Cytopathic and Plaque Assay of Rubella Virus in a Line of African Green Monkey Kidney Cells (Vero).* (32157)

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Since Weller and Neva first described the cytopathic effect (CPE) of Rubella virus in primary human amnion cell cultures(1), a number of investigators have reported CPE in several cell systems(2-7). The usefulness of these systems is limited, however, by the subtlety of the morphological changes, the slow appearance of the CPE, or the necessity for adaptation of the virus.

With the exception of the baby hamster kidney line BHK-21(4,5), none of these cell systems yields virus in high titer. Primary isolation, propagation and neutralization of rubella virus is still most widely done in primary cultures of African green monkey kidney (AGMK) cells(8). To detect rubella virus in these cells, it is necessary to show interference with the growth of a cytopathic virus(8). However, the interference method is time consuming and laborious. Moreover, the measurement of neutralizing antibody by interference is an indirect test which is subject to the influences of a number of variables which limit reproducibility and precision(8).

In this and the accompanying report†(9), we describe CPE and plaque formation of rubella virus in an African green monkey (*Cercopithecus aethiops*) kidney cell line (Vero)(10) which we used successfully for assay of the Tacaribe group of arbovirus(11). We have found that Vero cells not only support rubella virus growth, but also develop CPE with extensive destruction of the cell sheet. Rubella virus also produces plaques in monolayer cultures of Vero cells under agar overlay.

Materials and methods. The virus used in this study was "RV" strain supplied by 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(5). The RK-13-adapted rubella virus strain "M-33"(8) supplied by G. C. Cole, and the high passage attenuated rubella virus strain "DBS-HPV-77"(12) supplied by the Division of Biological Standards, were also used.

We obtained the Vero cells from Y. Yasumura, Chiba University, Chiba, Japan, in 1964, and maintained them first at the Laboratory of Tropical Virology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, and later in this laboratory.

The Vero cells were subcultured and maintained in Medium 199 supplemented with 5% fetal bovine serum. They were dispersed with 0.25% trypsin and diluted in the same medium. Cell suspension was adjusted to contain 1.5×10^5 cells per milliliter of medium. They were seeded into tubes (1 ml), 2 oz bottles (5 ml), or 32 oz bottles (40 ml) and were incubated for 3 to 4 days at 37°C. The medium was then changed, and the cultures were incubated for an additional 2 to 3 days and were used for inoculation. Medium 199 without serum was used as diluent. Assays of rubella virus were also done by the ECHO 11 interference technique in AGMK cells(8) and expressed as 50% interfering doses (InD_{50}). The AGMK cells were obtained from Microbiological Associates, Inc., and were maintained in Eagle's minimum essential medium in Earle's balanced salt solution containing 3% agamma calf serum,‡ 4 mM glutamine, and antibiotics (penicillin, 100 units and streptomycin 100 mg per milliliter).

Four tubes were used for each 10-fold serial dilution tested. Observations for CPE in Vero cells were made for 14 to 21 days following inoculation, and fluids were changed

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† Dr. Dorothy M. Horstmann, Yale Univ. School of Medicine, New Haven, Conn., reported obtaining cytopathic effect with rubella virus in Vero cells at a meeting at Nat. Inst. Health, Jan. 1966.

‡ Hyland Laboratories, Inc., Los Angeles.

weekly. The 50% endpoint was calculated by the Reed-Muench formula(13).

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 were 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. The second overlay was similar to the first, except that it contained neutral red at 1:45,000.

Neutralization tests. Plaque reduction and tube neutralization tests were performed with acute and convalescent human sera and with rabbit antiserum to the RV strain. Equal volumes of 10-fold serial dilutions of virus and diluted inactivated serum(1:5) were mixed. Serum-virus mixtures and control virus preparations were held at room temperature for 1 hour before inoculation of 0.2 ml amounts into each of 4 cultures. Vero tube cultures were read on day 4 through 12 without changing medium; neutralization of rubella virus was indicated by inhibition of CPE. Plaques were read after incubation at 37°C for 10-12 days and plaque-forming units (PFU) were determined. Parallel titrations of the same sera were also performed by ECHO-11 interference method in AGMK (12).

