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Plaque Development and Induction of Interferon Synthesis by RMC Poliovirus

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ABSTRACT

JOHNSON, TERRY C. (University of Minnesota, Minneapolis), AND LEROY C. McLAREN. Plaque development and induction of interferon synthesis by RMC poliovirus. J. Bacteriol. 90:565-570. 1965.—Plaque development by RMC poliovirus on human amnion cell monolayers was investigated with regard to autointerference and to the effect of acid-agar overlay on plaquing efficiency. The virus was inhibited by acid-agar overlay, thereby exhibiting the d^- marker typical of attenuated poliovirus strains. In addition, a lack of RMC poliovirus plaque development on HeLa cell monolayers was shown to be the result of an agar inhibitor which could be removed by NaCl extraction. By use of a simplified plaque reduction assay, it was shown that interferon production was responsible for the autointerference phenomenon. Interferon synthesis did not correlate with the ages in vitro of human amnion cell cultures. Fibroblasts originating from the chorionic membrane produced negligible amounts of the inhibitor. Interferon synthesis by human amnion cells infected with RMC poliovirus was inhibited by actinomycin D. The addition of guanidine hydrochloride to infected cultures immediately after RMC poliovirus adsorption markedly inhibited interferon synthesis, although after 2 hr (postadsorption) guanidine had no effect on interferon production.

Substantial evidence has been provided to show that interferon synthesis is a discrete mechanism that allows animal cells to be refractory to viral infections. Many investigators have reported the detection of interferon in several host cell-virus systems (Ho, 1962). However, despite the numerous reports on the detection of interferon, few studies have involved attempts to elucidate the mechanisms of interferon induction and synthesis.

The purpose of this study was to employ a dependable plaque assay to investigate the quantitative aspects of type 2, RMC strain poliovirus plaque development and the effect of multiplicity of infection on the autointerference phenomenon. In addition, the investigation provided an opportunity to study RMC poliovirus plaque development with regard to the d^- marker. Ho and Enders (1959*a*, *b*) reported that human amnion cells infected with RMC poliovirus produce interferon. This paper provides evidence that the interferon synthesis is host-cell directed, although a short period (postinfection) of virus action is required for the induction of the inhibitor.

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MATERIALS AND METHODS

Tissue cultures. For the preparation of primary human amnion cell cultures, amniotic membranes were trypsinized by the methods described by Lehmann-Grube (1961), and Blake bottles were seeded with approximately 50 million cells per culture. Primary cultures were propagated with Eagle's minimal essential medium (MEM) supplemented with 15% human serum, 10% calf serum, and antibiotics (growth medium).

Secondary cultures were prepared by removing the cells from the glass by trypsinization, resuspending them in fresh medium, and seeding plaque bottles with approximately 3,000,000 cells per culture.

HeLa cell cultures were propagated by routine methods previously described (McLaren, Holland, and Syverton, 1959).

Viruses. Attenuated type 2 poliovirus, strain MEF1 (RMC), obtained from Herald R. Cox as a 20% chick embryo suspension, was grown in primary human amnion cultures. Virus from infected cell monolayers was harvested by three cycles of freezing and thawing, followed by removal of cellular debris by centrifugation at 500 × *g*.

Type 1 (Mahoney) poliovirus was propagated in human amnion cells, and pools were obtained as described for RMC poliovirus.

Vaccinia virus (Lederle seed strain), previously grown in chick chorioallantoic membrane, was passed once in human amnion cell cultures, and

the resulting pool was employed in the following studies.

Virus titrations. Serial dilutions of RMC poliovirus were prepared in Hanks' balanced salt solution (BSS), and washed monolayers were infected with 0.1 ml of the appropriate dilution. The virus was allowed to adsorb for 1 hr at 37 C, and after adsorption the cell monolayers were overlaid with Eagle's MEM supplemented with 10% calf serum and antibiotics (maintenance medium). When cell monolayers were employed for plaque assays, they were overlaid with the maintenance medium supplemented with 0.6% agar, as a solidifying agent. Plaque bottle cultures, infected with RMC poliovirus, were incubated at 37 C for 48 to 72 hr and were stained with crystal violet (Holland and McLaren, 1959). Virus concentrations were recorded as the number of plaque-forming units (PFU) per milliliter of original suspension.

