Properties of Rhinovirus Plaque Mutants[1] (34859)

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(Introduced by H. G. Cramblett)

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Since application of a plaque method for assaying animal viruses (1, 2), numerous reports have described the occurrence of plaque type mutants in almost every virus group infecting man (3, 4). Among human picornaviruses, plaque type mutants have been observed with many members of the enterovirus subgroup (5–7), but not with the rhinovirus subgroup.

Following the development of a numbering system for rhinoviruses (8), we reported on the use of Ionagar as well as starch overlay media in plaquing all 55 serotypes (9). Under starch gel medium, there was no indication that any serotype contained a mixture of distinct plaque types. With Ionagar overlay medium, rhinovirus type 34 showed two distinct plaque populations. The large plaques were designated m^+ and small plaques m. The purpose of this report is to describe some of the biological and biophysical properties of these rhinovirus plaque type mutants.

Materials and Methods. Viruses. Prototype rhinovirus 34 (strain 137-3), from the fourth HeLa passage in this laboratory was the source of the plaque type mutants. The method for preparing plaque-purified stocks of virus has been described previously (9). The original type 34 virus was received from Dr. Dorothy Hamre and had been through 32 passages in human diploid cell cultures in her laboratory.

Cell cultures. The materials and methods used for growth and maintenance of HeLa and WI38 tube and bottle cultures have been reported elsewhere (10).

Overlay media and plaque assays. The preparation of starch gel and Ionagar No. 2 overlay media and the plaque assay method have been described (9). Agarose (General Biochemicals) overlay medium was prepared as described for Ionagar medium, but at a final concentration of 0.5%. Additives (protamine sulfate and DEAE-dextran) were used at concentrations of 500 and 100 μg/ml, respectively.

Agar extract. The method of Takemoto and Liebhaber (11) was used. Briefly, Ionagar No. 2 at a concentration of 0.6% was prepared in demineralized-distilled water. After solidification, it was frozen and thawed three times and centrifuged for 10 min at low speed. The supernatant fluid was removed and designated agar extract (AE). Assuming that all of the inhibitor was contained in the extract, sufficient AE was added to agarose overlay medium to give a final concentration of inhibitor one-half that found in a comparable volume of Ionagar.

Kinetics of adsorption. The methods used for determining viral adsorption rates have been described in detail (9).

Replication at 37°. To determine if differences could be detected between m^+ and m viruses incubated at different temperatures, plaque-purified stocks of each mutant were diluted and 10 cell culture tubes were inoculated with each $\log_{10}$ dilution of virus; 5 tubes were incubated at 33° and 5 at 37°. On the fifth day postinoculation, the cultures were observed for CPE. At 37° incubation, positive cultures obtained from the highest dilution of each virus inoculated were harvested, pooled and re-titrated at 33 and 37°.

[1] This investigation was supported by U.S. Public Health Research Contract No. NIH 69-2062. The competent technical assistance of G. Van Winkle, S. Thibaut, J. Gebhart and J. Halstead is gratefully acknowledged.

