Differentiation of Temperature Variants of Influenza A2 Viruses on the Basis of Plaque Formation

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The ability of temperature variants of influenza A2 virus to produce plaques in chick embryo kidney tissue culture was studied at different temperatures. Definite differences in efficiency of plaque formation by cryophilic and thermophilic strains were observed at low and high temperatures. Differentiation of the temperature variants appears to reside in a number of genetic markers, designated *rct-40*, *rct-28*, and plaque size (*S*). Virulence of influenza A2 virus, enhanced after prolonged cultivation at high temperature, is probably related to its greater efficiency of plating at 40 C (*rct-40*⁺), formation of larger plaques at optimal (36 C) and high (40 C) temperatures, and to loss of ability to form plaques at 28 C (*rct-28*⁻).

It has been reported earlier that plaque formation occurs with influenza A2 virus in tissue cultures of rhesus monkey kidney (3, 4, 14), calf kidney (9), chick fibroblasts (10), and a heteroploid epithelioid cell line obtained from cornea (12, 13). A serious disadvantage of the above-mentioned methods was variability of results and irregularity of plaque formation with chick embryo-passaged lines of influenza virus.

The present report deals with a study of plaque formation in tissue culture of chick embryo kidney infected with influenza A2 virus, and with the differentiation of cryophilic and thermophilic variants of the virus obtained after prolonged multiplication at temperatures above (supraoptimal) or below (suboptimal) those optimal for wild-type virus.

MATERIALS AND METHODS

Viruses. Two inhibitor-resistant strains of influenza A2 virus (A2/65/151 and A2/65/147) were studied; both were obtained during the 1965 epidemic, and were passaged three times in chick embryos at 36 C. Their cryophilic and thermophilic variants were obtained after 45 passages of the original viruses at low (28 to 26 C) and high (40 to 41 C) temperatures. Control lines of these viruses were passaged 45 times at 36 C. All viruses were used in the form of allantoic fluids collected from infected chick embryos.

Preparation of monolayers of trypsinized kidney cultures of 18-day-old chick embryos (KEC). Trypsinization was done by the method of Buthala and Mathews (2) and Lippelt and Mannweiler (8). Cells were grown in medium containing Hanks solution with 0.5% lactalbumin hydrolysate and 10%

bovine serum. Formation of monolayers in plates and tubes occurred, as a rule, in 48 to 72 hr.

Method of obtaining plaques. After removal of the growth medium, plates with tissue culture KEC were inoculated with 1 ml of virus diluted 10-fold in maintenance medium (Table 1). After 1.5 hr of adsorption at 36 C, the inoculum was removed and 5 ml of the first agar overlay was added to every plate. Infected cultures were incubated at 28, 32, 36, and 40 C in the dark for 3 to 4 days. A second agar overlay, containing neutral red indicator (1:10,000), was added after 3 to 4 days of incubation. Recording of plaques and measurement of their diameters were done on the day after the second agar overlay.

Method of virus cloning. With the help of a stretched curved Pasteur pipette, a bar of agar, 0.4 to 0.5 mm in diameter, was removed from the center of an isolated plaque and suspended in 0.5 ml of maintenance medium. The virus-containing suspension was titrated in serial decimal dilution in chick embryos. After 48 hr of incubation at 36 C, the embryos were cooled and allantoic fluid from each embryo was collected separately. Virus-containing allantoic suspensions, infected at limiting dilutions of the virus, were collected and used in each experiment.

Titration of the virus. Infective virus was inoculated in chick embryos or tubes of KEC tissue culture and estimated by hemadsorption with 0.4% suspensions of chick erythrocytes at 36 C. The 50% embryo infective dosage (EID₅₀) or tissue culture dosage (TCID₅₀) was calculated by the method of Reed and Muench.

Pathogenicity of variants of influenza A₂ virus for 11-day-old embryos. Groups of 16 to 18 chick embryos were inoculated at the same time with 10-fold dilutions of the variants tested and incubated at 36 C. At 3, 5, and 7 days, the number of deaths was recorded.

TABLE 1. *Constituents of media used for obtaining plaques with influenza A2 virus in chick embryo kidney culture*

Constituents	Growth medium	Maintenance medium	Agar overlay	
			No. 1	No. 2
Medium 199, double concentration.....			40.0	
Distilled water.....			48.4	88.2
NaHCO ₃ (5%).....	0.5	0.5	1.8	
Bovine serum.....	10.5	1.0		
Bovine serum heated at 60 C for 1 hr.....			10.0	1.0
Hydrolysed lactalbumin (0.5%) in Hanks solution.....	90.0			
Earle's solution.....		49.5		
Neutral red (1:1,000)...				10.0
Medium 199.....		49.5		
Agarose.....			1.6	0.8 ^a

^a Agarose was frequently substituted for Noble Agar in agar overlay no. 2.

