Plaque Formation by Rhinoviruses* (32941)

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(Introduced by H. G. Cramblett)
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The human rhinovirus subgroup now includes 55 distinct serotypes plus one subtype (1). Attempts to plaque some of these rhinoviruses have been limited to a few serotypes and have met with varying degrees of success (2–7). Recently, using a HeLa cell line, Fiala and Kenny (8) described plaque formation with 9 rhinovirus strains in the presence of Mg^{2+}. However, these workers could not plaque all strains examined. In addition, Abo-Ahmed *et al.* (9) have described plaque formation in human diploid cells with 16 rhinovirus strains. The present report describes a method for producing plaques with all currently recognized rhinovirus serotypes.

Materials and Methods. Viruses. Sixty rhinovirus strains representing 55 distinct serotypes and one subtype were used in these studies (1). The materials and methods used for propagation of viruses in HeLa and WI-38 cell cultures have been described elsewhere (10). Unless noted, all viruses were propagated in HeLa cell cultures.

Cell cultures. The techniques for growth and maintenance of HeLa cultures have been reported (10). Growth medium was Eagle's minimum essential medium (EMEM) in Earle's balanced salt solution (EBSS), 10% fetal bovine serum, 0.15% sodium bicarbonate. Penicillin, streptomycin, and chlortetracycline were added to final concentrations of 100 units, 100 μg and 40 μg/ml, respectively.

Overlay media. Starch gel. Starch gel medium was prepared by the method of De Maeyer and Schonne (11) as modified by Lehane *et al.* (12). The medium consisted of EMEM in colorless EBSS, 10% starch, 10% fetal bovine serum, 0.15% sodium bicarbonate, and penicillin and streptomycin at 100 units and 100 μg/ml, respectively. *Agar.* Agar overlay medium was prepared by combining one volume of 1.2% Ionagar no. 2 (Oxoid-Consolidated Laboratories) with one volume of double strength EMEM in colorless EBSS containing 4% fetal bovine serum, 0.30% sodium bicarbonate and the above antibiotics. Overlay media contained either 0.8 mM Mg^{2+} as a constituent of EBSS or 30.8 mM Mg^{2+} with $MgCl_2$ as the additive (8).

Plaque assay. HeLa bottle cultures (3 ounce prescription) were used for plaque assays. These were prepared by implanting 2×10^6 cells contained in 10 ml of growth medium. Bottle cultures were used between the second and sixth day after planting. After removal of growth medium, each bottle culture was inoculated with 0.5 ml of virus dilution. Colorless EBSS containing 0.3% sodium bicarbonate was used as diluent. Following incubation at 33°C for 2 hours, residual inoculum was decanted and either 5 ml of agar overlay or 6 ml of starch gel overlay was added. Cultures were incubated overnight with the cell agar layer down and inverted the following morning. After 4- to 6-days incubation at 33°C the cells were stained by adding 5 ml of 1% agar medium containing 0.007% neutral red. The bottles were covered with aluminum foil, incubated overnight, and examined for plaques the following morning.

Tube assay (infectivity titrations). Infectivity titers were determined in HeLa cell cultures by inoculating 0.1 ml of the appropriate virus dilution (tenfold) into each of 5 tubes. The diluent was as described above. Inoculated tube cultures were incubated on a roller apparatus at 33°C. Final readings were taken after 5 days of incubation and infectivity end points were determined by the method of Reed and Muench.

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TABLE I. Rhinoviruses Producing Uniform Small Plaques (2 mm or less) not Affected by Mg^{2+} Concentration.

1B (B632)[a]
6 (Thompson)[a]
7 (68)
8 (MRH)
9 (211)[a]
11 (1)
13 (353)[a]
19 (6072)
20 (15)
21 (47)[a]
22 (127)[a]
31 (140F)[a]
41 (56110)
42 (56822)
44 (71560)
45 (Baylor 1)[a]
53 (FO1-3928)
54 (FO1-3774)

[a] These viruses produced small plaques under starch gel and larger plaques under agar overlay medium. The remaining viruses produced small plaques under both starch gel and agar media.

Results. Rhinovirus plaque formation. Sixty rhinovirus strains representing 55 distinct serotypes and one subtype formed plaques under starch gel and Ionagar overlay. Considerable heterogeneity of size within a plaque population was observed. Density of the HeLa cell sheet did not appear to contribute to this variation in size.