Media processing for interferon assay. Media to be tested for interferon and control media were centrifuged at 500 × *g* to remove cell debris, and the supernatant fluid was centrifuged at 105,500 × *g* for 2 hr to sediment the bulk of the RMC poliovirus. The remaining virus was neutralized by the addition of type 2 specific antiserum. Before use in interferon assays, the processed media were tested for the presence of RMC poliovirus by plaque assay.

Plaque reduction assay for interferon. Secondary human amnion monolayers were infected with approximately 100 PFU of type 1 (Mahoney) poliovirus. After a 60-min adsorption period, 2 ml of interferon-containing medium, appropriately diluted in Hanks BSS, were added to each culture; immediately thereafter, 2 ml of double-strength maintenance medium, containing double-strength antibiotics and 1.2% agar, were added. The cultures were incubated at 37 C for 48 hr to allow for plaque development and were then stained with crystal violet.

RESULTS

Titration of RMC poliovirus by plaque assay. Human amnion cell monolayers, grown in plaque bottles, were infected in triplicate with serial dilutions of RMC poliovirus. After 70 hr of incubation, the monolayers were stained with crystal violet, and the number of plaques resulting was plotted against the reciprocal of the dilution (Fig. 1). It was readily apparent that the number of plaques was not always directly proportional to the dilutions of the virus preparation. In addition to fewer plaques, the 1:10 dilution resulted in much smaller plaques than the 1:20 dilution (autointerference).

Influence of multiplicity of infection on plaquing efficiency. To quantitate the effect of virus (PFU) input on the plaque assay for RMC poliovirus, one set of human amnion cell cultures was prepared by seeding 3,000,000 cells per bottle, and a second set was prepared by seeding smaller vessels with 2,500,000 cells per culture. Two separate RMC poliovirus pools were employed for the experiment. Complete inhibition of plaque development resulted when a multiplicity of 1 PFU per 1,000 host cells was employed (Fig. 2). Monolayers infected with this multiplicity have been incubated as long as 7 days without any apparent signs of cellular destruction by the virus infection. In contrast to the previous observation, an infection multiplicity of 1 PFU per 10,000 host

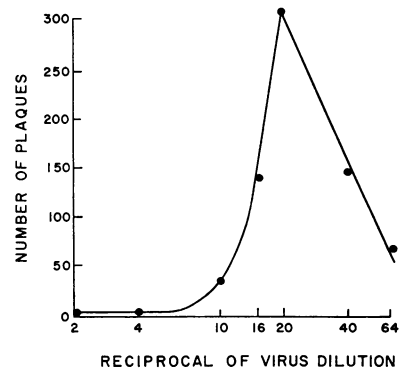

FIG. 1. *Titration of RMC poliovirus by plaque assay with human amnion cell monolayers. Triplicate human amnion plaque bottle cultures were infected with 0.1 ml of a dilution of a stock RMC poliovirus pool as described in the text.*

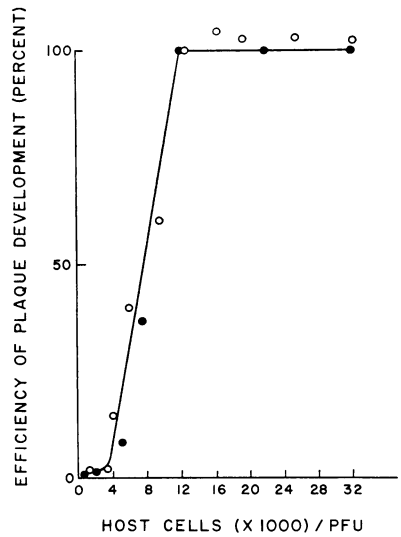

FIG. 2. *Effect of the multiplicity of infection (host cell/PFU) on the efficiency of RMC poliovirus plaque development with 3,000,000 cells per culture* (●) *and 2,500,000 cells per culture* (○).

