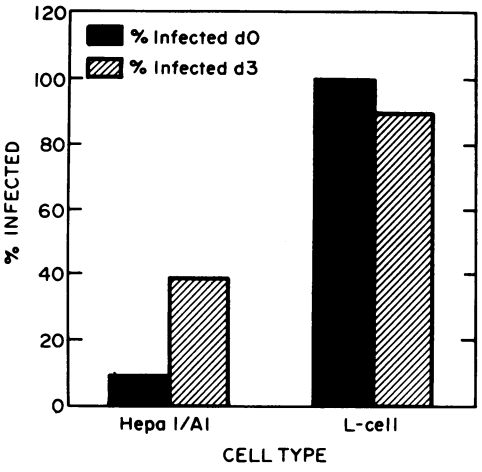

Figure 2. Infectious center assay comparing reovirus infection of L and Hepa 1/A1 cells. Cells were exposed to virus at an MOI of 5 at room temperature for 45 min, washed twice and then incubated with polyclonal rabbit anti-reovirus IgG to neutralize non-internalized virus and washed an additional two times. Cells then were allowed to adhere to 12-well plates and overlaid with monolayers of L cells. The number of infectious centers was determined by dividing the number of plaques obtained by the number of cells plated. In one set of experiments cells were assayed for reovirus infected cells after virus internalization (*d0*), whereas in the second experiment, cells were assayed for infectious centers after 48 h (*d3*).

To determine whether the additional infectious centers observed in Hepa 1/A1 cells with time was due to slower replication of virus in some cells, thereby decreasing our ability to score infectious centers during a reovirus plaque assay, we examined the capacity of Hepa 1/A1 and L cells to be lysed by virus. In addition, we protected Hepa 1/A1 and L cells from noninternalized reovirus with high titer rabbit anti-reovirus antibodies thereby providing another measure of whether both cell types are equally susceptible to reovirus. Rows of subconfluent L cell and Hepa 1/A1 cell monolayers in 96-well plates were infected with serial dilutions of reovirus type 1. In some rows of cells neutralizing rabbit anti-reovirus antibody was added. At the termination of the experiment at 4 d, wells that had the highest titers of reovirus, MOI = 160 to MOI = 5, had sufficient virus for most of the L cells to be infected. Moreover, the integrity of L cell monolayers infected with reovirus type 1 is independent of treatment with anti-reovirus antibodies, with similar degree of lysis occurring in both conditions (Fig. 3, *a* and *b*). In comparison, reovirus type 1 infected Hepa 1/A1 cell monolayers were protected from lysis when the cells were incubated with anti-reovirus antibodies for only 1 h (Fig. 3 *a*). There was an increase in infection of Hepa 1/A1 monolayers in the absence of anti-reovirus antibodies (Fig. 3 *b*). However, substantially less Hepa 1/A1 cells were infected than L cells, consistent with the results obtained in the infectious center assay. To demonstrate that the effectiveness of anti-reovirus antibodies to neutralize virus is not just cell dependent, incubation of reovirus with anti-reovirus antibodies for 1 h before infection of monolayers protected both cell types (Fig. 3 *c*). Therefore, nearly all L cells in any population adsorb, internal-

Table I. Cell Cycle Analysis of Cells after Various Culture Conditions

	Cell cycle analysis	
	G0/G1	S/G2/M
	%	%
Subconfluent	*49*	*51*
Confluent	*82*	*18*
2% DMSO	*63*	*37*

The effect of varying culture conditions on the percent of cells in different compartments of the cell cycle was determined by staining cells with propidium iodide, and performing flow cytometric analysis. Rapidly growing cultures (50% confluent) contained the fewest cells in G0/G1, while confluent cultures contained the highest number of resting cells. DMSO treated cultures had an intermediate number of cells in resting phase.

ize, and support viral replication. Despite comparable binding of virus by Hepa 1/A1, the proportion containing infectious virus is much lower. However, this phenotype is not stable, and additional cells are infected over time.

Efficiency of replication of reovirus is affected by factors which influence cell cycling in Hepa 1/A1 cells. To determine if the yield of reovirus may be affected by cell type, the total amount of virus present in each cell type was determined after lysis of infected cells at 48 h. The total number of infected cells was determined by infectious center assay, and the number of virus infected cells was correlated to the total amount of reovirus present. It was found that infectious centers from L cells and Hepa 1/A1 cells each contain ~ 300–400 pfu of reovirus. Therefore, given a Hepa 1/A1 cell competent for reovirus type 1 replication, virus grew as well as in L cells.

