A Plaque Assay for Rubella Virus Based upon Hemadsorption[1] (34164)

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Several technics for plaque assay of rubella virus have been described over the past few years (1–7). Those procedures based upon the production of a viral cytopathic effect in various cell culture systems require 7–9 days before results are obtainable, and most of the methods require the use of a second overlay to reveal the plaques. While the hemadsorption–negative plaque assay (5) requires only 4–5 days for completion, it is based upon interference, and thus the test is influenced by variables of both the rubella system and the challenge virus system; further, the method is not suitable for use in plaque purification of rubella virus. It has been demonstrated in this laboratory that cell cultures infected with rubella virus will hemadsorb certain species of erythrocytes (8), and the present report describes the development of a rapid and sensitive plaque assay for rubella virus based upon hemadsorption.

Materials and Methods. Virus strains. The laboratory strain of rubella virus employed for these studies was the RV strain, obtained from Drs. John Sever and Gilbert Schiff of the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland. It had been passed 10 times in primary grivet monkey kidney cells, 14 times in the RK-13 line of rabbit kidney cells and then 7–10 times in the BHK-21 line of baby hamster kidney cells. Field strains of rubella virus were isolated in RK-13 cells or the BS-C-1 line of grivet monkey kidney cells as previously described (9); they were used at the first or second passage level in these cell lines.

Cell lines. Most of the plaquing experiments were performed in the BHK-21 line of baby hamster kidney cells; some studies were done with the 0-853 line of baby hamster kidney cells, established by Dr. John L. Riggs of this laboratory, and with the Vero line of grivet monkey kidney cells. All cell lines were carried on growth medium consisting of 10% fetal bovine serum and 90% Eagle's minimum essential medium (MEM).

Interference tests. Detection and assay of rubella virus by the interference technic was performed in tube cultures of BS-C-1 cells using echovirus type 11 as a challenge virus (10).

Plaque assay for rubella virus. Initial attempts to demonstrate plaque formation by hemadsorption were made by infecting confluent monolayers of cells in petri dishes. However, it was found that plaques could be demonstrated more satisfactorily by infecting cells in suspension, plating them in petri dishes and then testing for hemadsorption after the cells had grown into monolayers. The following procedure was developed for plaque assays.

The BHK-21 cells were dispersed from monolayer cultures with trypsin and then suspended and counted in a small volume of plating medium consisting of 5% fetal bovine serum and 95% fortified Eagle's MEM (prepared with 2-times the standard concentrations of amino acids and vitamins, and with the ingredients dissolved in Earle's balanced salt solution). The concentration of the cell suspension was then adjusted with plating medium to give 1×10^6 cells/ml. For each 15 × 60-mm petri dish to be seeded,

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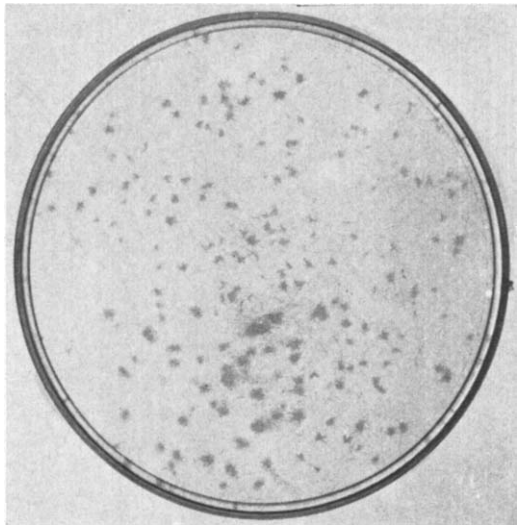

FIG. 1. Rubella virus plaques demonstrated by hemadsorption of pigeon erythrocytes onto infected BHK-21 cells.

0.5 ml of the cell suspension (500,000 cells) was mixed with 0.1 ml of viral inoculum. Each combination of cell suspension and viral inoculum was incubated in a tube at 37° with occasional shaking for 1.5 hr. Infected cell suspensions were then added in volume of 0.6 ml (500,000 cells) to 15 × 60-mm plastic petri dishes[2] containing 5 ml of plating medium and the dishes were incubated in a humidified CO_2 incubator at 36° for 72 hr; two or four dishes were inoculated with each virus dilution. Without removing the medium, 2.0 ml of a 0.75% suspension of pigeon erythrocytes was added to each dish. The erythrocyte suspension was prepared in dextrose–gelatin–Veronal (DGV) buffer containing 10^{-3} *M* $CaCl_2$ and having a pH of 7.4. After overnight incubation at 36° the medium was aspirated, the dishes were drained, and the foci (plaques) of hemadsorbing cells were counted. Plaques were 1 to 2 mm in diameter. Figure 1 shows plaques demonstrated by hemadsorption of pigeon erythrocytes to rubella-infected BHK-21 cells, and Fig. 2 shows a photomicrograph of a single plaque.