Effect of Mg^{2+} on plaque formation. All viruses formed plaques independent of Mg^{2+} concentration. However, most strains produced larger plaques in the presence of the 30.8 mM Mg^{2+}. Exceptions to this were noted with 18 types under starch gel and for 10 of these types under agar overlay. These types produced uniform plaques (2 mm or less) whose size was not influenced by Mg^{2+} concentration (Table I). Similarly, plaques produced by type 14 (1059) virus, although as large as 8 mm in diameter, also were not affected by the higher level of Mg^{2+}. Little quantitative difference was observed in plaque forming units (pfu) using either medium and either Mg^{2+} concentration (Table II).

Since plaques were obtained with greater technical ease under agar overlay, subsequent experiments to define the plaquing properties of rhinoviruses were done with this medium. In addition, because of the larger plaques obtained with most of the virus strains, overlay medium containing extra Mg^{2+} was used. Representative plaque sizes under these conditions may be seen in Fig. 1.

TABLE II. Representative Plaque Titers of Rhinoviruses under Starch Gel and Agar Overlay Media with and without Extra Mg^{2+}.

Virus type and strain	pfu/ml			
	Starch overlay		Agar overlay	
	30.8 m*M* Mg^{2+}	No Mg^{2+}[a]	30.8 m*M* Mg^{2+}	No Mg^{2+}[a]
5 (Norman)	3.4×10^6	2.8×10^6	3.4×10^6	4.4×10^6
8 (MRH)	11×10^6	5.2×10^6	4.7×10^6	2.7×10^6
9 (211)	3.4×10^6	1.2×10^6	5.6×10^6	3.6×10^6
11 (1)	2.2×10^5	5×10^5	14×10^5	2.4×10^5
12 (181)	8×10^6	2×10^6	3.6×10^6	12×10^6
14 (1059)	6×10^6	10×10^6	4×10^6	7.6×10^6
28 (6101)	6×10^6	4.8×10^6	3×10^6	2×10^6
32 (363)	4.8×10^6	2.2×10^6	1.6×10^6	2.4×10^6
42 (56822)	1×10^6	2.8×10^6	1.2×10^6	4×10^6
48 (1505)	3.6×10^6	2.4×10^6	1.4×10^6	2.4×10^6
50 (A2 no. 58)	1.4×10^6	2×10^6	1.3×10^6	1.5×10^6
55 (Wis 315E)	3×10^6	3×10^6	0.6×10^6	1.7×10^6

[a] Mg^{2+} present at 0.8 m*M* concentration as a component of Earle's BSS as compared to the 30 m*M* Mg^{2+} added to overlay media.