Hepa 1/A1 cells exposed to DMSO differentiate and to a lesser extent stop dividing. The proportionate decrease in cellular division after DMSO exposure is manifest by the number of resting cells in G0/G1, 63%, compared with subconfluent cells in G0/G1, 49% (Table I). DMSO treatment of Hepa 1/A1 cells resulted in a proportionate decrease in the number of infectious centers compared to DMSO nontreated cells (Fig. 4), consistent with the effect of DMSO on the total number of cells in G0/G1 compared with control cells. The difference in infectious centers between those obtained from subconfluent cells was statistically different from all other groups ($P < 0.001$). In addition, the number of infectious centers obtained from confluent cells was statistically different from those obtained from cells grown in the presence of 2% DMSO ($P < 0.003$) or 3% DMSO ($P < 0.05$) demonstrating the additive effect of DMSO treatment. Furthermore, a considerable decrease in the pfu per infectious center was observed after DMSO treatment (Fig. 5). These results were confirmed by immunohistochemical visualization of infected cells for the presence of reovirus antigen (Fig. 6). Hepa 1/A1 cells cultured in the presence of DMSO had minimal discernible concentrations of virus antigen compared to sub-confluent cultures. DMSO did not affect virus recovery from L cells similarly treated (data not shown), nor did DMSO affect the binding of reovirus to Hepa 1/A1 cells (Fig. 7).

Reovirus replication is affected by cellular DNA replication in Hepa 1/A1 cells. To examine whether the replicative state

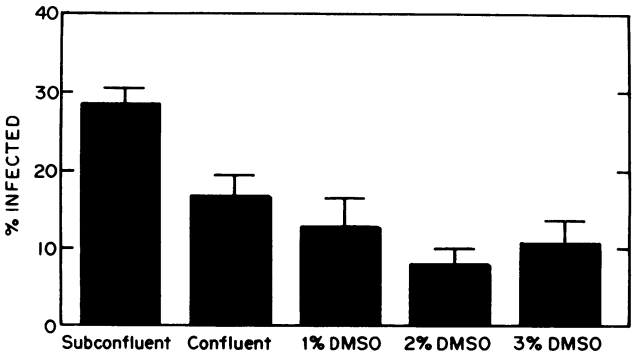

Figure 4. Infectious center assay comparing reovirus infection of Hepa 1/A1 cells in the presence and absence of DMSO. Hepa 1/A1 cells at 2×10^5 (subconfluent) to 2.5×10^6 (confluent) cells were seeded into T-25 tissue culture flasks (Costar Corp.). In some flasks, subconfluent cells were grown in media supplemented with 2 or 3% dimethyl sulfoxide (DMSO) for 24 to 48 h before infection with reovirus. Cells were exposed to virus at a MOI of 5 at room temperature for 45 min, washed twice and then incubated with polyclonal rabbit anti-reovirus IgG to neutralize non-internalized virus and washed an additional two times. 10-fold dilutions of cells were allowed to adhere to 12-well plates and overlaid with sufficient L cells to form monolayers. The number of infectious centers was determined by dividing the number of plaques obtained by the number of cells plated. The data represents the mean of four separate experiments±standard error of the mean.

of the cell influences reovirus antigen production, Hepa 1/A1 cells were arrested in phase by the addition of Aphidicolin and infected with reovirus. Before the addition of Aphidicolin, cells were pulsed with BrdU for 2 h, and the incorporation of BrdU is indicative of S-phase growth. 48 h after virus infection, cells arrested in S-phase were separated from cells that did not incorporate BrdU by fluorescent-activated cell sorting and examined for reovirus antigen using immunohistochemical methods after fixing cells onto glass slides. It was found that ~ 30% of the cells that incorporated BrdU expressed reovirus antigen compared with 5% of the BrdU negative cells (Fig. 8). Therefore, cells in S-phase are considerably more susceptible to infection with reovirus type 1/L than resting cells.

