Reovirus Type 2: Induction of Viral Resistance and Interferon Production in Fathead Minnow Cells[1] (35266)

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The mammalian reoviruses are known to infect many species of homeothermic animals (1). Whether the host range of these viruses extends to animals of lower toxonomic classifications has not been determined. Several mammalian viruses have been reported to successfully replicate *in vitro* in cells derived from poikilohermic animals (2). Therefore, attempts were made to infect cells from the fathead minnow (FHM), *Pimephalis promelas,* with reovirus type 2 (reo-2). Although these attempts were not successful, the FHM cells cultures exposed to reo-2 developed resistance to virus challenge and produced an antiviral substance which possessed many interferon-like properties. These findings are described below.

Materials and Methods. Cell cultures. Monolayer cultures of human amnion cells (RA) and baby hamster kidney cells (BHK/21) were serially propagated as previously described (3). A continuous line of fathead minnow cells (FHM) (Microbiological Associates, Inc.) was routinely cultured at 26° in Eagle's basal medium (EBM) plus 10% fetal bovine serum and antibiotics. The experimental medium consisted of EBM plus 2% fetal bovine serum and antibiotics (EBM2).

Viruses. Reovirus type 2, strain D-5, was prepared in HeLa cells as described elsewhere (3). The titer, as determined by the immunofluorescent plaque technique (4), was $10^{7.5}$ immunofluorescent units/ml (IU/ml). Purified ^{32}P-labeled reo-2 was prepared and furnished by Dr. K. Camyre of this laboratory. Frog virus 3 (FV3), obtained from Dr. A. Granoff (St. Jude Hospital, Memphis, Tennessee), was prepared in FHM cells. Briefly, monolayers of FHM cells were infected with FV3 at an input multiplicity of infection (MOI) of 0.5. When the cultures showed complete cytopathic effect (CPE), they were frozen and thawed, sonicated at 20 kcps for 30 sec, clarified by centrifugation at 2000 rpm for 10 min, and stored at —60°. This FV3 pool was plaque titrated by the fluid overlay method (5) in Leighton tube cultures of FHM and had a titer of $10^{7.6}$ plaque-forming units/ml (pfu/ml).

Infection procedure. RA and FHM cells were infected with reo-2 or FV3 by the following procedure: the cell cultures were washed 2 to 3 times with Hanks' balanced salt solution (BSS) and exposed to the virus inoculum for 2 hr at 26°. After the residual virus was removed by 3 additional washings with BSS, the cell cultures were fed with EBM2 and incubated at 26°.

Assay for antiviral activity. Since in a previous study reo-2 was found to be resistant to treatment at low pH values (3), reo-2 was removed from the inhibitor preparations prior to antiviral assay by filtration through Millipore filters (pore size 0.05 μ) using a Swinny hypodermic adapter. Preliminary studies had shown that this was an easy, efficient method for the removal of reo-2 from small volumes (up to 2 ml) of fluid. No infectivity was detected in the filtrate from a 2-ml volume of virus preparation which originally contained more than 8 $\log_{10}$ IU of reo-2. As a precautionary measure, all filtrates were treated with antireo-2 serum for 1 hr at 37° before being tested for viral inhibitory activity. The inhibitor preparations were assayed for antiviral activity by the plaque reduction method using a fluid overlay (6). Leighton tube cultures of FHM cells were exposed for 18 to 24 hr at 26° to serial 5-fold dilutions of the antiviral preparations. Then, the inhibi-

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tor was removed and the cells were washed twice with BSS before they were challenged with 60 to 80 pfu of FV3. After 48 hr of incubation at 26°, the cell sheets were stained with crystal violet and the plaques were counted. The titer of the inhibitor was considered to be the reciprocal of the highest dilution of the preparation which reduced FV3 plaque counts by 50%.

Staining techniques. The procedures for the preparation of reo-2-infected coverslip cultures of RA and FHM cells and for the staining of these cells with acridine orange and reo-2-specific fluorescent antibody have been previously described (3).

Radioactivity assay. The procedures for spotting strips of filter paper with samples and processing them for radioactivity measurements have been reported elsewhere (7).

Results. Adsorption. ^{32}P-labeled reo-2 was added to a suspension of FHM cells at a virus:cell ratio of approximately 10. This mixture was rotated on a gyro-rotary shaker immersed in a 26° water bath. At predetermined intervals of time, 0.5-ml samples were removed and centrifuged at 1500 rpm for 3 min. The residual radioactivity in the supernatant fluid was determined and the cell-adsorbed radioactivity was calculated as the difference in radioactive counts between time 0 and the subsequent time periods. The amount of cell-adsorbed radioactivity after 2.5 hr of adsorption (approx 30% of the input radioactivity) was considered as the 100% value and the percentage of adsorbed virus for all the other time periods was calculated accordingly. Figure 1 shows an adsorption curve constructed by plotting the percent of ^{32}P-labeled virus adsorbed to FHM cells against time. The results show that maximum adsorption was attained in approximately 1 h. The amount of ^{32}P-labeled reo-2 adsorbed in 1 hr was about 98% of that adsorbed in 2.5 hr. Based on this study, the use of a 2-hr adsorption period in all the experiments described here was considered more than adequate for maximum virus adsorption.