A 50% lethal dosage (ELD₅₀) was calculated separately for each time period.

Temperature characteristics. The *rct-28* and *rct-40* characteristics were determined by the difference in plaque formation titers at optimal (36 C), supra-optimal (40 C), and suboptimal (28 C) temperatures.

Average plaque size (S). The diameters of 500 plaques obtained at different temperature of cultivation were measured and the average calculated.

RESULTS

All strains of influenza A2 virus investigated formed plaques in tissue culture under agar (Fig. 1). Plaques became macroscopically visible after 72 hr of incubation of infected plates at 36 C, and their number was dose-dependent. After inoculation of 10³ TCID₅₀, it was possible to count as many as 200 plaques; when the amount of virus in the inoculum was decreased, discrete plaques were noted, usually with uneven borders. Microscopically, plaques appeared as unstained areas of degenerated cells that differed sharply from normal surrounding cells, which contained deep red granules of stain. When the concentration of neutral red in the second agar overlay was increased to 1:6,000, it was possible to see macroscopically transparent spots, surrounded by a "red border" (12). Average values of efficiency of plaque formation reached as high as 10⁶ plaque-forming units (PFU) in 1 ml of infected allantoic fluid (Table 2).

Cultivation of viruses at 40 and 28 C influenced plaque formation of cryophilic variants in different ways and permitted the differentiation

of these temperature variants of influenza A2 virus. Cryophilic strains completely lost their ability to form plaques at the supraoptimal temperature (40 C), whereas the original strains plaqued with somewhat less efficiency than the thermophilic variants. The thermophilic strains showed optimal plaque-forming activity at 40 C, but did not form plaques at 28 C (Table 2).

To confirm the differences in temperature sensitivity among the variants under study, we carried out a determination of *rct-40* and *rct-28* characteristics on the basis of difference in plaque-forming activity of the virus at 36, 40, and 28 C. This method was used by Benyesh-Melnick and Melnick (1) for temperature differentiation of attenuated and virulent strains of poliomyelitis virus. Our strains described as *rct-40*⁺ showed titration differences equal to or less than 2 logs, and *rct-40*⁻ showed titration differences greater than 5 logs. If the titration

FIG. 1. *Plaque size of cryophilic (left) and thermophilic (right) variants of influenza A2/65/151 virus.*

TABLE 2. Efficiency of plaque formation of cryophilic and thermophilic variants of influenza A2 virus in chick embryo kidney culture incubated at 36, 28, and 40 C

Virus strain	Virus variant	Virus concn (log ₁₀ PFU/ml)			Titration difference at 28 and 36 C	Titration difference at 40 and 36 C
		28 C	36 C	40 C		
A2/65/147	Initial	4.1	6.4	4.3	2.3	2.1
	Cryophilic	5.8	6.5	0	0.7	6.5
	Thermophilic	0	7.1	6.9	7.1	0.2
A2/65/151	Initial	4.4	6.8	4.1	2.4	2.7
	Cryophilic	5.4	6.7	0	1.3	6.7
	Thermophilic	0	7.3	6.8	7.3	0.5

values differed within 2.5 to 5.0 logs, the strain was designated *rct-40*[±].

In the same way that we calculated *rct-40*, a determination of the *rct-28* genetic marker was done by comparing plaque titration values of viruses at 36 and 28 C (Table 2). The difference in titration values at these temperatures was 2.4 logs for original strains, 0.2 to 0.5 log for cryophilic strains, and 7.0 to 7.1 logs for thermophilic variants. Consequently, cryophilic viruses were designated *rct-28*⁺ and thermophilic strains, *rct-28*⁻.

Thus, cryophilic and thermophilic strains of influenza A2 virus showed no difference in their plaque-forming activity in KEC tissue cultures incubated at optimal temperature, but showed differential sensitivity to cultivation at high or low temperature.

Differences between cryophilic and thermophilic variants of influenza A2 virus became apparent also from measurement of plaque size in infected cultures incubated both at optimal temperature and at 40 or 28 C. Plaque diameters of original strains reached 1.0 to 1.5 mm at 36 C and increased to 1.5 to 2.5 mm by the end of 5 days (average, of 1.7 mm). Thermophilic viruses formed the largest plaques at 36 C (2.5 to 3.8 mm). Plaques of cryophilic strains were smaller at the same interval after infection. They varied from microscopic size to 0.7 to 1.0 mm after 4 to 5 days of incubation, with an average diameter of about 0.8 mm.

Thus, cryophilic and thermophilic variants did not differ in their plaque numbers at the optimal temperature, although diameters of thermophilic strains exceeded by more than three times the plaque diameters of cryophilic strains.