Discussion

Reovirus infection of mice has served as a useful model for studies of viral pathogenesis (21). Models of infection that

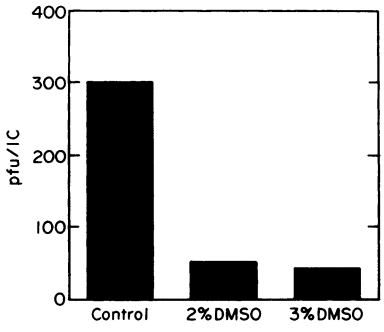

Figure 5. Concentration of infectious reovirus per infectious center following DMSO treatment. The quantity of reovirus present per infectious center was determined in cultures of Hepa 1/A1 cells 48 h after inoculation with virus. Cells were counted and divided into a group that was assayed for infectious centers and group cells that were lysed and total virus present titered on L cell monolayers. Treatment of Hepa 1/A1 cells with DMSO dramatically decreased the amount of virus replicating in the hepatocarcinoma cells.

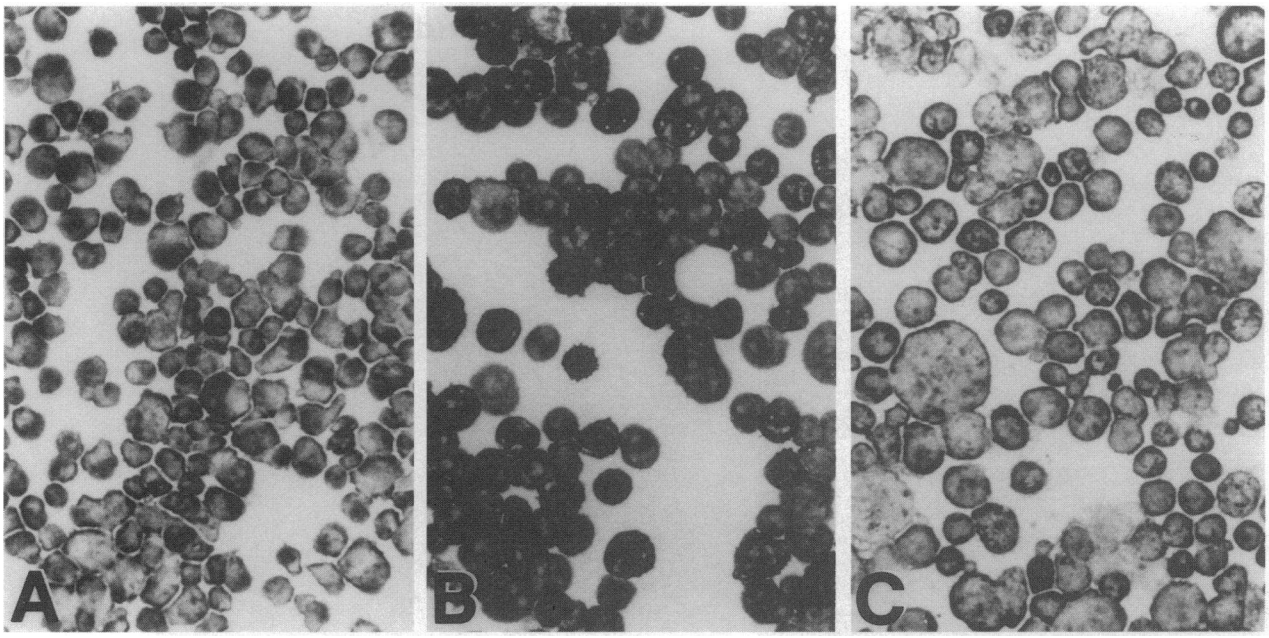

Figure 6. Immunohistochemical visualization of reovirus antigen in Hepa 1/A1 cells exposed to DMSO. Reovirus type 1, MOI of 5, was adsorbed to Hepa 1/A1 cells exposed to DMSO or control cells. The presence of reovirus antigen was assessed by immunohistochemical staining. Reovirus non-infected cells are shown which are representative of the level of background staining of immunohistochemical reagents (*A*), reovirus antigen is present in control, sub-confluent culture (*B*), and is barely detectable in DMSO exposed cells (*C*).