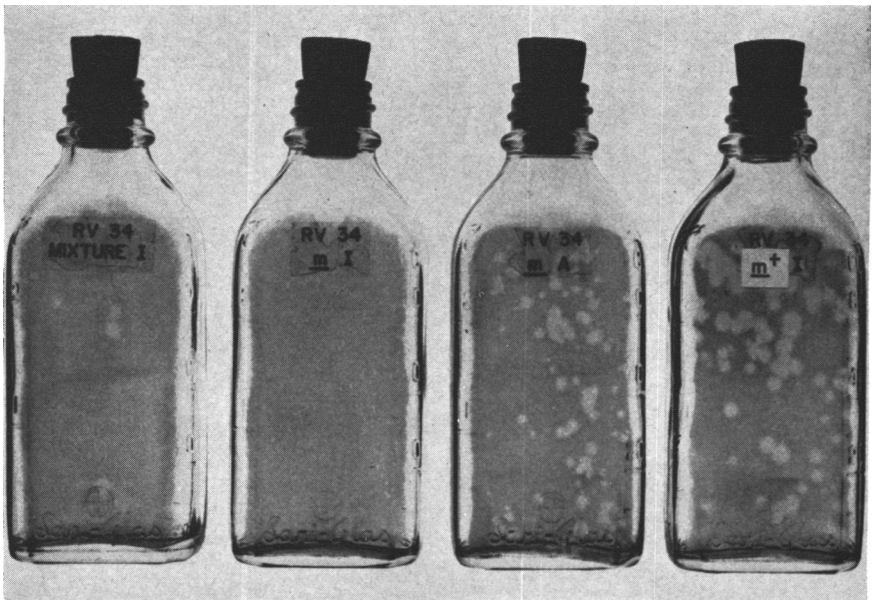

FIG. 1. Plaque types of rhinovirus 34 observed in mixture and after plaque purification under Ionagar (I), and agarose (A) overlay media.

This procedure was repeated through five consecutive passages. Cultures incubated at 33° served as controls. Infectivity assays by TCD_{50} determinations were done as described previously (10).

Density determinations. Pools of each mutant, as well as a pool of the fourth HeLa passage of rhinovirus 34 from which each mutant was obtained, were centrifuged in a Spinco Model L ultracentrifuge at 30,000 rpm for 4 hr using a number 30 rotor. The pellet was resuspended by soaking overnight in 0.01 *M* Tris buffer (pH 8.1) at 4° followed by freezing and thawing. The mean density of the suspension was adjusted to 1.40 with solid CsCl (General Biochemicals) and centrifugation was repeated at 39,000 rpm for 18–20 hr using the SW-39 rotor. Fractions were collected and used for plaque assays and density determinations (12).

Antisera. Immune sera were prepared in guinea pigs according to the procedure of Ketler *et al.* (13), except that the second injection consisted of 2.0 ml of aqueous virus given intraperitoneally.

Serology. Serum neutralization tests were performed as described previously (10). The preparation of antigens and a micromethod for double diffusion gel precipitation employing 0.6% agarose in 0.15 *M* NaCl have been reported elsewhere (14).

Results. Plaque types. Although only large plaques were observed with rhinovirus type 34 under starch medium, two distinct plaque populations were obtained with Ionagar No. 2. A plaque from each population was picked and purified by three consecutive plaque passages in HeLa cells. Representative plaques, including those present in the original mixture, are shown in Fig. 1. In the mixture, the large plaques were round, turbid, and measured 2 to 5 mm in diameter. Also, there were many small, irregular plaques, measuring 0.5 mm or less. The ratio of m^+/m plaques was approximately 1:10. The *m* mutant formed much larger plaques (Fig. 1) when agarose was substituted for Ionagar. Although not shown, plaque size of *m* virus also was increased when either DEAE-dextran or protamine sulfate was incorporated into Ionagar.

The inhibitor present in Ionagar was extractable and effective when incorporated into agarose (Fig. 2). The plaque size of *m* virus was reduced significantly in the presence of AE.

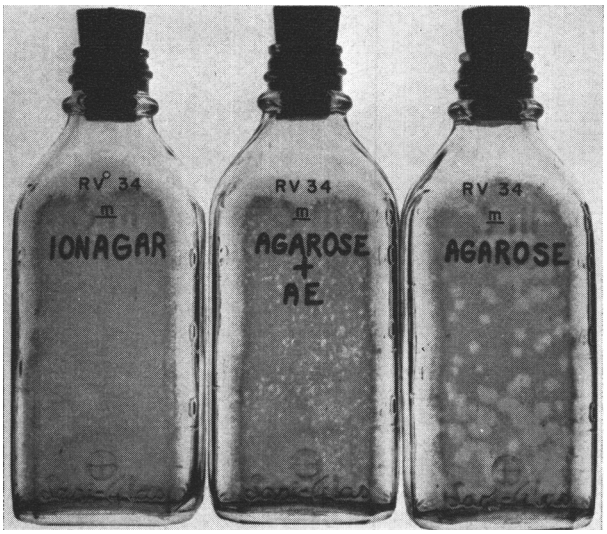

FIG. 2. Effect on *m* plaque size of agar extract (AE) incorporated into purified agar (agarose).