Complement fixing antigen. Preparation of rubella virus CF antigen from infected Vero cultures was done according to the method described previously(5).

Results. Fig. 1 shows CPE produced by rubella virus in Vero cells. With low virus dilutions, early CPE was evident within 2 to 4 days. Focal cell rounding with granulation was the most prominent feature of the early CPE. The 10^{-5} dilution of the virus produced detectable CPE by the 7th to 9th day after inoculation. CPE progressed to an extensive or total degeneration of the cell sheet after an additional 2-5 days. At the end of a titration, CPE-negative Vero tubes were challenged with ECHO-11 virus. There was no interference with ECHO-11 CPE. For comparison, the virus was titrated simultaneously in different passage levels of Vero cells (passage 112 and passage 168) and in different incubation situations (stationary and rotating) at 37°C. The cytopathic process progressed at the same rate whether the tubes were incubated in roller drums or in stationary racks. However, a significant virus titer difference was observed in different passage levels of Vero cells (Fig. 2). The virus was passed in Vero cells 5 times without affecting cytopathogenicity. Cytopathic effects were also observed in cultures inoculated with "M-33" and "DBS-HPV-77." They were identical, but "DBS-HPV-77" progressed at a slower rate.

Infectivity titers of 3 strains of non-adapted rubella virus were compared in AGMK and Vero cells. Titers obtained in the 2 cell cultures were similar for each of the 3 rubella virus strains (Table I).

Cell pack (20×) preparations from infected Vero cultures on the 10th day after inoculation contained specific CF antigen.

Small, circular plaques were visible as early as the 8th and 9th day after inoculation of rubella virus (RV strain). They increased in diameter to 0.5-1.0 mm by the 12th day. There was little or no increase in number after the 12th day. It should be noted that Vero bottle cultures which received a single overlay containing neutral red often became decolonized before plaques could develop. Repeated endpoint titration of the same virus stock in Vero cells indicated that titers by plaque assay give results at least 1 log lower

TABLE I. Rubella Virus Titers in AGMK and Vero Cells.

Virus strain	History	Infectivity titer* in AGMK	Vero
RV	AGMK/11:RK-13/1: BHK-21/7	6.0	6.4
M-33	AGMK/11:RK-13/9	5.8	6.2
DBS-HPV-77	AGMK/77	3.7	3.5

* Infectivity titer expressed as $\log_{10} TCD_{50}$/ml.