have been developed in adult immunocompetent mice demonstrate that reovirus serotypes 1 and 3 have limited capacity to infect adult tissues that are susceptible during neonatal development (3–5). In adult mice infected with reovirus type 1, crypt cells within the intestine appeared to be susceptible, but not mature villus cells (4). Additionally, tissues such as liver, that can be forced to undergo de-differentiation and replication, appear to be more susceptible to reovirus infection than quiescent tissues (8, 22). In the scid mouse, reovirus infection is predominantly limited to dividing cells within the intestine and liver. In this model, liver cells had the major change in susceptibility to reovirus infection, with a profound increase in the number of cells infected over a period of several weeks. Based upon the data obtained in scid mice and observations made on liver and intestinal infection of reovirus in conventional mice, we developed the hypothesis that reovirus replication was limited to replicating or activated cells in vivo.

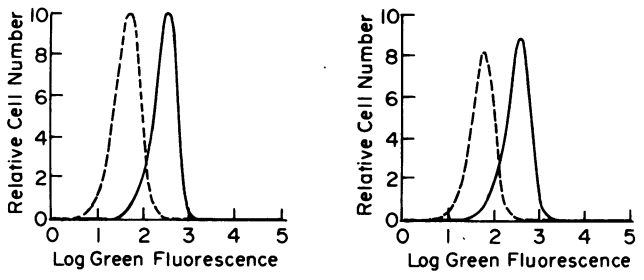

Figure 7. Reovirus effect of DMSO on reovirus binding to Hepa 1/A1 cells. The capacity of reovirus to bind to Hepa 1/A1 cells with or without treatment of cells with DMSO. Cells were incubated with reovirus, washed, then incubated with rabbit anti-reovirus type 1 followed by fluorescein-conjugated goat anti–rabbit IgG. The number of positive cells was quantitated with flow cytometry. Profiles of populations of cells without (*left*) or with DMSO (*right*) treatment demonstrate similar binding of reovirus (*solid lines*). Dashed lines are control populations of cells in which virus was not present before incubation with immunofluorescent reagents.

To confirm this hypothesis in vitro and to examine further the relative importance of cellular division or differentiation in the capacity of reovirus to replicate, we selected a hepatocyte cell line that differs from L cells by its properties of contact inhibition of growth and its capacity to differentiate. Preliminary experiments found that reovirus' capacity to infect Hepa 1/A1 cells differed from L-cells. For L cells, virtually all cells appear susceptible to infection, whereas for Hepa 1/A1 cells only a portion of the population of cells are susceptible. During prolonged incubation of Hepa 1/A1 cells with reovirus, additional cells become infected. The increase in the number of Hepa 1/A1 cells infected over time was inhibited by the addition of rabbit anti-reovirus antibody to the media. These data are consistent with anti-reovirus antibodies inhibiting infection due to a delay in entry of virus in some cells. Anti-reovirus antibody would not protect cells from infection if all cells internalized virus similarly and some cells replicated virus more slowly. Therefore, it is unlikely that slower replication of reovirus in most Hepa 1/A1 cells accounts for differences observed in replication of reovirus in Hepa 1/A1 and L cells.

Thus, the capacity to resist infection is not a stable phenotype as additional cells are infected as a function of time. This partial resistance to infection is different from resistance to infection that was recently reported for murine erythroleukemia (MEL) cells to reovirus type 1 or 3. In MEL cells, receptor mediated binding of virus is limited to reovirus type 3 strains that express the hemagglutinin domain (20). Reovirus type 1 and 3 strains that do not have the amino acid sequence that corresponds to this domain do not bind to MEL cells, and the MEL cells are uniformly resistant to infection (20). Therefore,

