JOURNAL OF BACTERIOLOGY, Dec., 1966
Vol. 92, No. 6


# Enhancement of Rhinovirus Plaque Formation in Human Heteroploid Cell Cultures by Magnesium and Calcium[1]

MILAN FIALA[2] AND GEORGE E. KENNY

*Department of Preventive Medicine, University of Washington School of Medicine, Seattle, Washington*

Received for publication 21 July 1966

## ABSTRACT

FIALA, MILAN (University of Washington, Seattle), AND GEORGE E. KENNY. Enhancement of rhinovirus plaque formation in human heteroploid cell cultures by magnesium and calcium. J. Bacteriol. **92**:1710–1715. 1966.—A reproducible macroplaque assay for six M and three H strains of rhinoviruses has been developed in several human heteroploid cell lines. Plaques were produced only with suitable solidifying agents: purified agar (Ionagar, Agarose) or methylcellulose. Plaque development was greatly enhanced by increasing $Mg^{+2}$ to 30 to 40 mM. Diethylaminoethyl (DEAE) dextran also increased plaque sizes, and the effects of $Mg^{+2}$ and DEAE dextran were additive. In addition, $Ca^{+2}$ substituted for $Mg^{+2}$. The suitability of human heteroploid cell lines for rhinovirus plaque assay varied greatly, ranging from insensitivity through partial to complete sensitivity. This assay was six to seven times more sensitive than an end point tube assay. These results indicate that potentiation of plaque formation by $Mg^{+2}$ known for some enteroviruses can also be extended to the rhinovirus group of picornaviruses.

Laboratory research on the properties of rhinoviruses has been hampered by the lack of a convenient plaque assay. Presently reported methods include: a microplaque assay in rhesus monkey kidney (7), a macroplaque assay in human embryonic lung (8), and a macroplaque test in WI-26 cells (14). These systems have been used for rhinovirus strains HGP, B632, "Norman," 363, and 1200.

This paper describes a reproducible macroplaque assay for rhinovirus M strains (and some H strains) in human heteroploid cell lines with three required conditions: (i) increased $Mg^{+2}$; (ii) employment of purified agar or methylcellulose; and (iii) a sensitive cell line. The experiments on enhancement of rhinovirus plaque formation by increased $Mg^{+2}$ were prompted by the demonstration of Wallace and Melnick (11) and Wallis et al. (13) that plaque formation of some enteroviruses in primary monkey kidney cell cultures is enhanced by increased $Mg^{+2}$.

[1] Some of the data in this paper were presented in preliminary communications: Clin. Res. **14**:143, 1966; Federation Proc. **25**:492, 1966. This paper represents a thesis submitted by Milan Fiala in partial fulfillment of the requirements for the M.S. degree in Preventive Medicine, University of Washington, Seattle.

[2] Present address: The John Herr Musser Department of Research Medicine, Hospital of the University of Pennsylvania, Philadelphia.

## MATERIALS AND METHODS

*Cells.* Cell cultures were grown on glass in Eagle's minimum essential medium (MEM). Hanks balanced salt solution (BSS) was employed as diluent, and the medium was supplemented with 10% fetal bovine serum (MEM FBS10). Cells were dispersed with 0.1% trypsin (Difco, 1:250) in glucose-potassium-sodium phosphate solution (GKNP) (BSS without $Ca^{+2}$ or $Mg^{+2}$ salts) after one or two rinses with 0.1% trypsin. Amounts of 1.5 or 2 million cells were seeded per 60-mm plastic petri dish (Falcon Plastics, Los Angeles, Calif.) and used for plaque assay the next day. Cell cultures used are listed in Table 1.

*Overlay for the plaque assay.* Overlay was prepared by combining 1 volume of BSS containing twice the MEM formulation of amino acids and vitamins with 1 volume of 0.8% Ionagar No. 2 (Consolidated Laboratories, Chicago Heights, Ill.) or 0.6% Agarose (SeaKem Brand, Bausch & Lomb, Inc., Rochester, N.Y.) in BSS. The following components were also added to the overlay at the final concentrations indicated in parentheses: fetal calf serum (2%), $NaHCO_3$ (5.6 mM), penicillin (100 units per ml), streptomycin (100 μg/ml), and nystatin (10 units per ml). Additional components, $MgCl_2$, $CaCl_2$, and diethylamino-

ethyl (DEAE) dextran, were added at concentrations indicated in the Results.