For assay of rubella antibodies by plaque reduction, equal volumes of the test dose of virus and dilutions of the test serum (inactivated at 56° for 30 min) were incubated at 37° for 30 min and then, for each culture dish to be prepared, 0.2 ml of each serum–virus combination was mixed with 0.5 ml of cell suspension (500,000 cells). The mixtures of inoculum and cells were incubated in tubes at 37° for 1.5 hr with occasional shaking. The cells were then sedimented by centrifugation at 1000 rpm for 10 min and washed by suspending in 5 ml of plating medium and centrifuging at 1000 rpm for 10 min. After removal of the wash fluid the cells were suspended in plating medium to a concentration of 1×10^6 cells/ml. Petri dishes containing 5 ml of plating medium were then seeded with 0.5 ml of the cell suspensions; two culture dishes were prepared for each serum–virus mixture. The dishes were incubated, erythrocytes were added, and plaques were enumerated as described above for virus assays. Antibody titers were expressed in terms of the highest initial serum dilution producing a 70% reduction in plaque count of the test dose of virus.

Results. Relationship between plaque numbers and virus concentration. Experiments were conducted to determine the proportionality between the number of plaques detectable by hemadsorption and the relative concentration of virus used to infect the cells. Starting with a $1 \times 10^{-4.5}$ dilution of rubella stock virus, successive twofold dilutions were

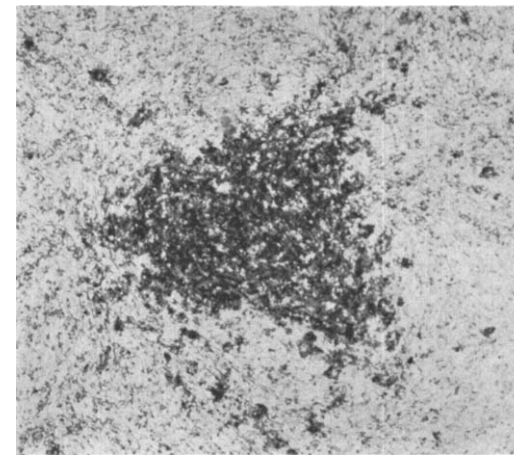

FIG. 2. Photomicrograph of a single hemadsorbing plaque, magnification 10×.

[2] Falcon Pastics, 5500 West 83rd Street, Los Angeles, California 90045.

TABLE I. Comparison between Rubella Virus Infectivity Titers Obtained by the Interference Technic and by the Hemadsorption Plaque Assay.

Virus lot no.	Passage history	Infectivity titers/ml	
		InD_{50}[a]	PFU[b]
475	$GMK_{10}RK\text{-}13_{14}BHK\text{-}21_7$	$10^{6.5}$	4×10^6
487	$GMK_{10}RK\text{-}13_{14}BHK\text{-}21_8$	$10^{7.5}$	3×10^7
492	$GMK_{10}RK\text{-}13_{14}BHK\text{-}21_9$	$10^{7.5}$	2×10^7
497	$GMK_{10}RK\text{-}13_{14}BHK\text{-}21_{10}$	$10^{6.5}$	6×10^6
499	$GMK_{10}RK\text{-}13_{14}BHK\text{-}21_8Vero_1$	$10^{7.0}$	5×10^6
485	$GMK_{10}RK\text{-}13_{14}BHK\text{-}21_6Vero_2$	$10^{6.0}$	6×10^5

[a] InD_{50} = 50% interfering doses; tests conducted in BS-C-1 cells, challenged with 100 TCD_{50} of echovirus type 11 seven days after infection with rubella virus.

[b] PFU = plaque-forming units.

prepared and each was used to infect BHK-21 cells in suspension as described above. Cells infected with each virus dilution were then plated into 4 petri dishes. Figure 3 shows that there was a linear relationship between the average number of plaques obtained for each virus dilution and the relative concentration of virus, indicating that each plaque was produced by a single infectious virus particle.

