Plaque Assays of Rubella Virus in Cultures of Rabbit Cornea (SIRC) Cells.* (32332)

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Rubella virus (RV) has recently been shown to produce cytopathic effect (CPE) in cultures of rabbit cornea (SIRC) cells(1). Rapid and reproducible appearance of readily detectable CPE in SIRC cells has been confirmed(2). In addition, the SIRC cell line was compared with the widely used primary African green monkey kidney (AGMK) cells(3), and shown to be useful for primary isolation, propagation and neutralization of RV(2). This report describes plaque formation with RV in monolayer cultures of SIRC cells. It was found that RV could produce distinct plaques with clear centers and sharp boundaries.

Materials and methods. Virus: The virus used in this study was "RV" strain supplied by Dr. J. L. Sever and was passed 11 times in primary AGMK cells, once in RK-13 cells, and 7 times in BHK-21 cells. It was used recently for the study of rubella CF antigen production in this laboratory(4).

Tissue cultures: Cell cultures of AGMK, SIRC, continuous embryonic rabbit kidney (MA-111)(5), and RK-13(6) cells were obtained from Microbiological Associates. These cells were maintained in Eagle's minimum essential medium in Earle's balanced salt solution supplemented with 3% fetal bovine serum and antibiotics (penicillin, 100 units, and streptomycin, 100 mg, per milliliter). A cell line of AGMK cells (Vero)(7) was obtained from Dr. Y. Yasumura, Chiba University, Chiba, Japan, in 1964, and maintained, first at the Laboratory of Tropical Virology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., and later in this laboratory. The Vero cells were subcultured and maintained in Medium 199 supplemented with 5% fetal bovine serum and antibiotics.

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Virus assay: Four tubes of SIRC cells were used for each 10-fold serial dilution tested. Observations for CPE were made for 10 to 14 days following inoculation, and fluids were changed every 5 to 7 days. The 50% endpoint was calculated by the Reed-Muench formula(8).

For *plaque assay,* monolayers in 2 oz. bottles were inoculated with 0.2 ml of appropriate dilutions of virus after removal of the medium. The inoculated bottles were incubated for 1 hour at 37°C with occasional rocking to prevent drying of cells and to improve distribution of the virus. After 1 hour, 5 ml of the first agar overlay was added. When the agar had solidified, the bottles were turned over and placed at 37°C. For the first agar overlay, the medium consisted of 0.5% lactalbumin hydrolysate in Earle's salt solution, 2% fetal bovine serum, 0.23% $NaHCO_3$, antibiotics, and 1.5% agar. On the eighth day of incubation, a second overlay of 5 ml was added. This was similar to the first, except that it contained neutral red at 1:45,000.

Neutralization tests were performed by mixing 0.4 ml of virus containing an estimated 50 PFU/0.2 ml with 0.4 ml of 2-fold serial dilutions of serum. After incubation at room temperature for 1 hour, 0.2 ml of the mixture was inoculated per bottle and the overlay was added as described above. A control titration using serial virus dilutions was carried out at the same time. Final readings were made on days 10 through 12. Serum titers were expressed as the final serum dilution producing 70% plaque reduction. Comparative titers by the CPE method, using 100 TCD_{50}, were performed in tubes at the same time. SIRC tube cultures were read on day 2 through 10; neutralization of rubella virus was indicated by inhibition of CPE. Parallel titrations of the same sera were also performed by the ECHO-11 inter-

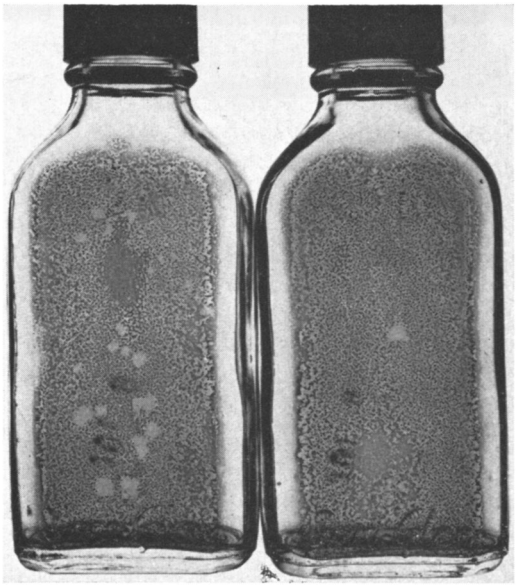

FIG. 1. Plaque formation of a 10^{-3} and a 10^{-4} dilution of rubella virus, RV strain, 10 days after seeding, 0.2 ml/2 oz bottle.

ference method in AGMK(9).