*Viruses.* Rhinoviruses were obtained through the courtesy of D. Hamre (HGP, B632, C328F, and C151) and J. P. Fox (another HGP virus, 140F, echo 28, 5582, FEB, 211, 5870, 1134, and 1059). The viruses received from D. Hamre had passage histories in human diploid or human embryonic kidney cells. All viruses received from J. P. Fox had been propagated in M-HeLa cells.

*Tube assay of infectivity ($ID_{50}$ assay).* Monolayer tube cultures of WI-38 or HeLa cells were employed. For virus assay, growth medium was discarded, and 0.1-ml samples of serial 10-fold dilutions of rhinovirus were permitted to adsorb for 1 hr at 34 C. A 1-ml amount of growth medium was then added, and the cultures were rolled (0.2 rev/min) at 34 C. Tubes were termed positive if any definite cytopathic effect (CPE) was observed.

*Plaque assay.* After aspiration of growth medium, monolayers planted the previous day were inoculated with 0.1 ml of virus appropriately diluted. Diluent was 0.2% fetal calf serum in BSS with *p*H adjusted to 7.2 to 7.3 by adding a 1:200 dilution of 0.83 M tris(hydroxymethylaminomethane (Tris) buffer (*p*H 10) and a 1:250 dilution of 0.8 N HCl. After incubation at 34 C for at least 0.5 hr, 10 ml of overlay was added. Dishes were incubated at 34 C in an incubator continuously flushed with 2.5% $CO_2$ in air and stained on the 3rd or 4th day, depending on the cell line used, with 1% gentian violet in 20% alcohol (5).

## RESULTS

*Preliminary experiments.* In preliminary experiments with rhesus monkey, African green monkey, and baboon kidney cultures, plaques were obtained with HGP virus. Secondary cell cultures were usually superior to primary cultures for plaque formation. Even though plaque production was enhanced by magnesium chloride, the reproducibility of results in these cell systems was poor. Occasional lots of cells were completely insensitive; in one case, a latent virus was present in rhesus cultures. A long incubation period of 8 to 11 days was required for plaque formation. Furthermore, the sensitivity of the assay was about fivefold less than that of tube assay in human diploid cells (WI-38). Therefore, to improve the assay, a number of human heteroploid cell lines were tested (Table 1) for plaque formation with rhinovirus HGP. Of the cell lines tested initially, one HeLa subline (calf HeLa) was most suitable for plaque formation with HGP virus. Accordingly, the conditions for an optimal plaque assay were investigated with calf HeLa cells.

*Magnesium requirement in overlay.* Plaques were not observed when the magnesium concentration was 0.8 mM in Hanks BSS (Table 2). Optimal plaque formation was obtained with 30 to 40 mM $MgCl_2$. Plaques appeared on the 3rd day. After 4 days of incubation with the use of purified

TABLE 1. *Description of cell lines and strains*

| Cell line or strain | Tissue source | Cell type | Suitability for plaque assay[a] |
|---|---|---|---|
| Calf HeLa | Cervical carcinoma (human) | Human heteroploid | +++ |
| HeLa 229 | Cervical carcinoma (human) | Human heteroploid | — |
| M-HeLa[b] | Cervical carcinoma (human) | Human heteroploid | ++++ |
| HeLa (human)[c] | Cervical carcinoma (human) | Human heteroploid | +++ |
| Intestine[c] | Human embryonic intestine | Human heteroploid | +++ |
| Chang's conjunctiva[c] | Human conjunctiva | Human heteroploid | — |
| Hep-2 | Human liver | Human heteroploid | — |
| WISH[c] | Human amnion | Human heteroploid | +++ |
| Clonal esophageal epithelium (EE) | Tracho-esophageal fistula (human) | Human heteroploid | — |
| KB | Human carcinoma of nasopharynx | Human heteroploid | — |
| WI-38 | Human lung | Human diploid | +++ |
| Human amnion | Human amnion | Primary cells | — |
| LLC-MK-2 | Rhesus monkey kidney | Monkey heteroploid | — |
| Primary rhesus kidney | Rhesus monkey kidney | Primary cells | ++ |
| Primary baboon kidney | Baboon kidney | Primary cells | ++ |
| Primary African green monkey kidney | African green monkey kidney | Primary cells | ++ |
| L-60 | $C_3H$ mouse | Mouse heteroploid | — |

[a] Symbols: ++++, optimal line; +++, good results with some rhinoviruses; ++, irregular results; —, unsuitable.

