

## Isolation, Propagation and Neutralization of Rubella Virus in Cultures Of Rabbit Cornea (SIRC) Cells.\* (31251)

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Rubella virus has recently been shown to produce direct cytopathic effect (CPE) in cultures of rabbit cornea (SIRC) cells(1). This virus has been shown to produce direct CPE in other cell lines, including rabbit kidney (RK-13), primary human amnion (PHA), and the AH-1 line of green monkey kidney (GMK) cells(2-4). Several disadvantages have been noted using RK-13, PHA, and AH-1 cells, including slow appearance and difficulty in detecting CPE. In this report, the rapid appearance of readily detectable CPE in SIRC cells is confirmed using several strains of primary GMK-adapted rubella virus isolated in Houston during the recent outbreak of congenital rubella syndrome(5). In addition, the SIRC cell line is compared with the widely used primary GMK cells(6), and shown to be useful for primary isolation, propagation, and neutralization of rubella virus.

*Materials and methods.* Rabbit cornea (SIRC) cells were kindly supplied by Dr. Jorgen Leerhoy of the Statens Seruminstitut, Copenhagen, Denmark. These cells were grown in Eagle's medium supplemented with 10% fetal bovine serum, 0.075% NaHCO<sub>3</sub>, 300 µg per ml glutamine, 100 units per ml of penicillin and 100 µg per ml of streptomycin. Prior to inoculation, when the cells reached a thin monolayer, medium was changed to Eagle's medium with 2% fetal bovine serum, 0.22% NaHCO<sub>3</sub>, 100 units per ml of penicillin and 100 µg per ml of streptomycin. GMK cultures were grown in M-H medium as previously described(7). After a monolayer formed, for these comparative experiments they were maintained in the same medium as were the SIRC cells.

Rubella virus strains used in the neutralization tests were obtained from Houston in-

fants with congenital rubella syndrome born during the fall of 1964 or the winter and spring of 1965(5) or from adults with rubella during the epidemic of 1964(8). Rubella virus was passed at least 4 times in SIRC cells before being used in neutralization tests in these cultures.

Specimens used for comparing the sensitivity of GMK and SIRC cells for primary isolation of rubella virus were throat swabs, rectal swabs, urine, cerebrospinal fluid, and lens tissue collected from infants with suspected congenital rubella syndrome. Swabs, in Hanks' balanced salt solution containing penicillin and streptomycin, and other specimens were stored at -30°C until tested. After specimens were thawed, 0.2 ml of inoculum was added to each of 4 GMK and 4 SIRC cultures. GMK cultures were inoculated after they had reached a full, well-populated monolayer while SIRC cells were inoculated when a sparse monolayer was present. Virus was detected in GMK cells by its ability to interfere with the CPE of a challenge virus, echovirus 11(6), while it was detected in SIRC cells by the direct appearance of CPE(1). For GMK culture, titers are given as TCInD<sub>50</sub> (50% tissue culture interfering doses), whereas for SIRC cells, they are given as TCD<sub>50</sub> (50% tissue culture doses).

Sera collected from infants with congenital rubella syndrome and from their mothers were tested for the presence of neutralizing antibody against rubella virus in GMK and SIRC cells. Serial 2-fold dilutions of test sera (1:8 to 1:28) were made in Eagle's medium containing 0.22% NaHCO<sub>3</sub> and 10% normal rabbit serum (for stabilizing the virus). Serum dilutions were incubated for 2 hours at room temperature with 50 TCD<sub>50</sub> of rubella virus, strain Mayor. The dilution of virus was based on a series of titrations of the virus stock. The serum-virus mixtures