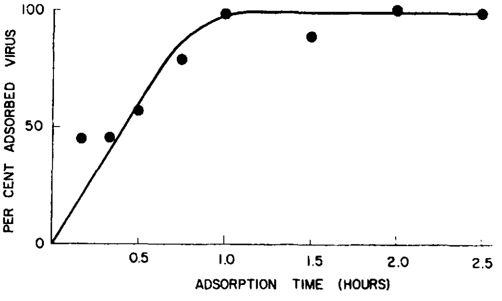

FIG. 1. Adsorption of reovirus type 2 (reo-2) to fathead minnow cells (FHM). Amount of ^{32}P-labeled reo-2 adsorbed at each time interval expressed as percentage of that adsorbed in 2.5 hr.

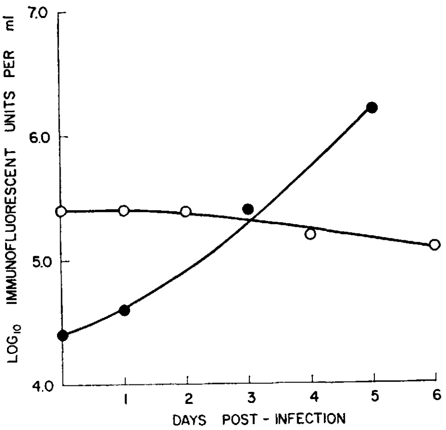

FIG. 2. Single cycle growth curves of reovirus type 2 (reo-2) in human amnion cells (RA) (●); and in fathead minnow cells (FHM) (○).

Growth studies. Single cycle growth studies of reo-2 in FHM and RA cells were done to determine whether virus replication can occur in these cells at 26°. Leighton tube cultures of FHM and RA cells were infected with reo-2 at a MOI of approximately 10. At predetermined times after infection, 2 tubes were removed and subjected to 2 cycles of freezing and thawing. The contents from the tubes of each time interval were then pooled, sonicated at 20 kcps for 1 min and centrifuged at 1000 rpm for 10 min. The supernatant fluids were harvested and assayed for infectious virus by the immunofluorescent plaque technique.

Figure 2 shows the resultant growth curves from one of the experiments. An increase of nearly 2 log$_{10}$ in virus titers was seen in RA cells by the fifth day postinfection (pi). In contrast, no virus replication was observed in reo-2-infected FHM cells during the 6-day duration of the experiment as indicated by the lack of increase in infectious virus titers.

A previous study had shown that reo-2 was very stable at 25°, suffering only slight losses in titers over a period of more than 2 months. Therefore, the infectious virus found in reo-2-infected FHM cell cultures could either be residual virus from the inoculum which was not removed by the washing process, adsorbed virus which had not undergone penetration and uncoating, or both.

FHM cultures contained 20- to 30-fold more cells/culture than RA cultures, thus requiring 20- to 30-fold more virus to obtain a MOI of 10. The high level of reo-2 titers found in FHM cultures at the initial time periods compared to that found in RA cultures was probably due to the larger doses of virus used to infect the fish cells.

Since FHM cells grow maximally at 34° (5), a subsequent experiment on reo-2 growth in fish cells was done at 33°. Even at this higher temperature, no reo-2 replication was observed in FHM cells.

Attempts were also made to demonstrate the production of viral antigen and viral inclusion in FHM cells infected with reo-2. Coverslip preparations of FHM cells infected with reo-2 were stained with acridine orange and reo-2-specific fluorescent antibody and examined microscopically for viral inclusion and viral antigen, respectively. Cultures infected for periods up to 6 days were all found to be negative for viral antigen and viral inclusion.

Viral resistance and viral inhibitor production. Monolayer cultures of FHM cells infected with reo-2 showed no CPE even after 7 days of incubation at 26°. The fluids from infected and uninfected cultures were harvested on the seventh day pi and frozen at —20° until assayed for antiviral activity. The cells from these cultures were challenged with $10^{6.0}$ pfu of FV3 to determine whether any viral resistance had developed as a consequence of reo-2 infection. In 2 to 3 days, control cultures were completely destroyed by FV3. The reo-2-infected cultures, however, showed virtually no visible effect of the FV3 challenge. When FV3 yields were determined, the reo-2-infected cultures were found to have produced only $10^{2.0}$ to $10^{2.5}$ pfu/ml of FV3, while the yields from control cultures were greater than $10^{6.5}$ pfu/ml. When the media from reo-2-infected and control cultures were tested for antiviral activity, 40 to 50 units/ml of the inhibitor was found in the media from reo-2-infected cultures, whereas no antiviral activity was detected in the fluids from control cultures.