[b] Courtesy of J. P. Fox, who received this line from the Merck Institute, West Point, Pa.

[c] Flow Laboratories, Inc., Rockville, Md.

TABLE 2. *Effect of $MgCl_2$ on plaque production by rhinovirus HGP in calf HeLa cell cultures*[a]

| $MgCl_2$ concn | No. of plaques | Avg plaque diameter |
|---|---|---|
| *mM* | | *mm* |
| 0.8[b] | None | |
| 10.8 | 20 | 0.3 |
| 20.8 | 48 | 0.6 |
| 30.8 | 57 | 1.3 |
| 40.8 | 47 | 2.7 |
| 50.8 | —[c] | >3.0 |

[a] Overlay contained 0.8% Ionagar.
[b] Concentration in Hanks BSS.
[c] Confluent plaques on poor cell monolayer.

agar and 30 mM $MgCl_2$ in overlay, the average plaque size was 1.3 mm, with a range of 0.5 to 3 mm. Higher concentrations of $MgCl_2$ allowed the formation of even larger plaques, but the cell monolayer appeared poor. When 30 mM $MgCl_2$ was used, about 50 plaques could be counted on a 60-mm plate without plaque overlap.

*Solidifying agents.* A comparison of solidifying agents, ordinary agar (Difco), purified agar (Ionagar No. 2), Agarose (SeaKem brand), and methylcellulose (Fischer Scientific Co., Pittsburgh, Pa.), is presented in Table 3. Plaques produced with Ionagar or Agarose overlay were of equal size, whereas plaques were much smaller with methylcellulose overlay. Plaques were not observed with ordinary agar unless DEAE dextran (Pharmacia, Uppsala, Sweden) was included in the overlay (100 μg/ml).

*Specificity of magnesium effect.* Experiments were conducted to determine whether the magnesium effect was a result of the magnesium ion itself, hypertonicity, or increased chloride. Plaque formation in calf HeLa cells was potentiated by 30 mM $MgSO_4$, but the plaques were smaller than those in the same concentration of $MgCl_2$. Increasing the tonicity of the medium by adding 80 to 160 milliosmolar NaCl or KCl did not potentiate plaque formation. Another divalent cation, $Ca^{+2}$, substituted for $Mg^{+2}$ at an approximately equimolar concentration.

*Linearity and sensitivity of the assay.* A closely spaced series of dilutions of a rhinovirus HGP pool was assayed on calf HeLa cells. A linear relationship of the number of plaques with the relative virus dose was found (Fig. 1), which suggests that each plaque was originated by one virus particle (2). Sensitivity of the assay was variable when calf HeLa cells were used. However, when M-HeLa cells were employed, the reproducibility and sensitivity of assay were much improved (Table 4). The plaque assay was six to seven times more sensitive than the tube assay in diploid or heteroploid cells (Table 5).

*Effect of volume of virus inoculum on the number of plaques.* To determine the optimal volume of virus inoculum from which the maximal number of viral particles would adsorb during the adsorption period, the effect of volume of virus inoculum was investigated. Equal virus doses were added to cell monolayers on which were previously distributed increasing volumes of diluent. Virus inoculum was allowed to stand on monolayers for 1 hr; the cells were then overlaid without washing. An inverse relationship of number of plaques and the volume in which the dose was contained was observed (Fig. 2).