TABLE 1. *Plaque development of RMC poliovirus under alkaline and acid agar overlay media*

Initial *p*H of overlay medium*	No. of plaques	Plaque diam†
		mm
6.6	3, 1, 1	0.5
7.0	68, 62, 66	2.0
7.5	70, 68, 64	2.0

* Initial *p*H of the media was adjusted with 5% $NaHCO_3$.

† Average plaque size.

cells resulted in a direct correlation between virus dilution and the number of resulting plaques.

Growth of RMC poliovirus under overlays of alkaline and acid agar. Vogt, Dulbecco, and Wenner (1957) reported that some strains of poliovirus, with reduced neuropathogenicity, are inhibited by acid-agar overlay. The effect of acid-agar overlay on RMC plaque development was determined by adjusting the initial *p*H of the maintenance medium with 5% $NaHCO_3$. The infected cell monolayers were gassed with 5% CO_2-95% air as an aid in maintaining the desired *p*H during incubation. RMC poliovirus plaque development was markedly inhibited by acid-agar (*p*H 6.6) overlay medium, and, therefore, the virus exhibits the d^- marker typical of attenuated poliovirus strains (Table 1).

Plaque assay of RMC poliovirus on HeLa cell monolayers. HeLa cell cultures, grown in plaque bottles, were infected with RMC poliovirus, and resulting cultures were incubated at 37 C. Unlike strains of virulent poliovirus, the plaques of RMC poliovirus developed very slowly, and even after 72 hr the foci were only 0.5 mm in diameter. To determine whether the inhibition of plaque development was a result of the large amounts of acid produced by HeLa cells, a sample of agar (Difco) was extracted with 0.9% NaCl (Agol and Chumkova, 1962). The extracted agar provided a solidifying agent that was not inhibitory to RMC plaque development, and subsequently HeLa cell monolayers could be employed for RMC poliovirus plaque assays.

Assay for interferon. Since interferon production by human amnion cells infected by RMC poliovirus was previously reported (Ho and Enders, 1959*a*, *b*), we attempted to determine whether the inhibitor played a role in the autointerference we observed. Primary human amnion cell monolayers were infected with a multiplicity of at least one PFU per 2,000 host cells. The infected cultures were incubated with maintenance medium for 5 days at 37 C, and the media from the cultures were processed as previously described. Dilutions of the processed media in Hanks BSS were tested, in triplicate, by the plaque-reduction assay. The per cent plaque reduction decreased linearly with the dilution of the media (Fig. 3), and the titer of interferon could be expressed by the dilution that produced a 50% plaque reduction of type 1 (Mahoney) poliovirus as compared with virus controls. Interferon was detected in the media, and subsequent experiments on interferon production were performed with this multiplicity of infection. In contrast, when a multiplicity of 1 PFU per 10,000 cells was employed, the cells were destroyed and the resulting medium did not contain detectable concentrations of interferon.

Effect of the age in vitro of human amnion cells on their ability to produce interferon. Ho and Enders (1959*b*) suggested that the age in vitro of human amnion cell cultures may influence their ability to produce interferon. Amnion cultures of various ages in vitro were tested to determine their ability to synthesize interferon. The results (Table 2) indicated that the final titer of interferon was not correlated with the age in vitro, although there was variation which reflected the individuality of the different primary cultures.

It was possible that enhanced interferon synthesis by older cultures (Ho and Enders, 1959*b*) may have been due to fibroblast-like cells originating from the chorionic membrane. Fibroblasts, unlike the epithelial cells derived from the amniotic membrane, rapidly multiply, and may become plentiful in older cultures. Cultures of chorionic membrane cells were prepared, and