In plates infected with cryophilic and thermophilic variants, single plaques could frequently be found with diameters that varied from the average values. Thus, in cultures infected with thermophilic variants, 3 to 5% of the plaques

were 1.6 to 3.0 mm in diameter; among very small plaques of cryophilic viruses, 2% were 1.6 to 2.0 mm in diameter. These atypical plaques were cloned, and they showed quite typical size for every one of the investigated variants. This proved that formation of plaques atypical in size was not a consequence of the appearance of heterogeneity of virus populations but was connected with some labile phenotypic factors that were not inherited in the course of subsequent passages.

Growth of cryophilic and thermophilic variants at supra- or suboptimal temperatures influenced the size of plaques and made it possible to differentiate temperature variants of influenza A2 virus (Table 3). The largest plaques formed by wild-type viruses were 0.92 to 1.2 mm. Cryophilic viruses formed larger plaques at low temperatures (28 C) than at 36 C (mean, 1.1 to 1.22 mm). Thermophilic viruses formed plaques of the same size at both 36 and 40 C.

It is known that plaque-forming activity at supra- and suboptimal temperatures (*rct-40* and *rct-28*), as well as the sizes of plaques (*S* character), serve as important genetic markers for differentiation of enterovirus strains pathogenic for humans and animals (6, 11). The cryophilic

TABLE 3. Average plaque size of thermophilic and cryophilic variants of influenza A2 virus in tissue culture KEC

Virus strain	Virus variant	Avg plaque size (mm)		
		28 C	36 C	40 C
A2/65/147	Initial	1.12	1.66	0.92
	Cryophilic	1.1	0.74	0
	Thermophilic	0	2.51	2.41
A2/65/151	Initial	1.2	1.69	1.1
	Cryophilic	1.22	0.82	0
	Thermophilic	0	2.44	2.38

TABLE 4. *Multiplication ability, pathogenicity for chick embryos, and plaque size in different temperature variants of influenza A2 virus*

Virus strain	Virus variant	Multiplication ^a	Pathogenicity ^a	Genetic marker in KEC tissue culture		Plaque size		
				<i>rct-40</i>	<i>rct-28</i>	28 C	36 C	40 C
A2/65/147	Initial	8.0	5.0	±	±	Small	Medium	Small
	Cryophilic	8.0	3.0	—	+	Small	Small	None
	Thermophilic	8.5	8.2	+	—	None	Large	Large
A2/65/151	Initial	8.0	4.8	±	±	Small	Medium	Small
	Cryophilic	8.0	3.0	—	+	Small	Small	None
	Thermophilic	8.5	8.5	+	—	None	Large	Large

^a Determined in chick embryos incubated at 36 C, expressed as log₁₀ EID/ml.

and thermophilic variants of influenza virus studied by us showed variable pathogenicity for the chick embryo (Table 4). Strains adapted to multiplication at 40 C showed higher levels of virulence for chick embryos than did newly isolated viruses and variants obtained after 45 subsequent passages at 26 to 28 C.

Increase in pathogenicity of viruses during the course of passages at high temperature, and decrease in pathogenicity at low temperature, were in good correlation with characteristics of plaques formed during virus multiplication in KEC cultures. The most virulent thermophilic viruses reproduced better and formed larger plaques at supraoptimal temperature. Low-virulence cryophilic variants obtained after 45 subsequent passages at 28 C developed normal plaques at 28 C but not at 40 C.

DISCUSSION

Prolonged cultivation of influenza A2 virus at supra- or suboptimal temperatures was followed by a change in biological properties and particularly of virulence for developing chick embryos. Increase in virulence during the course of passages at high temperature, and decrease in virulence at low temperature, was accompanied also by distinct changes in patterns of virus reproduction at 40 or 28 C. After 45 consecutive passages at 40 C, a thermophilic variant was obtained with high plaque-forming activity at 40 C. After the same number of passages at 28 C, cryophilic variants were obtained which completely lost the ability to reproduce at 40 C but multiplied well at 28 C.

High plaquing efficiency of influenza A2 virus in chick embryo kidney cells allowed us to differentiate cryophilic and thermophilic variants by their plaque-forming activity and plaque sizes at different temperatures of cultivation.

Cryophilic strains completely lost their ability to form plaques at 40 C and formed small plaques at 36 C. Thermophilic variants multiplied effectively and formed the largest plaques at 36 and 40 C, but showed no capacity to form plaques at low temperatures of cultivation.

A correlation among plaque-forming activity, size of plaques, and virulence of influenza A2 strains for chick embryos has been shown. These studies are analogous to those with virulent and attenuated variants of poliomyelitis virus (11), measles (5), vaccinia (7), and other viruses. The most virulent thermophilic viruses had *rct-40*⁺, *rct-28*[—], and *S*⁺ characteristics.

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