*Importance of the age and density of monolayers.* In some experiments, plaques were not seen despite the use of high magnesium overlay. After investigation of the variables between experiments, such as age of HeLa cell monolayers, method of removing cells from glass, and change in virus susceptibility of the parent cell line, it was found that sensitivity of monolayers to rhino-

TABLE 3. *Effect of solidifying agent on plaque production by rhinovirus HGP in calf HeLa cell cultures*[a]

| Solidifying agent (per cent concn) | No. of plaques | Avg plaque diameter |
|---|---|---|
| | | *mm* |
| Ionagar (0.4) | 47 | 2.7 |
| Agarose (0.25) | 41 | 2.6 |
| Methylcellulose (1) | 59 | 0.5 |
| Agar (0.5) | None | |

[a] Concentration of $MgCl_2$ in overlay was 40 mM.

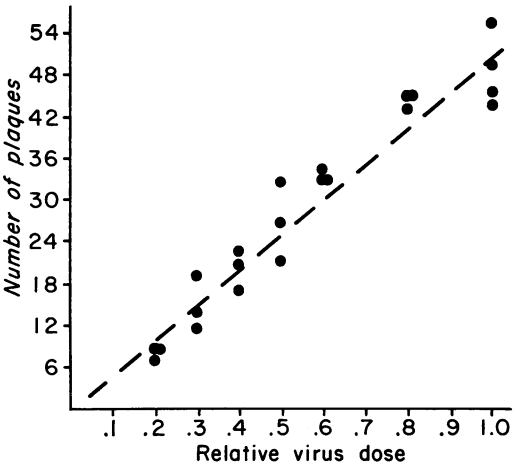


FIG. 1. *Linear relationship of number of plaques to virus dose.*

TABLE 4. *Reproducibility of titers of two HGP virus pools (one pool for each cell line) in two different cell lines*

| Cells | Titers (PFU/ml) |
|---|---|
| Calf HeLa[a] | $4.85 \times 10^6$ |
| | $1.40 \times 10^6$ |
| | $1.90 \times 10^6$ |
| | $0.75 \times 10^6$ |
| | $2.02 \times 10^6$ |
| M-HeLa[b] | $4.1 \times 10^6$ |
| | $4.05 \times 10^6$ |
| | $3.15 \times 10^6$ |
| | $3.2 \times 10^6$ |
| | $2.72 \times 10^6$ |
| | $2.48 \times 10^6$ |

[a] Average titer, $2.18 \times 10^6$; standard deviation, $1.57 \times 10^6$; coefficient of variation (standard deviation divided by average titer), 72%.

[b] Average titer, $3.28 \times 10^6$; standard deviation, $0.67 \times 10^6$; coefficient of variation, 20.3%.

TABLE 5. *Comparison of titers of HGP virus pool obtained by plaque assay (PFU/ml) and by end point tube assay ($TCID_{50}$/ml)*

| Titer in PFU/ml[a] (A) | 95% confidence limits of the titer | Titer in $TCID_{50}$ per ml/1.44[b] (B) | Significance of difference (A) − (B) |
|---|---|---|---|
| $10^{6.18}$[c] | $10^{5.87}$–$10^{6.86}$ | $10^{5.42 \pm 0.115}$[e] | $P < 0.01$ |
| $10^{6.45}$[d] | $10^{6.43}$–$10^{6.47}$ | $10^{5.58 \pm 0.164}$[d] | $P < 0.001$ |

[a] Titer was calculated as average of three plaque counts multiplied by dilution factor.

[b] Titer was calculated by use of Kärber's method, twofold dilution series, and four tubes per dilution, and was divided by 1.44 to account for the smaller size of $TCID_{50}$ units compared with PFU units.

[c] Assayed in calf HeLa.

[d] Assayed in M-HeLa.

[e] Assayed in diploid cells.

viruses declined rapidly following the 2nd day after the cells were seeded. A twofold increase in the number of cells initially added to a petri dish resulted in the reduction of the average diameter of plaques by 30 to 50%.

*Identity of the plaques of HGP virus.* To demonstrate that the plaques which were produced after seeding with the HGP virus pool were, indeed, caused by rhinovirus HGP, the following experiments were carried out.

*Plaque neutralization with specific antiserum.* In the experiments with specific antiserum (bovine, Abbott Laboratories, North Chicago, Ill.), plaques were not formed after preincubation with a 1:200 dilution of antiserum, and 90% plaque reduction was produced by preincubation with a 1:1,000 dilution of antiserum.

*Acid lability.* After 5 hr of incubation at *p*H 3.8 and 40 C, the viral inoculum produced no plaques, whereas control virus kept at physiological *p*H and 40 C showed high titer.