Relationship of virus dilution to plaque number. Starting at 10^{-2} dilution of virus stock, 0.2 ml of serial 2-fold virus dilutions were inoculated into each bottle. After 1 hour, bottles were removed from the incubator, washed once with diluent, and overlayed with agar medium as described above.

Results. Plaque assay. Round and clear plaques with sharp boundaries were visible as early as 3-4 hours after overlay with neutral red included in the second agar overlay, and they increased to 2-4 mm in diameter by the 10th day (Fig. 1). There was little increase in number after the tenth day. On the other hand, SIRC bottle cultures which received a single overlay containing neutral red often became decolorized before plaques could develop. To obtain well-defined and maximum plaque production, neutral red should be omitted from the first agar overlay; this has been shown in other virus-plaquing systems(10,11,12).

Plaques were also obtained with the same virus stock in Vero(13), MA-111, and RK-13 cell cultures by using the overlay described above. The plaques were not as clear and boundaries were not as distinct as those shown in SIRC monolayers. They were hazy, with less distinct margins, and included a number of cells which retained the neutral red stain in the center of the plaques, a phenomenon not observed in the plaques produced in SIRC monolayers.

Neutralization tests. Ten individual adult human sera, as well as a pool of adult human sera, have been tested against rubella virus. Table I shows in detail the results with a serum and a pool of adult human sera which had a high neutralizing capacity, and one serum that was negative.

One serum was titrated on 3 occasions. Each time, a 70% reduction in PFU count occurred at a dilution of 1:64. The use of challenge doses as low as 10 PFU or as high as 52 PFU did not seem to introduce significant variation in the results obtained. These results suggest that the neutralization test by plaque reduction gives a sharp and reproducible endpoint. This has also been

TABLE I. Rubella Neutralizing Antibody Titers of Human Sera Determined by Plaque Reduction.

Serum No.	Sera heated*	Test No.	PFU challenge dose	Antiserum dilution 1:4	1:8	1:16	1:32	1:64	1:128	1:256	70% serum titer‡
R-9377	—	1	10	0†	0	0	0	3	—	—	64
	+	2	14	—	0	0	1	3	—	—	64
	—	3	52	—	—	0	4	18	32	49	64
S547	+	1	10	0	2	4	10	11	—	—	16
S-1018	—	1	10	10	11	—	—	—	—	—	<4
	+	2	14	10	12	—	—	—	—	—	<4
	—	3	52	—	40	—	—	—	—	—	<8

* Heated at 56°C for 30 min.
† Average PFU/bottle.
‡ Titer shown as reciprocal of highest serum titer resulting in 70% reduction of PFU.

TABLE II. Comparison of Plaque and CPE Methods of Demonstrating Titers of Serum-Neutralizing Antibody to Rubella Virus (RV Strain).

Virus challenge dose	Antiserum dilution 1:8	1:16	1:32	1:64	1:128	1:256
52 PFU	0 *	0	4	18	32	49
32 TCD_{50}	4/4†	4/4	4/4	4/4	4/4	4/4

* Average PFU/bottle.
† No. of tube cultures showing CPE/No. inoculated at each dilution.

shown in the Rubella virus-hemadsorption negative plaque assay system(14). Leerhøy (15) found that heating sera at 56°C for 30 minutes reduced the rubella antibody titers. In contrast, our results indicate that reduction of rubella antibody titer did not occur with the use of heated sera, as shown in Table I.

The plaque reduction method was compared further with the tube neutralization test in SIRC cells. Results are presented in Table II. The 50% protection titer of serum against 100 TCD_{50} was less than 1:8 when tested by the tube neutralization method; however, a 70% plaque reduction was obtained at a dilution of 1:64 by the plaque technique.

In addition, the plaque reduction method was compared with the tube neutralization test, which is dependent on interference with ECHO virus 11 in AGMK cells. Table III summarizes the results. These comparative studies suggest that the plaque reduction method was a more sensitive means of detecting rubella antibodies.