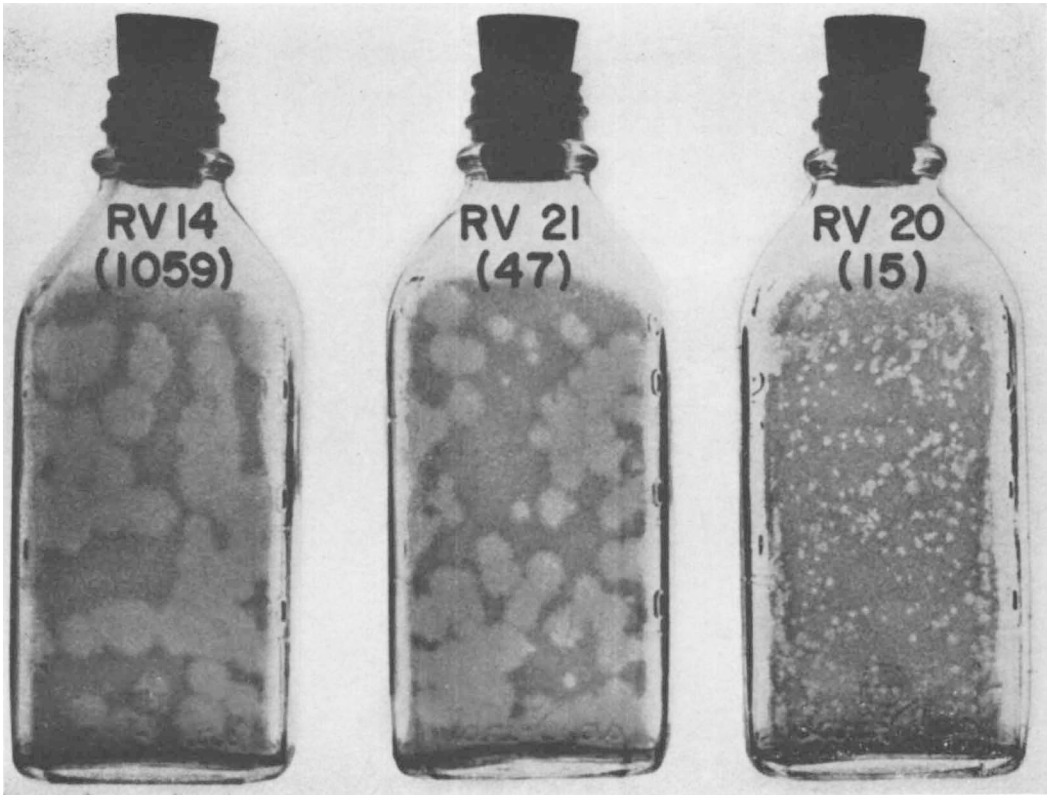

FIG. 1. Rhinovirus plaques appearing in HeLa cells. Virus strains represent large, intermediate, and small plaque formers.

Inhibition of type 1A (2060) in the presence of agar. Using 30.8 mM Mg^{2+} in the medium, type 1A (2060) produced plaques 1–5 mm in size under starch gel and plaques 1 mm or less in diameter under agar medium. However, if protamine sulfate (50 mg/100 ml) was incorporated into the Ionagar overlay or if Agarose (General Biochemicals Company) at a final concentration of 0.4% were used, plaques of 5–7 mm in diameter were formed.

Relationship of virus concentration to pfu. Different volumes (0.1, 0.2, 0.3, 0.5, and 1.0 ml) of 3 virus dilutions were each inoculated into 4 bottle cultures. All cultures were incubated for 2 hours at 33°C prior to addition of the agar overlay. A linear relationship was observed between the number of plaques and viral input (Fig. 2). Essentially identical results were obtained in similar experiments with other rhinovirus serotypes.

Relation of pfu to TCD_{50}. In preliminary experiments, the ratio between pfu and TCD_{50} titers was close to one. Based on this observation, all rhinovirus serotypes had been plaqued using a single dilution of virus. Additional experiments were done with 18 viruses to determine definitively the relationship between titers obtained by plaque assay and by TCD_{50} determinations in tube cultures.

For plaque assays, 0.5-ml amounts of serial tenfold dilutions of virus were inoculated into each of 2 bottle cultures. The bottles were overlayed after 2 hours' incubation at 33°C. Tube assays were done using the same dilution series. The results of assays with several viruses can be seen in Table III.

In most cases, pfu titers were slightly higher than TCD_{50} titers suggesting that the plaque assay was somewhat more sensitive. With one exception (type 14 virus), ratios of

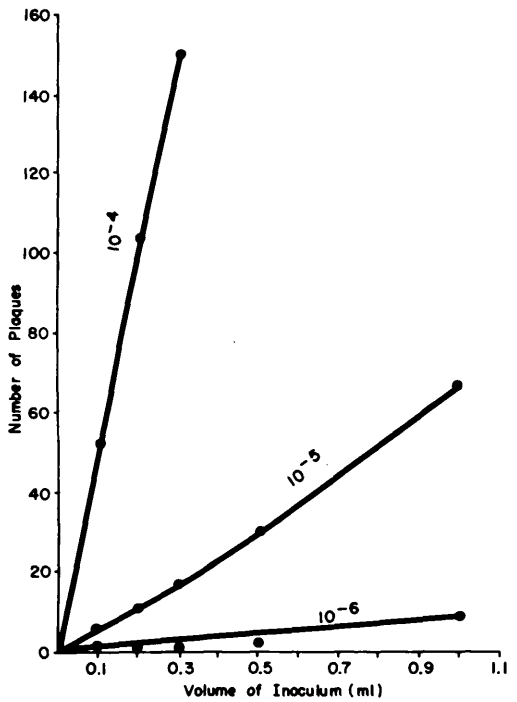

FIG. 2. Linearity of plaque assay with rhinovirus type 17 (33342). Each point represents the average of four determinations.

pfu/TCD_{50} ranged between 0.91 and 1.10. Using all values, the geometric and arithmetic mean ratios were each 1.03.