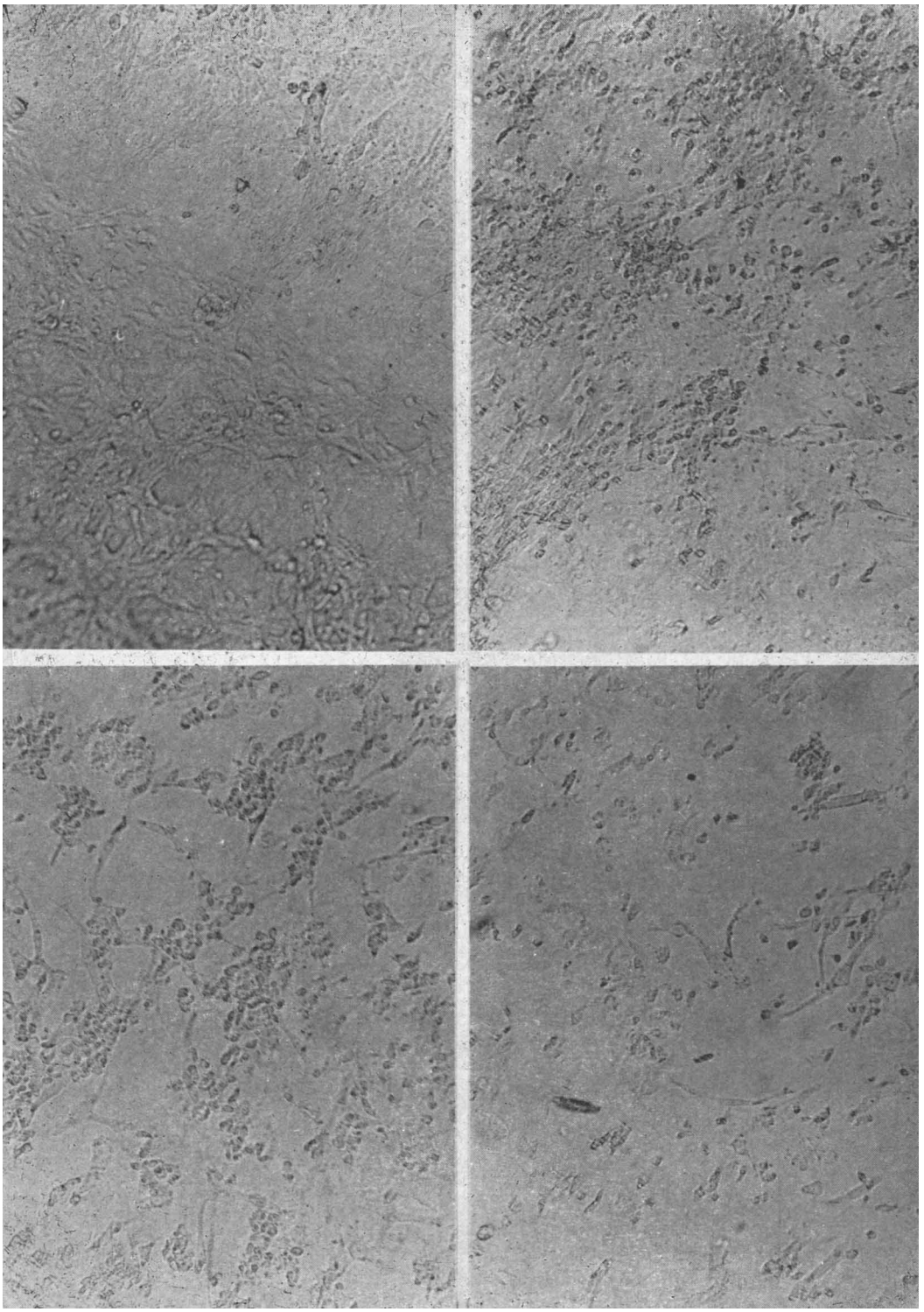

FIG. 1. Different stages of Rubella virus CPE in Vero cell cultures inoculated with 10 AGMK ID_{50}. Upper left, control: Upper right, 9th day: lower left, 10th day: lower right, 12th day after infection.

TABLE II. Rubella Neutralizing Antibody in Human and Rabbit Immune Sera Using CPE and Plaque Methods in Vero Cells.

Serum No.	Neutralizing antibody titer in AGMK‡	Log_{10} virus titer: TCD_{50} Serum + RV (1:5)	TCD_{50} RV control	PFU Serum + RV (1:5)	PFU RV control
1*	0	5.5	5.5	4.3	4.3
2*	8–16	3.0	5.0	2.3	4.3
3*	20	3.0	>5.5	2.3	4.3
4†	>16	3.0	>5.5	<2.0	4.0

* Human serum. RV = Rubella virus.
† Hyper-immune rabbit serum.
‡ Neutralizing antibody titer against 5-10 InD_{50} of rubella virus in AGMK cells.

than those done by the tube culture method.

Using plaque reduction and CPE methods, neutralization tests were carried out in Vero cells as described above. Table II summarizes the results. Sera from the convalescent, but not acute, phase, and rabbit antiserum to the RV strain neutralized the CPE and plaques. The log neutralization indices were 2.0 and $\geqq 2.5$, respectively.

Discussion. A line of African green monkey kidney cells (Vero) has been shown to produce clear and marked CPE when inoculated with rubella virus. These changes are easy to detect and are also produced by virus adapted to other cell lines. Vero cells were maintained in excellent condition for 12-14 days without changing medium: a period found to be sufficient for assay completion. Rubella virus also produces plaques in monolayer cultures of Vero cells under agar overlay. These observations have been confirmed in the accompanying report(9). Vero cells may be used for detecting rubella neutralizing antibody in human sera and for production of CF antigens.