Characterization of the viral inhibitor. A preparation containing approximately 45 units/ml of antiviral substance was used for the characterization of the viral inhibitor. The physical, chemical, and biological properties of the antiviral substance are summarized in Table I. This viral inhibitor was (i) not sedimented by centrifugation at 45,000

TABLE I. Properties of the Viral Inhibitor Produced by Fathead Minnow Cells Infected with Reovirus Type 2 (D-5).

pH 2 treatment for 24 hr at 4°	Stable
Heating at 56° for 30 min	Stable
Reovirus type 2 antiserum for 1 hr at 37°	Not neutralized
Repeated washings of treated cells	Activity not removed
Ultracentrifugation (45,000 rpm for 1.5 hr)	Not sedimented
Trypsin (0.2 mg/ml for 1 hr at 37°)	Activity completely destroyed
Host specificity	
Human amnion cells (RA)	Negative
Baby hamster kidney cells (BHK/21)	Negative
Fathead minnow cells (FHM)	Positive
Virus inhibition	
Frog virus 3 (FV3)	Positive

rpm for 1.5 hr, (ii) not affected by antireovirus serum, (iii) stable at pH 2 for 24 hr at 4°, (iv) not dialyzable, and (v) stable to heating at 56° for 30 min. After exposure of FHM cells to the inhibitor, the antiviral activity induced in the cells could not be removed by repeated washings. In addition, exposure of the inhibitor to 0.2 mg/ml of trypsin at 37° for 1 hr completely destroyed its biological activity, indicating the protein nature of the substance. Furthermore, the protective activity against FV3 challenge was demonstrable only in FHM cells and not in RA or BHK/21 cells, indicating the species specificity of its antiviral action.

Discussion. Although adsorption of reo-2 to FHM cells at 26° was found to occur, no detectible infectious virus, viral antigen, or viral inclusion was produced. The lack of reovirus replication in FHM cells cannot be solely attributed to the low temperature (26°) at which the growth studies were conducted, since (a) parallel studies with RA cells at 26° showed definite production of infectious virus (an increase in titer of 2 $\log_{10}$ in 5 days), and (b) no reo-2 replication could be demonstrated even at 33°, which is nearly the maximal temperature at which FHM cells will grow. The FHM cells are capable of supporting mammalian virus replication, since ECHO-11, adenovirus type 3, and poliovirus type 1 have been successfully grown in this cell system (2). Therefore, the reason(s) for the failure of reo-2 to replicate the FHM cells in spite of measurable adsorption is, as yet, not known.

The production of an interferon-like viral inhibitor suggests that a firm interaction occurred between virions and cells. Whether it is necessary for virus penetration and uncoating to occur in order to induce inhibitor production is yet to be determined. Reovirus can induce interferon production both *in vitro* and *in vivo* (8, 9), and the ribonucleic acid has been implicated as the interferon-inducing component of the virion (9). If uncoating of the viral geonome is essential for interferon induction, it must be assumed that the virion gets into the cell. Then, the reason(s) for the lack of synthesis of reo-2 progeny in FHM cells must be sought among the events of viral replication beyond penetration and uncoating. On this assumption, it may be fruitful to determine the intracellular fate of the reo-2 nucleic acid in FHM cells.

The antiviral substance produced by reo-2-infected FHM cells has many interferon-like properties. Similar inhibitors of virus replication have been reported to be produced *in vitro* by virus-infected poikilothermic cell systems: grunt fin cells chronically infected with the grunt fin agent (10); tortoise cells infected with parainfluenza virus type 1 (11); and fathead minnow cells infected with the infectious pancreatic necrosis virus (2). Also, an interferon-like viral inhibitor has been reported to be produced *in vivo* by the blue striped grunts inoculated with the endotoxin from *Escherichia coli* (12). In all the *in vitro* systems described so far, successful replication of the inducing virus was evident. Since in the present system, induction of interferon synthesis was accomplished without virus replication, reo-2 might be a very useful inducer for interferon studies in poikilothermic systems.

The role of interferon or interferon-like substances in the defense against or recovery from viral diseases in poikilothermic animals has not been explored. Since there is some evidence that the immune mechanism in fishes is not as well developed as in homeothermic animals (12), the nonimmune mechanisms such as the interferon system may play an important role in the fish defense against viral infections. Interferon may also play a role in the establishment of persistent or chronic viral infections in poikilothermic systems. Such a role is suggested by the finding of interferon in the medium from grunt fin cells chronically infected with the grunt fin agent (10).

Summary. Reovirus type 2 was found to adsorb to cells derived from a poikilothermic animal, the fathead minnow. Although this interaction between cell and virus did not result in the production of infectious virus, viral antigen, or viral inclusion, a state of viral resistance was developed and an antiviral substance was produced. This viral inhibitor was shown to be interferon based on the possession of common characteristics.

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