Comparison of rubella virus infectivity titers obtained by interference and by hemadsorption plaque assay. Several different rubella virus preparations propagated in either BHK-21 or Vero cells were assayed for infectivity both by the interference technic in BS-C-1 cells and by the hemadsorption plaque assay in BHK-21 cells. Table I shows

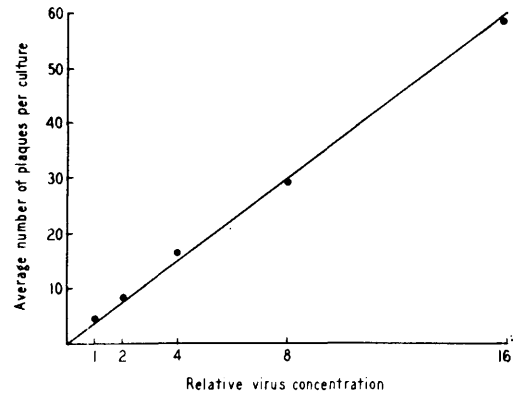

FIG. 3. Proportionality between number of hemadsorbing plaques and relative concentration of rubella virus.

that comparable infectivity titers were obtained with the two assay methods.

Rubella antibody assays by plaque reduction technic. Preliminary studies have indicated that rubella neutralizing antibody can be assayed by the plaque reduction technic described above in "Materials and Methods." Plaque reduction neutralization titers obtained for a representative rabbit immune serum and for paired sera from a human rubella infection are shown in Table II and compared with titers obtained by hemagglutination inhibition (HI) and complement fixation (CF). As in the case of certain other rubella neutralization tests, plaque reduction titers are lower than those demonstrated by HI, but higher than CF antibody titers (11).

Plaque formation by field strains of rubella virus. Demonstration of plaque formation by hemadsorption was found to be a sensitive technic for detecting small amounts of

TABLE II. Comparison of Rubella Neutralization Antibody Titers Obtained by Plaque Reduction with Titers Obtained by HI and CF Tests.

Serum examined	Time of collection	Rubella antibody titers by:		
		Plaque reduction neutralization[a]	HI	CF
Rabbit no. 1529	Preimmunization	<1:8	<1:8	<1:4
	Postimmunization	1:64	1:512	1:32
Rubella patient, BaBr	2 days after onset	<1:8	<1:8	<1:4
	18 days after onset	1:128	1:1024	1:16

[a] Based upon 70 percent plaque reduction.

TABLE III. Results of Plaque Assays on Cell Culture Materials from Rubella Virus Isolation Attempts.

Virus strain	Specimen no.	Passage level	Rubella virus detected by: HAd plaque formation[a]	HAd	Interference	FA staining[b]
Morales	T8-4635	RK-13$_1$	TNTC[c]	+	+	+
		BS-C-1$_1$	4	+	+	+
Cristian	T8-4313	BS-C-1$_1$	0	0	0	0
		BS-C-1$_2$	2	0	+	+
Riva	T8-4627	RK-13$_1$	0	0	0	0
		RK-13$_2$	TNTC	+	+	+
		BS-C-1$_1$	0	0	0	0
		BS-C-1$_2$	15	+	+	+
Steward	T8-3973	BS-C-1$_1$	0	0	0	0
		BS-C-1$_2$	TNTC	+	+	+
McIntosh	T9-0352	RK-13$_1$	TNTC	+	+	+
		BS-C-1$_1$	TNTC	+	+	+
	T9-0650	RK-13$_1$	2	0	0	+
Belotz	T8-3813	RK-13$_1$	4	0	0	0
		RK-13$_2$	ND[d]	+	+	+
		BS-C-1	0	0	0	0
19 RK-13 culture materials from negative isol. attempts		RK-13$_1$	0	0	0	0
20 BS-C-1 culture materials from negative isol. attempts		BS-C-1$_1$	0	0	0	0

[a] Number of plaques per 0.1 ml of inoculum.
[b] FA tests performed on second passage materials.
[c] Too numerous to count.
[d] ND = not done.

rubella virus in low passage materials from rubella virus isolation attempts. The RK-13 and BS-C-1 cell cultures which had been inoculated with clinical materials were harvested after the standard 7-day incubation period and then inoculated in parallel into BHK-21 cells for plaque assay, into BHK-21 tube cultures for demonstration of hemadsorption (HAd), and into BS-C-1 cells for interference test; second passages were made in the same cell types for examination by fluorescent antibody (FA) staining. Results are shown in Table III. All specimens in which rubella virus was demonstrable by HAd, interference, or FA staining produced plaques; specimens which failed to show the presence of rubella virus by any of the first three procedures also failed to produce plaques. The sensitivity of the plaque assay is illustrated in the case of specimen number T9-0650 which failed to produce HAd or interference, but produced 2 plaques and showed rubella antigen by FA staining at the second passage, and also in the case of specimen number T8-3813 which at the first RK-13 passage level was negative for HAd, interference, and FA staining, but produced 4 plaques; with subpassage, virus was demonstrable by the other technics.