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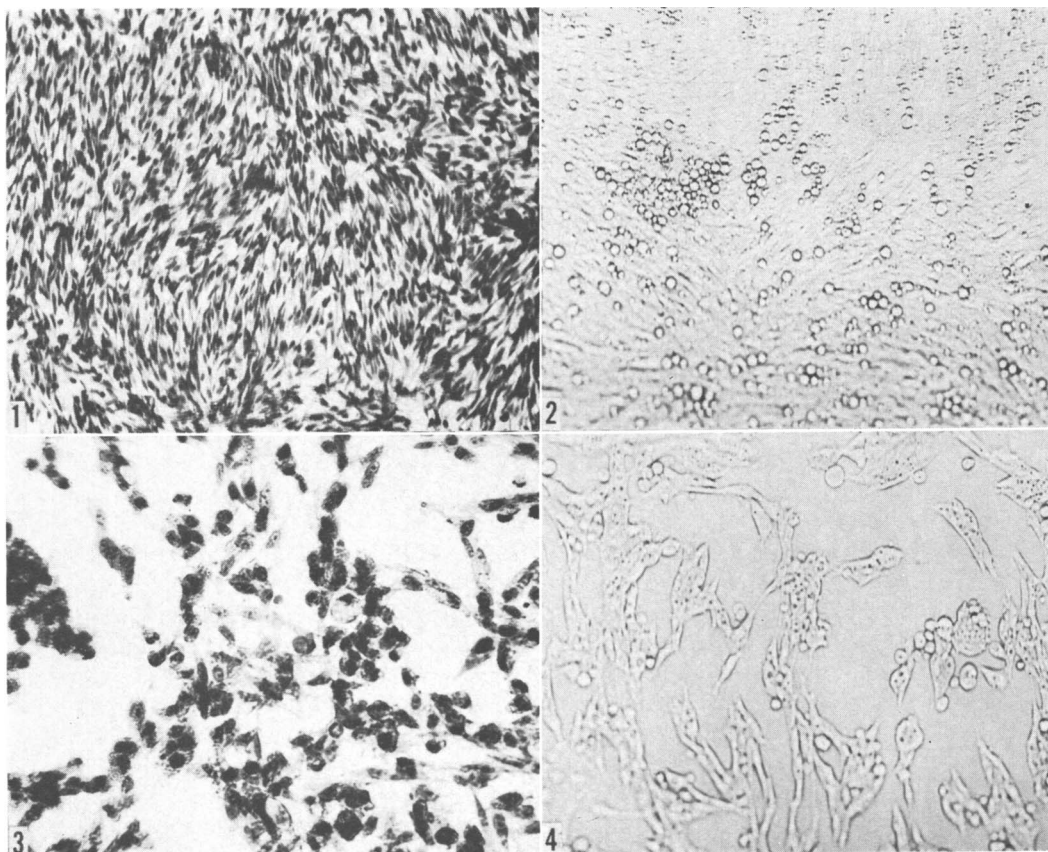


FIG. 1. Stained culture, uninoculated. H+E. 78  $\times$ .  
 FIG. 2. Live culture showing early rounding of fibroblast-like cells. 78  $\times$ .  
 FIG. 3. Stained culture showing extensive degeneration of the cell monolayer. H+E. 234  $\times$ .  
 FIG. 4. Live culture showing extensive degeneration of the cell monolayer. 78  $\times$ .

were then inoculated in 0.2 ml amounts into each of 4 tissue cultures of GMK and SIRC and the cultures were incubated at 37°C. SIRC cultures were read on days 2 through 6; neutralization of rubella virus was indicated by complete inhibition of CPE. GMK cultures were challenged on day 6 with echovirus 11; neutralization of rubella virus was indicated by the appearance of echovirus CPE. The serum endpoint in SIRC cells was defined as the dilution where 3 or 4 tubes showed no rubella CPE while in GMK it was defined as the dilution where 3 or 4 tubes showed at least 75% degeneration after echovirus challenge.

*Results.* Healthy monolayers of SIRC cells (Fig. 1) were inoculated with several strains of GMK-adapted rubella virus and early CPE was noted on day 5 (Fig. 2). Rounding

of the oblong, fibroblast-like cells was the most prominent feature of the early CPE. By day 7 extensive degeneration was noted (Fig. 3 and 4). After 4 or 5 passages of rubella virus in SIRC cells, early CPE could be detected on day 2 after inoculation. Maximum CPE was obtained by day 5. Serial passage of virus in SIRC cells was found to increase titers by about 1 log. Inoculation of at least 2000 TCD<sub>50</sub> of rubella virus was required to cause complete degeneration of the cell monolayer, while inoculation of 10 to 50 TCD<sub>50</sub> of rubella virus caused degeneration of 50% of the monolayer.

Thirty-one specimens collected from 13 infants with suspected congenital rubella syndrome were inoculated simultaneously into GMK and SIRC cells. Specimens included throat and rectal swabs, urine, cerebrospinal

TABLE I. Isolation of Rubella Virus in Green Monkey Kidney (GMK) and Rabbit Cornea (SIRC) Cells.

Cell culture	No. specimens tested	No. positive on primary passage	Additional No. positive on subcultivation	Total No. positive
GMK	31	13	3	16
SIRC	31	14	3	17

fluid, and lens tissue. On primary passage in GMK cells, 13 of 31 specimens yielded rubella virus while 14 of 31 specimens inoculated in SIRC cells yielded rubella virus (Table I). On subcultivation, 3 additional isolates were made in each of the cell lines. No further passage of negative specimens was attempted. Rubella virus was detected by direct CPE on days 5 to 7 in SIRC cells and frozen for passage on days 7 to 9. Inoculated cultures of GMK were allowed to incubate for 7 days at 37°C before challenge with echovirus 11. After challenge, tubes were read for 3 additional days and rubella virus was detected by its ability to interfere with the CPE of echovirus 11. Thus an average of 8 days was required to isolate rubella virus using SIRC cells while 10 days was required for isolation of the virus in GMK cells.

Infectivity titers of 4 strains of rubella virus were compared in GMK and SIRC cells after isolation and passage in the respective cell line. After 4 passages, somewhat higher titers were obtained in the SIRC cells (Table II).