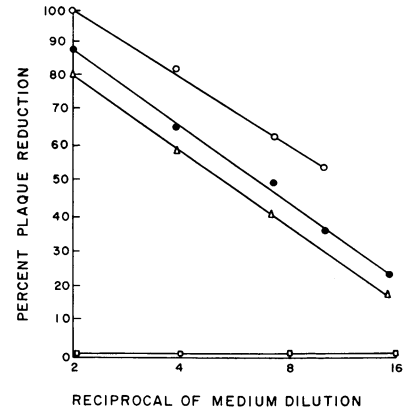

FIG. 3. *Assay of interferon concentration by the ability of media to reduce type 1 (Mahoney) poliovirus plaque development. Three pools of interferon* (○, ●, △) *and control media* (□) *were processed and assayed as described in the text. The interferon titer is expressed as the dilution that provides a 50% plaque reduction.*

TABLE 2. *Effect of the age in vitro of human amnion cell cultures on their ability to synthesize interferon*

Age in vitro of culture	Interferon titer*
days	
43	1:8
34	1:6
31	1:12
30	1:16
29	1:8
23	1:6
23	1:8
19	1:12
18	1:2
18	1:16
16	1:8
13	1:4
13	1:12
12	1:8
10	1:12

* Interferon titer expressed as the dilution that provided a 50% plaque reduction of type 1 (Mahoney) poliovirus on human amnion monolayers.

TABLE 3. *Effect of actinomycin D on interferon production by human amnion cells infected with RMC poliovirus*

Actinomycin D	Interferon titer	Effect on vaccinia virus plaque development* (per cent reduction)
μg/ml		
None	1:8	0 (control)
0.01	1:8	0
0.02	1:8	0
0.04	<1:2	80
0.10	Cells destroyed by toxicity	—

* Vaccinia virus plaque development was employed as a control to determine the effective concentrations of actinomycin D.

TABLE 4. *Effect of guanidine hydrochloride on interferon production by human amnion cells infected with RMC poliovirus*

Time guanidine HCl added to medium*	Interferon titer
hr	
None	1:16 (control)
0	1:2
2	1:16
4	1:16
24	1:16

* Guanidine HCl (50 μg/ml of maintenance medium) was added at the time indicated (post-viral adsorption).

monolayers of amnion cells, from the same placenta, were employed as controls. Interferon production by the chorionic fibroblasts was negligible when compared with the epithelial cells originating from the amniotic membrane of the same placenta.

Effect of actinomycin D on RMC poliovirus synthesis and interferon production. To determine the effect of actinomycin D on interferon synthesis, several concentrations of the antibiotic were added to human amnion cell monolayers previously infected with RMC poliovirus. As a control, the same contrations of actinomycin D were added to maintenance media employed for plaque assays of vaccinia virus in human amnion cells. No effect on interferon synthesis was detected with 0.01 and 0.02 μg of actinomycin D per ml of medium, but these concentrations also had no effect on plaque development of vaccinia virus, which is sensitive to the antibiotic (Table 3). However, the addition of 0.04 μg/ml, which reduced the number of vaccinia plaques by 80%, also markedly reduced the titer of interferon. Unfortunately, human amnion cells were very sensitive to prolonged incubation in the presence of higher concentrations of actinomycin D, and uninfected cell monolayers were destroyed within 60 hr.

Effect of guanidine hydrochloride on interferon synthesis. To determine the effect of guanidine on interferon synthesis, primary human amnion cultures were infected with RMC poliovirus as previously described, and one uninfected culture was maintained as a medium control. Immediately after the viral adsorption period, a final concentration of 50 μg of guanidine per ml of medium was added to one infected monolayer and to the medium-control monolayer. Similar concentrations were added to the remaining cultures at the times indicated in Table 4. All cultures were incubated for 6 days, at which time the media were removed, dialyzed against Hanks BSS at 4 C to remove the guanidine, and processed for interferon by our standard method. The addition of guanidine immediately after poliovirus adsorption grossly inhibited interferon synthesis (Table 4). However, addition of the agent 2 hr or longer after virus adsorption had no detectable effect on interferon production.