Effect of inoculum size on number of plaques. The relationship between the number of plaques and the volume in which the virus is contained was examined in our system. A dilution of virus calculated to contain between 30 and 50 pfu per 0.1 ml was prepared. Appropriate amounts of diluent were added to aliquots of this dilution to give the same number of pfu in 5 different volumes. Each of these preparations was inoculated into 6 plaque bottles, incubated for 2 hours at 33°C and overlayed. No significant difference could be observed between the number of plaques present and the varying volumes of inoculum (Table IV).

Influence of inoculum size on rate of adsorption. Volumes of 0.1, 0.5, and 1.0 ml, each containing the same number of pfu, were inoculated into replicate bottles which were incubated at 33°C for varying periods of time up to 180 min. At intervals, each bottle was washed 3 times with 5 ml of diluent. Uninoculated controls were treated similarly. Following washing, bottles were left at room temperature and overlayed after the 180-min interval. The results obtained in 3 experiments can be seen in Fig. 3. It appears that the adsorp-

TABLE III. Comparison of Rhinovirus Infectivity Titers Obtained by Plaque Assay and End Point Tube Assay (TCD_{50}).

Rhinovirus type and strain	Infectivity titer[a] $\log_{10}$ pfu/ml	Infectivity titer[a] $\log_{10}$ TCD_{50}/ml	Ratio pfu/TCD_{50}
1A (2060)	6.09	5.88	1.04
1B (B632)	6.16	6.80	0.91
2 (HGP)	6.11	6.00	1.02
7 (68)	5.28	5.00	1.06
10 (204)	6.60	6.35	1.04
14 (1059)	6.95	5.70	1.22
17 (33342)	6.37	6.00	1.06
20 (15)	6.51	6.65	0.98
21 (47)	6.01	5.46	1.10
23 (5124)	5.55	5.23	1.06
25 (5426)	5.60	5.37	1.04
30 (106F)	6.65	6.30	1.06
31 (140F)	5.53	5.90	0.94
34 (137-3)	5.08	5.60	0.91
35 (164A)	5.84	5.50	1.06
41 (56110)	6.05	5.90	1.03
49 (8213)	5.80	5.57	1.04
53 (FO1-3928)	5.96	6.09	0.98

[a] All infectivity titers represent geometric means of 2–6 determinations with each virus.

TABLE IV. Effect of Volume of Inoculum on Efficiency of Plating.

Volume of inoculum (ml)	Rhinovirus type and strain: 2 (HGP) Av no. of plaques	2 (HGP) Range[a]	14 (1059) Av no. of plaques	14 (1059) Range
0.1	40	36–44	34	33–37
0.3	44	38–49	34	31–36
0.5	45	39–49	33	30–35
0.7	49	43–55	34	30–37
1.0	44	40–49	34	30–38
SD		4.70		1.98

[a] Six replicate bottles inoculated with each volume.

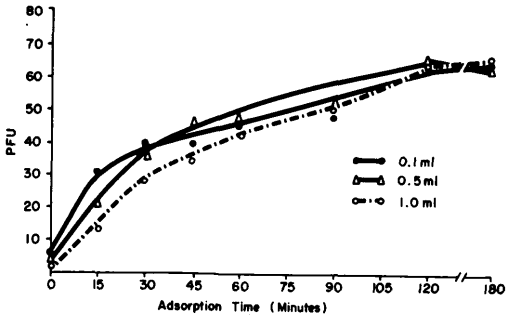

FIG. 3. Relationship of volume of inoculum to adsorption rate with rhinovirus type 2 (HGP).

tion rate is a function of inoculum size when a constant input of virus is used. The rate of adsorption with 0.5 ml inoculum was somewhat less than with 0.1 ml for the first hour and adsorption with the 1.0 ml inoculum was slowest. With all volumes inoculated, adsorption was maximal at 120 min.

Passage and identification of plaques. A bacteriological loop was used to remove agar plugs over isolated viral plaques. Three consecutive plaque purification steps were carried out with 6 rhinoviruses. Viruses obtained from the third plaque passage were inoculated into tube cultures, harvested after CPE was maximal and re-identified with homologous antisera. All types tested were readily "purified" and identified in this manner.

Plaque formation in HeLa cells by rhinoviruses propagated in human diploid cells. Six rhinovirus types propagated in WI-38 cells were examined for ability to form plaques on HeLa cell monolayers. All viruses produced plaques and in each case, virus could be recovered from the plaques. The size and morphology of plaques in first passage into HeLa cells was similar to those observed when HeLa adapted rhinoviruses were used for plaque production. Type 14 (1059) virus propagated in WI-38 cells, titered as high in HeLa cells (pfu) on first passage as did virus which had been through several passages in HeLa cells. With the exception of type 14 (1059), the only difference noted was quantitative. With one exception (type 14), HeLa adapted strains titered 1–3 logs higher by plaque assay.