It was found that rubella virus could be readily recovered from cell culture materials containing only a few infectious particles by plaquing and then "picking" the plaques and subpassaging them. The host cells from hemadsorbing plaques were drawn into a capillary pipette containing a small amount of medium and inoculated into tube cultures of the desired cell type. This permits recov-

ery of greater amounts of virus than would be transferred by subpassage of the supernatant fluids from the culture dishes.

Effect of certain variables on plaque formation. It has been reported that slightly higher titers of rubella virus are attained in BHK-21 cultures incubated at 35° than in those incubated at 37° (6). However in parallel experiments using incubation at 35 and 37°, it was found that a few more plaques were produced at 37° than at the lower temperature. Equal numbers of plaques were produced in cultures prepared from cells which had been washed to remove the viral inoculum prior to plating and in cultures from which the inoculum had not been removed. The presence of phenol red at a concentration of 0.002% in the plating medium had no adverse effect on plaque formation; equal numbers of plaques were obtained in cultures plated in Eagle's MEM containing phenol red and in those plated in Eagle's MEM prepared without phenol red.

The time after infection at which cultures were examined for hemadsorption was found to be an important factor in demonstrating plaques. Plaques of maximum size were demonstrable when the erythrocyte suspension was added 72 hr after plating of infected cells. If the erythrocytes were not added until 4 or 5 days after plating, there was a decreased amount of hemadsorption and an increased amount of hemagglutination in the supernatant fluids of the cultures.

Rubella virus plaques were also demonstrable by hemadsorption using the O-853 line of baby hamster kidney developed in this laboratory and the Vero line of grivet monkey kidney. However, these cell lines were not as satisfactory as the BHK-21 line due to the fact that smaller plaques were obtained, and also the cells tended to hemadsorb the test erythrocytes nonspecifically with prolonged incubation.

Discussion. The hemadsorption method for demonstrating rubella virus plaques is a sensitive technic for quantitative assay of the virus which can be completed within 4 days and does not require the use of solidifying overlays or a challenge virus. Although cells must be infected in suspension and then plated, the additional manipulations involved in infecting the cells are offset by the fact that monolayer cultures do not have to be prepared in advance. The hemadsorbed erythrocytes do not elute spontaneously from the infected foci, and plaques remain stable at 4° for hours and even for a few days; this is in contrast to the hemadsorption–negative plaque test in which erythrocytes adsorbed to the challenge Newcastle disease virus tend to elute rather rapidly, and thus it is necessary to enumerate plaques soon after addition of the erythrocyte suspension.

The fact that macroscopically visible hemadsorbing foci of infected cells can be demonstrated by infecting cells in suspension and then plating, but not by infecting intact monolayers, suggests that the plaques are produced by division of the initially-infected cells rather than by cell-to-cell transfer of virus. In already confluent monolayers the crowding of cells may limit further division of the infected cells, and thus plaques are minute in size. If plaques arise by division of infected cells rather than by cell-to-cell spread of virus this might explain the enhanced production of plaques at 37° (the optimal temperature for cellular proliferation) rather than at 35° (reportedly the optimal temperature for rubella virus replication).

Plaque production has been shown to be a highly sensitive method for detecting small amounts of rubella virus in cell culture materials, and the possibility of detecting virus by direct plaquing of clinical materials is being investigated.

Summary. A plaque assay for rubella virus was developed using hemadsorption of pigeon erythrocytes to demonstrate foci of infected cells. Tests conducted by this method can be completed more rapidly than previously-described plaquing procedures based upon a cytopathic effect of the virus, and they are not beset with the variables inherent in interference technics. The number of plaques demonstrable by hemadsorption was proportional to the concentration of virus used to infect the cells, indicating that each plaque was produced by a single infectious particle. Infectivity titers of rubella prepara-

tions obtained by plaque assays were comparable to those obtained by the interference technic. Rubella antibody could be assayed by a plaque reduction technic, and plaque production could be used to detect small amounts of virus at low passage levels of field strains of rubella virus.

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