DISCUSSION

The present investigation substantiated the findings of Ho and Enders (1959*a*, *b*), who reported that human amnion cells infected with RMC poliovirus often are not destroyed but are spared by the induction of cellular interferon synthesis. By the development of a reproducible

plaque assay for the quantitativion of RMC poliovirus, with its obvious advantages over less quantitative techniques involving roller tube cultures, it was found that the host-parasite relationship is one of delicate balance. A slight shift in the ratio of cells to input virus meant the difference between no visible manifestations of infection and virtually complete destruction of the cell monolayer (Fig. 2). Inhibition of plaque development, or autointerference, occurred at a multiplicity of infection (PFU per host cell) which induced interferon synthesis. However, the multiplicity necessary to induce interferon synthesis (1:1,000) also indicates that "nonplaque-forming" poliovirus particles may play a role in the induction. Previous investigators have reported that inactivation of poliovirus with heat, formalin, or irradiation renders the virus incapable of inducing interferon or viral interference (Ho and Enders, 1959*b*; Ledinko and Melnick, 1954). Therefore, if "nonplaque-forming" poliovirus particles are involved in the induction we have observed, they are probably genetically deficient or incomplete rather than being in the same state as experimentally altered poliovirus.

RMC poliovirus plaques on human amnion cell monolayers developed somewhat slower than those of the virulent poliovirus strains. Macroscopically, RMC poliovirus plaques often appeared as hazy foci of incompletely destroyed cells, and even after 60 hr at 37 C many plaques contained normal-appearing cells, apparently unaffected by the virus.

RMC poliovirus plaque development was markedly inhibited by acid agar overlay medium, thereby exhibiting the d^- marker typical of other strains of attenuated poliovirus (Vogt et al., 1957). Infection of HeLa cell monolayers with RMC poliovirus resulted in plaques which developed to only 0.5 mm within 60 hr of incubation. Since HeLa cells produce relatively large quantities of acid, the inhibition could be explained in part by the sensitivity of RMC poliovirus plaque development to acid-agar overlay (d^- marker). Agol and Chumkova (1962) reported that the acid-agar sensitivity of attenuated polioviruses was the result of a sulfated polysaccharide present in Difco agar. Extraction of the agar by the method of Agol and Chumkova (1962) provided a solidifying agent that was not inhibitory to RMC poliovirus plaque development on HeLa cell monolayers.

Although Ho and Enders (1959*b*) reported that human amnion cell cultures of older age in vitro were better interferon producers than younger cultures, the present investigation did not substantiate their findings. In fact, no correlation between age in vitro and interferon production could be established, although individual primary cultures varied in the concentration of interferon produced in response to infection with RMC poliovirus. In addition, fibroblastic cells, which originate from the chorionic membrane, produced negligible amounts of interferon when compared with the amnion cells from the same placenta.

Actinomycin D, an antibiotic shown to inhibit cellular deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) and subsequent protein synthesis (Reich et al., 1961, 1962), was employed to determine whether interferon synthesis was cell-directed. Poliovirus replication is resistant to the action of the antibiotic (Reich et al. 1961, 1962), and therefore the compound offers a method to distinguish between proteins synthesized under control of the host-cell and viral genomes. Despite the high toxicity of the antibiotic to amnion monolayers, 0.04 μg/ml of maintenance medium, a quantity sufficient to reduce vaccinia virus plaques by 80%, was shown to inhibit interferon synthesis. These data correlate with previous studies involving other cell-virus systems; i.e., interferon synthesis is sensitive to actinomycin D (Gifford and Heller, 1963; Heller, 1963) and, therefore, is apparently under the control of the host-cell genome.