Neutralization tests were carried out simultaneously in GMK and SIRC cells as outlined under *Materials and methods*. Table III summarizes the results. The titers obtained with unheated human sera were comparable in both cell lines tested.

*Discussion and summary.* A tissue culture cell line of rabbit cornea (SIRC) has been

TABLE II. Comparison of Rubella Virus Titers in GMK and SIRC Cells After 4 Passages.

Virus strain	Infectivity titer* in	
	GMK	SIRC
Wis	4.5	4.8
Or	3.5	4.8
Ma	2.5	4.0
El	4.5	5.5

\* Infectivity titer expressed as log<sub>10</sub> TCD<sub>50</sub>/ml.

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

Serum	GMK cells		SIRC cells	
	TCD <sub>50</sub> rubella virus	Neutralizing antibody titer	TCD <sub>50</sub> rubella virus	Neutralizing antibody titer
1	50	64	10*	64
2	50	64	10	64
3	50	8	10	8
4	50	<8	10	<8
5	50	128	50	64
6	50	64	50	64
7	50	<8	50	8
8	50	<8	50	<8

\* The dilution of virus used in the test is based on a number of previous titrations and is calculated to contain approximately 50 TCD<sub>50</sub> of virus. The TCD<sub>50</sub> reported is calculated from the titration carried out at the time of the neutralization test.

discovered by Leerhoy(1) to show clear and readily detectable CPE when inoculated with rubella virus. This observation has been confirmed and extended. The SIRC cells have been shown to be as effective for primary isolation of rubella virus from throat swabs, rectal swabs, urine, cerebrospinal fluid, and lens tissue as the more widely used GMK cells. As previously noted for both GMK and primary human amnion cells(9), subcultivations are necessary with SIRC cells for maximum isolation of rubella virus. SIRC cells may be used for detecting rubella neutralizing antibody in human sera and titers obtained are comparable to those obtained in GMK. Serial passage of rubella virus in SIRC cells appears to yield higher virus titers than those obtained after serial passage in GMK.

SIRC cells offer several advantages. They are a continuous cell line easily maintained in the laboratory. They produce direct CPE, eliminating the need for challenge of cultures with a second virus, and they reduce the time necessary to obtain test results. They should prove useful in rubella virus diagnosis and investigation.

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### Antimetabolic Antibodies to *Mycoplasma Pneumoniae* Measured by Tetrazolium Reduction Inhibition.\* (31252)

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Antigenic experience with *Mycoplasma pneumoniae* results in the development of several kinds of antibody whose role in immunity is still being investigated. Recent reviews(1,2) describe the serological technics which have been applied to the study of this organism and to primary atypical pneumonia. Of particular interest in the development of vaccines are procedures which measure antibody capable of inhibiting replication of the organism. Because technics utilizing suppression of colonial growth are laborious and relatively insensitive(3,4,5) we have investigated more rapid and simpler procedures based upon the observation that broth cultures of *M. pneumoniae* reduced 2, 3, 5-triphenyl tetrazolium chloride to its red formozan(6). The presence of specific antiserum inhibits metabolism and thus stops tetrazolium reduction. The measurement of this phenomenon with standardized quantities of reagents provides a sensitive and reproducible means of quantitating metabolism-inhibiting antibody. Its application to the study of serological patterns in natural and vaccine-induced immunity are described below.

**Materials and methods. Medium.** The medium was that described by Chanock *et al* (7) consisting of 7 parts PPLO broth (Difco) supplemented with 1% dextrose, 1 part

yeast extract (25%), 2 parts horse serum with 1,000 U/ml penicillin and 500 ppm thallium acetate. Stock aqueous solutions of 2, 3, 5-triphenyl tetrazolium chloride (Fisher or National Aniline), protected from light, were added at 0.05% final concentration to the broth immediately prior to use.

***Mycoplasma.*** The 2 strains (PI-1428 and FH) of *M. pneumoniae* were obtained from R. M. Chanock, National Institute of Allergy and Infectious Diseases, N.I.H., and had been serially transferred on the standard agar or broth medium a total of 13 and 189 times, respectively.

***Antisera.*** Pre- and post-vaccination sera were from rabbits and cynomolgus monkeys injected with formaldehyde-killed preparations of the FH strain grown in a serum-free medium(8). Hamster sera were obtained both post-vaccination and post-infection. Human sera were collected during an outbreak of mycoplasmal infections in children and adults. All sera were heated at 56°C for 30 minutes before testing.

***Serologic methods.*** Complement fixation tests were performed as described by Sever with phenol treated or lipid antigens extracted by the chloroform-alcohol procedure of Kenny and Grayston(10). The IHA procedure was that of Dowdle and Robinson (11) as modified by Taylor-Robinson *et al* (12) for the preparation of sensitized eryth-

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