Discussion. Using HeLa bottle cultures, high concentrations of Mg^{2+} are not required for rhinovirus plaque formation and do not increase the number of plaques produced. All rhinoviruses formed plaques under starch gel or agar overlay medium independent of Mg^{2+} concentration. Although plaques produced by most rhinoviruses were larger in the presence of 30.8 mM Mg^{2+}, the minute plaques produced by 10 rhinoviruses under agar overlay and by 10 viruses under starch overlay medium apparently were not affected by Mg^{2+} concentration. With these minute plaque formers (Table III), it is possible that size differences occurred but were not detectable.

With one exception (rhinovirus type 1A), plaques under agar overlay were larger than those under starch gel medium. The results with starch could be due to mechanical inhibition of virus spread in the presence of the high concentration of starch (10%) needed to form a firm gel.

Only limited attempts were made to define the specific conditions necessary for plaque formation by rhinoviruses. However, the HeLa cell line used is known to support the growth of 129 rhinovirus strains (10). As yet, no human rhinovirus strains have been encountered which do not propagate in this cell line. A wide spectrum of sensitivity of other human heteroploid cell lines in respect to plaque formation has been reported by Fiala and Kenny (8). Furthermore, these authors reported that rhinovirus passage history did not appear to influence plaque production in HeLa cells (8). We also found that rhinoviruses propagated in human diploid cells (WI-38) are capable of producing plaques directly in HeLa cell cultures.

Among the rhinoviruses, type 14 virus appears to differ in its plaquing characteristics. This virus produced large, uniform plaques under any of the conditions used. Further, the pfu/TCD_{50} ratio for this strain was much higher than those obtained with other rhinoviruses tested. Presently, no ready explanation is available for these findings.

Certain rhinoviruses like other picornaviruses, appear to be inhibited by a component of agar. Type 1A virus which produced plaques 1 mm or less under Ionagar–Mg^{2+} medium, formed plaques 5–7 mm in diameter when protamine sulfate (50 mg/100 ml) was

incorporated into the overlay. Furthermore, if Agarose was substituted for Ionagar, large plaques also could be obtained. Other workers (4–9) have shown that DEAE dextran is essential for plaque production with certain rhinoviruses when ordinary agar is used in the overlay. Thus, present knowledge indicates that the conditions required for rhinovirus plaque formation include a sensitive cell system such as the HeLa cells used in this study, and an inhibitor free overlay medium. Additives, such as $MgCl_2$, DEAE dextran, or protamine sulfate can be used to facilitate plaque formation.

Summary. A reliable plaque assay is described for 60 rhinovirus strains representing 55 distinct serotypes and one subtype. Using HeLa cell bottle cultures, plaques were produced under starch gel and Ionagar overlay media. Employing the methodology described in this report, certain basic aspects of rhinovirus plaque formation were investigated.

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Unusually Large-Plaque Mutant of Sindbis Virus (32942)

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In the course of a study on the plaque formation of various viruses at elevated temperatures, we noticed that Sindbis virus plaques formed at 40°C in primary chick embryo fibroblasts (CEF) revealed a pleomorphism, small plaques of various sizes mixing with the parent-type plaques. Then, it was attempted to separate the small-plaque clones by plaque purification. Thereby, a few of the small-plaque clones yielded progenies producing unusually large plaques. Analyses of some properties of this mutant have been done in comparison with the parent type and small-plaque type clones.

Materials and Methods. Virus. Egypt Ar339 strain of Sindbis virus was supplied by Dr. T. Okuno, the National Institute of Health, Tokyo, as a lyophilized suspension of infected baby mouse brains. It was passaged through CEF bottle cultures 61 times before starting the present experiments. The diluent for virus was 0.1% yolk–saline (1).

Plaque technique. The technique of plaque titration was essentially the same as described in a previous report (1). The CEF monolayer sheets formed were washed once with phosphate buffered saline (PBS) (2) and inoculated with serial decimal dilutions of Sindbis virus in the amount of 0.05 ml per dish. After an incubation at 37°C for 1 hour, the dishes were overlaid with an agar medium which contained 2% calf serum and 0.0025 M Tris buffer of pH 7.2. The dishes were then

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