Inhibition of m virus by starch overlay medium. Attempts to plaque *m* virus using starch overlay were unsuccessful. However, plaques 1–2 mm in size were formed when either protamine sulfate or DEAE-dextran was incorporated into the starch medium. Experiments were performed to compare the influence of various overlay media and additives on the size of *m* and m^+ viruses. For comparative purposes, rhinovirus type 1A, known to be sensitive to inhibitors present in Ionagar (9) was included. The incorporation of additives to overlay media did not affect the average diameter of plaques produced by 34 m^+ virus (Table I). Under Ionagar, both viruses 1A and 34 *m* gave large plaques in the presence of additives. On the other hand, under starch overlay, type 34 *m* virus was unique in its failure to produce plaques in the absence of additives. Although a starch extract was prepared and used in the same manner as AE, unequivocal inhibition could not be demonstrated. It appears that the inhibitory principle in starch differs from that in Ionagar despite the finding that both were neutralized by the same additives.

The pH of the various overlay media did not appear to be a factor. The pH of starch, Ionagar, and agarose media, with or without AE and polycations, was between 6.9 and 7.1. Moreover, Ionagar overlay media adjusted with Tris buffer and used at either pH 7.4 or 8.0 did not alter the average diameter of *m* virus plaques.

Rate of CPE and infectivity yields. The cytopathic effect with *m* mutant progressed more rapidly and generally was more extensive than that observed with m^+ mutant.

TABLE I. Effect of Overlay Media on Plaque Production by Rhinovirus 34 *m*, 34 m^+, and 1A.

	Plaque size (mm)					
	10% Starch		0.6% Ionagar No. 2		0.5% Agarose	
Virus	No additive	Protamine or DEAE-dextran	No additive	Protamine or DEAE-dextran	No additive	AE
34 *m*	None detectable	1–2	0.5 or less	3–5	3–5	1–2
34 m^+	1–4	1–4	2–5	2–6	2–5	ND[a]
1A	1–5	1–5	0.5 or less	5–7	5–7	ND

[a] ND, not done.

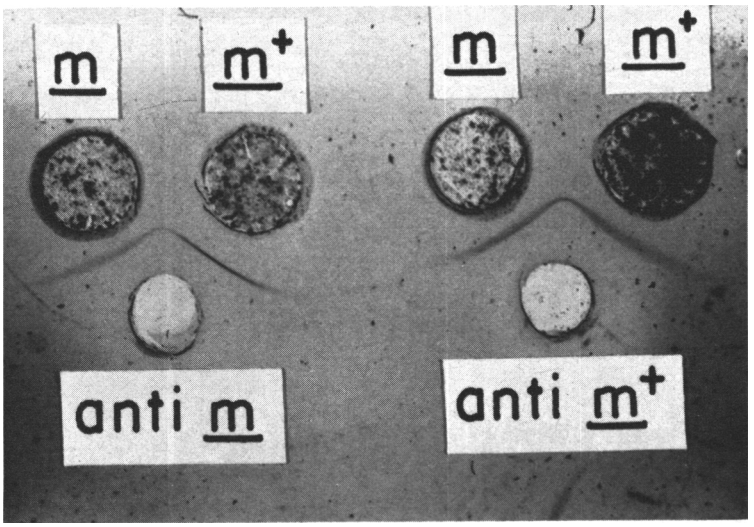

FIG. 3. Antigenic identity of m and m^+ mutants demonstrated by immunodiffusion. Undiluted HeLa propagated antigens and guinea pig immune sera diffused simultaneously.

However, yields of infectious virus, as determined in both plaque and TCD_{50} assays, were similar with both strains (m, 7.5 TCD_{50}/ml, 1.2×10^7 pfu/ml; m^+, 7.1 TCD_{50}/ml, 1.0×10^7 pfu/ml).