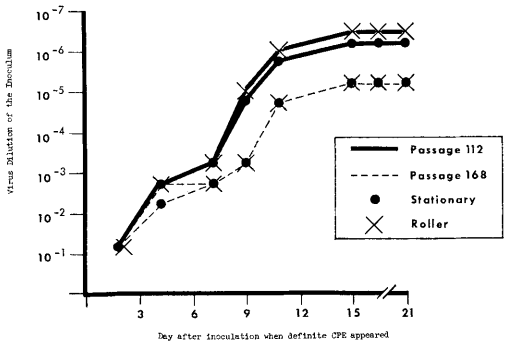

FIG. 2. Appearance of definite CPE after inoculation with various dilutions of rubella virus in stationary and rolled Vero cultures with different passage levels. Rubella virus titering $10^{6.2}$ InD_{50}/ml by interference in primary African green monkey kidney cells.

Vero cells offer several advantages. They are a continuous cell line easily maintained in the laboratory. Extensive and marked CPE and plaque assay make possible an efficient and reproducible direct technique for demonstration of virus and of specific neutralizing antibodies. In addition, growth of the virus to high titer provides a convenient source of virus for other studies and may offer advantages for production of vaccines.

Summary. An African green monkey kidney cell line (Vero) has been found to support growth of rubella virus. Cytopathic changes are pronounced and appear within 7 to 9 days after inoculation with 10^{-5} dilutions of the virus. Rubella virus also produced plaques in monolayer cultures of Vero cells under agar overlay. This cell system has been successful for the direct, reproducible titration of rubella virus and the demonstration of neutralizing antibody.

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Effect of Dimethyl Sulfoxide on Plasma Enzyme Changes in X-Irradiated Rats. (32158)

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Dimethyl sulfoxide (DMSO) has been found to have a radioprotective effect in mice(1) and rats(2). It has also been shown that DMSO, although causing little or no change in certain serum enzyme levels in normal animals(2,3,4), greatly augments the sharp rise in these enzyme levels occurring in rats subjected to various stresses such as arduous exercise(2) or exposure to high altitude(3) or a cold environment(4).

A number of investigators(6-11) have noted changes in serum enzyme levels following X-irradiation, but reports concerning the nature, degree and significance of some of these changes, particularly of serum transaminases, have varied and appear in part conflicting (8,9). The purpose of the present study was to determine the effect of DMSO on the enzyme levels in rats subjected to X-irradiation. It was thought that DMSO, perhaps by augmenting the changes, might aid in characterizing the alterations attributable to X-irradiation and indicate possible mechanisms involved.

Materials and methods. Several series of young adult male Sprague-Dawley rats weighing 275-375 g were divided into a saline group and a group treated with purified DMSO, provided by the Crown Zellerbach Corp., Camas, Wash. The number in the saline group was larger to compensate for a much higher mortality rate following X-irradiation (2). Each rat in the control group received an intraperitoneal injection of 10 ml/kg body weight of 0.85% saline (NaCl), while each rat in the other group was injected intraperitoneally with an equal volume of a 50% aqueous solution of DMSO, 5.5 g/kg.

The rats of both groups were given 800 r of whole-body X-irradiation beginning 5 to 15 minutes after the intraperitoneal injection. Irradiations were carried out at 23°C with an X-ray unit operated at 300 kvp and 20 ma with added filtration of 2 mm Cu. The target skin distance was 96 cm and the average dose rate about 38 r/min. Four saline and 4 DMSO-treated rats were irradiated at one time in a wooden cage partitioned into 8 compartments placed under the beam.

The animals were housed in a room maintained at 23°C and were given Purina laboratory chow and water *ad libitum*. Groups of rats were bled and sacrificed at 6 hours and 1, 2, 3, 6, 9, and 12 days after irradiation. Generally, the groups consisted of 3 saline- and 3 DMSO-treated irradiated rats and one non-irradiated control rat from each treatment group.

A blood sample for plasma enzyme determinations was obtained from each rat by cardiac puncture under light ether anesthesia immediately before sacrifice, using

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