The relationship between plaque count and virus concentration is shown in Fig. 2. A linear relationship was observed between number of plaques and virus concentration, indicating that each plaque was produced by a single virus particle(16).

Discussion and summary. Rubella virus produced distinct plaques with sharp boundaries in monolayer cultures of SIRC cells under a double agar overlay. Rubella virus plaques were also obtained in Vero, MA-111, and RK-13 cell cultures by using the same overlay, but the plaques were not clear and boundaries were not as distinct as those found in SIRC monolayers. A linear relationship was observed between plaque counts and virus concentration, permitting precise quantitative virus assays. Plaque reduction neutralization tests for rubella virus gave

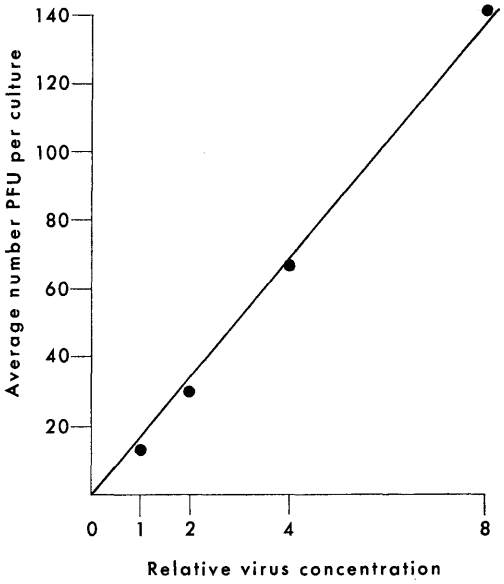

FIG. 2. Proportionality between number of plaques and virus concentration.

TABLE III. Rubella Neutralizing Antibody in Human Sera Using AGMK and SIRC Cells.

Serum	AGMK cells: TC InD_{50} rubella virus	AGMK cells: Neutralizing antibody titer	SIRC cells: TCD_{50} rubella virus	SIRC cells: Neutralizing antibody titer	SIRC cells: PFU rubella virus	SIRC cells: Neutralizing antibody titer†
1	10	0*	10	0	45	0
2	10	0	10	0	45	0
3	30	10	10	0	35	20
4	30	16	10	0	35	80
5	30	<10	10	0	35	40
6	30	64	10	>10	47	256

* Less than 1:5.
† Titer shown as reciprocal of highest serum titer resulting in 70% reduction in PFU.

reproducible titers, and proved to be more sensitive than those done by the CPE method using monolayers in tubes with fluid medium. The virus dose employed also appeared to be less critical.

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Histoplasmosis: To Skin Test or Not to Skin Test? (32333)

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The histoplasmin skin test has played a vital role in the present knowledge of histoplasmosis. Subsequent development of serologic tests provided a valuable diagnostic aid. That repeated skin tests are unnecessary and can stimulate antibody titer increases was first reported from this laboratory(1-3). As recently reviewed(4), there have been conflicting reports concerning the antibody stimulating effect of a single skin test on serologic results. Previous studies suggested that a single skin test did not alter serologic results in the tests employed in this laboratory(1,2,5). The interpretation of the significance of the serologic data in 2 suspected cases of histoplasma pericarditis, to be described separately, suggested reevaluation of the effect of a single skin test on serologic data obtained in this laboratory.

Materials and methods. A total of 187 persons consisting of 130 male prisoners 21-35 years of age and 57 medical technology students 18-22 years of age was used in this study. A single intradermal histoplasmin skin test employing a 1:100 dilution of the commercially-available antigen (Parke, Davis) routinely used in this institution was performed in all 187, and 35 of the 57 students also were skin tested concurrently with 1:100 dilutions of blastomycin (Park, Davis) and coccidioidin (Cutter). The test group was bled immediately prior to skin testing, at 8 weekly intervals thereafter, and then every 2 weeks through the 3rd month. Serum was separated promptly, stored at —30°C, and then all 11 samples from each subject were tested simultaneously.

The 2 serologic tests routinely employed in this laboratory, the collodion agglutination and yeast phase complement-fixation tests, were used. The collodion agglutination test was performed as originally described(6) employing an optimal dilution of histoplasmin prepared from the G-13 strain of *Histoplasma capsulatum*. A modification of the yeast phase complement-fixation test originally described