*Ether stability.* After 16 hr of incubation at 4 C, the viral inoculum both in 20% ethyl ether and in 0.2% fetal calf serum in BSS showed the usual titer.

*Plaque assay of other rhinoviruses and use of other human heteroploid lines.* Of the other rhinovirus M strains tested, only echo 28 produced plaques reliably in calf HeLa with 30 mM $Mg^{+2}$ in overlay. Plaque production with strains B632, 5582, and 140F of rhinoviruses in calf HeLa cells was irregular when optimal conditions for HGP were used. Consequently, other cell lines were tested. First it was found that "WISH" line gave very reproducible results with HGP, echo 28, and B632. Another HeLa subline (M-HeLa) was even more sensitive: at 2.5 days, plaques were between 2 and 4 mm in diameter. The spectrum of rhinoviruses which produced plaques in this cell line, with 30 mM $MgCl_2$ and 30 $\mu$g/ml of DEAE dextran in overlay, was larger, including six rhinovirus M strains (HGP, echo 28, 140F, 5582, B632, and C328F) and three H strains (5870, FEB, and 211). Three of the H strains tested (C151, 1059, and 1134) did not produce plaques under these conditions, although they are known to be viable by WI-38 assay. Magnesium chloride potentiated plaque sizes of all rhinoviruses which produced plaques successfully in any of the sensitive cell

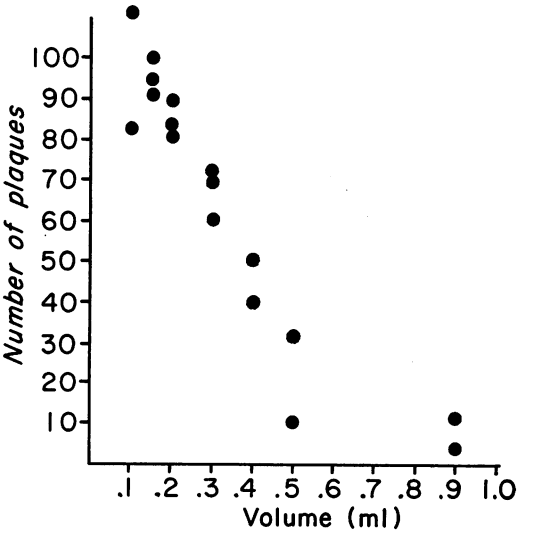


FIG. 2. *Effect of volume of virus inoculum on the number of plaques.*

lines (Table 1). Attempts at plaque formation with HGP virus in HeLa 229, HeLa S-3, HEp-2, EE, KB, secondary human amnion, and Chang's conjunctiva were unsuccessful.

*Effect of rhinovirus passage history on plaque formation.* HGP virus serially propagated in M-HeLa produced plaques as well as another strain of HGP virus propagated in human diploid cells after six passages in human kidney. On the other hand, two rhinovirus strains (1059 and 1134) propagated in M-HeLa did not produce plaques in the system described. These results would indicate that serial propagation of a rhinovirus in M-HeLa with resultant CPE did not necessarily lead to production of plaques in this system. Likewise, it was not necessary first to propagate a virus serially in M-HeLa or other human heteroploid cells before plaque production would occur.

*Potentiation of plaque formation with DEAE dextran.* DEAE dextran was found to be essential for plaque production with ordinary agar (as reported above). However, when DEAE dextran was used with Agarose at a final concentration of 30 μg/ml, plaque size was potentiated by DEAE dextran alone, and its effect was additive to that of 30 mM $MgCl_2$ when both were used together (Fig. 3).