Cell tropism. To determine if the mutants could be classified as M (monkey) or H (human) strains (15), we attempted to grow the viruses in cell cultures of Grivet monkey kidney. Using inputs of 7.0 $\log_{10}$ TCD_{50}/ml, both m and m^+ viruses failed to replicate.

Cross neutralization tests. Several guinea pigs were immunized with each plaque mutant and cross neutralization tests were performed with these immune sera and viruses (Table II). Using HeLa cells for both propagation of virus and antibody assays, no significant (4-fold or greater) differences in antibody sensitivity could be detected for the m, m^+, or original virus. The results were the same when a human diploid cell strain (WI-38) was used for preparing virus and for performing neutralization tests. These relationships are currently being reexamined by the kinetics of neutralization.

Immunodiffusion. Antigenic analysis was performed with m and m^+ viruses using double diffusion gel precipitation (Fig. 3). Reactions of complete identity were obtained as evidenced by fusion of lines produced by each mutant when diffused simultaneously against their respective animal immune sera. The specificity of these reactions was verified by testing several heterologous picornavirus antigens, including rhinoviruses, against homologous antisera and antisera for m and m^+ viruses. Reactions occurred only with homologous immune serum.

Adsorption kinetics. The kinetics of adsorption with each mutant were examined. The results obtained were similar to those previously reported for rhinoviruses from this laboratory (9). In each case, adsorption was

TABLE II. Representative Results of Cross Neutralization Tests with m, m^+, and m^0[a] Rhinoviruses Propagated in HeLa or WI-38 Cell Cultures and Tested in Homologous Cell System.

Virus	Cell system	Guinea pig immune sera[b]	
		m	m^+
m	WI-38	1440	3840
	HeLa	850	2130
m^+	WI-38	2240	6400
	HeLa	1600	3840
m^0	WI-38	1120	5120
	HeLa	1760	6720

[a] Seed from which m and m^+ were derived.

[b] Results expressed as reciprocal of serum dilution end points.

50% complete by 30 min. For both *m* and m^+, maximal adsorption occurred by 2 hr. No significant difference in adsorption rates was detected.

Replication at 37°. Incubation of rhinovirus-infected cells is commonly carried out at 33°. The effect of incubation at 37° as manifested by infectious yield of *m* and m^+ viruses was examined. Temperature of incubation had little if any effect on replication of *m* virus. The amount of infectious virus produced at 33° was similar to that at 37° and did not change through 5 consecutive passages in HeLa cells. On the other hand, through three consecutive passages, the amount of infectious m^+ virus produced was at least 1.0 $\log_{10}$ lower at 37° than at 33°. At the fourth and fifth passages, infectivity yields at 37° increased and were comparable to those obtained at 33°.

With both *m* and m^+ viruses, the extent of CPE produced was always greater at 37°. No changes in plaque morphology were noted with either virus after five consecutive passages at 37°.

Buoyant density. No difference in buoyant density could be detected between *m* and m^+ viruses. The maximum peak of infectivity with each mutant coincided with a density of 1.40 g/cm^3. Similar results were obtained using the original mixture of virus.

Discussion. We have described the observation and characterization of large (m^+) and small (*m*) plaque type mutants of rhinovirus type 34. Two differentiating characteristics were readily apparent between the two viruses: the effect of various overlay media on plaque size, and growth at 37°.

As originally reported by Takemoto and Liebhaber (11) for EMC virus, the reduction in size of rhinovirus *m* plaques under Ionagar is most likely due to the presence of inhibitors in the form of sulfated polysaccharides. Accordingly, plaque size was not reduced when inhibitor-free agarose was used or when polycations (DEAE-dextran, protamine sulfate) were incorporated into Ionagar. In addition, relatively low concentration of an Ionagar extract incorporated into agarose caused a reduction in size of *m* plaques.