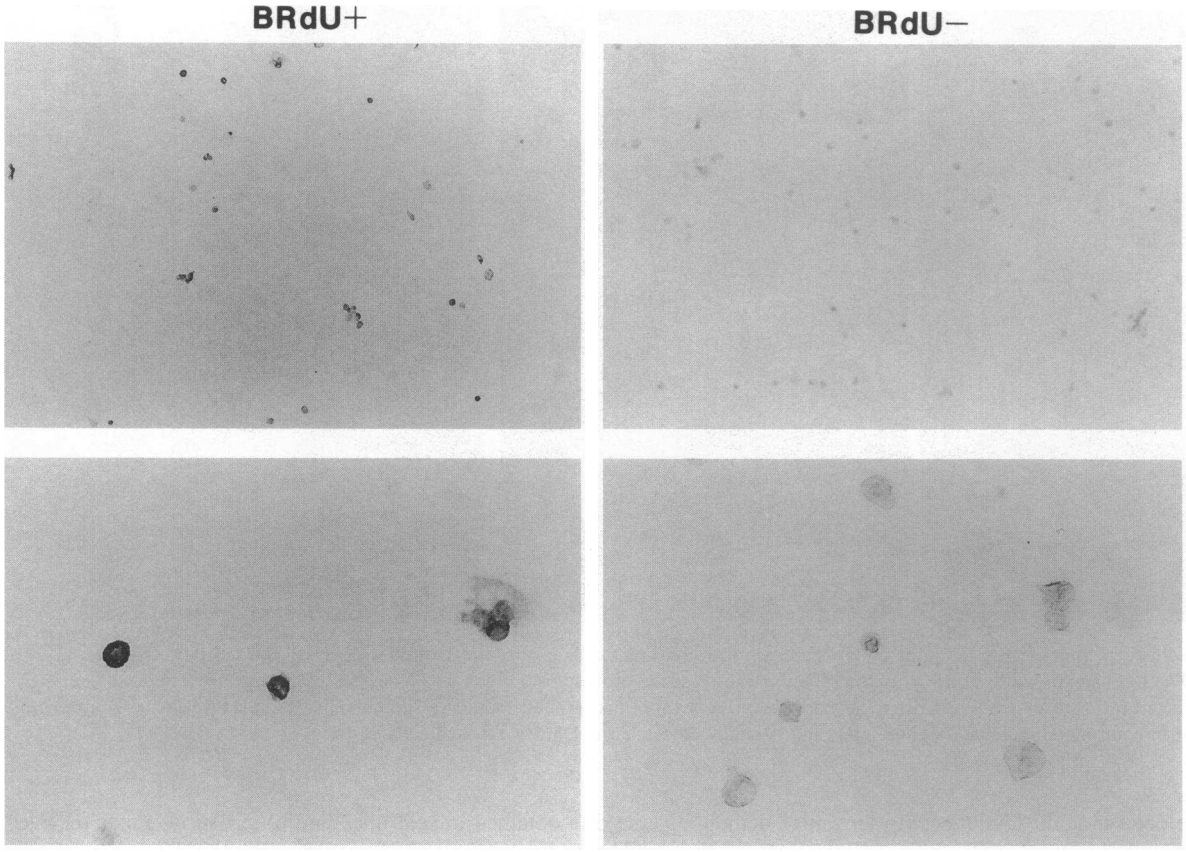

Figure 8. BrdU labeling of cells in S phase defines a population of cells capable of replicating reovirus type 1. Hepa 1/A1 cells were pulsed with BrdU for 2 h, washed twice and incubated with reovirus, MOI of 5, for 2 h at 37°C. Aphidicolin was added to culture, and 48 h later cells were fixed and stained with fluorescein-conjugated anti-BrdU antibodies (Becton-Dickinson). Sorted cells were separated into $BrdU^+$ and $BrdU^-$ populations by flow cytometry, followed by cytospinning each population onto slides and were then stained for reovirus antigen with immunoperoxidase technique. The top panels show low powered (×100), and the bottom panels show high powered (×400) cytospun and stained cells. Antigen positive cells were enumerated in a blinded fashion.

receptor-mediated binding is the major determinant of MEL cells' susceptibility to reovirus infection. Based upon indirect immunofluorescent techniques and flow cytometry, there was no indication of sub-populations of Hepa 1/A1 cells which do not bind reovirus. Indeed, reovirus' capacity to bind to populations of L cells and Hepa 1/A1 cells were quite similar. This data was supported by the observation that infectious virus, which accounts for ~ 1% of the virus particles, also bound to Hepa 1/A1 and L cells similarly. Therefore, any differences in susceptibility to reovirus of Hepa 1/A1 cells compared with L cells would be likely to occur after adsorption of virus to the cell surface, and is not due to differential binding of infectious virus to a subset of Hepa 1/A1 cells.