## DISCUSSION

A plaque assay for certain M and H rhinoviruses has been developed by use of these specific conditions: (i) sensitive human heteroploid cell lines (HeLa and WISH); (ii) an overlay with Ionagar, Agarose, or methylcellulose; and (iii) 30 mM $MgCl_2$. The effect of $Mg^{+2}$ on plaque size was proportionate to dose up to 40 mM when incorporated into methylcellulose overlay. The potentiating effect of magnesium was replaceable by another divalent cation, calcium. Monovalent cations, such as $Na^+$ or $K^+$, when tested at even higher osmolarity (180 milliosmolar) or same molarity (30 mM) did not replace magnesium. $MgSO_4$ did increase plaque sizes, although not as markedly as $MgCl_2$. Plaques were also produced when total osmolarity was kept constant by lowering NaCl concentration to balance the osmotic tension of 30 mM $MgCl_2$. These results indicate that the potentiation of plaque production was associated with the presence of magnesium or calcium, and not hypertonicity or some specific effects of $MgCl_2$. The finding that magnesium potentiates plaque formation of rhinoviruses resembles the findings of Wallis and Melnick (11) and Wallis et al. (13) with some enteroviruses. However, contrary to the findings of Wallis, Melnick, and Bianchi (12) with other picornaviruses and monkey kidney cells, agar (Difco) was not suitable for plaque formation with rhinoviruses. The findings of optimal plaque formation under Agarose and enhancement of plaque formation under ordinary agar overlay with DEAE dextran remind one of the situation with many other viruses inhibited by an agar factor (1, 2, 6). The effect of DEAE dextran was found to be additive with the effect of $MgCl_2$. Plaques were always smaller under methylcellulose (autoclaved during preparation) than under Agarose.

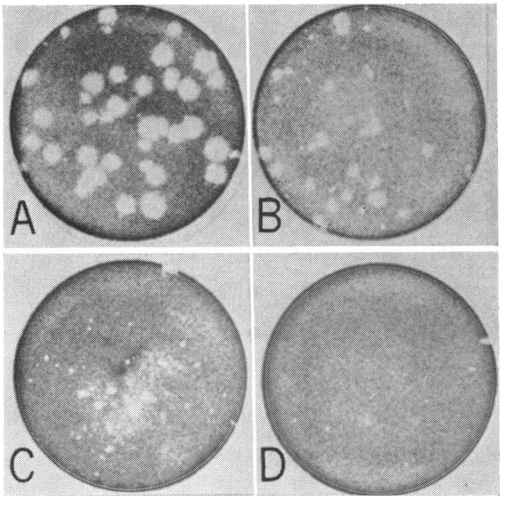


FIG. 3. *Plaques of HGP virus in M-HeLa cells with the following additions in overlay: (A) 30 mM $MgCl_2$, 30 μg/ml of DEAE dextran; (B) 30 mM $MgCl_2$; (C) 30 μg/ml of DEAE dextran; (D) no addition.*

Occasional unreliability of calf HeLa cell line in plaque assay was found to be most closely correlated with the the use of cell monolayers more than 1 day after seeding. The cell line in question was mycoplasma-free, and sensitivity of monolayers could not be increased by pretreatment of insensitive monolayers with trypsin or conditions that stop cell division (5-fluorodeoxyuridine or chilling). This time limitation appeared to be related to increasing cell density, because plaque sizes showed inverse relationship to the number of cells seeded initially per dish.

Differences in plaque morphology of different M strains were striking. Although B632 and echo 28 reportedly are serologically related (10), echo 28 plaques were two to three times as large as those of B632.

Reproducible counting of microfoci of HGP (7) required experience. In our macroplaque assay, counting of plaques was reproducible, although sometimes the wide spectrum of plaque sizes necessitated low magnification (dissecting microscope). The assay of HGP virus described

by Porterfield (8) involved the use of human embryonic lung cells. In our experience, plaques produced in fibroblastic cells were generally difficult to count accurately, because plaques were poorly delineated against the light fibroblast monolayer. The plaque assay described here may well be the first practical plaque assay for rhinoviruses which has a fairly wide range of application, currently including six M and three H strains of rhinoviruses.

A striking finding in this study was the wide spectrum of sensitivity of human heteroploid cell lines with regard to plaque formation. Sublines of the HeLa cell line showed a range of responses from maximal sensitivity (M-HeLa) to average (CaHeLa) and no sensitivity (HeLa 229 and HeLa S3).

Using the assay described in the present report, we have been able to titrate antibodies against HGP, 104F, and echo 28 in human sera and, also, to study some basic aspects of rhinovirus adsorption, growth, and neutralization by antibody.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research contract PH-43-63-562, and by training grant TI-AI-206 from the National Institute of Allergy and Infectious Diseases.

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