In contrast to actinomycin D, guanidine hydrochloride has been demonstrated to be a potent inhibitor of poliovirus synthesis at concentrations that have no demonstrable effect on host-cell metabolism (Crowther and Melnick, 1961; Loddo, 1961; Rightsel et al., 1961). If guanidine was added to the medium immediately after RMC poliovirus adsorption, interferon synthesis was markedly reduced. However, if the addition of guanidine was delayed for at least 2 hr after viral adsorption, interferon concentrations equal to infected control cultures (without guanidine) were obtained. This observation suggests that guanidine does not inhibit the cellular synthesis of interferon, but rather the presence of the compound during the early infection cycle of the virus inhibits the induction of interferon synthesis. The nature of the guanidine-sensitive period is unknown, although it may involve the formation of a poliovirus RNA polymerase or a period where poliovirus RNA is synthesized to a concentration that is capable of inducing cellular interferon synthesis. In any event, interferon induction involves an early event associated with viral infection, and continued synthesis of the inhibitor is the result of an unsuccessful attempt by the virus to completely monopolize host-cell metabolism. When HeLa cells are infected with virulent poliovirus, the host-cell genome is rendered nonfunctional, although the functional capacity of the DNA remains (Holland, 1962). This is also evident when

human amnion cells are infected with RMC poliovirus, since at least a portion of the host-cell DNA is able to function and to direct interferon synthesis.

ACKNOWLEDGMENTS

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LITERATURE CITED

AGOL, V. I., AND M. Y. CHUMKOVA. 1962. An agar polysaccharide and the d marker of poliovirus. Virology **17**:221–223.

CROWTHER, D., AND J. L. MELNICK. 1961. Studies of the inhibitory action of guanidine on poliovirus multiplication in cell cultures. Virology **15**:65–74.

GIFFORD, G. E., AND E. HELLER. 1963. Effect of actinomycin D on interferon production by 'active' and 'inactive' Chikungunya virus in chick cells. Nature **200**:50–51.

HELLER, E. 1963. Interferon: its production, action, properties and significance. Biochem. J. **87**:18–19.

HO, M. 1962. Interferons. New Engl. J. Med. **266**:1258–1264, 1313–1318, 1367–1371.

HO, M., AND J. F. ENDERS. 1959*a*. An inhibitor of viral activity appearing in infected cell cultures. Proc. Natl. Acad. Sci. U.S. **45**:385–389.

HO, M., AND J. F. ENDERS. 1959*b*. Further studies on the inhibitor of viral activity appearing in infected cell cultures and its role in chronic viral infections. Virology **9**:446–477.

HOLLAND, J. J. 1962. Inhibition of DNA-primed RNA synthesis during poliovirus infection of human cells. Biochem. Biophys. Res. Commun. **9**:556–562.

HOLLAND, J. J., AND L. C. MCLAREN. 1959. Improved method for staining cell monolayers for virus plaque counts. J. Bacteriol. **78**:596–597.

LEDINKO, N., AND J. L. MELNICK. 1954. Interference between poliomyelitis viruses in tissue culture. J. Exptl. Med. **100**:247–267.

LEHMANN-GRUBE, F. 1961. Preparation of cell cultures from human amniotic membrane. Arch. Ges. Virusforsch. **11**:258–275.

LODDO, B. 1961. Inibizione della moltiplicazione *in vitro* di virus poliomielitici ad opera della guanidia. Boll. Soc. Ital. Biol. Sper. **37**:395–397.

MCLAREN, L. C., J. J. HOLLAND, AND J. T. SYVERTON. 1959. The mammalian cell-virus relationship. I. Attachment of poliovirus to cultivated cells of primate and non-primate origin. J. Exptl. Med. **109**:475–485.

REICH, E., R. M. FRANKLIN, A. J. SHATKIN, AND E. L. TATUM. 1961. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. Science **134**:556–557.

REICH, E., R. M. FRANKLIN, A. J. SHATKIN, AND E. L. TATUM. 1962. Action of actinomycin D on animal cells and viruses. Proc. Natl. Acad. Sci. U.S. **48**:1238–1245.

RIGHTSEL, W. A., J. R. DICE, R. J. MCALPINE, E. A. TIMM, I. W. MCLEAN, G. J. DIXON, AND I. W. SHABEL. 1961. Antiviral effect of guanidine. Science **134**:558–559.

VOGT, M., R. DULBECCO, AND H. A. WENNER. 1957. Mutants of poliomyelitis viruses with reduced efficiency of plating in acid medium and reduced neuropathogenicity. Virology **4**:141–155.