A second step that may be involved in the ability of reovirus to infect Hepa 1/A1 cells may be the capacity of the hepatoma cells to internalize virus. Indeed, after binding of reovirus type 1 and internalization, anti-reovirus antibodies inhibited 90% of the uninfected cells from becoming infected. These results are consistent with a block in infection at a step subsequent to adsorption. In murine thymoma (R1.1) cells, reovirus type 3 binds but does not infect cells due to a failure to internalize virus (23). However, for R1.1 cells, the failure of cells to internalize virus results in a uniformly resistant population of cells (23). Data exists to suggest that some cellular receptors are regulated by the cell cycle (24–26), and it is possible that for Hepa 1/A1 cells reovirus-receptor–mediated internalization is cell cycle dependent. Further studies are in progress to assess the role of internalization in reovirus replication in Hepa 1/A1 cells.

The capacity of reovirus to replicate in Hepa 1/A1 cells is not only limited by the finite number of cells initially infected. We found that reovirus yield per cell in Hepa 1/A1 cells is profoundly affected by the presence of DMSO in the media, which affects cellular differentiation (10, 11). Subconfluent cell populations had discernible differences in the number of cells in G0/G1 compared with DMSO-treated cells. These differences were enough to allow us to observe diminished numbers of virally infected cells, as determined by infectious centers following DMSO treatment. In Hepa 1/A1 cells treated with DMSO there was a striking decrease in the total amount of infectious virus present compared to subconfluent cultures. The data obtained from studies determining the yield of virus per infectious center was further supported by immunohistochemical visualization of viral antigen in DMSO treated cells compared to sub-confluent cultures. These data suggest a direct correlation between the presence of reovirus antigen and infectious virus yields. The concomitant decrease in reovirus antigen and infectious virus in DMSO-treated cells suggests that the

decrease in pfu per infectious center is due to a step in morphogenesis prior to translation. If a late step in morphogenesis was involved, reovirus proteins would accumulate within the cell out of proportion to the quantity of infectious virus. Moreover, these data suggest that it may be possible to separate the effect of the state of differentiation from the effect of cell cycle on reovirus replication.

The state of differentiation of the cells is not the only factor which affects reovirus replication in Hepa 1/A1 cells, as is suggested by data obtained from cells arrested in cell cycle by Aphidicolin (17, 27). Reovirus type 1 antigen was found predominantly in cells that were in S-phase at the time of reovirus adsorption. These data are consistent with the above notion that an early step after adsorption, such as virus internalization, may be cell cycle regulated in hepatocytes. Alternatively, factors that effect DNA replication may be required for a phase of reovirus infection. In this case, our findings would be consistent with data from other researchers who have found a requirement for cellular factors that are organ specific, developmentally, or cell cycle regulated for other viruses. These viruses include: hepatitis B virus (HBV) in which replication occurs in mature, differentiated hepatocytes (26, 28), hepatitis A virus in which trans-acting translation factors from liver specifically stimulate translation (29), papillomavirus, in which replication is dependent upon cellular differentiation factors (30), and human immunodeficiency virus (HIV), in which cellular factors, such as NFκB play a role in HIV transcription (31). Moreover, for HBV, DMSO stimulates virus growth consistent with the observations of enhanced growth of HBV in differentiated hepatocytes (32). Further studies in progress are aimed at defining whether specific transcription or translation factors are needed in reovirus replication.

The dependence of reovirus replication on both the state of differentiation and cell cycle is consistent with the observation of reovirus induced disease in neonatal but not adult mice. The decrease in viable progeny of viruses in differentiating cells confirms in vivo data obtained in intestinal infection with reovirus (4, 5). Additionally, it has been found that the size of the initial inoculum determines the extent of reovirus disease in adult mice inoculated parenterally, but not in parenterally inoculated neonates (3, 5, 33). These data suggest that infection is not self-sustaining, in part, due to the small quantity of infectious virus released as a result of cellular differentiation. Whether this phenomena may occur in other cell types is being studied.

The liver has the capacity to replicate in response to injury and to be stimulated to alter its metabolism in response to cytokines (34–36). Recently, several studies have found an association between hepatitis C and a hepatic toxin, alcohol, in the development of alcoholic hepatitis (37, 38), or hepatocarcinoma (39). In addition, the chronic liver disease associated with α1-antitrypsin deficiency may be related to increased susceptibility to virus infection (40). Whether the liver disease observed in these situations is due to similar processes to that described for reovirus remains to be determined.

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