Wallis and Melnick (16, 17) have suggested that purified agars are more alkaline than crude agars and that the pH, not the presence of sulfated polysaccharides, influences picornavirus plaque size. They also have implied that the enlarged plaques resulting from addition of polycations to crude agar, starch, and methylcellulose overlays are due to a more positively charged environment which increases the rate of rival diffusion. In our study, pH of overlay media did not appear to be an important factor in the inhibition of *m* plaque size.

There have been no reports of viral inhibitors present in starch. Some investigators (16–18), have used starch gel as an inhibitor-free medium when studying viruses affected by agar inhibitors. Our finding that the inhibitory activity of starch gel is "neutralized" by either DEAE-dextran or protamine sulfate suggests that it might be due to anionic substances. It is also possible that the effect produced by starch is limited to the rhinovirus subgroup of picornaviruses.

Although not reported here, the presence of an inhibitor in serum (19) was considered because of the relatively high concentration (10%) used in initial studies with starch. However, complete inhibition of *m* virus occurred when the serum concentration was reduced to 2%, the same concentration used in agarose and Ionagar media.

The CPE produced by the *m* and m^+ mutants differed in extent and time of appearance. Using primate cell cultures, similar observations have been reported for Coxsackie B_4 virus (6) and Echovirus 6 (7). However, the minute *m* mutants of these viruses produced greater infectivity yields than their respective m^+ mutants. In addition, differences in yields between mutants of Echovirus 6 have been observed in human diploid cells (WI-38) by Suto *et al.* (20). Nevertheless, when other human cell systems were tested, including HeLa, no differences were obtained. In contrast, both rhinovirus mutants produced comparable amounts of infectious virus in either HeLa or WI-38 cell cultures.

By cross neutralization, no significant differences in antibody sensitivity were observed with *m* and m^+mutants of RV 34. This was

not the case with plaque type mutants of Coxsackie B_4 (6), ECHO 6 (7), and an agar inhibited strain of $ECHO_4$ (21). Furthermore, antigenic analysis of these rhinovirus mutants by immunodiffusion did not reveal any differences.

There was a marked difference in the replication of *m* and m^+ viruses at 37°. The yield of infectious virus obtained when *m* mutant was incubated at 37° equaled that obtained at 33°. No passages were necessary to obtain optimum growth at 37°. Possibly related to this finding is the observation that the initiation of viral synthesis with *m* mutant, as determined in one-step-growth curves, preceeds that of m^+ mutant by at least 1 hr. Studies concerning this difference will be the subject of a later report. At 37°, the m^+ mutant required an adaptation period of three to four cell culture passages before maximum yields were obtained. A similar period of adaptation to replication at 37° has been reported for rhinovirus types 7 and 8 by Hamparian *et al.* (22). The occurrence in nature of rhinovirus mutants which replicate at 37° without adaptation, raise interesting questions concerning the pathogenesis of these viruses at sites other than the upper respiratory tract.

We do not know which plaque type of rhinovirus 34 is representative of the "wild" virus. The virus had been passaged several times in human diploid cells prior to being received in this laboratory. Neither clinical nor early passage material is available for examination.

Summary. Under Ionagar overlay medium, the prototype strain of rhinovirus type 34 was found to consist of a mixture of two plaque types designated *m* (minute) and m^+ (large). Large plaques were obtained with *m* virus when agarose was used or when polycations (protamine or DEAE-dextran) were incorporated into Ionagar medium. Starch gel medium completely inhibited the appearance or *m* but not m^+ plaques. However, the *m* virus formed plaques under starch when polycations were incorporated. The nature of the inhibitory principle in starch is unknown. The pH of overlay media did not appear to influence the plaque size of either mutant.

Temperature of incubation had little if any effect on replication of *m* virus. At 37°, the m^+ mutant required an adaptation period of three to four cell culture passages before maximum yields of infectious virus were obtained.

No differences between the mutants were observed in buoyant density, rate of adsorption, host cell tropism, or antibody sensitivity.

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Received Mar. 9, 1970. P.S.E.B.M., 1970, Vol. 134.